PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N

A2

(11) International Publication Number: **WO 97/26321**

(43) International Publication Date: 24 July 1997 (24.07.97)

(21) International Application Number: PCT/US97/00761

(22) International Filing Date: 17 January 1997 (17.01.97)

(30) Priority Data:

08/589,028	19 January 1996 (19.01.96)	US
60/028,427	15 October 1996 (15.10.96)	US

(60) Parent Applications or Grants
(63) Related by Continuation

US	08/589,028 (CIP)
Filed on	19 January 1996 (19.01.96)
US	60/028,427 (CIP)
Filed on	15 October 1996 (15.10.96)

(71) Applicants *(for all designated States except US)*: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US). BETAGENE, INC. [US/US]; Suite 125, 2600 Stemmons Freeway, Dallas, TX 75207 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NEWGARD, Christopher, B. [US/US]; 4415 Ridge Road, Dallas, TX 75229 (US). HALBAN, Philippe, A. [CH/CH]; 41, chemin de Grange-Canal, CH-1208 Geneva (CH). NORMINGTON, Karl, D. [US/US]; 10918 Aladdin Drive, Dallas, TX 75229 (US). CLARK, Samuel, A. [US/US]; 1603 S. Alamo, Rockwell, TX 75087 (US). THIGPEN, Anice, E. [US/US]; 9014 San Leandro, Dallas, TX 75218 (US). QUAADE, Christian [DK/US]; 6258 Velasco Avenue, Dallas, TX 75214 (US). KRUSE, Fred [US/US]; 10438 Silverock Drive, Dallas, TX 75218 (US). MCGARRY, Dennis [US/US]; 4908 Forest Bend, Dallas, TX 75244 (US).

(74) Agent: HIGHLANDER, Steven, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RECOMBINANT EXPRESSION OF PROTEINS FROM SECRETORY CELL LINES

(57) Abstract

The present invention provides methods for production of heterologous polypeptides, for example amylin, using recombinantly engineered cell lines. Also described are methods engineering cells for high level expression, methods of large scale heterologous protein production, methods for treatment of disease *in vivo* using viral delivery systems and recombinant cell lines, and methods for isolating novel amylin receptors.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

RECOMBINANT EXPRESSION OF PROTEINS FROM SECRETORY CELL LINES

I. BACKGROUND OF THE INVENTION

The present application is a continuation-in-part of U.S. Patent Application Serial No. Serial Number 08/589,028, filed January 19, 1996, and U.S. Provisional Patent Application Serial No. Serial Number 60/028,279, filed October 11, 1996. The entire text of each of the above-referenced disclosures is specifically incorporated by reference herein without disclaimer.

A. Field of the Invention

The present invention is related to the recombinant expression of proteins from eukaryotic cells. More particularly, the invention relates to the production of recombinant proteins from genetically engineered secretory cells. Methods for use of the cells also are provided.

B. Related Art

Mammalian cells of neuroendocrine origin have been used extensively over the last fifteen years as systems for the study of pathways and mechanisms of polypeptide secretion (Burgess and Kelly, 1987 and Chavez *et al.*, 1994). Examples of cell lines in which such studies have been carried out include the mouse pituitary line AtT-20 (ATCC CCL 89), the rat pituitary growth hormone secreting lines GH3 (ATCC CCL 82.1), the insulin secreting βTC lines derived from transgenic mice expressing SV40 T antigen (Efrat *et al.*, 1988), radiation induced, rat islet cell tumor derived RIN lines (Gazdar *et al.*, 1980) and the rat adrenal pheochromocytoma cell line PC12 (ATCC CRL 1721). These cell lines maintain many of their endogenous functions, including synthesis of peptide hormones destined for the regulated secretory pathway. These cell lines also are transfectable, allowing expression of novel transgenes for studies of heterologous protein systems.

Three major areas have been studied using these heterologous systems. The first is the study of the sorting mechanism, whereby a given protein, destined for secretion, is targeted to the regulated secretory pathway or the default constitutive secretory pathway. The second relates to understanding the complex process of secretory protein maturation. This would include the

specific steps of protein folding, disulfide bond formation, glycosylation, endoproteolytic processing and post-translational modifications of specific amino acids as well as understanding the enzymes involved in these processes. And the third relates to control of the regulated release of peptide hormones from secretory granules following physiological stimuli.

Neuroendocrine cell lines have been generated in which genes encoding specific peptide hormones have been stably inserted. These enzymes include insulin (Moore *et al.*, 1983, Powell *et al.*, 1988 and Gross *et al.*, 1989), somatostatin (Sevarino *et al.*, 1987), thyrotropin-releasing hormone (Sevarino *et al.*, 1989), neuropeptide Y (Dickerson *et al.*, 1987), insulin-like growth factor-1 (Schmidt and Moore, 1994), proopiomelanocortin (Thorne *et al.*, 1989), glucagon (Drucker *et al.*, 1986 and Rouille *et al.*, 1994), pancreatic polypeptide (Takeuchi *et al.*, 1991) and growth hormone (Moore and Kelly, 1985). In general, heterologous expression of these proteins has demonstrated faithful sorting to the regulated secretory pathway, as well as maturation of the proteins in the secretory granules. However, the expression levels of the heterologous proteins have generally been low when compared to normal endogenous expression of the same proteins in a homologous system

Neuroendocrine cell lines expressing the enzymes involved in the processing of peptide hormones in secretory granules also have been generated. These include the endoproteases PC2 and PC3 (Ohagi *et al.*, 1992, Benjannet *et al.*, 1993, and Rouille *et al.*, 1995) and peptidylglycine alpha-amidating monooxygenase (PAM) (Milgram *et al.*, 1992 and Yun and Eipper, 1995). Overexpression of these processing enzymes has helped dissect their relative contributions to peptide hormone processing as well as their intracellular sites of action. These studies demonstrate the academic use of neuroendocrine cells in studying the regulated secretory pathway.

A series of papers over the last five years has addressed the possibility of production of heterologous peptide hormones in neuroendocrine cells. Three of these reports (Sambanis *et al.*, 1990 and 1991, Grampp *et al.*, 1992) use previously established AtT-20 lines expressing either insulin (Moore *et al.*, 1983) or growth hormone (Moore and Kelly, 1985). The highest level of

secretion of insulin under stimulated conditions was in the range of 35 to 144 microunits/million cells/hour (equivalent to 1 to 5 ng insulin/million cells/hr). Growth hormone secretion under stimulated conditions was 130 to 340 ng/million cells/hour. These levels of production are well below those reported in the literature for growth hormone production from other recombinant systems (Pavlakis and Hamer, 1983 and Heartlein *et al.*, 1994). Another study dealing with protein production from a neuroendocrine cell makes use of an insulinoma line engineered to express prolactin (Chen *et al.*, 1995). Absolute levels of production of prolactin on a per cell basis are not reported. A neuroendocrine cell-based system for either *in vitro*, biologically active peptide hormone production or for *in vivo*, cell-based delivery of biologically active peptide hormones has not been achieved in any of these earlier studies.

A number of important features must be addressed before a neuroendocrine cell-based system for protein production can be developed. The first feature is the absolute level of production of the polypeptide in question. A sufficiently high level of production to make either *in vitro* purification or *in vivo* efficacy must be achieved. As stated above, while many groups have reported expression of recombinant proteins in neuroendocrine lines, the proteins are produced at very low levels.

A second feature is quantitative processing of the peptide to their biologically active forms. Neuroendocrine cell lines maintain variable levels of the enzymes responsible for peptide hormone processing and in many lines the enzyme levels may be insufficient to ensure sufficient processing. This is a critical parameter, especially as attempts are made to engineer high level production of specific peptide hormone transgenes.

A third feature is the maintenance of a dynamic response of the regulated secretory pathway. For both *in vivo* and *in vitro* use of a neuroendocrine cell-based system, the ability to quickly release high concentrations of the biologically active peptide by extracellular stimuli is important. *In vivo* modulation of peptide hormone release is required for titrating the biological efficacy of the cell-based delivery. *In vitro* modulation of peptide hormone release establishes efficient production of highly enriched fractions of starting material for subsequent purification.

Yet another feature is the ability to further engineer functions into neuroendocrine cells other than just the high-level production of a given polypeptide. This further engineering could involve augmenting the cells capabilities such that any of the three previous points are improved or stabilized (*i.e.*, increased protein levels, increased processing efficiencies or increased dynamic regulated secretory response).

A final engineering maneuver that may prove significant is the ability to reduce or completely ablate the endogenous expression of an unwanted gene product. Reduction or ablation may result in an improved capability to produce, process or release the heterologous polypeptide. This also may confer advantages by removing unwanted or contaminating biological properties of the endogenous peptide hormone. Endogenous peptide production also might counteract the biological activities of the exogenous peptide hormone being produced, resulting in unwanted immunological reactions, reducing the capacity of the engineered lines to quantitatively produce the exogenously engineered protein or complicating purification of the exogenously produced protein. Because all of the existing neuroendocrine cell lines produce endogenous secreted proteins, these concerns are significant.

Thus, despite the benefits of developing a secretory cell line in which the protein synthetic machinery has been commandeered for the production of a heterologous polypeptide, there appear to be significant technical obstacles that are not addressed by the art. As a result, there currently exist no engineered cells that address all of these problems.

II. SUMMARY OF THE INVENTION

The present invention pertains to the engineering of mammalian cells for production of heterologous proteins, for example, in the production of secreted peptide hormones. In particular the present invention describes methods of engineering cells for high level expression of a variety of proteins including amylin and methods of large scale protein production. Further, the present

invention describes methods of treatment of disease and also methods of isolating novel amylin receptors.

There is provided, according to the present invention, a method of engineering a mammalian cell comprising providing a starting cell, introducing into the starting cell an amylin-encoding gene operatively linked to a first promoter; and selecting a cell that exhibits increased amylin production as compared to the starting cell. In other embodiments, the method of engineering a mammalian cell further comprises introducing into the selected cell an insulin-encoding gene operatively linked to a second promoter.

In particular embodiments, the starting cell produces amylin, and in other embodiments the starting cell does not produce amylin either naturally or as a result of engineering. The starting cell may be human or non-human. The starting cell may be secretory or non-secretory. The starting cell may be a neuroendocrine cell, a beta cell, or a pituitary cell. It also may be secretagogue-responsive, glucose-responsive or non-glucose-responsive. In preferred embodiments of the present invention the starting cell is derived from a βTC, RIN, HIT, BHC, CM, TRM, TRM6 AtT20, PC12 or HAP5 cell.

The amylin produced by the engineered mammalian cells may be proteolytically processed, amidated, and/or glycosylated. Glycosylated amylin species of the present invention may be O-glycosylated or N-glycosylated. When the amylin species of the present invention are glycosylated, such glycosylation may comprise an oligosaccharide linked to threonine-9 of an amylin of SEQ ID NO:51 or SEQ ID NO:53. Alternatively, the glycosylated amylin comprises an oligosaccharide linked to threonine-6 of an amylin of SEQ ID NO:51 or SEQ ID NO:53.

In certain embodiments the glycosylated amylin of the present invention comprises an oligosaccharide structure having between about 3 and about 10 saccharide units. The saccharides may be selected from the group consisting of ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, fructose, gulose, idose, galactose, talose, ribulose, sorbose, tagatose, gluconic acid, glucuronic acid, glucaric acididuronic acid, rhamnose, fucose, N-acetyl glucosamine, N-

acetyl galactosamine, N-acetyl neuraminic acid, sialic acid, amino glycal and a substituted amino glycal or any other saccharide unit commonly present in glycoproteins. In preferred embodiments the glycosylated amylin species of the present invention comprise [NeuAc,$HexNAc_2$]Gal(β1-3)GalNac.

In particular embodiments, the first promoter is selected from the group consisting of CMV IE, SV40 IE, RSV LTR, RIP, modified RIP, POMC and GH. In other embodiments the second promoter is selected from the group consisting of CMV IE, SV40 IE, RSV LTR, RIP, modified RIP, POMC and GH.

The amylin encoding gene that is engineered into the mammalian cells may be a human amylin-encoding gene, and may be linked to a selectable marker. The selectable marker may be selected from a group consisting of hygromycin resistance, neomycin resistance, puromycin resistance, zeocin, gpt, DHFR and histadinol. Likewise, the insulin gene in other embodiments may be linked to a selectable marker selected from a group consisting of hygromycin resistance, neomycin resistance, puromycin resistance, zeocin, gpt, DHFR and histadinol.

The amylin of the present invention may be an analog of human amylin and may also be a non-amyloidogenic analog. Alternatively, the amylin gene used in the present invention is a rat amylin-encoding gene or a rat amylin analog encoding gene.

Also provided is a method of providing amylin to a mammal comprising providing a starting cell, introducing into the starting cell an amylin-encoding gene operatively linked to a first promoter; selecting a cell that exhibits increased amylin production as compared to the starting cell, and administering the selected cell to a mammal.

In certain embodiments, it is envisioned that the mammal to which the amylin is provided exhibits at least one pathologic condition selected from the group consisting of angiogenesis, gastric emptying, anorexia, obesity, hypertension, hypercalcemia, Pagets disease and osteoporosis.

The administering of the selected cell may be achieved by (i) encapsulating the selected cell in a biocompatible coating or (ii) placing the cells into a selectively permeable membrane in a protective housing. Alternatively, the cell is administered intraperitoneally, subcutaneously or via the CNS. In administering the selected cell to the mammal, the cell may be contained within a selectively semi-permeable device, the device being connected to the vasculature of the mammal.

Other embodiments provide a method of producing mammalian amylin comprising providing a starting cell, introducing into the starting cell an amylin-encoding gene operatively linked to a first promoter; selecting a cell that exhibits increased production of amylin as compared to the starting cell and culturing the selected cell. The method of producing amylin may further comprise the step of purifying the amylin.

Also contemplated in the present invention is an amylin produced according to a process comprising the steps of providing a starting cell, introducing into the starting cell an amylin-encoding gene operatively linked to a first promoter, selecting a cell that exhibits increased production of amylin as compared to the starting cell and culturing the selected cell. The amylin produce may be secreted and the process may also comprise the step of purifying the amylin.

The process may further comprise the step of introducing into the cell an insulin-encoding gene operatively linked to a second promoter. In preferred embodiments, where amylin and insulin are both encoded within the cell, the amylin-to-insulin content of the selected cell is between about 0.002 to about 10.0. In other embodiments, the amylin-to insulin ratio may be between about 0.005 to about 9 in yet other embodiments the ratio may be between about 0.01 and 8, in other embodiments the ratio may be between about 0.05 to about 7 in other embodiments the ratio may be between about 1 and about 6 in further embodiments the ratio may be between about 2 and about 5 or between about 3 and about 4.

Also provided is a method of regulating blood glucose levels in a mammal comprising providing a starting cell, introducing into the starting cell an amylin-encoding gene operatively linked to a first promoter; selecting a cell that exhibit increased amylin secretion as compared to the starting cell, and administering the selected cell to the mammal, whereby the secreted amylin regulates blood glucose levels of the mammal. In preferred embodiments this method may further comprise providing the cell with an insulin-encoding gene operatively linked to a second promoter.

In still other embodiments, there is provided a method for modulating the circulating levels of insulin in a mammal comprising the steps of providing a starting cell, introducing into the cell an amylin-encoding gene operatively linked to a promoter; selecting a cell that exhibits increased amylin secretion as compared to the starting cell and administering the selected cell to the mammal, whereby the secreted amylin modulates glucose-stimulated insulin secretion in the mammal.

Also provided is a method for decreasing glycogen synthesis in a mammal comprising the steps of providing a starting cell, introducing into the cell with an amylin-encoding gene operatively linked to a first promoter; selecting a cell that exhibits increased amylin secretion as compared to the starting cell and administering the selected cell to the mammal, whereby the secreted amylin reduces glycogen synthesis in the mammal. In other embodiments, the secretory cell further is transfected with an insulin-encoding gene operatively linked to a promoter.

There also is provided a method of screening for an amylin receptor comprising obtaining amylin from a recombinant amylin expressing secretory cell; admixing the amylin with a composition comprising a putative amylin receptor; and detecting an amylin receptor bound to the amylin. In preferred embodiments, the composition comprising a putative amylin receptor is a composition comprising a population of recombinant cells transfixed with portions of a DNA library. In preferred embodiments the method further comprises obtaining a DNA segment from the DNA library that expresses an amylin receptor.

In certain embodiments, there is provided an amylin receptor gene, prepared by the process of obtaining amylin from a recombinant amylin expressing secretory cell, admixing the amylin with a composition comprising a putative amylin receptor the composition comprising a population of recombinant cells transfixed with portions of a DNA library and obtaining a DNA segment from the DNA library that expresses an amylin receptor.

Other embodiments of the present innovation provide an amylin receptor-like gene, wherein at least a portion of the gene hybridizes to at least a portion of the amylin receptor gene identified under low stringency hybridization conditions. In still other embodiments, the present invention provides a purified amylin composition comprising an amidated and glycosylated amylin polypeptide.

In other aspects the present invention pertains to the engineering of mammalian cells for production of heterologous proteins, for example, in the production of secreted peptide hormones. These mammalian cells also may be engineered such that production of at least one endogenous gene is blocked by molecular engineering, *i.e.*, permitting the usurping of the machinery for the production of the heterologous protein.

Therefore, in one embodiment, there is provided a method for producing a polypeptide comprising providing a secretory host cell, blocking the production of an endogenous, secreted polypeptide, contacting with the host cell an exogenous polynucleotide comprising a gene encoding an exogenous polypeptide, wherein the gene is under the control of a promoter active in eukaryotic cells, and culturing the secretory host cell under conditions such that the exogenous polynucleotide expresses the exogenous polypeptide.

In particular embodiments, the promoter is selected from the group consisting of CMV, SV40 IE, RSV LTR, GAPHD and RIP1. The exogenous polynucleotide may further comprise an adenovirus tripartite 5′ leader sequence and intron, and the intron may comprise the 5′ donor site of the adenovirus major late transcript and the 3′ splice site of an immunoglobulin gene. The exogenous polynucleotide may further comprise a polyadenylation signal.

The secretory host cell may be a neuroendocrine cell, such as an insulinoma, more particularly, a rat insulinoma cell or a human insulinoma cell. It also may be glucose responsive or non-glucose responsive.

The exogenous polypeptide may be secreted, amidated or a fusion protein. Amidated polypeptides include calcitonin, calcitonin gene related peptide (CGRP), β-calcitonin gene related peptide, hypercalcemia of malignancy factor (1-40) (PTH-rP), parathyroid hormone-related protein (107-139) (PTH-rP), parathyroid hormone-related protein (107-111) (PTH-rP), cholecystokinin (27-33) (CCK), galanin message associated peptide, preprogalanin (65-105), gastrin I, gastrin releasing peptide, glucagon-like peptide (GLP-1), pancreastatin, pancreatic peptide, peptide YY, PHM, secretin, vasoactive intestinal peptide (VIP), oxytocin, vasopressin (AVP), vasotocin, enkephalins, enkephalinamide, metorphinamide (adrenorphin), alpha melanocyte stimulating hormone (alpha-MSH), atrial natriuretic factor (5-28) (ANF), amylin, amyloid P component (SAP-1), corticotropin releasing hormone (CRH), growth hormone releasing factor (GHRH), luteinizing hormone-releasing hormone (LHRH), neuropeptide Y, substance K (neurokinin A), substance P and thyrotropin releasing hormone (TRH).

The exogenous polypeptide may be a hormone, such as growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, thyroid-stimulating hormone, leptin, adrenocorticotropin (ACTH), angiotensin I, angiotensin II, β-endorphin, β-melanocyte stimulating hormone (β-MSH), cholecystokinin, endothelin I, galanin, gastric inhibitory peptide (GIP), glucagon, insulin, lipotropins, neurophysins and somatostatin. In the case of insulin, recombinant cells having an insulin content of at last about 1000, 1250, 1500 and 2500 ng per 10^6 cells are provided. Recombinant cells producing 200, 300, 400, 500 and 1000 ng of insulin per 10^6 cells per hour also are provided. Recombinant cells secreting at least 25 μg of human growth hormone per 10^6 cells per hour, at least 50 μg of human growth hormone per 10^6 cells per hour and about 200 μg of human growth hormone per 10^6 cells per hour are provided.

The exogenous polypeptide may be a growth factor, such as epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor and insulin-like growth factor 1.

In a particular embodiment, the endogenous, secreted polypeptide and the exogenous polypeptide are the same, for example, where both the endogenous, secreted polypeptide and the exogenous polypeptide are insulin.

In another embodiment, the exogenous polypeptide enhances the production and/or secretion of at least one polypeptide produced by said cell, for example, a protein processing enzyme, a receptor and a transcription factor. Examples include hexokinase, glucokinase, GLUT-2, GLP-1, IPF1, PC2, PC3, PAM, glucagon-like peptide I receptor, glucose-dependent insulinotropic polypeptide receptor, BIR, SUR, GHRFR and GHRHR.

Other elements that may be included in the construct are a selectable marker and an internal ribosome entry site.

Methods for blocking of production of an endogenous, secreted polypeptide include expression of an RNA antisense to the DNA or mRNA corresponding to the endogenous, secreted polypeptide, production of ribozyme specific for the mRNA of the endogenous, secreted polypeptide, interruption of the gene encoding said endogenous, secreted polypeptide by homologous recombination genomic site directed mutagenesis or random integration. . As used herein, genomic site directed mutagenesis may employ RNA:DNA oligonucleotides or DNA:DNA oligonucleotides.

Also contemplated by the present invention are large scale production methods including stirring a suspension of the secretory host cell, gas stream agitation of a suspension of the secretory host cell, incubation of the secretory host cell in a non-perfused attached cell container or a perfused attached cell container, culture on microcarriers, microencapsulation of the

secretory host cell, followed by cell culture and incubation of the secretory host cell in a perfused packed bed reactor.

Also provided is a method of preventing type I diabetes comprising identifying a subject at risk of type I diabetes and providing to the subject a polynucleotide comprising a human insulin β-chain gene, wherein the β-chain gene is under the control of a promoter active in eukaryotic cells. The providing may comprise introducing the polynucleotide to a cell of the subject *in vivo*. Alternatively, the providing comprises contacting with a secretory host cell *ex vivo* and administering the secretory host cell to the subject. Further, the expression of the endogenous insulin β-chain in said secretory host cell may be blocked. An advantageous vehicle for providing of the polynucleotide is in a packageable, replication defective adenoviral expression construct.

A further embodiment includes a method for treating a subject afflicted with diabetes comprising identifying a subject afflicted with diabetes and providing to the subject a secretory host cell, wherein (i) the production of an endogenous, secreted polypeptide has been blocked and (ii) wherein the secretory host cell comprises an exogenous polynucleotide comprising a gene encoding insulin, wherein the gene is under the control of a promoter active in eukaryotic cells.

In yet another embodiment, there is provided a method for providing a polypeptide to an animal comprising the step of providing to the animal a secretory host cell, wherein (i) the production of an endogenous, secreted polypeptide in the secretory host cell has been blocked and (ii) wherein the secretory host cell comprises an exogenous polynucleotide comprising a gene encoding the polypeptide, wherein the gene is under the control of a promoter active in eukaryotic cells.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention,

are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

III. BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein:

FIG. 1: Map of wild-type HKI allele, vector for replacement, and disrupted HKI allele. Arrows indicate the direction of transcription of hexokinase 1 (E1 for exon 1 shown), neomycin resistance (positive selection gene) and the hsv-tk (negative selection gene). Oligos 1, 2, 3 and 4 used in PCR™ analysis are indicated. Capital bold letters indicate restriction enzyme sites introduced by the knock-out vector and lower case letters indicate sites in the endogenous gene. b, B = *Bam*HI; e = *Eco*RI; k = *Kpn*I; N = *Not*I; X = *Xho*I. The 16 kB *Kpn*I fragment cloned from RIN 1046-38 genomic DNA is indicated as well as the probe used in genomic Southerns (FIG. 2).

FIG. 2: Genomic Southern confirming hexokinase I gene disruption. The probe (hatched rectangle, Fig. 1) is a 1 kB *Pst* I fragment upstream of the recombination site. Genomic DNA was digested with *Not*I and *Eco*RI. The DNA in each lane is as follows: first lane, RIN 1046-38; second lane, RIN-52/17 containing a randomly integrated HKI replacement vector; and lane 3, RIN-52/17 containing a disrupted allele of the HKI gene (clone 86/X4).

FIG. 3: Rat insulin 1 gene knockout strategy. Map of wild-type RIN insulin I (RINS-I) allele, vector for replacement, and disrupted RINS-I allele. Restriction enzyme sites are shown. Capital bold letters indicate sites introduced by the replacement vector and lower case letters indicate sites in the endogenous gene. b = *Bam*HI; bg = *Bgl*II; N = *Not*I; P =

*Pac*I ; s = *Spe*I; x, X = *Xho*I. The coding region for RINS-1 gene is indicated by the rectangle with an arrow showing the direction of transcription. The hatched rectangle indicates the sequence used as a probe in genomic Southerns. The arrows, 1 and 2, show the locations of the primers used to amplify genomic DNA specifically recombined at the RINS-1 gene.

FIG. 4A: Insulin content in engineered cell lines. Immunoreactive insulin was determined from acid extracts prepared from the following cell lines: RIN 1046-38, R5C.I-17, R5C.I-17 chronically treated with 1.0 mM butyrate, and 11/3E9. Values are reported as μg of insulin per million cells.

FIG. 4B: Basal and stimulated insulin secretion from cell lines engineered to produce human insulin. Secreted immunoreactive insulin was determined from the following cell lines: RIN 1046-38, R5C.I-17, R5C.I-17 chronically treated with 1.0 mM butyrate, and 11/3E9. Basal samples are from a one hour incubation in media lacking glucose and containing 100 μM diazoxide. Stimulated samples are from cells incubated for one hour in media containing 5 mM glucose, 100 μM carbachol, 100 μM IBMX and amino acids. Values are reported as ng of insulin per million cells per hour.

FIG. 5A, FIG. 5B, FIG. 5C: Human proinsulin is efficiently processed to mature insulin. Immunoreactive insulin was determined from HPLC fractionated acid/ethanol extracts prepared from RIN 1046-38 (FIG. 5A), R5C.I-17 (FIG. 5B) and EP11/3E9 (FIG. 5C). Arrows indicate positions where the following standards elute: mature rat and human insulin (RI and HI), rat and human proinsulin (RPI and HPI), and rat and human processing intermediates des-31,32- and des-64,65-split proinsulin (R 3132, R 6465, H 3132, and H 6465).

FIG. 6A and FIG. 6B: Blood glucose levels of nude rats injected with human insulin-producing cells. Nude rats were injected with either 3 million R5C.I-17 cells (NR1-4, FIG. 6A) or EP11/3E9 cells (NR21-24, FIG. 6B) on day 0. NR5 is an uninjected control

animal. Blood glucose was determined on the indicated days. NR1, NR2 and NR23 died prematurely from severe hypoglycemia.

FIG. 7: Insulin message analysis from tumors explanted from nude rats injected with R5C.I-17 cells (see NR1-4, FIG. 6). Primer extension analysis of endogenous rat insulin produces a 91 base extended product (lower band) while the human insulin transgene produces a 101 base extended product (upper band). Analysis of in vitro maintained RIN 1046-38 is shown in the first lane and *in vitro* maintained R5C.I-17 is shown in the second and last lanes. The day of tumor explant is indicated for each *in vivo* sample.

FIG. 8: *In vivo* potency of engineered RIN cell lines. The *in vitro* stimulated insulin secretion values of RIN 1046-38, R5C.I-17 and EP11/3E9 (see FIG. 4B) are compared to the explanted tumor mass at initial onset of hypoglycemia in nude rats (see FIG. 6). Individual tumor masses are indicated.

FIG. 9: Gene expression of many endogenous genes is stable *in vitro* versus *in vivo* with the noted exception of GLUT-2. Northern analysis of RNA from *in vitro* maintained cells *versus* day 25 *in vivo* tumors (R5C.I-17 cells). Signals on Northerns are running at correct sizes relative to published messages- islet GK - 2.8 kB (Hughes *et al.*, 1991); GAPDH - 1.3 kB (Fort *et al.*, 1985); amylin - 0.9 kB (Leffert *et al.*, 1989); IPF1 - 1.4 kB (Leonard *et al.*, 1993 and Miller *et al.*, 1994); Sulfonylurea receptor - 5.1 kB (Aguilar-Bryan *et al.*, 1995); HK1 - 3.7 kB (Schwab and Wilson, 1989); GLUT-2 - 2.6 kB (Thorens, *et al.*, 1988); human insulin transgene - 0.7 kB (this study); and Neo transgene - 1.6 kB (this study).

FIG. 10A: GLUT-2 transgene expression as driven by the CMV promoter is stable *in vitro* and *in vivo*. Northern analysis of GLUT-2 transgene expression of a cell line expressing high levels of GLUT-2 (49/206) is maintained *in vivo* following a 16 or 34 day passage of the insulinoma in a nude rat model.

FIG. 10B: Low level of endogenous GLUT-2 expression seen in the parental RIN cells maintained *in vitro* (Lane 1, Panels A and B) is lost following a 24 day passage of the cells *in vivo*. The message for GAPDH serves as a loading control.

FIG. 11: Increased insulin content resulting from expression plasmids containing internal ribosome entry sites (IRES). Immuno reactive insulin was determined from acid/ethanol extracts from 29 independent G418 resistant clones (EP18/3 clones) generated from pCMV8/INS/IRES/NEO. Values are reported as a percentage of the insulin content in R5C.I-17 cells.

FIG. 12A: Higher human insulin-producing clones generated by iterative engineering of RIN clones with IRES-containing insulin expression plasmids. Northern analysis of EP18/3E1 (FIG. 11), a clone expressing a human insulin/IRES/NEO transgene (first lane) and clones of EP18/3E1 expressing a second transgene encoding human insulin/IRES/Puromycin (EP111/205, 206, 227, and 230). The neomycin containing message is 1.9 kB while the puromycin containing message is 1.7 kB. Messages were detected with a probe specific for human insulin.

FIG. 12B: Increase in insulin content following iterative engineering of RIN clones. Insulin content was determined from acid/ethanol extracts of 18/3E1 cells and 5 clones derived from 18/3E1 expressing a second human insulin transgene (EP111/205, 206, 220, 228 and 230). Cell counts were determined as values are reported as ng insulin per million cells.

FIG. 13: Northern blot analysis of promoter activity in stably transfected RIN lines. Different promoters were driving expression of the common transgene, INS/IRES/NEO, were constructed. For RIP/RIPi, the 5′ generic intron from INS/IRES/NEO was replaced with the rat insulin 1 gene intron (RIPi). All lanes contained 10 micrograms of total cellular RNA. The lane labeled RIN38 contains RNA from untransfected cells. The lane

labeled PC (PolyClone) contains RNA from a pool of RIN38 clones transfected with pFFE3/RIP8/INS/IRES/NEO.

FIG. 14: Human growth hormone production in RIN cells. Secreted growth hormone was determined from six independent RIN clones. Conditioned media samples were collected from each following a one hour incubation in media lacking glucose and containing 100 μM diazoxide (Basal/hr), a one hour incubation in media containing 5 mM glucose, 100 μM carbachol, 100 μM IBMX and amino acids (Stimulated/hr), and a 24 hr collection in standard tissue culture media containing 11 mM glucose and 5% fetal calf serum. Cell counts were determined as described and values are reported as μg growth hormone per million cells.

FIG. 15A: Coexpression of PAM and amylin in cell lines. Endogenous levels of expression of PAM and amylin in a series of cell lines was determined by Northern analysis. Cell lines examined were RIN 1046-38, AtT-20, RIN 1027-B2 and RIN 1046-44 (Phillipe *et al.*, 1987), EP18/3G8 and EP53/114 (this study). Pam message runs at 3.5 to 4.0 kB (Stoffers *et al.*, 1989) while amylin message is 0.9 kB (Leffert *et al.*, 1989)

FIG. 15B: Northern analysis of RIN 1046-38 cells stably transfected with an amylin expression plasmid demonstrates high level expression of the transgene. Amylin is expressed as a amylin/IRES/NEO bicistronic message of 2.1 kB in the polyclone. Endogenous expression of amylin is present in the polyclone as well as RIN 1046-38 and R5C-I.17.

FIG. 16: Insulin Promoter Factor 1 (IPF-1) transgene expression in RIN cells. Levels of stably-transfected IPF-1 mRNA expressed in RIN 38 polyclones and monoclonal cell lines were determined by Northern blot analysis. All lanes contained 10 μg of total cellular RNA. The lane labelled RIN 38 contains RNA from untransfected cells. The lane labeled INS1 conatins RNA from another untransfected stable b cell line called

INS1. IPF-1 transgene mRNAs is denoted by IPF-1/IRES/NEO. Also shown are levels of endogenous IPF-1 in different RIN lines.

FIG. 17: Stimulated Secretion of Recombinant Rat Amylin from Engineered RIN Lines. Immunoreactive amylin species were determined for basal and stimulated media samples from parental RIN 38-1046 cells and five recombinant rat amylin producing RIN clone.

FIG. 18: Human Proinsulin is Efficiently Proteolytically Processed in the Rat Insulinoma. Immunoreactive insulin was determined from HPLC fractionated acid extracts prepared from RIN 1046-38 cells (FIG. 18A) and BG18/3E1 (FIG. 18B). Arrows indicate positions where standards elute: mature rat and human insulin (RI and HI), rat and human proinsulin (RPI and HPI), and rat and human processing intermediates des-31,32- and des-64,65-split proinsulin (R3132, R 6465, H 3132, and H6465).

FIG. 19: Secreted and Extractable Amylin Species from Engineered RIN Lines. A stimulated one hour secretion sample from either RIN 1046-38 (parental), EP97/134 (rat amylin), or EP182/13 (human amylin) is compared to the extracted intracellular material from the same cell lines. Cell extracts were prepared following either the acetic acid or the triflouroacetic acid/acetonitrile (TFA/ACN) extraction protocols described in Materials and Methods. Immunoreactive amylin is reported in ng/million cells/hour for the secreted samples and ng/million cells for the extracted material.

FIG. 20A and FIG. 20B: Rat and Human Amylin are Efficiently Proteolytically Processed in the Rat Insulinoma. FIG. 20A. Immunoreactive rat amylin was determined from HPLC fractionated acid extracts prepared from rat amylin producing BG 183/20 cells with comparison to fractionated rat amylin standard. Shown is a plot of the UV absorbance versus time in minutes and the amylin immunoreactivity data in the indicated fractions overlaid on the plot. FIG. 20B. Immunoreactive amylin was determined from HPLC fractionated TFA/ACN extracts prepared from either human amylin producing BG

182/12 cells or parental RIN 38 cells with comparison to fractionated human and rat amylin standards.

FIG. 21A and FIG. 21B: Immunoprecipitation of RIN Produced Human Amylin Species. FIG. 21A. Immunoprecipitates were prepared from metabolically labeled extracts prepared from parental RIN 1046-38 cells or human amylin producing BG 182/12 cells. Extracts were prepared using a NP-40 lysis buffer (1) or RIPA lysis buffer (2) as described. FIG. 21B. Pulse/ chase experiment demonstrates that BG 182/12 human amylin producing cells secrete immunoreactive amylin species. Cells were either labeled for four hours or labeled for three hours followed by a one hour chase by incubating in the stimulated secretion buffer described above. The stimulated media was collected and cell extracts prepared using the NP-40 lysis buffer followed by immunoprecipitation from the three samples. MW standards in kD are indicated and arrows indicate the relative migration of synthetic human and rat amidated amylin.

FIG. 22: Coexpression of Amylin Transgene with Endogenous Insulin, PAM and Amylin in Engineered RIN Cell Lines. Northern analysis of RNA prepared from four recombinant rat amylin producing RIN clones and RIN 1046-38 cells. Parallel filters were probed with rat amylin, PAM or insulin specific probes.

FIG. 23: Induction of Hypoglycemia in Nude Rats Bearing Amylin Producing Tumors. Daily blood glucose levels are plotted for an uninjected control and five nude rats injected with a rat amylin producing RIN clone (BG97/134), five animals injected with a human amylin producing RIN clone (BG182/12) and three animals injected with RIN 1046-38 cells.

FIG. 24: Amylin Transgene Expression is Stable *in vivo*. Northern analysis comparing RNA isolated from cells maintained in tissue culture (in vitro) or tumors following the indicated time of in vivo passage (days) using a rat amylin specific probe demonstrates no difference in either the low levels of endogenous amylin expression in either RIN 1046-

38 or BG97/134 cells or high levels of the rat amylin transgene in engineered BG97/134 cells .

FIG. 25: RIN Cells Engineered for Amylin or Insulin Expression Efficiently Reduce Blood Glucose Levels in Streptozotocin-Induced Diabetic Nude Rats. Daily blood glucose levels are plotted for an uninjected control and three streptozotocin (STZ) injected nude rats. Fifteen days following STZ injection, animals were injected with a rat amylin producing RIN clone (BG97/134), a human insulin producing RIN clone (BG111/228), or RIN 1046-38 cells.

FIG. 26: Blood Glucose Averages of Nude Rats Bearing Engineered Rat Insulinomas. Average daily blood glucose levels are plotted for uninjected controls (3 animals), and rats injected with an insulin producing RIN clone (EP18/3E1, 4 animals), an amylin producing RIN clone (EP97/134, 4 animals), an insulin and amylin producing RIN clone (EP181/59, 4 animals), and parental RIN 1046-38 cells (3 animals).

FIG. 27: Serum Amylin Levels in Nude Rats Bearing Engineered Rat Insulinomas. Average serum amylin levels from rats described in FIG. 26 legend. Amylin values were measured against a rat amylin standard as described in Materials and Methods.

FIG. 28: Serum Insulin Levels in Nude Rats Bearing Engineered Rat Insulinomas. Average serum insulin levels from rats described in FIG. 26 legend. Insulin values were measured against a human amylin standard as described in Materials and Methods.

FIG. 29: Serum Amylin/Insulin Ratios in Nude Rats Bearing Engineered Rat Insulinomas. Molar ratios of serum amylin to serum insulin were determined using values from FIG. 27 and FIG. 28.

IV. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Secretory cells, especially neuroendocrine cells, have several endogenous functions that make them uniquely suited for production of a wide range of proteins, including secreted peptide hormones. These specialized functions include the regulated secretory pathway. The regulated secretory pathway embodies the secretory granules of neuroendocrine cells which serve as the site of maturation and storage of a large class of peptide hormones with profound biological functions. Proper biological function of the peptides is due both to their secretion in a regulated and titratable manner as well as a complex set of post-translational modifications resulting in the final biologically active product. As a result, these cells can be used *in vitro* to produce large amounts of proteins, *in vivo* to supply therapeutic proteins, or *in vivo* to immunize hosts, for example, in the production of monoclonal antibodies.

The present invention is designed to take advantage of this secretory machinery for the purpose of producing heterologous proteins. A variety of different modifications may be made to increase the efficiency of the cell, one example is the blocking of production of an endogenous protein in the host cell. This will, in essence, "make room" for the heterologous protein and, hence, avoid competition between the endogenous and heterologous proteins during synthesis. The components for such a system, and methods of producing proteins therewith, are set forth in detail below.

In a particular embodiment, the present invention relates to the production of amylin species in a mammalian host cell system. These amylin species possess all the post-translational modifications of natural amylins. The availability of such amylins allows for the elucidation of the natural amylin receptors, the role of amylin secretion and the consequences of its absence. Further, a neuroendocrine cell-based system for either *in vitro* biologically active amylin production, alone or in combination with insulin, or for *in vivo* cell-based delivery of biologically active amylin alone or in combination with insulin, would provide an effective therapy in the treatment of diabetes, hypoglycemia and the restoration of β-cell function.

The present invention describes methods and compositions for generating amylin species from engineered eukaryotic cells. Further, the present invention details methods of elucidating the role of amylin secretion and amylin receptor(s). The methods and compositions described in this invention will be useful in the treatment of pathogenic states resulting from aberrations in amylin synthesis or secretion. The present invention for the first time describes methods of obtaining "natural" amylin species, that is to say, amylins that have been post-translationally modified in ways that result in natural amylin instead of a synthetic amylin substitute.

A. Host Cells

Engineering of secretory cells to synthesize proteins for either *in vitro* large scale production, or for *in vivo* cell-based delivery, will advantageously make use of many attributes of these cells. Regulated secretory cells present a natural bioreactor containing specialized enzymes involved in the processing and maturation of secreted proteins. These processing enzymes include endoproteases (Steiner *et al.*, 1992) and carboxypeptidases (Fricker, 1988) for the cleavage of prohormones to hormones and PAM, an enzyme catalyzing the amidation of a number of peptide hormones (Eipper *et al.*, 1992a). Similarly, maturation and folding of peptide hormones is performed in a controlled, stepwise manner with defined parameters including pH, calcium and redox states.

Complete processing requires sufficient levels of the processing enzymes as well as sufficient retention of the maturing peptides. In this way, physiological signals leading to the release of the contents of the secretory granules ensures release of fully processed, active proteins. This is important for both maximum production for *in vitro* purposes and for the possible use of cells for *in vivo* purposes.

All cells secrete proteins through a constitutive, non-regulated secretory pathway. A subset of cells are able to secrete proteins through a specialized regulated secretory pathway. Proteins destined for secretion by either mechanism are targeted to the endoplasmic reticulum and pass through the golgi apparatus. Constitutively secreted proteins pass directly from the golgi to the plasma membrane in vesicles, fusing and

releasing the contents constitutively without the need for external stimuli. In cells with a regulated pathway, proteins leave the golgi and concentrate in storage vesicles or secretory granules. Release of the proteins from secretory granules is regulated, requiring an external stimuli. This external stimuli, defined as a secretagogue, can vary depending on cell type, optimal concentration of secretagogue, and dynamics of secretion. Proteins can be stored in secretory granules in their final processed form for long periods of time. In this way a large intracellular pool of mature secretory product exists which can be released quickly upon secretogogue stimulation.

A cell specialized for secreting proteins via a regulated pathway can also secrete proteins via the constitutive secretory pathway. Many cell types secrete proteins by the constitutive pathway with little or no secretion through a regulated pathway. As used herein, "secretory cell" defines cells specialized for regulated secretion, and excludes cells that are not specialized for regulated secretion. The regulated secretory pathway is found in secretory cell types such as endocrine, exocrine, neuronal, some gastrointestinal tract cells and other cells of the diffuse endocrine system.

(i) Glucose Responsive Cells.

For delivery of some peptide hormones or factors, it may be desirable to cause the polypeptide to be released from cells in response to changes in the circulating glucose concentration. The most obvious example of a secretory cell type that is regulated in this fashion is the β-cell of the pancreatic islets of Langerhans, which releases insulin in response to changes in the blood glucose concentration. Engineering of primary β-cells for production of products other than insulin is not practical. Instead, a preferred vehicle may be one of the several cell lines derived from islet β-cells that have emerged over the past two decades. While early lines were derived from radiation- or virus-induced tumors (Gazdar *et al.*, 1980, Santerre *et al.*, 1981), more recent work has centered on the application of transgenic technology (Efrat *et al.*, 1988, Miyazaki *et al.*, 1990). A general approach taken with the latter technique is to express an oncogene, most often SV40 T-antigen, under control of the insulin promoter in transgenic animals, thereby generating β-cell tumors that can be used for propagating insulinoma cell lines (Efrat *et al.*, 1988,

Miyazaki *et al.*, 1990). While insulinoma lines provide an advantage in that they can be grown in essentially unlimited quantity at relatively low cost, most exhibit differences in their glucose-stimulated insulin secretory response relative to normal islets. These differences can be quite profound, such as in the case of RINm5F cells, which were derived from a radiation-induced insulinoma and which in their current form are completely lacking in any acute glucose-stimulated insulin secretion response (Halban *et al.*, 1983, Shimuzu *et al.*, 1988). RIN 1046-38 cells are also derived from a radiation-induced insulinoma but can be shown to be glucose responsive when studied at low passage numbers (Clark *et al.*, 1990). This response is maximal at subphysiological glucose concentrations and is lost entirely when these cells are cultured for more than 40 passages (Clark *et al.*, 1990). GLUT-2 and glucokinase are expressed in low passage RIN 1046-38 cells but are gradually diminished with time in culture in synchrony with the loss of glucose-stimulated insulin release (Ferber *et al.*, 1994). Restoration of GLUT-2 and glucokinase expression in RIN 1046-38 cells by stable transfection restores glucose-stimulated insulin secretion (Ferber *et al.*, 1994), and the use of these genes as a general tool for engineering of glucose sensing has been described in a previously issued patent (Newgard, US Patent 5,427,940). RIN 1046-38 cells transfected with the GLUT-2 gene alone are maximally glucose responsive at low concentrations of the sugar (approximately 50 μM), but the threshold for response can be shifted by preincubating the cells with 2-deoxyglucose, which when converted to 2-deoxyglucose-6-phosphate inside the cell serves as an inhibitor of low K_m hexokinase, but not glucose activity (Ferber *et al.*, 1994).

Recently, Asafari *et al.* have reported on the isolation of a new insulinoma cell line called INS-1 that retains many of the characteristics of the differentiated β-cell, most notably a relatively high insulin content and a glucose-stimulated insulin secretion response that occurs over the physiological range (Asafari *et al.*, 1992). This line was isolated by propagating cells freshly dispersed from an X-ray induced insulinoma tumor in media containing 2-mercaptoethanol. Consistent with the finding of physiological glucose responsiveness, a recent report indicates that INS-1 cells express GLUT-2 and glucokinase as their predominant glucose transporter and glucose phosphorylating enzyme, respectively (Marie *et al.*, 1993). INS-1 cells grow very slowly and require 2-mercaptoethanol. It remains to be determined whether glucose

responsiveness and expression of GLUT-2 and glucokinase are retained with prolonged culturing of these cells.

Cell lines derived by transgenic expression of T-antigen in β-cells (generally termed β TC cells) also exhibit variable phenotypes (Efrat *et al.*, 1988, Miyazaki *et al.*, 1990, Whitesell *et al.*, 1991 and Efrat *et al.*, 1993). Some lines have little glucose-stimulated insulin release or exhibit maximal responses at subphysiological glucose concentrations (Efrat *et al.*, 1988, Miyazaki *et al.*, 1990, Whitesell *et al.*, 1991), while others respond to glucose concentrations over the physiological range (Miyazaki *et al.*, 1990 and Efrat *et al.*, 1993). It appears that the near-normal responsiveness of the latter cell lines is not permanent, since further time in culture results in a shift in glucose dose response such that the cells secrete insulin at subphysiological glucose concentrations (Efrat *et al.*, 1993). In some cases, these changes have been correlated with changes in the expression of glucose transporters and glucose-phosphorylating enzymes. Miyazaki *et al.* isolated two classes of clones from transgenic animals expressing an insulin promoter/T-antigen construct. Glucose-unresponsive lines such as MIN-7 were found to express GLUT-1 rather than GLUT-2 as their major glucose transporter isoform, while MIN-6 cells were found to express GLUT-2 and to exhibit normal glucose-stimulated insulin secretion (Miyazaki *et al.*, 1990). More recently, Efrat and coworkers demonstrated that their cell line bTC-6, which exhibits a glucose-stimulated insulin secretion response that resembles that of the islet in magnitude and concentration dependence, expressed GLUT-2 and contained a glucokinase:hexokinase activity ratio similar to that of the normal islet (Efrat *et al.*, 1993). With time in culture, glucose-stimulated insulin release became maximal at low, subphysiological glucose concentrations. GLUT-2 expression did not change with time in culture, and glucokinase activity actually increased slightly, but the major change was a large (approximately 6-fold) increase in hexokinase expression (Efrat *et al.*, 1993). Furthermore, overexpression of hexokinase I, but not GLUT-1, in well-differentiated MIN-6 cells results in both increased glucose metabolism and insulin release at subphysiological glucose concentrations. Similar results have been obtained upon overexpression of hexokinase I in normal rat islets (Becker *et al.*, 1994b). These results are all consistent with the observations of Ferber, *et al.* described

above in showing that a high hexokinase:glucokinase ratio will cause insulin-secreting cells to respond to glucose concentrations less than those required to stimulate the normal β-cell.

(ii) Non-glucose Responsive Cells

An alternative to insulinoma cell lines are non-islet cell lines of neuroendocrine origin that are engineered for insulin expression. The foremost example of this is the AtT-20 cell, which is derived from ACTH secreting cells of the anterior pituitary. A decade ago, Moore *et al.* demonstrated that stable transfection of AtT-20 cells with a construct in which a viral promoter is used to direct expression of the human proinsulin cDNA resulted in cell lines that secreted the correctly processed and mature insulin polypeptide (Moore *et al.*, 1983). Insulin secretion from such lines (generally termed AtT-20ins) can be stimulated by agents such as forskolin or dibutyryl cAMP, with the major secreted product in the form of mature insulin. This suggests that these cells contain a regulated secretory pathway that is similar to that operative in the islet β-cell (Moore *et al.*, 1983, Gross *et al.*, 1989). More recently, it has become clear that the endopeptidases that process proinsulin to insulin in the islet β-cell, termed PC2 and PC3, are also expressed in AtT-20ins cells (Smeekens and Steiner., 1990, Hakes *et al.*, 1991). AtT-20ins cells do not respond to glucose as a secretagogue (Hughes *et al.*, 1991). Interestingly, AtT-20 cells express the glucokinase gene (Hughes *et al.*, 1991, Liang *et al.*, 1991) and at least in some lines, low levels of glucokinase activity (Hughes *et al.*, 1991 and 1992, Quaade *et al.*, 1991), but are completely lacking in GLUT-2 expression (Hughes *et al.*, 1991 and 1992). Stable transfection of these cells with GLUT-2, but not the related transporter GLUT-1, confers glucose-stimulated insulin secretion, albeit with maximal responsiveness at subphysiological glucose levels, probably because of a non-optimal hexokinase:glucokinase ratio (Hughes *et al.*, 1992, 1993).

The studies with AtT-20ins cells are important because they demonstrate that neuroendocrine cell lines that normally lack glucose-stimulated peptide release may be engineered for this function. Other cell lines that are characterized as neuroendocrine, but lacking in endogenous glucose response include PC12, a neuronal cell line (ATCC CRL 1721) and GH3, an anterior pituitary cell line that secretes growth hormone (ATCC CCL82.1). It is not possible to determine whether such cell lines will gain glucose responsiveness by engineering

similar to that described for the AtT-20ins cell system without performing the experiments. However, these lines do exhibit other properties important for this invention such as a regulated secretory pathway, expression of endopeptidases required for processing of prohormones to their mature hormone products, and post-translational modification enzymes. In sum, all neuroendocrine cell lines are useful for the essential aspect of this invention, which is the production of heterologous products in a cell line in which the natural product (insulin, growth hormone, ACTH, *etc.*) has been eliminated. Some or all of these lines will also be useful for glucose-regulated product delivery, using the methods described in U.S. Patent 5,427,940 to generate such responsiveness.

(iii) Methods for Blocking Endogenous Protein Production

Blocking expression of an endogenous gene product may be an important modification of host cells according to the present invention. The targeted endogenous gene encodes a protein normally secreted by the host cell. Blocking expression of this endogenous gene product, while engineering high level expression of genes of interest, represents a unique way of designing cells for protein production.

Cells generated by this two-step process express heterologous proteins, including a variety of natural or engineered proteins (fusions, chimeras, protein fragments, *etc.*). Cell lines developed in this way are uniquely suited for *in vivo* cell-based delivery or *in vitro* large-scale production of defined peptide hormones with little or no contaminating or unwanted endogenous protein production.

Five basic approaches are contemplated for blocking of expression of an endogenous gene in host cells. First, constructs are designed to homologously recombine into particular endogenous gene loci, rendering the endogenous gene nonfunctional. Second, constructs are designed to randomly integrate throughout the genome, resulting in loss of expression of the endogenous gene. Third, constructs are designed to introduce nucleic acids complementary to a target endogenous gene. Expression of RNAs corresponding to these complementary nucleic acids will interfere with the transcription and/or translation of the target sequences. Fourth,

constructs are designed to introduce nucleic acids encoding ribozymes - RNA-cleaving enzymes - that will specifically cleave a target mRNA corresponding to the endogenous gene. Fifth, endogenous gene can be rendered dysfunctional by genomic site directed mutagenesis

Antisense. Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function

is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

Ribozymes. Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cook *et al.*, 1981). For example, U.S. Patent No. 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990; Sioud *et al.*, 1992). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

Homologous Recombination. Another approach for blocking of endogenous protein production involves the use of homologous recombination. Homologous recombination relies, like antisense, on the tendency of nucleic acids to base pair with complementary sequences. In this instance, the base pairing serves to facilitate the interaction of two separate nucleic acid molecules so that strand breakage and repair can take place. In other words, the "homologous" aspect of the method relies on sequence homology to bring two complementary sequences into close proximity, while the "recombination" aspect provides for one complementary sequence to replace the other by virtue of the breaking of certain bonds and the formation of others.

Put into practice, homologous recombination is used as follows. First, a target gene is selected within the host cell. Sequences homologous to the target gene are then included in a genetic construct, along with some mutation that will render the target gene inactive (stop codon, interruption, *etc.*). The homologous sequences flanking the inactivating mutation are said to "flank" the mutation. Flanking, in this context, simply means that target homologous sequences are located both upstream (5′) and downstream (3′) of the mutation. These sequences should correspond to some sequences upstream and downstream of the target gene. The construct is then introduced into the cell, thus permitting recombination between the cellular sequences and the construct.

As a practical matter, the genetic construct will normally act as far more than a vehicle to interrupt the gene. For example, it is important to be able to select for recombinants and, therefore, it is common to include within the construct a selectable marker gene. This gene permits selection of cells that have integrated the construct into their genomic DNA by conferring resistance to various biostatic and biocidal drugs. In addition, a heterologous gene that is to be expressed in the cell also may advantageously be included within the construct. The arrangement might be as follows:

...vector•5′-flanking sequence•heterologous gene• selectable marker gene•flanking sequence-3′•vector...

Thus, using this kind of construct, it is possible, in a single recombinatorial event, to (i) "knock out" an endogenous gene, (ii) provide a selectable marker for identifying such an event and (iii) introduce a heterologous gene for expression.

Another refinement of the homologous recombination approach involves the use of a "negative" selectable marker. This marker, unlike the selectable marker, causes death of cells which express the marker. Thus, it is used to identify undesirable recombination events. When seeking to select homologous recombinants using a selectable marker, it is difficult in the initial screening step to identify proper homologous recombinants from recombinants generated from random, non-sequence specific events. These recombinants also may contain the selectable marker gene and may express the heterologous protein of interest, but will, in all likelihood, not have the desired "knock out" phenotype. By attaching a negative selectable marker to the construct, but outside of the flanking regions, one can select against many random recombination events that will incorporate the negative selectable marker. Homologous recombination should not introduce the negative selectable marker, as it is outside of the flanking sequences.

In a particular aspect of this embodiment, the negative selectable maker is GLUT-2. It is also contemplated that GLUT-5 would function in a similar manner to GLUT-2. Therefore, the selection protocols described are intended to refer to the use of both GLUT-2 and GLUT-5.

In a first embodiment, a target gene within a GLUT-2$^-$ host cell is selected as the location into which a selected gene is to be transferred. Sequences homologous to the target gene are included in the expression vector, and the selected gene is inserted into the vector such that target gene homologous sequences are interrupted by the selected gene or, put another way, such the target gene homologous sequences "flank" the selected gene. In preferred embodiments, a drug selectable marker gene also is inserted into the target gene homologous sequences. Given this possibility, it should be apparent that the term "flank" is used broadly herein, namely, as describing target homologous sequences that are both upstream (5′) and downstream (3′) of the selected gene and/or the drug selectable marker gene. In effect, the flanking sequences need not directly abut the genes they "flank."

The construct for use in this embodiment is further characterized as having a functional GLUT-2 gene attached thereto. Thus, one possible arrangement of sequences would be:

... 5′-GLUT-2•flanking target sequences•selected gene•drug-selectable marker gene•flanking target sequences-3′ ...

Of course, the GLUT-2 could come at the 3′-end of the construct and the selected gene and drug-selectable marker genes could exchange positions.

Application of a drug to such cells will permit isolation of recombinants, but further application of Streptozotocin (glucopyranose, 2-deoxy-2-[3-methyl-e-nitrosourido-D]; STZ) to such cells will result in killing of non-homologous recombinants because the incorporated GLUT-2 gene will produce GLUT-2 transporter, rendering the cells susceptible to STZ treatment (the original cell was GLUT-2$^-$).

On the other hand, site-specific recombination, relying on the homology between the vector and the target gene, will result in incorporation of the selected gene and the drug selectable marker gene only; GLUT-2 sequences will not be introduced in the homologous recombination event because they lie outside the flanking sequences. These cells will be drug resistant and but not acquire the GLUT-2 sequences and, thus, remain insensitive to STZ. This double-selection procedure ($drug^{res}/STZ^{res}$) should yield recombinants that lack the target gene and express the selected gene. Further screens for these phenotypes, either functional or immunologic, may be applied.

A modification of this procedure is one where no selected gene is included, *i.e.*, only the selectable marker is inserted into the target gene homologous sequences. Use of this kind of construct will result in the "knock-out" of the target gene only. Again, proper recombinants are screened by drug resistance and STZ resistance (the original cell was GLUT-2$^-$).

Genomic Site-Directed Mutagenesis with Oligonucleotides. Through analysis of radiation-sensitive mutants of *Ustilago maydis*, genes have been characterized that participate in DNA repair (Tsukuda *et al.*, 1989; Bauchwitz and Holloman, 1990). One such gene, *REC2*, encodes a protein that catalyzes homologous pairing between complementary nucleic acids and is required for a functional recombinational repair pathway (Kmiec *et al.*, 1994; Rubin *et al.*, 1994). *In vitro* characterization of the REC2 protein showed that homologous pairing was more efficient between RNA-DNA hybrids than the corresponding DNA duplexes (Kmiec *et al*, 1994; PCT, WO 96/22364). However, efficiency in pairing between DNA:DNA duplexes could be enhanced by increasing the length of the DNA oligonucleotides (Kmiec et al., 1994). These observations led investigators to test the use of chimeric RNA-DNA oligonucleotides (RDOs) in the targeted modification of genes in mammalian cell lines (Yoon *et al.*, 1996; Cole-Strauss *et al.*, 1996; PCT WO95/15972). The RNA-DNA oligonucleotides that were used to test this application contained self-annealing sequences such that double-hairpin capped ends are formed. This feature is thought to increase the in vivo half-life of the RDO by decreasing degradation by helicases and exonucleases. Further, the RDOs contained a single base pair that differs from the target sequence and otherwise aligns in perfect register. It is believed that the single mismatch

will be recognized the DNA repair enzymes. And the RDOs contained RNA residues modified by 2'-O-methylation of the ribose sugar. Such modification makes the RDO resistant to degradation by ribonuclease activity (Monia *et al.*, 1993).

Two separate experimental systems have been used to test the use of RDOs for targeted gene disruption in mammalian cell lines. In one system RDOs were used to target and correct an alkaline phosphatase cDNA in that was maintained in the episomal DNA of Chinese hamster ovary cells. An inactive form of alkaline phosphatase was converted to a wild-type form with an efficiency of about 30% (Yoon *et al.*, 1996). In a second system, a genetic mutation within chromosomal DNA was targeted and corrected. A lymphoid blast cell line was derived from a patient with sickle cell disease who was homozygous for a point mutation in the β–globin gene. Here again the overall frequency of gene conversion from the mutant to the wild-type form was very high and was found to be dose-dependent on the concentration of the RDOs (Cole-Strauss *et al.*, 1996).

If the use of RDOs or DNA oligonucleotides for the purposes of targeted gene conversion is broadly applicable to various mammalian cell lines, then it offers several advantages to current technologies that have been used to accomplish gene disruption such as homologous recombination. First, if gene conversion by RDO or DNA oligonucleotides occurs in various cell lines at an efficiency of 30% then this will represent a much higher rate than has been reported for targeted gene disruption via homologous recombination. Secondly, only short sequences are required for gene disruption by RDOs or DNA oligonucleotides(typically 60-mers to 70-mers); whereas homologous recombination requires very long stretches of complementary sequences. Homologous sequences from 9 to 15 kilobases are typically recommended in the construction of targeting vectors. As a result, construction of DNA vectors for homologous recombination usually involves extensive gene mapping studies and time consuming efforts in the isolation of genomic DNA sequences. Such efforts are unnecessary if RDOs are used for targeted gene conversions. Thirdly, assays for gene conversion by RDOs can be performed 4 to 6 hours following introduction of the RDOs or DNA oligonucleotides into the cell. In contrast, gene

conversion by homologous recombination requires a relatively long period of time (days to weeks) between the time of introducing the targeting vector DNA and assaying for recombinants.

Random Integration. Though lacking the specificity of homologous recombination, there may be situations where random integration will be used as a method of knocking out a particular endogenous gene. Unlike homologous recombination, the recombinatorial event here is completely random, *i.e.*, not reliant upon base-pairing of complementary nucleic acid sequences. Random integration is like homologous recombination, however, in that a gene construct, often containing a heterologous gene and a selectable marker, integrates into the target cell genomic DNA via strand breakage and reformation.

Because of the lack of sequence specificity, the chances of any given recombinant integrating into the target gene are greatly reduced. Also possible is integration into a second loci, resulting in the loss of expression of the gene of interest. This second locus could encode a transcription factor needed for expression of the first gene, a locus control region needed for the expression of the first gene, *etc.* As a result, it may be necessary to "brute force" the selection process. In other words, it may be necessary to screen hundreds of thousands of drug-resistant recombinants before a desired mutant is found. Screening can be facilitated, for example, by examining recombinants for expression of the target gene using immunologic or even functional tests; expression of the target gene indicate recombination elsewhere and, thus, lack of suitability.

(iv) Methods for Increasing Production of Recombinant Peptides from Secretory Cells

The present invention also contemplates augmenting or increasing the capabilities of cells to produce biologically active polypeptides. This can be accomplished, in some instances, by overexpressing the proteins involved in protein processing, such as the endoproteases PC2 and PC3 (Steiner *et al.*, 1992) or the peptide amidating enzyme, PAM (Eipper *et al.*, 1992a) in the case of amidated peptide hormones.

Expression of proteins involved in maintaining the specialized phenotype of host cells, especially their secretory capacity, is important. Engineering the overexpression of a cell type-specific transcription factor such as the Insulin Promoter Factor 1 (IPF1) found in pancreatic b-cells (Ohlsson *et al.*, 1993) could increase or stabilize the capabilities of engineered neuroendocrine cells. Insulin promoter factor 1 (IPF-1; also referred to as STF-1, IDX-1, PDX-1 and bTF-1) is a homeodomain-containing transcription factor proposed to play an important role in both pancreatic development and insulin gene expression in mature b cells (Ohlsson et al., 1993, Leonard et al., 1993, Miller *et al.*, 1994, Kruse *et al.*, 1993). In embryos, IPF-1 is expressed prior to islet cell hormone gene expression and is restricted to positions within the primitive foregut where pancreas will later form. Indeed, mice in which the IPF-1 gene is disrupted by targeted knockout do not form a pancreas (Jonsson *et al.*, 1994). Later in pancreatic development, as the different cell types of the pancreas start to emerge, IPF-1 expression becomes restricted predominantly to b cells. IPF-1 binds to TAAT consensus motifs contained within the FLAT E and P1 elements of the insulin enhancer/promoter, whereupon, it interacts with other transcription factors to activate insulin gene transcription (Peers *et al.*, 1994).

Stable overexpression of IPF-1 in neuroendocrine β cell lines will serve two purposes. First, it will increase transgene expression under the control of the insulin enhancer/promoter. Second, because IPF-1 appears to be critically involved in β cell maturation, stable overexpression of IPF-1 in β cell lines should cause these mostly dedifferentiated β-cells to regain the more differentiated function of a normal animal β cell. If so, then these redifferentiated β cell lines could potentially function as a more effective neuroendocrine cell type for cell-based delivery of fully processed, bioactive peptide hormones.

Also, further engineering of cells to generate a more physiologically-relevant regulated secretory response is claimed. Examples would include engineering the ratios of glucokinase to hexokinase in rat insulinoma cells that also overexpress the Type II glucose transporter (GLUT-2) such that a physiologically-relevant glucose-stimulated secretion of peptide hormones is achieved. Other examples include engineering overexpression of other signalling proteins known to play a role in the regulated secretory response of neuroendocrine cells. These include

cell surface proteins such as the β-cell-specific inwardly rectifying potassium channel (BIR; Inagaki *et al.*, 1995), involved in release of the secretory granule contents upon glucose stimulation, the sulfonylurea receptor (SUR), and ATP sensitive channel. Other cell surface signalling receptors which help potentiate the glucose-stimulated degranulation of β-cells including the glucagon-like peptide I receptor (Thorens, 1992) and the glucose-dependent insulinotropic polypeptide receptor (also known as gastric inhibitory peptide receptor) (Usdin, 1993) can be engineered into neuroendocrine cells. These β-cell-specific signaling receptors, as well as GLUT-2 and glucokinase, are involved in secretory granule release in response to glucose. In this way, glucose stimulated release of any heterologous peptide targeted to the secretory granule can be engineered. Alternatively, other cell surface signaling proteins involved in non-glucose-stimulated release of secretory granule contents can be engineered into neuroendocrine cells. Examples would include releasing factor receptors such as Growth Hormone Releasing Factor Receptor (Lin *et al.*, 1992) and Somatostatin or Growth Hormone Releasing Hormone Receptor (Mayo, 1992).

One potential target for genetic engineering to improve cell characteristics for protein production is hexokinase I. It now has been determined that interfering with hexokinase I function reduces the growth rate of cells. The following is a discussion of engineering of hexokinases according to the present invention.

Mitochondrial Binding. Low K_m hexokinases are distinguished from glucokinase in that they are allosterically regulated by glucose-6-phosphate and by binding to mitochondria (Wilson, 1968; 1973; 1985; 1995). Micromolar concentrations of glucose-6-phosphate inhibit the activities of hexokinases I, II, and III, but appreciable inhibition of glucokinase requires glucose-6-phosphate concentrations in excess of 10 mM. Binding of hexokinases I and II to mitochondria alters their kinetic properties (Wilson, 1968; 1985; 1995), while glucokinase does not appear to be capable of binding to mitochondria at all (Becker *et al.* 1996).

When bound to mitochondria, hexokinase I undergoes an increase in affinity (a decrease in K_m) for its substrate ATP (Wilson, 1985). In addition, the enzyme becomes far less inhibitable

by glucose-6-phosphate, as indicated by a several-fold increase in K_i for this ligand (Wilson, 1985). Studies with hexokinase I have revealed the existence of two types of mitochondrial binding sites (Kabeer and Wilson, 1994). Glucose-6-phosphate causes displacement of a proportion of mitochondrially-bound hexokinase from one type of site. The enzyme that remains bound to mitochondria after glucose-6-phosphate treatment is considered to occupy the second site, from which it can be removed by treatment with 0.5 M KSCN.

It has been known for some time that limited digestion of hexokinase I with chymotrypsin yields an enzyme fragment that retains catalytic activity but that loses its capacity for mitochondrial binding, and that enzyme treated in this manner is lacking in a portion of its N-terminal domain (Polakis and Wilson, 1985). The N-terminal sequences of both hexokinases I and II are relatively hydrophobic, and it has been shown that the hydrophobic N-terminus of hexokinase I is capable of insertion into the lipid bilayer of the mitochondrial membrane (Xie and Wilson, 1988).

Subsequently, Gelb *et al.*, (1992) demonstrated that a chimeric protein consisting of the N-terminal 15 amino acids of hexokinase I fused to chloramphenicol acetyltransferase was capable of binding to rat liver mitochondria, and that this binding was competitive with authentic hexokinase I (Gelb *et al.* 1992). Although Gelb *et al.* (1992) have suggested that the first 15 amino acids of hexokinase are sufficient to target such a chimeric protein to mitochondria, these studies were not designed to attempt to alter metabolic regulation in target cell lines. Thus, the elements required to effect displacement of endogenous hexokinase from its mitochondrial binding site were not unequivocally identified in the study of Gelb and co-authors as discussed below.

While the results of Gelb *et al.* (1992) argue for the importance of this small N-terminal segment in targeting of hexokinase to mitochondria, others have suggested that other regions of the molecule may also be important in stabilizing the interaction (Polakis and Wilson, 1985; Felgner and Wilson, 1977; Smith and Wilson, 1991). This is based on studies showing that hexokinase I binding to mitochondria is stabilized by Mg^{2+}, an effect likely reflecting

electrostatic interactions between the enzyme and the outer mitochondrial membrane (*i.e.*, not involving the N-terminal 15 amino acids that are intercalated into the membrane). Therefore, the mitochondrial binding regions of HK have not been clearly identified to date, and there is even less information available on the issue of HK displacement.

At least part of hexokinase binding to mitochondria is via interactions with members of a family of proteins known as voltage-dependent anion channels (VDAC) or porins (Fiek *et al.*, 1982; Linden *et al.*, 1982). These porins form a channel through which metabolites such as ATP and various anions traverse the outer mitochondrial membrane. Binding of hexokinases to porin thus may ensure a supply of intramitochondrially-generated ATP as substrate.

Constructs of the present invention may comprise the N-terminal 15 amino acids of a hexokinase enzyme, preferably hexokinase I or II, since this segment should be easily expressed in cells and retained as a stable peptide. Constructs comprising the entire N-terminal domain of either hexokinase I or hexokinase II, or the intact, full-length hexokinase I or II proteins that have been rendered inactive by site-directed mutagenesis of amino acids that are important for the enzyme's catalytic function are also contemplated. Constructs based upon hexokinase I will be particularly, or even exclusively, preferred in certain embodiments.

The reason for preferring the N-terminal domain construct is that this element seems to comprise a complete structural domain, based upon studies in which this domain can be expressed in bacteria and shown to bind glucose-6-phosphate (Wilson, 1994; Arora *et al.*, 1993; White and Wilson, 1987; White and Wilson, 1990). This suggests that the intact N-terminal domain should fold and form a structure analogous to its structure in the full-length hexokinase I or II protein. As the present inventors contemplate that this structure mediates attachment of the intact hexokinase protein to mitochondria, the intact, correctly folded N-terminal domain is a preferred embodiment of this invention.

For embodiments involving the N-terminal domain, a segment comprising amino acids 1-455 is preferred because of a naturally occurring *Nco*I restriction enzyme site in the DNA

sequence corresponding to amino acid 482. This *Nco*I site allows the fragment encoding the N-terminal domain to be easily isolated and subcloned, and also allows direct fusion of the N-terminal domain of hexokinase to the intact functional sequence of glucokinase via an *Nco*I site located at the AUG start codon of this gene.

Of course, it will be understood that peptides, polypeptides and protein domains of any intermediate length between about 15 amino acids and about 455 amino acids, and longer proteins, may be used in displacing endogenous hexokinase from the mitochondria. Accordingly, constructs comprising about 20, about 50, about 100, about 150, about 200, about 300 or about 400 amino acids in length may be used for these purposes. It is also contemplated that an intact hexokinase protein that is rendered catalytically inactive will interact with mitochondria in a manner identical to the active proteins. Expression of such a HK variant is therefore another method for inhibiting endogenous HK (Baijal and Wilson, 1992). Inactivated, hexokinase proteins include those that have been subjected to chemical mutagenesis and also those produced using molecular biological techniques and recombinant protein production.

The identification of appropriate polypeptide regions and/or particular amino acid sites that may be targeted in order to inactivate hexokinase will be known to those of skill in the art. The crystal structure of certain hexokinase enzymes is available. Coupling the crystal structure information with a comparison of the primary sequence information for various distinct hexokinases will allow one to identify those regions and sites that are important for hexokinase activity, such as the binding sites for ATP, glucose and glucose-6-phosphate. This has been discussed in detail in various publications, such as Printz *et al.* (1993), incorporated herein by reference, which information can be used in connection with preparing mutants and variants for use herewith. Deletion of certain amino acids or peptide segments, as may be achieved by molecular biological manipulation, is another contemplated method for preparing inactive hexokinases.

The enzyme glycerol kinase is another protein thought to bind to mitochondria via porins or VDACs (Adams *et al.*, 1991). Glycerol kinase catalyzes formation of glycerol phosphate

from glycerol, using ATP as phosphate donor. Thus, expression of glycerol kinase in cell lines represents an alternative to expression of inactive hexokinase proteins or fragments thereof which is also contemplated for use in the displacement of endogenous low-K_m hexokinases from their normal mitochondrial binding site.

A particularly powerful method of inhibiting hexokinase within a mammalian cell involves the displacement of hexokinase from the mitochondria and the concomitant provision of active glucokinase. This is advantageously achieved by providing to the cell a hexokinase-glucokinase chimera or fusion protein, in which the hexokinase portion is capable of binding to the mitochondria and yet does not exhibit hexokinase catalytic activity, and in which the glucokinase portion is catalytically active. Chemically-fused polypeptides are a possibility, but recombinant proteins are naturally most preferred for use in this manner. The identification of appropriate hexokinase fragments for use in such a chimera has been described herein above.

In terms of the glucokinase portions of these fusion proteins, any glucokinase-derived sequence that contains enough primary sequence information to confer glucokinase catalytic activity to the chimera will be useful in this context. However, it will often be preferred to use the entire glucokinase enzyme as this is more straightforward in terms of methodology. Again, one may look to the extensive information available in various published references in order to assist with the identification of appropriate glucokinase enzymes or fragments thereof.

At this point, a discussion of the kinetic properties of hexokinase and glucokinase is relevant. It will be understood that in providing a functional equivalent of a hexokinase or glucokinase enzyme, one would desire to provide a protein that has substantially the same kinetic parameters as the native enzyme. Equally, in providing a hexokinase mutant that is devoid of catalytic activity, one would provide an enzyme that is either completely inactivated or whose kinetic parameters have been shifted so that it is, in fact, distinct from the native enzyme.

Table 1, below, sets forth a comparison of glucokinase with hexokinases I-III. This information may be used in order to determine whether any particular variant is "equivalent", and also, to confirm that any inactive mutants have indeed been properly disabled.

TABLE 1

A Comparison of Glucokinase With Hexokinases

	GK	HK 1-111
Km glucose	5-12 mM	0.02-0.13 mM
Km ATP	0.5 mM	0.2-0.5 mM
Ki G-6-P	60 mM	0.2-0.9 mM
Molecular weight	52 kd	100 kd
Substrate preference		
Glucose	1	1
Mannose	0.8	1-1.2
2-Deoxyglucose	0.4	1-1.4
Fructose	0.2	1.1-1.3

The activity of glucose as a substrate is taken as 1. The other numbers are expressed in relation to the activity of glucose as a substrate.

Trehalose-6-Phosphate Metabolism. In Baker's yeast, glucose phosphorylation is also catalyzed by a family of hexokinases that are related in sequence and function to the mammalian hexokinase gene family. Yeast cells, however, contain other genes involved in carbohydrate metabolism for which there are no mammalian counterparts. The trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase complex is an example of such an activity.

The trehalose-6-phosphate synthase/phosphatase complex catalyzes the formation of trehalose, a disaccharide of two glucose molecules (α-D-glucopyranosyl (1-1) α-D-glucopyranoside) by first forming trehalose-6-phosphate by condensation of two molecules of glucose-6-phosphate and then using its phosphatase activity to remove the phosphate groups to

generate free trehalose (Bell *et al.*, 1992). Trehalose is thought to represent a form of storage polysaccharide in yeast, bacteria and other lower organisms, but neither the trehalose-6-phosphate synthase enzyme complex nor its products trehalose-6-phosphate or free trehalose are known to be present in mammalian cells.

Blazquez *et al.* have demonstrated that trehalose-6-phosphate can inhibit the activity of hexokinases from a variety of different organisms, including rat brain, which expresses predominantly hexokinase I (Blasquez *et al.*, 1993). This has led to the suggestion that trehalose-6-phosphate may be an important regulator of glycolytic flux in yeast cells. Consistent with this notion, the yeast gene known as *cif-1* was originally cloned from yeast that are unable to grow in glucose (Blasquez *et al.*, 1993) and subsequently shown to be identical to the smallest subunit (56 kD) of the trehalose phosphate synthase/trehalose-6-phosphate phosphatase complex (Bell *et al.*, 1992). Cells lacking in the CIF-1 gene product exhibit rapid depletion of ATP, presumably because they are unable to produce trehalose-6-phosphate that normally serves to moderate yeast hexokinase activity. It is believed that the 56 kDa CIF-1 gene product encodes the trehalose phosphate synthase activity (Bell *et al.*, 1992).

One of the three general methods described in this application for inhibiting low K_m hexokinase activity in mammalian cells is to express an enzyme, such as yeast trehalose-6-phosphate synthase, that will allow trehalose-6-phosphate to accumulate. This will have two effects. First, the accumulated trehalose-6-phosphate will serve to allosterically inhibit endogenous low K_m hexokinase activity. Second, where trehalose-phosphate synthase is used, this enzyme will divert glucose-6-phosphate into trehalose-6-phosphate at low, non-stimulatory glucose concentrations where low K_m hexokinases but not glucokinases are active, thereby "short-circuiting" metabolic signalling for insulin secretion, which is thought to require ATP produced via further glucose metabolism (Newgard and McGarry, 1995).

A currently preferred gene for use in these aspects is the *S. cerevisiae* gene encoding trehalose-6-phosphate synthase (TPS1). Genes from several other organisms encoding treholose-6-phosphate synthase have been isolated and the amino acid sequences deduced. These include

E. coli (Accession # X69160), *S. pombe* (# Z29971), *Mycobacterium laprae* (# U15187) and *Aspergillus niger* (# U07184). It is contemplated that any of the foregoing or other biological functional equivalents thereof may be used in the context to the present invention.

Hexokinase Inhibition at Nucleic Acid Level Several different ribozyme motifs have been described with RNA cleavage activity (reviewed in Symons, 1992). Examples that would be expected to function equivalently for the down regulation of low K_m hexokinases include sequences from the Group I self splicing introns including Tobaco Ringspot Virus (Prody *et al.*, 1986), Advocado Sunblotch Viroid (Palukaitis *et al.*, 1979 and Symons, 1981), and Lucerne Transient Streak Virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead hammerhead ribozyme based on a predicted folded secondary structure.

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity (Yuan *et al.*, 1992, Yuan and Altman, 1994), hairpin ribozyme structures (Berzal-Herranz *et al.*, 1992 and Chowrira *et al.*, 1993) and Hepatitis Delta virus based ribozymes (Perrotta and Been, 1990). The general design and optimization of ribozyme directed RNA cleavage activity has been discussed in detail (Haseloff and Gerlach, 1988, Symons, 1992, Chowrira *et al.*, 1994, and Thompson *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to a site by complimentary base pair interactions. Two stretches of homology are required for this targeting. These stretches of homologous sequences flank the catalytic ribozyme structure defined above. Each stretch of homologous sequence can vary in length from 7 to 15 nucleotides. The only requirement for defining the homologous sequences is that, on the target RNA, they are separated by a specific sequence which is the cleavage site. For hammerhead ribozyme, the cleavage site is a dinucleotide sequence on the target RNA is a uracil (U) followed by either an adenine, cytosine or uracil (A,C or U) (Perriman *et al.*, 1992 and Thompson *et al.*, 1995). The frequency of this dinucleotide occurring in any given RNA is statistically 3 out of 16. Therefore, for a

given target messenger RNA of 1000 bases, 187 dinucleotide cleavage sites are statistically possible. The message for low K_m hexokinases targeted here are greater than 3500 bases long, with greater than 500 possible cleavage sites.

The large number of possible cleavage sites in the low K_m hexokinases coupled with the growing number of sequences with demonstrated catalytic RNA cleavage activity indicates that a large number of ribozymes that have the potential to downregulate the low K_m hexokinases are available. Designing and testing ribozymes for efficient cleavage of a target RNA is a process well known to those skilled in the art. Examples of scientific methods for designing and testing ribozymes are described by Chowrira *et al.*, (1994) and Lieber and Strauss (1995), each incorporated by reference. The identification of operative and preferred sequences for use in hexokinase-targeted ribozymes is simply a matter of preparing and testing a given sequence, and is a routinely practiced "screening" method known to those of skill in the art.

Combination of Inhibitory Methods. Any of the three general methods of HK inhibition described above (Mitochondrial HK displacement, trehalose-6-phosphate generation and anti-HK ribozymes) may be combined with one another and/or with other engineering methods. It is particularly contemplated that these methods could be used in combination with glucokinase overproduction. Glucokinase overproduction alone is even thought to be a useful method of inhibiting hexokinase, as set forth below.

Low K_m hexokinases, including hexokinases I and II that are present at high levels in mammalian cell lines, are inhibited by glucose-6-phosphate. Thus, this invention also relates to methods for maintaining glucose-6-phosphate at high levels in cell lines. The preferred method for achieving consistently high levels of glucose-6-phosphate in cells is to overexpress glucokinase in such lines.

Expression of glucokinase is considered advantageous for two distinct reasons. First, as described in U.S. Patent 5,427,940 expression of glucokinase is part of an advantageous method for engineering of glucose-stimulated insulin secretion in cell lines. Glucokinase expression is

herein shown to have the added benefit of maintaining high levels of glucose-6-phosphate to keep low K_m hexokinases in an inhibited state. This advantage would become particularly relevant at glucose concentrations in the physiological range (4-9 mM), because glucokinase is active at these levels. Also, while glucokinase is a member of the hexokinase gene family, it is not itself inhibited by glucose-6-phosphate.

Advantages of Hexokinase Inhibition in Mammalian Cells. The various aspects of this invention focus specifically on reducing the levels of low K_m hexokinase activity in mammalian cells. A particular type of target cell is a neuroendocrine cell. There are at least two significant achievements accomplished by the hexokinase inhibition of the present invention, as set forth below.

In addition to the regulation of insulin secretion by glucose, the hexokinase gene family may also be important in the regulation of cell growth and proliferation. As described above, increases in low K_m hexokinase activity usually correlate with the transformation of cells from a normal to cancerous phenotype. However, the correlation has not been proven to exist as a cause and effect relationship. In addition, increases in mitotic activity are not universally linked to induction of low K_m hexokinases. The activity of these enzymes did not increase in preneoplastic mouse beta cell lines over-expressing simian virus 40 large T antigen (Tag) (Radvanyi *et al.*, 1993); nor are they universally elevated in fully transformed mouse β cells (Efrat *et al.*, 1993).

The reduction of hexokinase activity in a cell line by any suitable method, including any of the novel methods disclosed herein, is contemplated to be of use in inhibiting cell growth. Hexokinase I was discovered to be a regulator of cell growth during the inventors' studies in which a RIN cell line (86/X4) that contains a disrupted allele of the HKI gene was surprisingly found to grow about one-half as fast as clones containing the normal compliment of two HKI wild-type genes.

A relationship between low K_m hexokinase activity and cellular growth rates has three important implications relative to the application of cell-based therapies. First, from the perspective of iterative genetic engineering, an untimely or unregulated decrease of hexokinase activity will potentially hinder the growth and selection of clones possessing desired genotypes and traits. A cell line that over-expresses hexokinase I from a regulatable promoter may provide the optimal genetic background for engineering of gene targets. For example, a RIN cell line could be developed that transgenically expresses hexokinase under the control of the tetracycline (Tet)-resistance operon regulatory system (Gossen and Bujard, 1992). This expression system allows powerful transcription of gene products and permits the ablation of gene expression in the presence of Tet and Tet derivatives. Efrat *et al.* (1995) have demonstrated the feasibility of using this expression system to regulate large Tag gene expression. The expression of Tag caused transformation and expansion of mouse beta cells. A decrease of Tag expression, by the *in vitro* or *in vivo* administration of Tet, led to an inhibition of cellular proliferation.

A RIN or neuroendocrine cell line that expresses HKI from a repressible promoter could be further engineered to express high levels of human insulin, glucokinase, and GLUT-2. In addition, such a cell line would be an ideal host for the ablation or down regulation of low K_m hexokinases. Such engineering could be pursued without the hindering complication of slowed growth. Following a series of desired genetic manipulations, the growth of the cells and the glucose sensing ability could be modulated by down regulating hexokinase expression.

A second implication of low K_m hexokinase as a regulator of cellular growth concerns the use of engineered cells for *in vivo* therapies. It is envisioned that cell-based delivery will be conducted by maintenance of the cells *in vivo* in a perm-selective device. It is contemplated that cells with reduced levels of low K_m hexokinase activity will survive for longer periods of time in devices or capsules as a consequence of their reduced growth rates.

A third implication of low K_m hexokinases as regulators of cellular growth involves the creation of novel β-cell lines. The over-expression of HKI by introduction of exogenous DNA into a primary beta cell could be an essential ingredient of the transformation process. NIH-3T3

cells, an immortalized cell line, showed increases in glycolysis and growth rates following transfection with low K_m hexokinase (Fanciulli *et al.*, 1994). In a preferred embodiment, hexokinase I would need to be under the control of a promoter that can be down regulated. Such transcriptional regulation would allow the subsequent modulation of growth and glucose sensing.

A second important reason for reducing hexokinase activity is that it will contribute to the development of engineered cells that exhibit glucose-regulatable protein secretion, the most important aspect of which is presently the physiologically regulated release of insulin. Insulin release from the β-cells of the islets of Langerhans in the pancreas is prominently regulated by the circulating glucose concentration. Glucose stimulates insulin release over the physiological range of glucose concentrations (approximately 4-9 mM), with the amount of insulin secreted being proportional to the rate of glucose metabolism (Newgard and McGarry, 1995).

Glucose phosphorylation appears to play an important role in regulating glucose metabolism and insulin responsiveness (Meglasson and Matschinsky, 1986). Thus, while islet extracts contain approximately equal amounts of high K_m glucokinase and low K_m hexokinase activities (Meglasson and Matchinsky, 1986; Hughes *et al.*, 1992), the hexokinases appear to be inhibited in intact islets, presumably by glucose-6-phosphate, allowing the glucokinase activity to be predominant. Since glucokinase has a K_m for glucose (approximately 6-8 mM) that is within the physiological range, it is ideally suited for regulating glycolytic flux and insulin release in proportion to the extracellular glucose concentration.

The concept of a regulatory role for glucokinase, which has been developed over several years (Meglasson and Matschinsky, 1986; Matshchinsky, 1990), is supported by recent genetic and molecular studies, in which reduced expression of glucokinase was shown to result in less robust glucose-stimulated insulin secretion (Froguel *et al.*, 1993; Efrat *et al.*, 1994). Islet β-cells are also equipped with a specialized glucose transporter, GLUT-2, which like glucokinase is the high K_m member of its gene family.

One of the present inventors has shown that GLUT-2 and glucokinase work in tandem as the "glucose sensing apparatus" of the β-cell (U.S. Patent 5,427,940; Newgard *et al.*, 1990). U.S. Patent 5,427,940, incorporated herein by reference, describes methods for conferring glucose sensing in neuroendocrine cells and cell lines by transfection of such cells with one or more genes selected from the insulin gene, the glucokinase gene and the GLUT-2 glucose transporter gene, so as to provide an engineered cell having all three of these genes.

The overexpression of low K_m hexokinases is known to exert a dominant effect on the glucose concentration threshold for insulin secretion. Overexpression of a low K_m hexokinase from yeast in islet β-cells of transgenic animals results in increased rates of low K_m glucose metabolism and enhanced insulin release at subphysiological glucose concentrations (Epstein *et al.*, 1992; Voss-McGowan *et al.*, 1994). Similar changes were noted upon overexpression of hexokinase I in isolated rat islets (Becker *et al.*, 1994a) or in an ill-differentiated insulinoma cell line called MIN-6 (Ishihara *et al.*, 1994).

It has been shown that the neuroendocrine cell lines that are contemplated for use in engineering artificial β-cells generally have significantly higher low K_m hexokinase activity than normal islet β-cells (Hughes *et al.*, 1992; Efrat *et al.*, 1993; Hughes *et al.*, 1993; Ferber *et al.*, 1994; Knaack *et al.*, 1994), and that glucose metabolism in such cells is highly active at low glucose concentrations. As the glucokinase:hexokinase activity ratio is a critical determinant of the glucose response threshold in insulin secreting neuroendocrine cells, and as an imbalance in favor of hexokinase can cause insulin secretion to occur at glucose concentrations that are below the physiological threshold, it is evident that the most preferred artificial β cells should be further engineered to reduce hexokinase activity. The application of the methods of the present invention to the development of improved insulin secreting cells thus represents a significant advance.

Inhibition Levels As defined herein, the degree of inhibition of hexokinase that is preferred is that necessary to achieve a glucose responsive insulin secretion in the physiologic

range of 1.0 to 20 mM glucose. It will be understood by those working in this field that the absolute level of inhibition is difficult to predict. Measurements of hexokinase and glucokinase in freshly isolated islets as well as cell lines varies dramatically. Ratios of HK to GK can vary from 2.8 (Burch *et al.*, 1981) to 0.8 (Liang *et al.*, 1990) to 0.5 (Hosokawa *et al.*, 1995) in fresh islets all with "normal" glucose stimulated insulin secretion. Reports of incorporated herein by reference cell lines with "normal" secretion shows an HK to GK ratio of 0.6 (Efrat *et al.*, 1993) in the range of the fresh islets. These discrepancies illustrate the difficulties in specifying absolute numbers of glucokinase and hexokinase activities, hence the preference for using glucose responsive insulin secretion ranges as a meaningful parameter in this characterizing the cells invention.

(v) Methods for Re-engineering Engineered Cells

In many situations, multiple rounds of iterative engineering will be undertaken in generating the final cell lines. The events that may be conducted as separate construction events include blocking expression of endogenous gene products by molecular methods (including targeting of both copies of the endogenous gene), introducing a heterologous gene, and further modification of the host cell to achieve high level expression. The particular difficulty in performing multiple steps like this is the need for distinct selectable markers. This is a limitation in that only a few selectable markers are available for use in mammalian cells and not all of these work sufficiently well for the purposes of this invention.

The present invention therefore contemplates the use of the *Cre/Lox* site-specific recombination system (Sauer, 1993, available through Gibco/BRL, Inc., Gaithersburg, Md.) to rescue specific genes out of a genome, most notably drug selection markers. It is claimed as a way of increasing the number of rounds of engineering. Briefly, the system involves the use of a bacterial nucleotide sequence knows as a *Lox*P site, which is recognized by the bacterial *Cre* protein. The *Cre* protein catalyzes a site-specific recombination event. This event is bidirectional, *i.e.*, *Cre* will catalyze the insertion of sequences at a *Lox*P site or excise sequences that lie between two *Lox*P sites. Thus, if a construct containing a selectable marker also has *Lox*P sites flanking the selectable marker, introduction of the *Cre* protein, or a polynucleotide

encoding the *Cre* protein, into the cell will catalyze the removal of the selectable marker. If successfully accomplished, this will make the selectable marker again available for use in further genetic engineering of the cell. This technology is explained in detail in U.S. Patent No. 4,959,317, which is hereby incorporated by reference in its entirety.

It also is contemplated that a series of different markers may be employed in some situations. These markers are discussed in greater detail, below.

B. Proteins

A variety of different proteins can be expressed according to the present invention. Proteins can be grouped generally into two categories - secreted and non-secreted - discussions of each are detailed below. There are some general properties of proteins that are worthy of discussion at this juncture.

First, it is contemplated that many proteins will not have a single sequence but, rather, will exists in many forms. These forms may represent allelic variation or, rather, mutant forms of a given protein. Second, it is contemplated that various proteins may be expressed advantageously as "fusion" proteins. Fusions are generated by linking together the coding regions for two proteins, or parts of two proteins. This generates a new, single coding region that gives rise to the fusion protein. Fusions may be useful in producing secreted forms of proteins that are not normally secreted or producing molecules that are immunologically tagged. Tagged proteins may be more easily purified or monitored using antibodies to the tag. A third variation contemplated by the present invention involves the expression of protein fragments. It may not be necessary to express an entire protein and, in some cases, it may be desirable to express a particular functional domain, for example, where the protein fragment remains functional but is more stable, or less antigenic, or both.

(i) Secreted Proteins

Expression of several proteins that are normally secreted can be engineered into neuroendocrine cells. The cDNA's encoding a number of useful human proteins are available. Examples would include soluble CD-4, Factor VIII, Factor IX, von Willebrand Factor, TPA, urokinase, hirudin, interferons, TNF, interleukins, hematopoietic growth factors, antibodies, albumin, leptin, transferin and nerve growth factors.

Peptide Hormones. Peptide hormones claimed herein for engineering in neuroendocrine cells are grouped into three classes with specific examples given for each. These classes are defined by the complexity of their post-translational processing. Class I is represented by Growth Hormone, Prolactin and Parathyroid hormone. A more extensive list of human peptides that are included in Class I is given in Table 2. These require relatively limited proteolytic processing followed by storage and stimulated release from secretory granules. Class II is represented by Insulin and Glucagon. A more extensive list of human peptide hormones that are included in Class II are given in Table 3. Further proteolytic processing is required, with both endoproteases and carboxypeptidases processing of larger precursor molecules occurring in the secretory granules. Class III is represented by Amylin, Glucagon-like Peptide I and Calcitonin. Again, a more extensive list of Class III human peptide hormones is given in Table 4. In addition to the proteolytic processing found in the Class II peptides, amidation of the C-terminus is required for proper biological function. Examples of engineering expression of all three of these classes of peptide hormones in a neuroendocrine cell are found in this patent.

TABLE 2 - Class I Human Peptide Hormones

Growth Hormone
Prolactin
Placental Lactogen
Luteinizing Hormone
Follicle-stimulating Hormone
Chorionic Gonadotropin
Thyroid-stimulating Hormone
Leptin

TABLE 3 - Human Peptide Hormones Processed by Endoproteases from Larger Precursors

Adrenocorticotropin (ACTH)
Angiotensin I and II
β-endorphin
β-Melanocyte Stimulating Hormone (β-MSH)
Cholecystokinin
Endothelin I
Galanin
Gastric Inhibitory Peptide (GIP)
Glucagon
Insulin
Lipotropins
Neurophysins
Somatostatin

TABLE 4 - Amidated Human Peptide Hormones

Calcium Metabolism Peptides:
- Calcitonin
- Calcitonin Gene related Peptide (CGRP)
- β-Calcitonin Gene Related Peptide
- Hypercalcemia of Malignancy Factor (1-40) (PTH-rP)
- Parathyroid Hormone-related protein (107-139) (PTH-rP)
- Parathyroid Hormone-related protein (107-111) (PTH-rP)

Gastrointestinal Peptides:
- Cholecystokinin (27-33) (CCK)
- Galanin Message Associated Peptide, Preprogalanin (65-105)
- Gastrin I
- Gastrin Releasing Peptide

Glucagon-like Peptide (GLP-1)
Pancreastatin
Pancreatic Peptide
Peptide YY
PHM
Secretin
Vasoactive Intestinal Peptide (VIP)

Pituitary Peptides:
Oxytocin
Vasopressin (AVP)
Vasotocin

Enkephalins:
Enkephalinamide
Metorphinamide (Adrenorphin)

Alpha Melanocyte Stimulating Hormone (alpha-MSH)
Atrial Natriuretic Factor (5-28) (ANF)
Amylin
Amyloid P Component (SAP-1)
Corticotropin Releasing Hormone (CRH)
Growth Hormone Releasing Factor (GHRH)
Luteinizing Hormone-Releasing Hormone (LHRH)
Neuropeptide Y
Substance K (Neurokinin A)
Substance P
Thyrotropin Releasing Hormone (TRH)

(i) Non-Secreted Proteins

Expression of non-secreted proteins can be engineered into neuroendocrine cells. Two general classes of such proteins can be defined. The first are proteins that, once expressed in cells, stay associated with the cells in a variety of destinations. These destinations include the cytoplasm, nucleus, mitochondria, endoplasmic reticulum, golgi, membrane of secretory granules and plasma membrane. Non-secreted proteins are both soluble and membrane associated. The second class of proteins are ones that are normally associated with the cell, but have been modified such that they are now secreted by the cell. Modifications would include site-directed mutagenesis or expression of truncations of engineered proteins resulting in their secretion as well as creating novel fusion proteins that result in secretion of a normally non-secreted protein.

Cells engineered to produce such proteins could be used for either *in vitro* production of the protein or for *in vivo*, cell-based therapies. *In vitro* production would entail purification of the expressed protein from either the cell pellet for proteins remaining associated with the cell or from the conditioned media from cells secreting the engineered protein. *In vivo*, cell-based therapies would either be based on secretion of the engineered protein or beneficial effects of the cells expressing a non-secreted protein.

The cDNA's encoding a number of therapeutically useful human proteins are available. These include cell surface receptors, transporters and channels such as GLUT2, CFTR, leptin receptor, sulfonylurea receptor, β-cell inward rectifying channels, *etc.* Other proteins include protein processing enzymes such as PC2 and PC3, and PAM, transcription factors such as IPF1, and metabolic enzymes such as adenosine deaminase, phenylalanine hydroxylase, glucocerebrosidase.

Engineering mutated, truncated or fusion proteins into neuroendocrine cells also is contemplated. Examples of each type of engineering resulting in secretion of a protein are given (Stoller and Mains, 1989; Ferber *et al.*, 1991). Reviews on the use of such proteins for studying the regulated secretion pathway are also cited (Burgess and Kelly, 1987; Chavez *et al.*, 1994).

(ii) Amylin an amidated, secreted peptide hormone

Amylin is the major component of islet amyloid in the pancreas of patients with non-insulin dependent diabetes mellitus (NIDDM). Also known as diabetes associated peptide (DAP) or islet amyloid polypeptide (IAP), amylin is a 37 amino acid peptide with a 50% homology to calcitonin gene related peptide (CGRP). Amylin also has been found in normal islets in insulin-containing secretory granules of the β cells.

The role of amylin in diabetes remains unknown. However, since β-cells are proficient amylin secretory cells, and the loss of β-cell function accompanies insulin dependent diabetes, it is evident that there is a loss of amylin secretion as well as a loss of insulin secretion as a result of IDDM. Amylin also has been implicated in many other biological processes; however, its true role in metabolism has been difficult to define.

Two related factors have potentially contributed to the confusion surrounding amylin's role in metabolism and participation in disease processes. The first of these factors relates to the identity of the amylin species available to researchers for study. The amylin molecule that has been used for most *in vitro* studies, and currently is in phase III clinical trials for the treatment of NIDDM, is chemically synthesized and amidated, but it is not glycosylated. However, it has been shown from analysis of pancreatic extracts that about 50% of naturally occurring amylin is O-linked glycosylated (Rittenhouse, *et al.*, 1996), a modification that cannot be re-created with chemical synthesis methods.

The second factor that has contributed to the mysteries surrounding amylin's physiological role concerns the failure to isolate and characterize a receptor that binds amylin with specificity and high affinity. The potential interdependency of these two factors is evident, i.e., it is possible that isolation of an amylin receptor has been hampered by the failure to use its physiological ligand

Currently, amidated amylin is assayed by its interactions with receptors for which it has relatively low affinity. These include the calcitonin gene-related peptide (CGRP) receptor and

the calcitonin receptors (Pittner *et al.*, 1994). Amidated amylin binds these receptors with 100 - 1000 times lower affinity than their true ligands. The naturally occurring, glycosylated form of amylin does not bind these receptors (Rittenhouse, *et al.*, 1996). In addition, most of the roles that have been ascribed to amylin have been deduced from studies in which the doses of amylin given were pharmacologic, far exceeding normal circulating levels of the peptide (McGarry, 1994).

Given the indirect evidence for the existence of an amylin receptor, several groups have attempted to isolate a protein from membranes that has been described as an amylin-specific receptor. A report in 1992 identified several proteins in lung membranes that bound amylin with high affinity (Bhogal *et al.*, 1992). Kinetic studies by another group implicated a specific amylin receptor as being present on CHO-K1 Chinese hamster ovary cell line (D'Santos *et al.*, 1992). High affinity amylin binding sites in rat brain were described in 1993 (Beaumont *et al.*, 1993). None of these studies which claimed to identify high affinity, specific amylin receptors have come to fruition as evidenced by the failure to isolate such receptors.

The production of amylin from mammalian cell lines, in particular those that are derived from pancreatic beta cells, will allow for the large scale production of amylin species that faithfully mimic the chemical identity of naturally occurring amylins.

Due to the clear correlation between a potential role for amylin and the pathogenesis of diabetes, it is clear that there is a need to synthesize amylins from a mammalian system which will possess all the post-translational modifications of natural amylins. The availability of such amylins will allow for the elucidation of the natural amylin receptors, the role of amylin secretion, and the consequences of its absence. Further a neuroendocrine cell-based system for either *in vitro*, biologically active amylin production, alone or in combination with insulin or for *in vivo*, cell-based delivery of biologically active amylin alone or in combination with insulin would provide an effective amylin therapy in the treatment of diabetes, hypoglycemia and the restoration of β-cell function.

In the context of the present invention, the term "amylin" is intended to refer to a peptide or peptides having biological and/or immunological identity of the hormone amylin, or other peptides derived from the amylin cDNA for example, as exemplified by naturally occurring amylins such as those found in human rat, or other mammalian species.

Amylin is a 37 amino acid polypeptide hormone (SEQ ID NO:51) processed from a larger precursor polypeptide by proteolytic processing (Sanke, *et al.*, 1988) . Amylin is normally co-produced and co-secreted with insulin by beta cells at fairly constant ratios, acting as a hormone to regulate carbohydrate metabolism (Hoppener, Oosterwijk *et al.*, 1994) . Any variation in the sequence of amylin depicted in SEQ ID NO:54) that allows for the biological and/or immunological integrity of the amylin peptide to be maintained is incorporated as part of the present invention.

Amylin is structurally similar to calcitonin gene related peptide (CGRP) having an identity with human CGRP-2 (US Patent No. 5,124,314 incorporated herein by reference). Cooper *et al.*, showed that amylin is a major component of islet amyloid (1987). Soon thereafter it was demonstrated the presence of amylin in β-cells, thereby suggesting the co-secretion of amylin and insulin for such cells.

The biological effects of amylin are uncharacterized, however, a number of theories abound as to the biological action of amylin. It has been postulated that amyloid deposits result in the destruction of β-cells in the Islets of Langerhans. As these cells are involved in type 1 diabetes, it is likely that this type of diabetes is associated with a deficiency in the amylin secretion, as well as insulin secretion. Furthermore, amylin modulates the rate of glucose stimulated insulin secretion from islet β-cells (Ahren *et al.*, Diabetologia 1987; 30:354-359) and amylin reduces the rate of glycogen synthesis in both basal and insulin stimulated modes (US Patent No. 5,124,314).

Hence, it appears that amylin exerts a powerful force on insulin-induced storage of glucose as glycogen. This may well be a mechanism by which type-2 diabetes related insulin

resistance occurs. Thus amylin modulates and reduces the hypoglycemic effect of insulin both by reducing the release of insulin in response to a glucose stimulus and by reducing the rate of glucose storage.

Several reports have demonstrated the down-regulation of insulin secretion by amylin. This result has been seen in isolated islets (Ohsawa, *et al.*, 1989; Wang, *et al.*, 1993; Wagoner, *et al.*, 1993), perfused whole pancreases, and with a RIN cell line (Murakami, *et al.*, 1990). Although in many of these reports, pharmacological levels of amylin were needed (Ohsawa, *et al.*, 1989; Wang, *et al.*, 1993; Murakami, *et al.*, 1990), while in others physiologic levels of amylin (5 - 20 x 10^{-9} M) had significant effects on glucose-stimulated insulin secretion (Wagoner, *et al.*, 1993; Degano, *et al.*, 1993).

A physiologic role for amylin's ability to inhibit or modulate insulin secretion was supported by *in vivo* administration of amylin antagonists (Young, *et al.*, 1992; Gedulin, *et al.*, 1993). An enhanced insulin response following a glucose challenge was observed. These reports would suggest that engineering higher recombinant amylin levels into an insulin producing cell line would result in down-regulation of insulin secretion.

As stated above, amylin is a 37 amino acid polypeptide hormone processed from a larger precursor polypeptide by proteolytic processing (Sanke, Bell *et al.*, 1988). Amylin is normally co-produced and co-secreted with insulin by beta cells at fairly constant ratios, acting as a hormone to regulate carbohydrate metabolism (Hoppener, Oosterwijk *et al.*, 1994).

The present invention provides the ability to engineer a range of amylin-to-insulin ratios. This is an unexpected result since there are several reports demonstrating the ability of amylin to down regulate insulin in beta cells and beta cell lines, including RIN lines.

Co-expression of insulin and amylin at, conceivably, any ratio, and at therapeutically efficient levels, has several benefits in the context of cell based co-administration. Also novel is

the use of a novel expression plasmid leading to the efficient co-expression of insulin and amylin.

Engineered neuroendocrine cells represent a novel source for mammalian cell produced amylin and amylin related species that are similar to naturally-occurring forms seen in plasma of rats and humans. By producing amylin in neuroendocrine cells, important cell-specific processing steps are provided including dibasic amino acid proteolytic processing from larger proamylin precursor, proteolytic trimming by mono-basic amino acid endoproteases, amidation of the glycine-extended amylin precursor and O-linked glycosylation of amylin at Threonine residues in positions 6 and 9 of SEQ ID NOs: 59 and 62. RIN1046-38 cells have been demonstrated to produce proteins with all of the above modifications. Examples cited in this application include insulin (both rat and human), glucagon, glucagon-like peptide-1 (human wild type GLP-1 and site directed mutants), and amylin.

Glucagon, a 29 amino acid peptide hormone involved in the regulation of glucose and fatty acid metabolism (Unger and Orci *et al.*, 1981), is proteolytically processed from preproglucagon, a large polypeptide precursor. Expression of the mRNA encoding preproglucagon is found in a number of cell types, most notably alpha cells of the pancreas and L cells of the intestine. Preproglucagon posttranslational processing differs in these cell types, giving rise to predominantly glucagon from the alpha cells and glucagon-like peptides I and II (GLP-I and II) from L cells (Mojsov, Heinrich *et al.*, 1986). The reason for this differential production in alpha cells and L cells is due to differential levels of expression of the endoproteases PC2 and PC3 (Rouille, *et al.*, 1995). The expression of these endoproteases is known to vary in other cell types as well (Day, Schafer *et al.*, 1992), giving rise to cell specific posttranslational processing of POMC into distinct hormone peptides.

Amylin and GLP-1 are two peptide hormones that are amidated *in vivo*. Alpha-amidation is now appreciated as a critical determinant for biological activity of a large number of peptide hormones. The enzyme involved in alpha-amidation, peptidylglycine alpha-amidating monooxygenase (PAM) has been well characterized at the molecular level (reviewed in (Eipper

et al., 1992). Although there is only one gene in mammals encoding PAM (Ouafik, *et al.*, 1992), there are several forms of PAM due to alternative splicing and endoproteolytic processing (Stoffers, *et al.*, 1989; Stoffers, *et al.*, 1991; Eipper, *et al.*, 1992) leading to both membrane bound and secreted forms of this enzyme. PAM also is known to be developmentally regulated and differentially expressed in vivo (Ouafik *et al.*, 1989).

The importance of alpha-amidation of peptide hormones is such that the presence of the consensus amidation sequence (glycine followed by two basic amino acids, lysine and/or arginine) is usually predictive of that peptide functioning as a precursor to a bioactive polypeptide (Cuttitta 1993). Attempts at mammalian cell production of any amidated hormones requires endoproteolytic cleavage from larger precursors, carboxypeptidase trimming and alpha-amidation. For instance, GLP-1 is a peptide hormone secreted from the intestinal L cells in response to meals with powerful insulinotropic effects (Kreymann and Williams *et al.*, 1987). It is processed from a larger polypeptide precursor through steps that are very similar to the processing of amylin.

Processing involves the endoproteases PC2 and PC3 and carboxypeptidase from the same precursor that glucagon is produced from (Mojsov, Heinrich *et al.*, 1986) (Rouille, *et al.*, 1995). The final biologically active peptide is a mixture of GLP-1 7-37 and GLP-1 7-36 amide, a difference resulting from the alternative processing of the glycine at position 37 to an alpha-amidated form by peptidylglycine alpha-amidating monooxygenase (PAM) (Orskov, Bersani *et al.*, 1989) (Mojsov, Kopczynski *et al.*, 1990). Both GLP-1 7-37 and GLP-1 7-36 amide are both biologically active in humans (Orskov, Wettergren *et al.*, 1993). The rat insulinoma cell line used here, RIN 1046-38 has already been shown to express sufficient levels of PC2, PC3 and carboxpeptidase for complete processing of highly expressed human insulin.

Processing of insulin, GLP-1, glucagon, and amylin by PC2, PC3 and carboxypeptidases is demonstrated. Efficient RIN 1046-38 amidation of GLP-1 using two specific assays completely specific for amidated GLP-1 or non-amidated GLP-1 is demonstrated.

RIN cells also are known to synthesize and secrete glycosylated proteins. Examples of glycosylation includes N-linked as well as O-linked glycosylated proteins. Amylin is known to have O-linked glycosylation of specific residues leading to its heterogeneity in vivo (Rittenhouse, *et al.*, 1996).

Co-administration of amylin and amylin-related species with insulin to animals results in novel physiologic effects, including enhanced blood glucose lowering effects. Co-administration can be by injection of purified recombinant amylin and amylin related species formulated with or in conjunction with insulin. Alternatively, co-administration can be by *in vivo* cell-based delivery of amylin and amylin-related species with insulin. Because cell-based delivery of amylin provides the full spectrum of post-translational modifications of the peptide, biologic effects may be distinct from those achieved with injection of synthetic material. Co-administration of amylin and amylin related species with insulin can be accomplished over a large range of ratios (amylin/insulin ratios of .002 to 0.9).

The present invention allows for the production of mammalian cell produced recombinant amylin and amylin-related species. In further embodiments, these amylin species are used as a reagents for *in vitro* and *in vivo* drug testing, biological screens and a reagent for identification and isolation of novel receptors for amylin and amylin related peptides. The drugs and amylins produced by the present invention may be used in the treatment of diabetes mellitus, hypoglycemia, osteoporosis, Pagets disease, hypercalcemia, obesity, hypertension or any other disorder requiring amylin regulation. The methods of treatment of such disorders can be found in US Patent Numbers 5527771; 5508260; 5405831; 5376638; 5367052; 5364841; 5321008; 5281581; 5280014; 5266561 the entire text of each patent being specifically incorporated herein by reference.

The present invention allows for production of mammalian cell produced recombinant amylin and amylin related species post-translationally modified. These modifications include dibasic-amino acid proteolysis, carboxypeptidase trimming, amidation and glycosylation. There presently is no alternative recombinant production system for amylin that provides all of these

modifications. Glycosylation, and specifically O-linked glycosylation as has been described for naturally occurring forms of amylin (Rittenhouse, *et al.*, 1996), is a modification that is not possible by any existing methods of producing amylin, including synthetic methods for producing amylin.

O-linked glycosylation of amylin is an expected modification, as exemplified in this patent. Threonines at positions 6 and 9 would be the expected site for glycosylation, as these are the residues modified in naturally occurring amylin. O-linked glycosylation can be heterogeneous for naturally occurring recombinant proteins as well as recombinantly produced proteins (Jenkins, *et al.*, 1996). In fact, heterogeneity of recombinant proteins can be in part controlled by selection of appropriate expression systems, culturing conditions, *etc.* (Jenkins *et al.*, 1996). O-linked glycosylation generally refers to the modification of serine or threonine residues by addition of N-acetyl Galactose linked to galactose and neurominic acid residues.

The present invention further provides methods for the co-expression of insulin and amylin at conceivably any ratio and at therapeutically efficient levels. In light of the earlier discussion regarding the role of amylin in diabetes these methods undoubtedly have several benefits and possibly novel functions in the context of cell based co-administration. The present invention further provides for the use of a novel expression plasmid leading to the efficient co-expression of insulin and amylin.

(iii) Leptin - Engineering Leptin Expression in Cells

In another embodiment of the present invention, the engineered cells may express and overexpress the obesity-associated protein known as leptin. Leptin is a peptide hormone that controls body composition and is believed to do so, at least in part, via interaction with hypothalamic receptors that regulate food intake and body weight. The various isoforms of leptin receptor (Ob-R), including the long isoform (OB-Rb), are widely expressed in various tissues, suggesting that leptin may play an important role in actions on extraneural tissues as well.

Additional evidence that leptin has non-neural function comes from a report that extraordinary changes in body fat are seen in rats made chronically hyperleptinemic by treatment with an adenovirus vector expressing the leptin cDNA. Chen *et al.*, *Proc. Nat'l Acad. Sci. USA* 93:14795 (1996). In this report, rats lost all discernible body fat within 7 days of adenovirus infusion, while animals that were "pair-fed" at the same low rate of food intake as the hyperleptinemic animals retain more of their body fat. The magnitude and rapidity of the lipid depletion suggested the possibility of a direct "hormone-to-cell" action by leptin, in addition to effects cause through the sympathetic nervous system.

Chen *et al.* (1996) also examined the effects of leptin overexpression on plasma glucose, insulin, plasma triglycerides and free fatty acid levels. While glucose did not change, both plasma triglycerides and free fatty acids dropped by about 50% in adenoviral-leptin treated animals, when compared to controls (Ad-β-gal or saline). These studies now have been confirmed and extended with respect to phospholipids. No clear cut changes in phospholipid concentration was observed. However, using an *in vitro* system, it was established that reductions in triglyceride levels could be achieved in the absence of sympathetic nervous system effects. Studies performed to determine what pathways are involved in the triglyceride depletion indicated that leptin-induced triglyceride depletion involves a novel mechanisms by which triglyceride disappears through enhanced intracellular triglyceride metabolism, rather than through more traditional free fatty acid export pathways.

Insulin levels in adenovirus-leptin infected rats dropped even more dramatically than the fatty acids, being only about 1/3 of the amount seen in controls. As stated above, the glucose levels of these animals was normal, however. These findings are consistent with enhanced insulin sensitivity in treated animals. Pancreata were isolated from hyperleptinemic rats and examined for β-cell function and morphology. The most striking finding was the complete absence of insulin secretion in response to either glucose or arginine. The morphology appeared normal, and it was determined that insulin secretion could be reestablished following perfusion of pancreatic tissue in the presence of free fatty acids, thereby establishing an important role for these molecules in β-cell function. These studies also indicate that leptin-mediated reduction of

elevated tissue lipid levels will improve β-cell function, reduce insulin resistance and help restore abnormal glucose homeostasis in obese individuals.

A further connection between diabetes and leptin comes from studies with genetically obese ZDF rats, which contain mutant OB-R genes. The islets of these animals become overloaded with fat at the time that hyperglycemia begins. Because maneuvers that reduce islet fat content prevent diabetes in ZDF rats, it has been proposed that the accumulation of triglycerides in islets plays a causal role in β-cell dysfunction. Thus, the predisposition to diabetes in homozygous ZDF rats may reflect the fact that their tissue have been completely "unleptinized" throughout their life and therefore have accumulated high levels of TG. In normal rats, this accumulation is prevented by the action of leptin. It is expected that any therapy that reduces triglycerides in islets and in the target tissues of insulin will improve β-cell function and reduce insulin resistance.

In hyperleptinemic rats, every tissue that was examined was lipopenic. Thus, it is speculated that normal non-adipocytes carry a minute quantity of triglyceride, perhaps to serve as a reserve source of fuel in adipocytes that are depleted of fat by starvation and become unable to meet the fuel needs of certain tissues. It is suspected that this triglyceride storage function is closely regulated by leptin. In the obese ZDF rats, this regulatory control is absent, and these putative intracellular triglycerides reserves soar to levels of over 1000-times that of hyperleptinemic rats.

In light of these observations, the present application therefore encompasses various engineered cells which express leptin in amounts in excess of normal. The methods by which leptin genes may be manipulated and introduced are much the same as for other genes included herein, such as amylin. A preferred embodiment would involve the use of a viral vector to deliver a leptin-encoding gene, for example, an adenoviral vector. This approach may be exploited in at least two ways. First, in the engineering of cells to produce certain polypeptides *in vitro*, it may be desirable to express high levels of leptin in order to downregulate various cellular functions, including synthesis of certain proteins. Similarly, leptin overexpression may

synergize with cellular functions, resulting in the increased expression of an endogenous or exogenous polypeptide of interest.

Second, it may be desirable to use a leptin-overexpressing cell, or a leptin expression construct, such as a leptin-expressing adenovirus, in an *in vivo* context. This includes various "combination" approaches to the treatment of disease states such as obesity, hyperlipidemia and diabetes. For example, leptin expressing cell lines may provide for prolonged expression of leptin *in vivo* and for high level expression. Preliminary results indicate that injection of recombinantly produced leptin is less efficacious at achieving weight loss and reduction of lipids. Induction of hyperleptinemia using cells lines or expression constructs also may find use in reducing fat content in livestock just prior to slaughter. Moreover, because leptin-induced weight loss may act through different mechanisms than those currently employed, it may be possible to avoid related side effects such as diet-induced ketosis, heart attack and other diet-related symptoms. These regimens may involve combinations of other engineered cells, cells engineered with leptin and at least one other gene or genetic construct (knock-out, antisense, ribozyme, *etc.*), combination gene therapy or combination with a drug. The methods of delivering such pharmaceutical preparations are described elsewhere in this document.

D. Expression of Polypeptides

The amylin gene can be inserted into an appropriate expression system. The gene can be expressed in any number of different recombinant DNA expression systems to generate large amounts of the polypeptide product, which can then be purified and used to vaccinate animals to generate antisera with which further studies may be conducted.

In one embodiment, amino acid sequence variants of a polypeptide can be prepared. These may, for instance, be minor sequence variants of a polypeptide that arise due to natural variation within the population or they may be homologues found in other species. They also may be sequences that do not occur naturally but that are sufficiently similar that they function similarly and/or elicit an immune response that cross-reacts with natural forms of the polypeptide. Sequence

variants can be prepared by standard methods of site-directed mutagenesis such as those described below in the following section.

Amino acid sequence variants of a polypeptide can be substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the native protein which are not essential for function or immunogenic activity, and are exemplified by the variants lacking a transmembrane sequence described above. Another common type of deletion variant is one lacking secretory signal sequences or signal sequences directing a protein to bind to a particular part of a cell. An example of the latter sequence is the SH2 domain, which induces protein binding to phosphotyrosine residues.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide such as stability against proteolytic cleavage. Substitutions preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

Insertional variants include fusion proteins such as those used to allow rapid purification of the polypeptide and also can include hybrid proteins containing sequences from other proteins and polypeptides which are homologues of the polypeptide. For example, an insertional variant could include portions of the amino acid sequence of the polypeptide from one species, together with portions of the homologous polypeptide from another species. Other insertional variants can include those in which additional amino acids are introduced within the coding sequence of the

polypeptide. These typically are smaller insertions than the fusion proteins described above and are introduced, for example, into a protease cleavage site.

In one embodiment, major antigenic determinants of the polypeptide are identified by an empirical approach in which portions of the gene encoding the polypeptide are expressed in a recombinant host, and the resulting proteins tested for their ability to elicit an immune response. For example, PCR can be used to prepare a range of cDNAs encoding peptides lacking successively longer fragments of the C-terminus of the protein. The immunoprotective activity of each of these peptides then identifies those fragments or domains of the polypeptide that are essential for this activity. Further experiments in which only a small number of amino acids are removed at each iteration then allows the location of the antigenic determinants of the polypeptide.

Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson *et al.*, "Peptide Turn Mimetics" in *BIOTECHNOLOGY AND PHARMACY*, Pezzuto *et al.*, Eds., Chapman and Hall, New York (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule.

Successful applications of the peptide mimetic concept have thus far focused on mimetics of β-turns within proteins, which are known to be highly antigenic. Likely β-turn structure within an polypeptide can be predicted by computer-based algorithms as discussed above. Once the component amino acids of the turn are determined, peptide mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains.

Modification and changes may be made in the structure of a gene and still obtain a functional molecule that encodes a protein or polypeptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent,

or even an improved, second-generation molecule. The amino acid changes may be achieved by change the codons of the DNA sequence, according to the following data.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982).

TABLE 5

Amino Acids			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	AGC AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine *-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

E. Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is

generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

F. Genetic Constructs and Their Delivery to Cells

I. Formation of Genetic Constructs

Also claimed in this patent are examples of DNA expression plasmids designed to optimize production of the heterologous proteins such as amylin. These include a number of enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in neuroendocrine cells. Elements designed to optimize messenger RNA stability and translatability in neuroendocrine cells are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable neuroendocrine cell clones

expressing the peptide hormones are also provided, as is an element that links expression of the drug selection markers to expression of the heterologous polypeptide.

(i) Vector Backbone

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

In preferred embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene

and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid encoding a particular gene is not believed to be important, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the gene of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a gene of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of the gene product following transfection can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product. Tables 6 and 7 list several elements/promoters which may be employed, in the

context of the present invention, to regulate the expression of the gene of interest. This list is not intended to be exhaustive of all the possible elements involved in the promotion of gene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct (Table 6 and Table 7). Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 6

ENHANCER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
β-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene
α-Fetoprotein
τ-Globin
β-Globin
e-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α1-Antitrypsin
H2B (TH2B) Histone

ENHANCER
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 7

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
ß-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone
Insulin E Box	Glucose

In preferred embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into

mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kB of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

(ii) Other Regulatory elements

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. The inventors have employed the human Growth Hormone and SV40 polyadenylation signals in that they were convenient and known to function well in the target cells employed. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

(iii) Selectable Markers

In certain embodiments of the invention, the delivery of a nucleic acid in a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. The marker would result in an identifiable change to the transfected cell permitting easy identification of expression. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) (eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid

encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

(iv) Multigene constructs and IRES

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picanovirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

G. *In Vivo* Delivery and Treatment Protocols

It is proposed that engineered cells of the present invention may be introduced into animals with certain needs, such as animals with insulin-dependent diabetes. In the diabetic treatment aspects, ideally cells are engineered to achieve glucose dose responsiveness closely resembling that of islets. However, other cells will also achieve advantages in accordance with the invention. It should be pointed out that the experiments of Madsen and coworkers have shown that implantation of poorly differentiated rat insulinoma cells into animals results in a return to a more differentiated state, marked by enhanced insulin secretion in response to

metabolic fuels (Madsen *et al.*, 1988). These studies suggest that exposure of engineered cell lines to the *in vivo* milieu may have some effects on their response(s) to secretagogues.

The preferred methods of administration involve the encapsulation of the engineered cells in a biocompatible coating. In this approach, the cells are entrapped in a capsular coating that protects the contents from immunological responses. One preferred encapsulation technique involves encapsulation with alginate-polylysine-alginate. Capsules made employing this technique generally have a diameter of approximately 1 mm and should contain several hundred cells.

Cells thus may be implanted using the alginate-polylysine encapsulation technique of O'Shea and Sun (1986), with modifications, as later described by Fritschy *et al.* (1991). The engineered cells are suspended in 1.3% sodium alginate and encapsulated by extrusion of drops of the cell/alginate suspension through a syringe into $CaCl_2$. After several washing steps, the droplets are suspended in polylysine and rewashed. The alginate within the capsules is then reliquified by suspension in 1 mM EGTA and then rewashed with Krebs balanced salt buffer.

An alternative approach is to seed Amicon fibers with cells of the present invention. The cells become enmeshed in the fibers, which are semipermeable, and are thus protected in a manner similar to the micro encapsulates (Altman *et al.*, 1986). After successful encapsulation or fiber seeding, the cells may be implanted intraperitoneally, usually by injection into the peritoneal cavity through a large gauge needle (23 gauge).

A variety of other encapsulation technologies have been developed that are applicable to the practice of the present invention (see, *e.g.*, Lacy *et al.*, 1991; Sullivan *et al.*, 1991; WO 91/10470; WO 91/10425; WO 90/15637; WO 90/02580; U.S. Patent 5,011,472; U.S. Patent 4,892,538; and WO 89/01967; each of the foregoing being incorporated by reference).

Lacy *et al.* (1991) encapsulated rat islets in hollow acrylic fibers and immobilized these in alginate hydrogel. Following intraperitoneal transplantation of the encapsulated islets into

diabetic mice, normoglycemia was reportedly restored. Similar results were also obtained using subcutaneous implants that had an appropriately constructed outer surface on the fibers. It is therefore contemplated that engineered cells of the present invention may also be straightforwardly "transplanted" into a mammal by similar subcutaneous injection.

Sullivan *et al.* (1991) reported the development of a biohybrid perfused "artificial pancreas", which encapsulates islet tissue in a selectively permeable membrane. In these studies, a tubular semi-permeable membrane was coiled inside a protective housing to provide a compartment for the islet cells. Each end of the membrane was then connected to an arterial polytetrafluoroethylene (PTFE) graft that extended beyond the housing and joined the device to the vascular system as an arteriovenous shunt. The implantation of such a device containing islet allografts into pancreatectomized dogs was reported to result in the control of fasting glucose levels in 6/10 animals. Grafts of this type encapsulating engineered cells could also be used in accordance with the present invention.

The company Cytotherapeutics has developed encapsulation technologies that are now commercially available that will likely be of use in the application of the present invention. A vascular device has also been developed by Biohybrid, of Shrewsbury, Mass., that may have application to the technology of the present invention.

Implantation employing such an encapsulation technique are preferred for a variety of reasons. For example, transplantation of islets into animal models of diabetes by this method has been shown to significantly increase the period of normal glycemic control, by prolonging xenograft survival compared to unencapsulated islets (O'Shea and Sun, 1986; Fritschy *et al.*, 1991). Also, encapsulation will prevent uncontrolled proliferation of clonal cells. Capsules containing cells are implanted (approximately 1,000-10,000/animal) intraperitoneally and blood samples taken daily for monitoring of blood glucose and insulin.

An alternate approach to encapsulation is to simply inject glucose-sensing cells into the scapular region or peritoneal cavity of diabetic mice or rats, where these cells are reported to

form tumors (Sato *et al.*, 1962). Implantation by this approach may circumvent problems with viability or function, at least for the short term, that may be encountered with the encapsulation strategy. This approach will allow testing of the function of the cells in experimental animals but obviously is not applicable as a strategy for treating human diabetes.

Engineering of primary cells isolated from patients is also contemplated as described by Dr. Richard Mulligan and colleagues using retroviral vectors for the purposes of introducing foreign genes into bone marrow cells (see, *e.g.*, Cone *et al.*, 1984; Danos *et al.*, 1988). The cells of the bone marrow are derived from a common progenitor, known as pluripotent stem cells, which give rise to a variety of blood borne cells including erythrocytes, platelets, lymphocytes, macrophages, and granulocytes. Interestingly, some of these cells, particularly the macrophages, are capable of secreting peptides such as tumor necrosis factor and interleukin 1 in response to specific stimuli. There is also evidence that these cells contain granules similar in structure to the secretory granules of ß-cells, although there is no clear evidence that such granules are collected and stored inside macrophages as they are in ß-cells (Stossel, 1987).

It may ultimately be possible to use the present invention in combination with that previously described by the one of the present inventors (U.S. Patent 5,427,940, incorporated herein by reference) in a manner described for clonal cells to engineer primary cells that perform glucose-stimulated insulin secretion. This approach would completely circumvent the need for encapsulation of cells, since the patient's own bone marrow cells would be used for the engineering and then re-implanted. These cells would then develop into their differentiated form (*i.e.*, the macrophage) and circulate in the blood where they would be able to sense changes in circulating glucose by secreting insulin.

Alternatively, it may be desirable to introduce genetic constructs to cells *in vivo*. There are a number of way in which nucleic acids may introduced into cells. Several methods are outlined below.

(i) Adenovirus

One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense polynucleotide that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization or adenovirus, a 36 kB, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of

infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kB of DNA. Combined with the approximately 5.5 kB of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kB, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Recently, Racher *et al.*, (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding the gene of interest at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.*, (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

(ii) Retroviruses

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the

potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact- sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

(iii) Other Viral Vectors as Expression Constructs

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpes viruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.*, recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was co-transfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

(iv) Non-viral vectors

In order to effect expression of sense or antisense gene constructs, the expression construct must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo*, as in the treatment of

certain disease states. As described above, the preferred mechanism for delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression construct has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In one embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky *et al.*, (1984) successfully injected polyomavirus DNA in the form of calcium phosphate precipitates into liver and spleen of adult and newborn mice demonstrating active viral

replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of calcium phosphate-precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ, *i.e.*, *ex vivo* treatment. Again, DNA encoding a particular gene may be delivered via this method and still be incorporated by the present invention.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.*, (1980) demonstrated the feasibility of liposome-mediated

delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.*, (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Other expression constructs which can be employed to deliver a nucleic acid encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.*, (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a particular gene also may be specifically delivered into a cell type such as lung, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of a nucleic acid encoding a gene in many tumor cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues. Anderson *et al.*, U.S. Patent 5,399,346, and incorporated herein in its entirety, disclose *ex vivo* therapeutic methods.

H. Bioreactors and Large Scale Cultures

The ability to produce biologically active polypeptides is increasingly important to the pharmaceutical industry. Over the last decade, advances in biotechnology have led to the production of important proteins and factors from bacteria, yeast, insect cells and from mammalian cell culture. Mammalian cultures have advantages over cultures derived from the less advanced lifeforms in their ability to post-translationally process complex protein structures such as disulfide-dependent folding and glycosylation. Neuroendocrine cell types have added unique capacities of endoproteolytic cleaving, C-terminal amidation and regulated secretion. Indeed, mammalian cell culture is now the preferred source of a number of important proteins for use in human and animal medicine, especially those which are relatively large, complex or glycosylated.

Development of mammalian cell culture for production of pharmaceuticals has been greatly aided by the development in molecular biology of techniques for design and construction of vector systems highly efficient in mammalian cell cultures, a battery of useful selection markers, gene amplification schemes and a more comprehensive understanding of the biochemical and cellular mechanisms involved in procuring the final biologically-active molecule from the introduced vector.

However, the traditional selection of cell types for expressing heterologous proteins has generally been limited to the more "common" cell types such as CHO cells, BHK cells, C127 cells and myeloma cells. In many cases, these cell types were selected because there was a great deal of preexisting literature on the cell type (*e.g.*, "cookbook" methods for transfection of the cells) or the cell was simply being carried in the laboratory at the time the effort was made to express a peptide product. Frequently, factors which affect the downstream (in this case, beyond the T-75 flask) side of manufacturing scale-up were not considered before selecting the cell line as the host for the expression system. Also, development of bioreactor systems capable of sustaining very high density cultures for prolonged periods of time have not lived up to the increasing demand for increased production at lower costs.

The present invention will take advantage of the biochemical and cellular capacities of secretory cells as well as of recently available bioreactor technology. Growing cells according to the present invention in a bioreactor allows for large scale production and secretion of complex, fully biologically-active polypeptides into the growth media. By designing a defined media with low contents of complex proteins and using a scheme of timed-stimulation of the secretion into the media for increased titer, the purification strategy can be greatly simplified, thus lowering production cost.

(i) Anchorage-dependent versus non-anchorage-dependent cultures.

Animal and human cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing freely in suspension throughout the bulk of the culture; or as anchorage-

dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. Large scale suspension culture based on microbial (bacterial and yeast) fermentation technology has clear advantages for the manufacturing of mammalian cell products. The processes are relatively simple to operate and straightforward to scale up. Homogeneous conditions can be provided in the reactor which allows for precise monitoring and control of temperature, dissolved oxygen, and pH, and ensure that representative samples of the culture can be taken.

However, suspension cultured cells cannot always be used in the production of biologicals. Suspension cultures are still considered to have tumorigenic potential and thus their use as substrates for production put limits on the use of the resulting products in human and veterinary applications (Petricciani, 1985; Larsson and Litwin, 1987). Viruses propagated in suspension cultures as opposed to anchorage-dependent cultures can sometimes cause rapid changes in viral markers, leading to reduced immunogenicity (Bahnemann, 1980). Finally, sometimes even recombinant cell lines can secrete considerably higher amounts of products when propagated as anchorage-dependent cultures as compared with the same cell line in suspension (Nilsson and Mosbach, 1987). For these reasons, different types of anchorage-dependent cells are used extensively in the production of different biological products.

The current invention includes cells which are anchorage-dependent of nature. RIN cells, *e.g.*, are strictly anchorage-dependent, and when grown in suspension, the cells will attach to each other and grow in clumps, eventually suffocating cells in the inner core of each clump as they reach a size that leaves the core cells unsustainable by the culture conditions. Therefore, an efficient means of large-scale culture of anchorage-dependent cells is needed in order to effectively take advantage of these cells' capacity to secrete heterologous proteins.

(ii) Reactors and processes for suspension.

Large scale suspension culture of mammalian cultures in stirred tanks was undertaken. The instrumentation and controls for bioreactors adapted, along with the design of the fermentors, from related microbial applications. However, acknowledging the increased demand for contamination control in the slower growing mammalian cultures, improved aseptic designs were quickly implemented, improving dependability of these reactors. Instrumentation and controls are basically the same as found in other fermentors and include agitation, temperature, dissolved oxygen, and pH controls. More advanced probes and autoanalyzers for on-line and off-line measurements of turbidity (a function of particles present), capacitance (a function of viable cells present), glucose/lactate, carbonate/bicarbonate and carbon dioxide are available. Maximum cell densities obtainable in suspension cultures are relatively low at about 2-4 x 10^6 cells/ml of medium (which is less than 1 mg dry cell weight per ml), well below the numbers achieved in microbial fermentation.

Two suspension culture reactor designs are most widely used in the industry due to their simplicity and robustness of operation - the stirred reactor and the airlift reactor. The stirred reactor design has successfully been used on a scale of 8000 liter capacity for the production of interferon (Phillips *et al.*, 1985; Mizrahi, 1983). Cells are grown in a stainless steel tank with a height-to-diameter ratio of 1:1 to 3:1. The culture is usually mixed with one or more agitators, based on bladed disks or marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through seals on stirrer shafts.

The airlift reactor, also initially described for microbial fermentation and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design is the simplicity and lack of need for mechanical

mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easy, has good mass transfer of gasses and generates relatively low shear forces.

Most large-scale suspension cultures are operated as batch or fed-batch processes because they are the most straightforward to operate and scale up. However, continuous processes based on chemostat or perfusion principles are available.

A batch process is a closed system in which a typical growth profile is seen. A lag phase is followed by exponential, stationary and decline phases. In such a system, the environment is continuously changing as nutrients are depleted and metabolites accumulate. This makes analysis of factors influencing cell growth and productivity, and hence optimization of the process, a complex task. Productivity of a batch process may be increased by controlled feeding of key nutrients to prolong the growth cycle. Such a fed-batch process is still a closed system because cells, products and waste products are not removed.

In what is still a closed system, perfusion of fresh medium through the culture can be achieved by retaining the cells with a fine mesh spin filter and spinning to prevent clogging. Spin filter cultures can produce cell densities of approximately 5 x 10^7 cells/ml. A true open system and the simplest perfusion process is the chemostat in which there is an inflow of medium and an outflow of cells and products. Culture medium is fed to the reactor at a predetermined and constant rate which maintains the dilution rate of the culture at a value less than the maximum specific growth rate of the cells (to prevent washout of the cells mass from the reactor). Culture fluid containing cells and cell products and byproducts is removed at the same rate. These perfused systems are not in commercial use for production from mammalian cell culture.

(iii) Non-perfused attachment systems.

Traditionally, anchorage-dependent cell cultures are propagated on the bottom of small glass or plastic vessels. The restricted surface-to-volume ratio offered by classical and traditional techniques, suitable for the laboratory scale, has created a bottleneck in the production of cells

and cell products on a large scale. In an attempt to provide systems that offer large accessible surfaces for cell growth in small culture volume, a number of techniques have been proposed: the roller bottle system, the stack plates propagator, the spiral film bottles, the hollow fiber system, the packed bed, the plate exchanger system, and the membrane tubing reel. Since these systems are non-homogeneous in their nature, and are sometimes based on multiple processes, they suffer from the following shortcomings - limited potential for scale-up, difficulties in taking cell samples, limited potential for measuring and controlling the system and difficulty in maintaining homogeneous environmental conditions throughout the culture.

Despite these drawbacks, a commonly used process of these systems is the roller bottle. Being little more than a large, differently shaped T-flask, simplicity of the system makes it very dependable and, hence, attractive. Fully automated robots are available that can handle thousands of roller bottles per day, thus eliminating the risk of contamination and inconsistency associated with the otherwise required intense human handling. With frequent media changes, roller bottle cultures can achieve cell densities of close to 0.5×10^6 cells/cm^2 (corresponding to 10^9 cells/bottle or 10^7 cells/ml of culture media).

(iv) Cultures on microcarriers

In an effort to overcome the shortcomings of the traditional anchorage-dependent culture processes, van Wezel (1967) developed the concept of the microcarrier culturing systems. In this system, cells are propagated on the surface of small solid particles suspended in the growth medium by slow agitation. Cells attach to the microcarriers and grow gradually to confluency of the microcarrier surface. In fact, this large scale culture system upgrades the attachment dependent culture from a single disc process to a unit process in which both monolayer and suspension culture have been brought together. Thus, combining the necessary surface for a the cells grow with the advantages of the homogeneous suspension culture increases production.

The advantages of microcarrier cultures over most other anchorage-dependent, large-scale cultivation methods are several fold. First, microcarrier cultures offer a high surface-to-volume ratio (variable by changing the carrier concentration) which leads to high cell density yields and a

potential for obtaining highly concentrated cell products. Cell yields are up to 1-2 x 10^7 cells/ml when cultures are propagated in a perfused reactor mode. Second, cells can be propagated in one unit process vessels instead of using many small low-productivity vessels (*i.e.*, flasks or dishes). This results in far better utilization and a considerable saving of culture medium. Moreover, propagation in a single reactor leads to reduction in need for facility space and in the number of handling steps required per cell, thus reducing labor cost and risk of contamination. Third, the well-mixed and homogeneous microcarrier suspension culture makes it possible to monitor and control environmental conditions (*e.g.*, pH, pO_2, and concentration of medium components), thus leading to more reproducible cell propagation and product recovery. Fourth, it is possible to take a representative sample for microscopic observation, chemical testing, or enumeration. Fifth, since microcarriers settle out of suspension easily, use of a fed-batch process or harvesting of cells can be done relatively easily. Sixth, the mode of the anchorage-dependent culture propagation on the microcarriers makes it possible to use this system for other cellular manipulations, such as cell transfer without the use of proteolytic enzymes, cocultivation of cells, transplantation into animals, and perfusion of the culture using decanters, columns, fluidized beds, or hollow fibers for microcarrier retainment. Seventh, microcarrier cultures are relatively easily scaled up using conventional equipment used for cultivation of microbial and animal cells in suspension.

(v) Microencapsulation of mammalian cells

One method which has shown to be particularly useful for culturing mammalian cells is microencapsulation. The mammalian cells are retained inside a semipermeable hydrogel membrane. A porous membrane is formed around the cells permitting the exchange of nutrients, gases, and metabolic products with the bulk medium surrounding the capsule. Several methods have been developed that are gentle, rapid and non-toxic and where the resulting membrane is sufficiently porous and strong to sustain the growing cell mass throughout the term of the culture. These methods are all based on soluble alginate gelled by droplet contact with a calcium-containing solution. Lim (1982) describes cells concentrated in an approximately 1% solution of sodium alginate which are forced through a small orifice, forming droplets, and breaking free into an approximately 1% calcium chloride solution. The droplets are then cast in a layer of

polyamino acid that ionically bonds to the surface alginate. Finally the alginate is reliquefied by treating the droplet in a chelating agent to remove the calcium ions. Other methods use cells in a calcium solution to be dropped into a alginate solution, thus creating a hollow alginate sphere. A similar approach involves cells in a chitosan solution dropped into alginate, also creating hollow spheres.

Microencapsulated cells are easily propagated in stirred tank reactors and, with beads sizes in the range of 150-1500 μm in diameter, are easily retained in a perfused reactor using a fine-meshed screen. The ratio of capsule volume to total media volume can kept from as dense as 1:2 to 1:10. With intracapsular cell densities of up to 10^8, the effective cell density in the culture is 1-5 x 10^7.

The advantages of microencapsulation over other processes include the protection from the deleterious effects of shear stresses which occur from sparging and agitation, the ability to easily retain beads for the purpose of using perfused systems, scale up is relatively straightforward and the ability to use the beads for implantation.

(vi) Perfused attachment systems

Perfusion refers to continuous flow at a steady rate, through or over a population of cells (of a physiological nutrient solution). It implies the retention of the cells within the culture unit as opposed to continuous-flow culture which washes the cells out with the withdrawn media (*e.g.*, chemostat). The idea of perfusion has been known since the beginning of the century, and has been applied to keep small pieces of tissue viable for extended microscopic observation. The technique was initiated to mimic the cells milieu *in vivo* where cells are continuously supplied with blood, lymph, or other body fluids. Without perfusion, cells in culture go through alternating phases of being fed and starved, thus limiting full expression of their growth and metabolic potential. The current use of perfused culture is in response to the challenge of growing cells at high densities (*i.e.*, 0.1-5 x 10^8 cells/ml). In order to increase densities beyond 2-4 x 10^6 cells/ml (or 2 x 10^5 cells/cm^2), the medium has to be constantly replaced with a fresh supply in order to make up for nutritional deficiencies and to remove toxic products. Perfusion

allows for a far better control of the culture environment (pH, pO_2, nutrient levels, *etc.*) and is a means of significantly increasing the utilization of the surface area within a culture for cell attachment.

Microcarrier and microencapsulated cultures are readily adapted to perfused reactors but, as noted above, these culture methods lack the capacity to meet the demand for cell densities above 10^8 cells/ml. Such densities will provide for the advantage of high product titer in the medium (facilitating downstream processing), a smaller culture system (lowering facility needs), and a better medium utilization (yielding savings in serum and other expensive additives). Supporting cells at high density requires extremely efficient perfusion techniques to prevent the development of non-homogeneity. This means the use of highly sophisticated procedures and apparati and has, until recently, been confined to a relatively small scale.

(vii) CelliGen™ bioreactor system

The development of a perfused packed-bed reactor using a bed matrix of a non-woven fabric has provided a means for maintaining a perfusion culture at densities exceeding 10^8 cells/ml of the bed volume (CelliGen™, New Brunswick Scientific, Edison, NJ; Wang *et al.*, 1992; Wang *et al.*, 1993; Wang *et al.*, 1994). Briefly described, this reactor comprises an improved reactor for culturing of both anchorage- and non-anchorage-dependent cells. The reactor is designed as a packed bed with a means to provide internal recirculation. Preferably, a fiber matrix carrier is placed in a basket within the reactor vessel. A top and bottom portion of the basket has holes, allowing the medium to flow through the basket. A specially designed impeller provides recirculation of the medium through the space occupied by the fiber matrix for assuring a uniform supply of nutrient and the removal of wastes. This simultaneously assures that a negligible amount of the total cell mass is suspended in the medium. The combination of the basket and the recirculation also provides a bubble-free flow of oxygenated medium through the fiber matrix. The fiber matrix is a non-woven fabric having a "pore" diameter of from 10 μm to 100 μm, providing for a high internal volume with pore volumes corresponding to 1 to 20 times the volumes of individual cells.

In comparison to other culturing systems, this approach offers several significant advantages. With a fiber matrix carrier, the cells are protected against mechanical stress from agitation and foaming. The free medium flow through the basket provides the cells with optimum regulated levels of oxygen, pH, and nutrients. Products can be continuously removed from the culture and the harvested products are free of cells and produced in low-protein medium which facilitates subsequent purification steps. Also, the unique design of this reactor system offers an easier way to scale up the reactor. Currently, sizes up to 30 liter are available. One hundred liter and 300 liter versions are in development and theoretical calculations support up to a 1000 liter reactor. This technology is explained in detail in WO 94/17178 (August 4, 1994, Freedman *et al.*), which is hereby incorporated by reference in its entirety.

A number of culturing parameters, used in conjunction the CelliGen™ system, have been demonstrated to play a role in increased production. For example, the CelliGen™ Plus reactor system, including the use of non-woven polyester fiber matrix (preferably, Fibra-Cel™) and centrifugal lift impeller (preferably, Fibra-Cel™) are system components that give improved yields. Also, several media formulations have been employed with improved performance. For example, use of serum free medium is preferred, as is the use of cholesterol rich lipid extract (0.01% to 0.10%, volume to volume), ascorbic acid (from between about 0.001 to 0.100 mM), glutamate (rather than 2 mM glutamine) at 2 to 20 mM, preferably 4 mM, alpha ketoglutarate (rather than 2 mM glutamine) at 2 to 20 mM, preferably 4 mM, and the absence of growth promoting factors.

viii) CellCube™ bioreactor system

The Cellcube™ (Corning-Costar) module provides a large styrenic surface area for the immobilization and growth of substrate attached cells. It is an integrally encapsulated sterile single-use device that has a series of parallel culture plates joined to create thin, sealed laminar flow spaces between adjacent plates. The Cellcube™ module has inlet and outlet ports that are diagonally opposite each other and help distribute the flow of media to the parallel plates. The medium is constantly recirculated from the module through an oxygenator and back to the cube. The external oxygenator provides a bubble free stream of oxygenated medium and allows for the

additional control of the pH of the medium. With concurrent addition of fresh medium, medium with secreted product and wastes can be harvested continuously, retaining the cell population in the cube.

During the first few days of growth the culture is generally satisfied by the media contained within the system after initial seeding. The amount of time between the initial seeding and the start of the media perfusion is dependent on the density of cells in the seeding inoculum and the cell growth rate. The measurement of nutrient concentration in the circulating media is a good indicator of the status of the culture. When establishing a procedure it may be necessary to monitor the nutrients composition at a variety of different perfusion rates to determine the most economical and productive operating parameters.

Cells within the system reach a higher density of solution (cells/ml) than in traditional culture systems. Many typically used basal media are designed to support 1-2 x 10^6 cells/ml/day. A typical CellCube™ run with an 21 000 cm^2 surface, contains approximately 1.2 liters of media within the module. The final cell density can exceeds 2.5 x 10^6 cell/cm^2 or 5 x 10^7 cells/ml in the culture vessel. At confluence, depending on the cell line used, media required can vary anywhere form 4-16 module volumes per day.

The advantage of the CellCube™ system is that it to a large extent replicates the conditions the cells experience in T flask culture. This allows for very linear scale up of any culture that is successfully grown in flask culture without severe loss in per-cell performance.

I. Purification of Proteins

Protein purification techniques are well known to those of skill in the art. These techniques tend to involve the fractionation of the cellular milieu to separated the amylin form other components of the mixture. Having separated amylin from the other plasma components the amylin sample may be purified using chromatographic and electrophoretic techniques to achieve complete purification. Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel

electrophoresis; isolectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

The present invention isolates amylin from cells containing amylin by preparing acid/ethanol extracts of whole cells or conditioned media and analyzing the extracts by HPLC as described (Halban, *et al.*, 1986, Sizonenko and Halban, 1991). Solvent systems, gradients and flow rates used were as described by Halban, *et al.*, (1986) however it is well within the skill of the ordinary person ion the art to adapt the chromatography conditions to suit individual need. Standards may be used to obtain optimization of chromatography conditions and methods.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide " as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state, *i.e.*, in this case, relative to its purity within a hepatocyte or β-cell extract. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the number of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific

activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number". The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater -fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al., Biochem. Biophys. Res. Comm., 76*:425, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain and adequate flow rate. Separation can be accomplished in a matter of minutes, or a most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by

cyanogen bromide. Conconavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

J. Isolating Receptors That Bind Amylin Species With High Affinity And Specificity.

The present invention also outlines the use of amylin species of the present invention in isolating amylin-receptors. The methods for isolating the endogenous amylin receptors will generally involve:

1. Purification of amylin species from cell-source and develop assays that distinguish among various activities or chemical identities. These methods are set out elsewhere in the specification.

2. Radio-labeling amylin species of interest and using the labelled species to isolate or screen for a specific receptor.

Protein purification made feasible by amylin-receptor interactions in cell lysates: Protein purification techniques are well within the skill of the ordinary person skilled in the art and are described elsewhere in the specification. These techniques may well involve chemical cross-linking and protein purification, cross-linking or non-covalent interactions of amylin and its receptor followed by co-immunoprecipitation of the amylin bound-receptor complex. Once a protein has been purified, its amino acid sequence is determined and the cDNA isolated. This will facilitate the production of cDNA libraries for expression cloning and homology screening.

Expression cloning: In expression cloning it will be desirable to identify a cell type that binds amylin species of interest. This will generally be a receptor-rich cell. cDNA from receptor-rich cell type is then made by techniques well known to those of skill in the art. the cDNA is then transfected into a mammalian cell type that does not bind amylin or transform E. coli. In either case, the use of DNA vector systems described above that provide for protein expression will allow screening for cells that switch from a non-binding to a amylin-binding phenotype. These types of screening studies will be useful in isolating the cDNA that confers the phenotypic switch.

Homology screening: Amylin belongs to a family of related peptides and presumably so does its receptor. In order to perform homology screening studies of the amylin binding receptor it is necessary to identify a cell type that binds amylin species of interest with high affinity. It will then be possible to make cDNA from these cells and screen the cDNA population with low-stringency PCR that employs various oligonucleotide pairs for amplification of DNA that is conserved across family members. The DNA amplified by this procedure is sequenced and translated into protein for detection of novel types of receptors. Alternatively, conserved regions of receptor family members could be used in low-stringency hybridization screens with cDNA or genomic DNA from cells that contain amylin receptors.

K. Preparation of Antibodies Specific for Amylin Proteins

The protein species of the present invention, for example amylin, can be used to produce novel antibodies for use in immunoassays.

For some embodiments, it will be desired to produce antibodies that bind with high specificity to the protein product(s) generated by the present invention. Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

Methods for generating polyclonal antibodies are well known in the art. Briefly, a polyclonal antibody is prepared by immunizing an animal with an antigenic composition and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety

of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or in some cases the animal can be used to generate MAbs. For production of rabbit polyclonal antibodies, the animal can be bled through an ear vein or alternatively by cardiac puncture. The removed blood is allowed to coagulate and then centrifuged to separate serum components from whole cells and blood clots. The serum may be used as is for various applications or the desired antibody fraction may be purified by well-known methods, such as affinity chromatography using another antibody or a peptide bound to a solid matrix.

Monoclonal antibodies (MAbs) may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified expressed protein, polypeptide or peptide. The immunizing composition is administered in a manner that effectively stimulates antibody producing cells.

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep or frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

The animals are injected with antigen as described above. The antigen may be coupled to carrier molecules such as keyhole limpet hemocyanin if necessary. The antigen would typically

be mixed with adjuvant, such as Freund's complete or incomplete adjuvant. Booster injections with the same antigen would occur at approximately two-week intervals.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5 X 10^7 to 2 X 10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and have enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by

requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG. The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this low frequency does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and thus they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

Large amounts of the monoclonal antibodies of the present invention may also be obtained by multiplying hybridoma cells *in vivo*. Cell clones are injected into mammals that are histocompatible with the parent cells, *e.g.*, syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection.

In accordance with the present invention, fragments of the monoclonal antibody of the invention can be obtained from the monoclonal antibody produced as described above, by methods which include digestion with enzymes such as pepsin or papain and/or cleavage of disulfide bonds by chemical reduction. Alternatively, monoclonal antibody fragments

encompassed by the present invention can be synthesized using an automated peptide synthesizer, or by expression of full-length gene or of gene fragments in *E. coli*.

The monoclonal conjugates of the present invention are prepared by methods known in the art, *e.g.*, by reacting a monoclonal antibody prepared as described above with, for instance, an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers are prepared in the presence of these coupling agents or by reaction with an isothiocyanate. Conjugates with metal chelates are similarly produced. Other moieties to which antibodies may be conjugated include radionuclides such as ^{3}H, ^{125}I, ^{131}I ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, and ^{99m}Tc, are other useful labels that can be conjugated to antibodies. Radioactively labeled monoclonal antibodies of the present invention are produced according to well-known methods in the art. For instance, monoclonal antibodies can be iodinated by contact with sodium or potassium iodide and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Monoclonal antibodies according to the invention may be labeled with technetium-^{99} by ligand exchange process, for example, by reducing pertechnate with stannous solution, chelating the reduced technetium onto a Sephadex column and applying the antibody to this column or by direct labeling techniques, *e.g.*, by incubating pertechnate, a reducing agent such as $SNCl_2$, a buffer solution such as sodium-potassium phthalate solution, and the antibody.

It will be appreciated by those of skill in the art that monoclonal or polyclonal antibodies specific for the amylin and amylin-related compositions of the present invention will have utilities in several types of applications. These applications will include the production of diagnostic kits for use in detecting amylin or diagnosing deficiencies in amylin secretion. The skilled practitioner will realize that such uses are within the scope of the present invention.

L. Immunodetection Assays

The immunodetection methods of the present invention have evident utility in the detecting of conditions such as amylin and diagnosing diseases associated therewith. Here, a

biological or clinical sample suspected of containing either the encoded protein or peptide or corresponding antibody is used. However, these embodiments also have applications to non-clinical samples, such as in the titering of antigen or antibody samples, in the selection of hybridomas, and the like.

Those of skill in the art are very familiar with differentiating between significant expression of a protein, which represents a positive identification, and low level or background expression of such a protein. Indeed, background expression levels are often used to form a "cut-off" above which increased staining will be scored as significant or positive. Significant expression may be represented by high levels of antigens in tissues or within body fluids, or alternatively, by a high proportion of cells from within a tissue that each give a positive signal.

1. Immunodetection Methods

In still further embodiments, the present invention concerns immunodetection methods for binding, purifying, removing, quantifying or otherwise generally detecting biological components. The encoded proteins or peptides of the present invention may be employed to detect antibodies having reactivity therewith, or, alternatively, antibodies prepared in accordance with the present invention, may be employed to detect the encoded proteins or peptides. The steps of various useful immunodetection methods have been described in the scientific literature and are well known to those of skill in the art.

In general, the immunobinding methods include obtaining a sample suspected of containing a protein, peptide or antibody, and contacting the sample with an antibody or protein or peptide in accordance with the present invention, as the case may be, under conditions effective to allow the formation of immunocomplexes.

The immunobinding methods include methods for detecting or quantifying the amount of a reactive component in a sample, which methods require the detection or quantitation of any immune complexes formed during the binding process. Here, one would obtain a sample suspected of containing an amylin, an amylin-related peptide or a corresponding antibody, and

contact the sample with an antibody or encoded protein or peptide, as the case may be, and then detect or quantify the amount of immune complexes formed under the specific conditions.

In terms of antigen detection, the biological sample analyzed may be any sample that is suspected of containing an amylin antigen, such as a pancreatic β-cell, a homogenized tissue extract, an isolated cell, a cell membrane preparation, separated or purified forms of any of the above protein-containing compositions, or even any biological fluid that comes into contact with diabetic tissue, including blood.

Contacting the chosen biological sample with the protein, peptide or antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, *i.e.*, to bind to, any antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any radioactive, fluorescent, biological or enzymatic tags or labels of standard use in the art. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

The encoded protein, peptide or corresponding antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined.

Alternatively, the first added component that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the encoded protein, peptide or corresponding antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the encoded protein, peptide or corresponding antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under conditions effective and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if desired.

2. *Immunohistochemistry*

The antibodies of the present invention may also be used in conjunction with both fresh-frozen and formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). For example, each tissue block consists of 50 mg of residual "pulverized" diabetic tissue. The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and is well known to those of skill in the art.

Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen "pulverized" diabetic tissue at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and pelleting again by centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and cutting 25-50 serial sections.

Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 hours fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and embedding the block in paraffin; and cutting up to 50 serial permanent sections.

3. ELISA

As noted, it is contemplated that the encoded proteins or peptides of the invention will find utility as immunogens, *e.g.*, in connection with vaccine development, in immunohistochemistry and in ELISA assays. One evident utility of the encoded antigens and corresponding antibodies is in immunoassays for the detection of amylin and amylin-related peptides, as needed in diagnosis and prognostic monitoring of various diseased states.

Immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and western blotting, dot blotting, FACS analyses, and the like may also be used.

In one exemplary ELISA, antibodies binding to the encoded proteins of the invention are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition suspected of containing the amylin, such as a clinical

sample, is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound antibody may be detected. Detection is generally achieved by the addition of a second antibody specific for the target protein, that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of a second antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

In another exemplary ELISA, the samples suspected of containing the amylin antigen are immobilized onto the well surface and then contacted with the antibodies of the invention. After binding and washing to remove non-specifically bound immune complexes, the bound antigen is detected. Where the initial antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has binding affinity for the first antibody, with the second antibody being linked to a detectable label.

Another ELISA in which the proteins or peptides are immobilized, involves the use of antibody competition in the detection. In this ELISA, labeled antibodies are added to the wells, allowed to bind to the amylin protein, and detected by means of their label. The amount of marker antigen in an unknown sample is then determined by mixing the sample with the labeled antibodies before or during incubation with coated wells. The presence of marker antigen in the sample acts to reduce the amount of antibody available for binding to the well and thus reduces the ultimate signal. This is appropriate for detecting antibodies in an unknown sample, where the unlabeled antibodies bind to the antigen-coated wells and also reduces the amount of antigen available to bind the labeled antibodies.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described as follows:

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating of nonspecific adsorption sites on the immobilizing surface reduces the background caused by nonspecific binding of antisera to the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the control and/or clinical or biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween™. These added agents also tend to assist in the reduction of nonspecific background.

The "suitable" conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a

solution such as PBS/Tween™, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this label will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween™).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azido-di-(3-ethyl-benzthiazoline-6-sulfonic acid [ABTS] and H_2O_2, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer.

4. Use of Antibodies for Radioimaging

The antibodies of this invention will be used to quantify and localize the expression of antigens such as amylin, including receptor-bound amylin. The antibody, for example, will be labeled by any one of a variety of methods and used to visualize the localized concentration of the cells producing the encoded protein. Such an assay also will reveal the subcellular localization of the protein, which can have diagnostic and therapeutic applications.

In accordance with this invention, the monoclonal antibody or fragment thereof may be labeled by any of several techniques known to the art. The methods of the present invention may also use paramagnetic isotopes for purposes of *in vivo* detection. Elements particularly useful in Magnetic Resonance Imaging ("MRI") include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

Administration of the labeled antibody may be local or systemic and accomplished intravenously, intraarterially, via the spinal fluid or the like. Administration may also be intradermal or intracavitary, depending upon the body site under examination. After a sufficient time has lapsed for the monoclonal antibody or fragment thereof to bind with the diseased tissue, for example 30 minutes to 48 hours, the area of the subject under investigation is examined by routine imaging techniques such as MRI, SPECT, planar scintillation imaging or newly emerging imaging techniques. The exact protocol will necessarily vary depending upon factors specific to the patient, as noted above, and depending upon the body site under examination, method of administration and type of label used; the determination of specific procedures would be routine to the skilled artisan. The distribution of the bound radioactive isotope and its increase or decrease with time is then monitored and recorded. By comparing the results with data obtained from studies of clinically normal individuals, the presence and extent of the diseased tissue can be determined.

It will be apparent to those of skill in the art that a similar approach may be used to radio-image the production of the encoded amylin or amylin-related proteins in human patients. The present invention provides methods for the *in vivo* detection of amylin or amylin-related peptide with a view to correlating such detection to diagnosis diabetes in a patient. Such methods generally comprise administering to a patient an effective amount of an amylin antibody, to which antibody is conjugated a marker, such as a radioactive isotope or a spin-labeled molecule, that is detectable by non-invasive methods. The antibody-marker conjugate is allowed sufficient time to come into contact with reactive antigens that are present within the tissues of the patient, and the patient is then exposed to a detection device to identify the detectable marker.

5. Kits

In still further embodiments, the present invention concerns immunodetection kits for use with the immunodetection methods described above. As the encoded proteins or peptides may be employed to detect antibodies and the corresponding antibodies may be employed to detect encoded proteins or peptides, either or both of such components may be provided in the kit. The

immunodetection kits will thus comprise, in suitable container means, an encoded protein or peptide, or a first antibody that binds to an encoded protein or peptide, and an immunodetection reagent.

In certain embodiments, the encoded protein or peptide, or the first antibody that binds to the encoded protein or peptide, may be bound to a solid support, such as a column matrix or well of a microtiter plate.

The immunodetection reagents of the kit may take any one of a variety of forms, including those detectable labels that are associated with or linked to the given antibody or antigen, and detectable labels that are associated with or attached to a secondary binding ligand. Exemplary secondary ligands are those secondary antibodies that have binding affinity for the first antibody or antigen, and secondary antibodies that have binding affinity for a human antibody.

Further suitable immunodetection reagents for use in the present kits include the two-component reagent that comprises a secondary antibody that has binding affinity for the first antibody or antigen, along with a third antibody that has binding affinity for the second antibody, the third antibody being linked to a detectable label.

The kits may further comprise a suitably aliquoted composition of the encoded protein or polypeptide antigen, whether labeled or unlabeled, as may be used to prepare a standard curve for a detection assay.

The kits may contain antibody-label conjugates either in fully conjugated form, in the form of intermediates, or as separate moieties to be conjugated by the user of the kit. The components of the kits may be packaged either in aqueous media or in lyophilized form.

The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the antibody or antigen may be placed, and

preferably, suitably aliquoted. Where a second or third binding ligand or additional component is provided, the kit will also generally contain a second, third or other additional container into which this ligand or component may be placed. The kits of the present invention will also typically include a means for containing the antibody, antigen, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

M. Pharmaceutical Compositions

Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions - either gene delivery vectors or engineered cells - in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of the active ingredients as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent growth of microorganisms.

The expression vectors and delivery vehicles of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra*.

The vectors and cells of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection also may be prepared. These preparations also may be emulsified. A typical composition for such purposes comprises a 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate-buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters, such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components in the pharmaceutical are adjusted according to well-known parameters.

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch,

magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

An effective amount of the therapeutic agent is determined based on the intended goal. The term "unit dose" refers to a physically discrete unit suitable for use in a subject, each unit containing a predetermined quantity of the therapeutic composition calculated to produce the desired response in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject, and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

N. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Hexokinase I Targeted Disruption

Methods

Construction of gene replacement vector. A 15 kB clone, containing a portion of the rat hexokinase I (HKI) gene encompassing exon 1, about 0.2 kB of intron 1 and about 14.8 kB of sequence upstream of exon 1, was employed. Sequence and maps of this clone aided in the

mapping of the HKI gene and in the isolation of homologous isogenic sequences from RIN genomic DNA. The novel 1082 base sequence of the non-transcribed rat HKI genomic DNA as well as the first 170 bases of HKI transcribed DNA (Schwab and Wilson, 1989) is given as SEQ ID NO:13. A plasmid vector providing positive and negative selection, pPolIIshort-neobPA-HSV-tk, is derived from the pGEM3Zf(+) backbone and contains a neomycin phosphotransferase gene (positive selection) and two tandem copies of herpes simplex virus thymidine kinase gene (HSV-tk) that provide negative selection in the presence of ganciclovir (Ishibashi *et al.*, 1993). pPolIIshort-neobPA-HSV-tk was modified to create pAT9 by creating a unique *Not*I site 5′ of the Neo cassette (FIG. 1). A 873 base pair fragment was amplified from RIN genomic DNA using oligos (TTTCCCCTCGAGCACCGCCCGGAACAGTACC, SEQ ID NO:16 and GTTGCGCCTCGAGCATGCTGACGGTGGGGG, SEQ ID NO:17) to provide a short arm of homology to the HKI gene. The sequence extends 5′ from the first methionine of exon 1 and is flanked by engineered *Xho*I sites.

In addition, a 1121 base fragment was amplified from RIN genomic DNA using oligos (GTTGGACTCGAGAGTCACCTAAGGGCCTATG, SEQ ID NO:18 and GTTGCGCCTCGAGCATGCTGACGGTGGGGG, SEQ ID NO:17), providing a longer short arm to serve as a positive control for screening for homologous recombinants by PCR™. The 873 and 1121 base pair PCR™ fragments were restricted with *Xho*I and subcloned into pAT9 at a unique *Xho*I site which is flanked by the Neo cassette and the copies of HSV-tk (FIG. 1), generating pAT21 and pAT22, respectively.

Southern blot analysis in RIN 1046-38 genomic DNA with a probe within intron 1 revealed a 16 kB *Kpn*I fragment. This fragment was enriched by sucrose density ultracentrifugation, modified with adapters to create flanking *Not* I sites, and subcloned into lambda Dash II (Stratagene, La Jolla, CA). Recombinant phages containing the fragment were isolated by plaque screening. The 16 kB *Not*I fragment was cloned into the unique *Not* I site of pAT22 to provide a long arm of homology to the HKI gene (FIG. 1), generating pAT23, the HK1 replacement vector.

Cell culture, electroporation, and drug selection. Various cell lines derived from the rat insulinoma RIN 1046-38 line (Clark *et al.*, 1990) were grown in Medium 199 with Earle's salts, containing 11 mM glucose and 5% fetal bovine serum. Exogenous DNA was introduced into the cells by electroporation. RIN cell lines were grown to 50% to 75% confluence, harvested by trypsinization, washed once with phosphate-buffered saline (PBS), and resuspended in PBS for counting. For each electroporation, 1 X 10^7 cells were pelleted by centrifugation at 1000 rpm for 2 minutes and resuspended in 0.4 ml cold Electroporation Buffer (137 mM NaCl, 6.1 mM glucose, 5 mM KCl, 0.7 mM Na_2HPO4, 20 mM Hepes, pH 7.0). DNA was added to the cell suspension to achieve a final concentration of 30-50 micrograms per ml. DNA was electroporated into cells in a 2 mm cuvette at 170 volts and 510 microFaradies using an Electro Cell Manipulator 600 (BTX, Inc., San Diego, CA) Cells were plated in non-selective medium and cultured for 2 to 3 days. Medium containing G418 at a final concentration of 500 micrograms per ml was used for 14 days to select for clones integrated with the neomycin resistance marker. Following positive selection in G418, ganciclovir (Syntex Inc., Palo Alto, CA) at a final concentration of 6 μM was used to selectively kill clones expressing HSV-tk. Ganciclovir was applied for 3 days; cells were then maintained in medium containing G418.

PCR™ assay for targeted recombinants. Following positive selection in G418 and negative selection in ganciclovir, clones were grown until visible by the naked eye. Individual colonies were picked, dispersed in trypsin, and divided between duplicate cultures into 96-well plates. Following 10 to 15 days in culture, cells of one duplicate were rinsed in PBS and lysed by incubation at 37°C for 8 to 12 hours in fifty microliters of Lysis Buffer (16.6 mM ammonium sulfate, 67 mM Tris-HCl, 6.7 mM $MgCl_2$, 5.0 mM 2-mercaptoethanol, 6.7 μM EDTA, 1.7 μM SDS, 50 μg/ml proteinase K, pH 8.8), (Willnow and Herz, 1994). Five microliters of lysate were used as a template in a twenty-five microliter polymerase chain reaction (PCR™) in 16.6 mM ammonium sulfate, 67 mM Tris-HCl, 6.7 mM MgCl2, 5.0 mM 2-mercaptoethanol, 6.7 μM EDTA, 1 mM each dNTP, 80 μg/ml BSA, 0.8 μg/ml of each primer, and 2.5 units Taq DNA polymerase. The amplification program consisted of 92°C, 40 seconds, 57°C 40 seconds, 75°C, 1 minute (40 cycles) and a final extension for 5 minutes at 75°C. The oligonucleotides used to

amplify disrupted HKI included a primer in the 3′ end of the Neo cassette (5′GATTGGGAAGACAATAGCAGGCATGC3′ SEQ ID NO:19, primer 1, FIG. 1 Ishibashi *et al.*, 1993) and a primer in the HKI gene upstream of the putative recombination site (5′AGTCGCCTCTGCATGTCTGAGTTC3′ SEQ ID NO:20, primer 3, FIG. 1). The plasmid pAT22, containing the longer short arm of homology, served as a positive control in this PCR™ reaction. A second control PCR™ reaction was also included using primer 1 and a primer in the HK1 gene downstream of the recombination site (5′CTTGAGCTCTTACATGGTGTCACG3′ SEQ ID NO:21, primer 2, FIG. 1). This control PCR™ reaction should detect both homologous and random integrants of the HK1 replacement vector. Recombinants detected in the first screen were confirmed in a second PCR™ reaction for which no positive control plasmid exists. The absence of such a control negates the possibility of a false positive due to contamination. The primers in this secondary screen were primer 1 and primer 4 (5′TCCCCAGGCGTGGGGTAGAAG3′ SEQ ID NO:22), an oligonucleotide upstream of the recombination site in the HKI gene (FIG. 1). PCR™ products analyzed either by gel electrophoresis or a slot blot assay. For electrophoresis, reaction products were fractionated in 1% agarose gels in Tris-borate/EDTA buffer (9 mM Tris-borate, 0.2 mM EDTA). DNA was visualized by staining in ethidium bromide. For slot-blots, reaction products were denatured in 0.5 N NaOH, 1.5 M NaCl, neutralized in 1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl, and transferred to a nylon membrane using a 96-well blot apparatus (Scheichller and Schuell, Keene, N.H.). DNA was cross-linked to the membrane and HKI amplified products were detected by hybridization with ^{32}P-labelled oligonucleotides complementary to HKI and internal to primers used in the amplification reaction. Positive clones were replated in 96-well dishes to obtain densities of one cell per well. These clones were allowed to grow and assayed by PCR™ with the primers described above. This cycle of dilution cloning was repeated until all clones of a plating were positive in the assay.

Genomic Southern analysis. RIN clones that were positive by PCR™ for a disrupted allele of HKI were assayed by genomic Southern. Genomic DNA was isolated using reagents and protocols of the QIAamp Blood Kit (catalog number 29104, Qiagen, Inc., Chatsworth CA)

Five to ten micrograms of DNA were digested with enzymes as indicated and fractionated through 0.8% agarose gels using TEAN buffer (0.04 M Tris-HCl, 0.025 M sodium acetate, 0.018 M NaCl, 25 mM EDTA, pH 8.15). Electrophoresis was conducted for 12 to 16 hours at 25 to 35 volts with recirculation of the buffer. DNA was visualized by staining with ethidium bromide. DNA in the gel was denatured for 30 minutes in 0.5 N NaOH, 1.5 M NaCl. Following neutralization in 1 M Tris-HCl, pH 7.5, 1 M NaCl for 30 minutes, DNA was transferred to a nylon membrane (Hybond-N+, Amersham) in 10x SSC (1X: 0.15 M NaCl, 0.015 M sodium citrate) and cross-linked to the membrane by ultraviolet radiation (UV Stratalinker 2400, Stratagene, Inc.). Radiolabeled probes (^{32}P) for hybridization to and detection of genomic fragments were synthesized as directed using the rediprime Random Primer Labelling Kit (RPN 1633, Amersham Life Sciences). Membranes were prehybridized and hybridized in Rapid-hyb Buffer (NIF939, Amersham Life Sciences). All incubations and washes were performed in a Micro-4 Hybridization Oven (Hybaid Limited). Membranes were exposed to X-OMAT, AR5 film (Kodak) to obtain autoradiographic signals.

Results:

Prior to construction of a gene replacement vector, a comparison was made of the copy number of HKI alleles in rat versus RIN genomic DNA. DNA was digested with *Xba*I, Southern blotted, and probed with a radiolabeled fragment from intron 1 of the HKI gene. Autoradiography revealed equivalent signals derived from the rat and RIN HKI gene fragments. Presumably, these signals correspond to diploidy of the HKI gene in both the rat and RIN genomes. This conclusion is supported by data that show RIN-derived cell lines to have maintained a diploid state in their chromosomes. Karyotype analysis of RIN 1046-38 showed a distribution of 35 to 40 chromosomes with the normal rat compliment being 42 chromosomes.

The HKI replacement vector (FIG. 1) was transfected into RIN cells in three separate electroporations (EP): EP81, EP86, EP95. These electroporations differ from each other in their temporal distributions, the identity of the parental cell line, and the number of clones screened from each (Table 7). EP81 was derived from a low passage RIN 1046-38 cell line. Of the 500 colonies screened, none were positive for disruption of an HKI allele. RIN-52/17, a RIN 1046-

38 derived clone, was the parental line in EP86. One positive clone was detected in a screen of about 970 colonies. RIN-52/9, a cell line engineered to express high levels of rat glucokinase with pcb7/GK was used as a parental line in EP95. About 3200 clones were screened by PCR™ for the presence of a disrupted HKI allele. None were positive.

Potentially, the loss of an HKI allele could result in a growth disadvantage and thereby lead to a lower frequency of detecting HKI gene replacement events. To negate a potential metabolic disadvantage conferred by loss of HKI activity, efforts were made to create parental cell lines that overexpress rat glucokinase. Such parental lines could potentially serve two functions - first, to prevent metabolic stress should phosphorylation of glucose became rate-limiting in transformed cell lines with diminished HKI activity; and second, to restore a high K_m glucose-phosphorylating activity to the RIN lines to shift glucose-responsive insulin secretion towards a more physiological range. RIN-52/17, the parental cell line in EP86, had previously been electroporated with a plasmid conferring hygromycin resistance and containing a copy of the rat glucokinase (GK) cDNA. RIN-52/17 was hygromycin resistant and was thought to express moderate levels of glucokinase from the transgene. Subsequent data confirmed resistance to hygromycin, but disproved expression of GK from the transgene (Table 7). About 1000 individual clones were screened from EP86. From this screen one clone, 86/X4, was positive by PCR™. Clone 86/X4 was initially identified by amplification with primer 1 and primer 3. The molecular weight of the amplified product was equal to that derived from the plasmid control. Confirmation of this clone as containing a disrupted HKI allele was obtained by amplification with primer 1 and primer 4. No plasmid control exists for this PCR™ reaction, therefore, the product is not the result of contamination.

TABLE 7

Electroporation (EP) of RIN Cell Lines with a HK1 Replacement Vector

EP	Parental line	DrugR,Parental	Transgene	Clones screened	+ by PCR™
81	RIN 1047-38	(-)	(-)	500	0
86	RIN 52-17	HygroR	(-)	970	1
95	RIN 52-9	HygroR	rat GK	3200	0

Targeted disruption of HK1 was attempted in various RIN lines, in the absence of presence of high levels of expression of rat glucokinase (GK) from a transgene. Cells expressing the transgene were first selected for resistance to hygromycin (HygroR) and then assayed by Western blotting for expression of exogenous rat GK.

The original positive culture of 86/X4 was passaged several times prior to dilutional plating for assessing the purity of the clonal population. 197 individual colonies were cultured in 96-well plates, allowed to grow to 50-70% confluence, trypsinized, and split into duplicate cultures. Cells from one set of cultures were lysed and screened by PCR™ using primers 1 and 3 (FIG. 1) and then reaction products were analyzed by a slot assay. Two clones were confirmed as containing a disrupted allele of HKI. This result demonstrates two things. First, the original culture that was identified as 86/X4 was a polyclonal rather than a monoclonal population. Second, the clone containing the disrupted allele of HKI seems to have a growth disadvantage compared to other cells in the population. This latter possibility is supported by observations of the growth rates of the purified HKI replacement clone. The pure 86/X4 grows significantly slower (about one-half as fast) than clones randomly integrated with the replacement vector.

Additional data verifying the identity of clone 86/X4 were derived by analysis of genomic DNA by Southern blotting (FIG. 2). DNA was digested with *EcoR*I and *Not*I, blotted, and hybridized with a probe upstream of the recombination site (hatched rectangle, FIG. 1). DNA from RIN 1046-38 cells (lane 1) and from RIN-52/17 randomly integrated with pAT23 (lane 2) produce a predicted signal of about 5.5kB in the autoradiograph. This signal corresponds to a homozygous, wild-type HKI gene. Clone 86/X4 produces two autoradiographic signals in the

genomic Southern (lane 3): a 5.5 kB signal corresponding to a wild-type allele and an additional signal (about 4.6 kB), indicative of a HKI allele that has homologously recombined with the replacement vector.

EXAMPLE 2

Insulin Knockout

Methods:

Construction of gene replacement vector. The rat insulin I gene (Genbank accession number J00747) provided a template from which to create primers for amplifying sequences from RIN genomic DNA. A 590 base pair fragment 3′ of the rat insulin gene and corresponding to positions 4830 to 5420 was amplified by polymerase chain reaction (PCR™), subcloned, and used as radiolabeled probe. RIN genomic Southerns using this probe revealed a *Bgl*II fragment of about 12 kB that extends three prime from position 1176. This fragment was enriched by sucrose density ultracentrifugation and subcloned into *Bam*HI sites of lambda Dash II vector (Stratagene). Recombinant phages containing the fragment were isolated by plaque screening. A portion of this fragment extending from an internal SpeI site to a *Not*I site provided by the lambda Dash vector was used to provide a long arm of homology to RIN DNA in the context of a replacement vector (FIG. 3). A short arm of homology to RIN DNA (five prime of the rat insulin I gene) was derived by amplification of a fragment corresponding to nucleotides 1822 to 2860. This fragment, flanked by *Xho*I sites, was cloned into the replacement vector (FIG. 3).

The plasmid backbone (pSL9), used for creating a rat insulin I (RINS-1) replacement vector, provided several features designed to enhance and complement disruption of the rat insulin I gene. First, positive selection for integration of exogenous DNA into the RIN genome was provided by the gene encoding neomycin phosphotransferase. The expression of this gene is linked to the expression of human insulin by an internal ribosome entry site (IRES). This allows disruption of the rat insulin gene to be coupled to expression of human insulin cDNA. Secondly, negative selection, to allow enrichment of targeted over random integration events, was provided by the expression of the type 2 rat glucose transporter (GLUT-2). The presence of a functional GLUT-2 renders cells susceptible to streptozotocin (STZ) toxicity (Schnedl *et al.*, 1994).

Thirdly, a unique *Pac*I site at the distal end of the long arm of homology was used to linearize the vector prior to electroporation into RIN cells (FIG. 3).

Cell culture, electroporation, and drug selection. Culture conditions are as described above except that following positive selection in G418, STZ (1 mM for 2.5 h) was used to selectively kill clones expressing a functional Glut-2 transporter.

PCR™ assay for targeted recombinants. Following positive selection in G418 and negative selection in STZ, clones were cultured for about 3-4 weeks. Cells in each well were dispersed in trypsin and divided between duplicate cultures into 96-well plates. Following 10 to 15 days in culture, cells of one duplicate were rinsed in PBS and lysed by incubation at 37°C for 8 to 12 hours in 50 µl of Lysis Buffer (16.6 mM ammonium sulfate, 67 mM Tris-HCl, 6.7 mM $MgCl_2$, 5.0 mM 2-mercaptoethanol, 6.7 µM EDTA, 1.7 µM SDS, 50 µg/ml proteinase K, pH 8.8) (Willnow and Herz, 1994). Five microliters of lysate were used as a template in a 25 µl PCR™ in 16.6 mM ammonium sulfate, 67 mM Tris-HCl, 6.7 mM $MgCl_2$, 5.0 mM 2-mercaptoethanol, 6.7 µM EDTA, 1 mM each dNTP, 80 µg/ml BSA, 0.8 µg/ml of each primer, and 2.5 units Taq DNA polymerase. The amplification program consisted of 40 cycles at 92°C, 40 seconds, 57°C 40 seconds, 75°C, 1 minute and a final extension for 5 minutes at 75°C, and was performed in a 96-well thermocycler (HB-96V, MJ Research, Inc., Watertown, MA) The oligonucleotides used to amplify disrupted RINS-1 included a primer in the 3′-end of the Neo cassette (5′-CAACCGGTGGGACATTTGAGTTGC-3′ SEQ ID NO:23, primer 1, FIG. 3) and a primer in the RINS-1 gene upstream of the putative recombination site (5′-CCAAGTCATTATAGAATCATAGTC-3′ SEQ ID NO:24, primer 2, FIG. 3). The plasmid pRD1 was created to serve as a positive control in the PCR™ reaction. The backbone of pSL9 was ligated to an insert encompassing all of the short arm of homology and extending an additional 200 base pairs 5′. PCR™ products were analyzed using a slot-blot apparatus (part number 27560, Scheicher and Schuell). Reaction products were denatured in 0.5 N NaOH, 1.5 M NaCl, neutralized in 1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl, and transferred to a nylon

membrane. DNA was cross-linked to the membrane and RINS-1 amplified products were detected by hybridization with ^{32}P-labelled oligonucleotides complementary to RINS-1 and internal to primers used in the amplification reaction. Positive clones were replated in 96-well dishes to obtain densities of one cell per well. These clones were allowed to grow and assayed by PCR™ with the primers described above. This cycle of dilution cloning was repeated until all clones of a plating were positive in the assay.

RIN clones that were positive by PCR™ for a disrupted allele of RINS-1 were assayed by genomic Southern. Genomic DNA was isolated using reagents and protocols of the QIAamp Blood Kit (catalog number 29104, Qiagen, Inc., Chatsworth, CA). Five to ten micrograms of DNA was digested with enzymes as indicated and fractionated through 0.8% agarose gels using a TEAN buffer (0.04 M Tris-HCl, 0.025 M sodium acetate, 0.018 M NaCl, 25 mM EDTA, pH 8.15). Electrophoresis was conducted for 12 to 16 hours at 25 to 35 volts with recirculation of the buffer from the positive to the negative electrode. DNA was visualized by staining with ethidium bromide. DNA in the gel was denatured for 30 minutes in 0.5 N NaOH, 1.5 M NaCl. Following neutralization in 1 M Tris-HCl, pH 7.5, 1 M NaCl for 30 minutes, DNA was transferred to a nylon membrane (Hybond-N+, Amersham, Chicago, IL) in 10X SSC (1X: 0.15 M NaCl, 0.015 M sodium citrate) and cross-linked to the membrane by ultraviolet radiation (UV Stratalinker 2400, Stratagene, Inc.). Radiolabeled probes (^{32}P) were synthesized as directed using the rediprime Random Primer Labelling Kit (RPN 1633, Amersham Life Sciences). Membranes were prehybridized and hybridized in Rapid-hyb Buffer (NIF939, Amersham Life Sciences). All incubations and washes were performed in a Micro-4 Hybridisation Oven (Hybaid Limited). Membranes were exposed to X-OMAT, AR5 film (Kodak) to obtain autoradiographic signals.

EXAMPLE 3

Human Insulin Expression

Methods:

Expression plasmid construction, general design. Initial expression plasmids were based on pCB6 and pCB7 (Brewer, 1994). These plasmids utilize the strong promoter/enhancer of the

human Cytomegalovirus (CMV) immediate-early regulatory sequence to express inserted genes of interest. Efficient polyadenylation of transcribed messenger RNA is directed by the human growth hormone polyadenylation sequence. pCB6 encodes the Neomycin resistance gene conferring resistance to the neomycin analog G418, while pCB7 encodes the hygromycin resistance gene. Both resistant markers are transcribed by the SV40 early promoter.

A second expression plasmid was constructed with many of the same elements as pCB6. The open reading frame of the neomycin resistance gene was amplified with the polymerase chain reaction from pCB6 (Brewer, 1994) using oligos (CCGGATCCCATGATTGAACAAGAT, SEQ ID NO:25 and CCAAGATCTCGCTCAGAAGAACTC, SEQ ID NO:26). The resulting 816 bp amplified product was restricted with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pCMV8, generating pCMV8/NEO/hGH PolyA. pCMV8 was derived from pCMV4 (Anderson *et al.*, 1989) following removal of the alpha mosaic virus 4 RNA translational enhancer and replacing it with the 5′ leader sequence of the adenovirus tri-partite leader (+14 to +154 of major late transcript) fused to a hybrid intron composed of the adenovirus major late transcript 5′-donor site and a 3′-splice site from a variable region immunoglobulin gene on a 409 bp *Eco*RI/*Pst*I fragment (SEQ ID NO:14, Kaufman and Sharp, 1982). Secondly, a portion of the gene encoding the 5′-transcribed leader of the human Glucose Regulated Protein 78 (GRP78) was amplified using the polymerase chain reaction from pThu6.5 (corresponding to bases 372 to 594, Ting and Lee, 1988) using oligos (CCGGATCCAGGTCGACGCCGGCCAA, SEQ ID NO:27 and CGAGATCTTGCCAGCCAGTTGG, SEQ ID NO:28), generating SEQ ID NO:11. The 5′-leader of human GRP 78 has been shown to direct internal initiation of translation allowing for construction of functional polycistronic genes in mammalian cells (Macejak and Sarnow, 1991). The 235 bp amplified product (SEQ ID NO:11) was restricted with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pCMV8/NEO/hGH PolyA generating pCMV8/IRES/NEO/hGH PolyA (FIG. 4B). Unique restriction endonuclease sites exist (5′-*Sal*I/*Xba*I/*Bam*HI-3′) for subcloning fragments into this expression plasmid between the CMV promoter/intron and the internal ribosome entry site/NEO elements. cDNA's or other open reading frames cloned into

these sites are transcribed from the CMV promoter into a bicistronic message containing the cDNA as the upstream open reading frame and neomycin resistance (NEO) as the downstream open reading frame. Both open reading frames are translated efficiently, linking neomycin drug resistance and expression of the upstream gene of interest.

A final expression plasmid was designed for expression of genes of interest. The 5′ elements found in pCMV8 composed of the 5′ leader sequence of the adenovirus tri-partite leader (+14 to +154 of major late transcript) fused to a hybrid intron composed of the adenovirus major late transcript 5′ donor site and a 3′ splice site from a variable region immunoglobulin gene (SEQ ID NO:14, Kaufman and Sharp, 1982) was removed by endonuclease restriction by *Sna*B1 and *Bam*HI and ligated into *Sna*B1 and *Bgl*II restricted pCB6 (Brewer, 1994), generating pCB6/intron (FIG. 4A). *Sna*B1 cuts uniquely in both plasmids at identical positions in the CMV promoter sequence. pCB6/intron has several unique endonuclease restriction sites for subcloning fragments downstream of the intron sequence and upstream of the hGH PolyA sequence (5′-*XbaI/KpnI/MluI/ClaI/Bsp*DI/*XbaI/Bam*HI-3′). The neomycin resistance gene is transcribed using the SV40 promoter from an independent transcriptional unit encoded on the plasmid (Brewer, 1994).

Human insulin expression plasmid. A human insulin cDNA contained on a 515 base *Eco*RI fragment (SEQ ID NO:1, Bell *et al.*, 1979) encoding human preproinsulin (SEQ ID NO:2) was ligated into the *Eco*RI site of pBluescript (Stratagene, Inc., La Jolla, CA), generating pBS/INS. pBS/INS was digested with *Hin*DIII, located 5′ of the insulin open reading frame, and *Bam*HI, located 3′ of the Insulin open reading frame. The resulting 542 base fragment was ligated into pCB6 that had been restricted with *Hin*DIII and *Bam*HI, generating pCB6/INS. pCB6/INS was digested with *Bgl*II and *Bam*H1 and the resulting 549 base fragment containing the human insulin cDNA (SEQ ID NO:1) was ligated into the *Bam*H1 site pCMV8/IRES/NEO/hGH PolyA generating pCMV8/INS/IRES/NEO. The CMV promoter drives transcription of a bicystronic messenger RNA with human insulin encoded in the upstream open reading frame and the neomycin resistance gene encoded in the downstream open reading

frame. Stable transfectants from this plasmid are selected in G418. The same 542 base *Hin*DIII/*Bam*HI fragment was also ligated into *Hin*DIII/*Bam*HI digested pCB7 generating pCB7/INS. Stable transfectants from this plasmid are selected in hygromycin.

A third insulin expression plasmids was also constructed. pCB6/INS was digested with *Bgl*II and *Bam*HI and the resulting 549 base fragment containing the human insulin cDNA (SEQ ID NO:1) was ligated into the *Bam*HI site of pCMV8/IRES/PURO/hGH PolyA, generating pCMV8/INS/IRES/PURO. The CMV promoter drives transcription of a bicystronic messenger RNA with human insulin encoded in the upstream open reading frame and the puromyocin resistance gene encoded in the downstream open reading frame. Stable transfectants from this plasmid are selected in puromyocin.

Alternative promoter/enhancers utilized in human insulin expression plasmids. The rat insulin 1 promoter fragment was isolated from pAC/RIP (a derivative of pACCMV.pLpA in which the rat insulin 1 promoter was substituted for the CMV promoter, Becker *et al.*, 1994) as a *Kpn*I/*Hin*DIII fragment (SEQ ID NO:12) corresponding to bases -412 to +1 relative to the start site of transcription. This fragment was ligated into *Kpn*I/*Hin*DIII digested pBlueScript (Stratagene, Inc.), generating pBS/RIP. pBS/RIP was digested with *Kpn*I, treated with Klenow fragment to blunt the end, then digested with *Eco*RI, generating a 450 base pair fragment containing the rat insulin 1 promoter. This fragment was ligated into pCMV8/INS/IRES/NEO that had been previously digested with *Spe*I, treated with Klenow and then digested with *Eco*RI, generating pRIP8/INS/IRES/NEO.

The rat insulin 1 promoter fragment (441 base pair *Kpn*I/*Hin*DIII fragment, SEQ ID NO:12) was also ligated into both *Kpn*I and *Hin*DIII digested pCB6/INS and pCB7/INS generating pCB6/RIP.INS and pCB7/RIP.INS, respectively. The CMV promoter fragment of both of these plasmids was removed by digesting with *Spe*I and *Bgl*II (removing bases -585 to +1 of the CMV promoter), treating with Klenow fragment and ligating to close, generating pRIP6/INS and pRIP7/INS. Stable transformants of pRIP6/INS are selected in G418 while stable transformants of pRIP7/INS are selected in hygromycin.

The rat insulin 1 gene promoter fragment (RIP) was also modified in an attempt to strengthen its transcriptional activity. The principal modification involved the attachment of varying numbers of mutant Far-FLAT minienhancers (FFE minienhancer) (German, *et al.*, 1992) to different positions within an intact RIP or to a RIP truncated at -205 (-205RIP). FFE minienhancers were constructed by generating oligonucleotides corresponding to the region of RIP between -247 and -196 (top strand, 5′-GATCCCTTCATCAGGCCATCTGGCCCCTTGTTA ATAATCGACTGACCCTAGGTCTAA-3′ SEQ ID NO:29; bottom strand, 5′-GATCTTAGACCTAGGGTCA GTCGATTATTAACAAGGGGCCAGATGGCCTGATGAAGG-3′, SEQ ID NO:30). The underlined sequences at the ends of the oligonucleotides are *Bam*HI and *Bgl*II recognition sites. The oligonucleotides were annealed and ligated in the presence of restriction enzymes *Bam*HI and *Bgl*II. Since *Bam*HI and *Bgl*II produce compatible DNA ends but can no longer be digested by *Bam*HI or *Bgl*II, the only multimers that escaped *Bam*HI and *Bgl*II digestion were ligated head-to-tail. FFE minienhancer dimers, trimers, etc. were separated by polyacrylamide gel electrophoresis and blunt-end cloned into the transient transfection vector, pBS/RIP/hGH, at either a *Xho*I site immediately upstream of -415 of the intact RIP, into an *Avr*II site at -206 of an intact RIP, or into an *Apa*I site immediately upstream of -205RIP. The number and orientation of FFE minienhancer repeats were verified by DNA sequencing. The stable transfection vector, pFFE3/RIP8/INS/IRES/NEO containing three copies of FFE minienhancers (FFE3, SEQ ID NO:15), was generated by inserting a blunt-ended *KpnI/Hin*DIII FFE3/RIP into pCMV8/INS/IRES/NEO in which the CMV promoter was removed with *Spe*I and *Sac*I. pFFE6/RIP8/INS/IRES/NEO was constructed by inserting an *ApaI*/blunt-ended*Hin*DIII FFE6/RIP fragment into pRIP8/hGH polyA in which RIP was removed by *ApaI/Eco*RV. A *BglII/Stu*I INS/IRES/NEO fragment was then inserted into pFFE6/RIP8/hGH polyA to complete pFFE6/RIP8/INS/IRES/NEO.

The rat insulin 1 gene intron (RIPi) was obtained by polymerase chain reaction from rat genomic DNA using oligonucleotides CTCCCAAGCTTAAGTGACCAGCTACAA, SEQ ID NO:31 and GGGCAACCTAGGTACTGGACCTTCTATC, SEQ ID NO:32. These oligos

produced a 185 bp product containing the 119 base pair RIPi (Cordell *et al.*, 1979) and a HindIII site on the 5′-end and a *Bam*HI site on the 3′-end. The PCR™ product was digested with *Hin*DIII and *Bam*HI and ligated into pNoTA/T7, whereupon it was removed with *Xba*I blunt-ended with Klenow, treated with *Hin*DIII and inserted into *Eco*RV/*Hin*DIII digested pRIP8/INS/IRES/NEO to generate pRIP8/RIPi/INS/IRES/NEO. pFFE6/RIP8/RIPi/INS/IRES/NEO was constructed by replacing the 5′ adenovirus-immunoglobulin hybrid intron/INS/IRES of pFFE6/RIP8/INS/IRES/NEO with RIPi/INS/IRES from pRIP8/RIPi/INS/IRES/NEO. p(RIE)3/-85RIP/RIPi/INS/IRES/NEO contained three copies of the rat insulin 1 gene enhancer (RIE) fused to RIP truncated at -85. This plasmid was constructed by replacing a *Bsg*RI/*Hin*DIII RIP fragment from pRIP8/RIPi/INS/IRES/NEO with an *Apa*I/*Hin*DIII (RIE)3/-85RIP fragment. Both the *Bsg*RI and *Apa*I restriction sites were blunt-ended by Klenow polymerase.

The 2,000 base pair Class III human insulin-linked polymorphic region (ILPR), a region demonstrated to enhance transcriptional activity of the human insulin promoter (Kennedy *et al.*, 1995), was obtained from the phage lambda clone 1-H1-3 (Owerbach and Aagard, 1984). A *Pst*I/*Nco*I fragment containing the ILPR was treated with Klenow and inserted into a blunt-ended *Xho*I site immediately upstream of RIP to make pILPR/RIP8/INS/IRES/NEO. Orientation of the 14 bp repeats in the ILPR with respect to RIP was determined by DNA sequencing.

The human glyceraldehyde-3-phosphate dehydrogenase promoter (GAPDH) was isolated by the polymerase chain reaction from human genomic DNA using oligos (GGGTCTAGAGGACCTGTTCCCACCG, SEQ ID NO:33 and GCCGAATTCGAGGAGCAGAGAGCGAAGC, SEQ ID NO:34). These oligos generated a 1143 base product corresponding to bases -1121 to +22 of the published sequence (Ercolani *et al.*, 1988) with the introduction of a unique *Xba*I site at the 5′ end and a unique *Eco*RI site at the 3′ end. The PCR™ product was digested with *Pst*I (located at position -735 relative to start site of transcription), treated with Klenow, and then digested with *Eco*RI. The resulting 757 base fragment was ligated into pCMV8/INS/IRES/NEO that had been previously digested with *Spe*I, treated with Klenow and then digested with *Eco*RI, generating pGAPDH8/INS/IRES/NEO.

The Rous Sarcoma Virus Long Terminal Repeat (RSV) was isolated from pREP4 (Invitrogen, Inc., San Diego, CA). A 637 base pair *SalI/PvuII* fragment containing RSV was isolated, treated with Klenow to blunt the ends and ligated into pCMV8/INS/IRES/NEO that had been previously digested with *SpeI* and *EcoRI* and treated with Klenow, generating pRSV8/INS/IRES/NEO.

Bioreactor Inoculation and Culture. EP18/3E1 cells were grown, split, and maintained in RPMI-1640 medium with 2 mM glutamine (JRH Bioscience, Lenexa, KS) supplemented with 5% fetal calf serum (JRH) and 0.125 µg/ml G418 (Gibco BRL, Gaithersburg, MD) in T75 culture flasks as described previously. A large scale bioreactor (Celligen Plus™, New Brunswick Scientific (NBS), Edison, NJ) with dissolved oxygen electrode, pH electrode (both Ingold), and 4-gas proportional-integral-derivative (P-I-D) controller is set up for perfusion culture with a packed bed of polyester discs (Fibra-Cel®, Sterilin, England) and a centrifugal lift impeller (Cell Lift™, NBS). The reactor has a working volume of 1.25 liters and a packed bed volume of 0.7 liters containing 70 grams of polyester discs. Cells are trypsinized and seeded into the reactor containing the same media composition as the maintenance media at a density of approximately 10^6 cells per ml of working volume. After transfer, the cells are allowed to seed onto the bed material for 8 h with a low impeller speed of 50 rpm and no media perfusion. After seeding, the impeller speed is brought up to 80 rpm and the culture is maintained with no perfusion for approximately 75 hours. Media perfusion is started and the flow rate is brought from 0 working volumes per day (WV/d) to 4 WV/d over the course of the following 500 hours. The perfusion rate is thereafter maintained constant at 4 WV/d. The perfusion media is RPMI-1640 with 2 mM glutamine which is then supplemented with 2 g/l glucose (final concentration of 4 g/l), 0.10% fraction V bovine serum albumin, 10 µg/ml human apo-transferrin, 50 µM each of ethanolamine and o-phosphorylethanolamine, and 0.10% cholesterol rich lipids from adult bovine serum (Clark and Chick, 1990) (all Sigma Chemicals, St. Louis, MO). The perfusion media contains no fetal calf serum or other full sera. At approximately 600 hours of culture, the media was further enriched with glucose to a final concentration of 6 g/l. The impeller speed was increased to 100 rpm after 200 hours of culture, to 120 rpm after 300 hours, and to 150 rpm after 700 hours. The

culture temperature is maintained at 37°C, the dissolved oxygen level at 80% (indexed relative to saturation of air in 37°C phosphate-buffered saline), and the pH at 7.4. Glucose levels in the reactor are maintained in the range of 1-3 g/l by adjusting the perfusion rate and the glucose concentration in the fresh perfusion media. Cultures have been maintained successfully for as long as 2000 hours in the bioreactor under similar conditions.

Media samples were collected once daily and quantitatively analyzed for insulin secreted into the media by ELISA as previously described. Selected samples were qualitatively analyzed for insulin processing by HPLC analysis as previously described. Ammonia and lactate levels are monitored in the daily samples and analyzed using an automated analyzer (IBI Biolyzer, Johnson & Johnson, New Brunswick, NJ).

At the end of the culture, the reactor is opened and a representative number of polyester discs are sampled for quantitation of DNA and insulin content.

Cyclically Stimulated Secretion in the Bioreactor. At a point during the culture when the oxygen controller output has stabilized around 60, the culture is cyclically stimulated with addition of a 10X concentrated secretion-stimulation cocktail once every 24 hours. The addition of the cocktail yields final medium concentrations of 10 mM each of leucine, arginine, and glutamine, 100 μM IBMX, and 100 μM carbachol (all from Sigma). At the beginning of every cycle, approximately one-tenth of the working volume is replaced with the 10X cocktail while the perfusion of fresh media is left unchanged. At 4 l/d of perfusion, *e.g.*, the remaining concentration of cocktail after 24 h is less than 2% relative to the initial concentration due to the continuous dilution by the perfusion. Six samples were taken every 30 minutes, then four samples every hour.

Stable transfection of cell lines. Cells were transfected by electroporation as described above for the Hexokinase 1 knockout electroporations.

Insulin message primer extension analysis. Total RNA from RIN cell lines grown *in vitro* was isolated using RNAzol B RNA Isolation Reagent (Cinna/Biotex Laboratories Int.). Total RNA from RIN cell lines grown *in vivo* as tumors was isolated using TriReagent (Molecular Research Center, Inc.). Ten μg total RNA was hybridized to a 5′ digoxigenin-labeled oligo (GCCAGCAGGGGCAGGAGGCGCATCCACAGGGCCAT, SEQ ID NO:35, Genosys Biotechnologies, Inc.) in 0.25 M KCl at 68°C for 15 min. This oligo hybridizes to the first 35 bases of the endogenous rat insulin I as well as the human insulin open reading frames. Primer extension reactions were then carried out with 2.5 units AMV Reverse Transcriptase in the supplied buffer (Promega, Inc.) supplemented with 0.8 mM dNTP's (Pharmacia, Inc.) and 100 μg/ml Actinomycin D (Sigma Chemical Co.) at 42°C for one hour. Extension products were precipitated, resuspended in 40% water/60% Formamide, heated to 100°C for 5 min and run on a 8% acrylamide/7M urea/1X TBE denaturing gel. Electrophoresed products were transferred to Qiabrane Uncharged Nylon Membrane (Qiagen, Inc.) using a Transphor Unit, TE50X (Hoefer, Inc., San Francisco, CA). Digoxigenin-labeled products were detected using the Genius 7 Non radioactive Detection System (Boehringer Mannheim) followed by exposure to Xomat-AR auto radiography film (Kodak). Primer extension of endogenous rat insulin I message generates a 91 base product (Cordell *et al.*, 1979), the human insulin transgene expressed from pCB6 generates a 101 base product and the human insulin transgene expressed from pRIP7 generates a 68 base product. Primer extension of the human insulin transgene expressed from pCMV8/INS/IRES/NEO generates a primary signal of 280 bases with three other minor premature termination signals of approximately 190, 130 and 115 bases.

Northern analysis. Total RNA was isolated as described above for the primer extension protocol. Ten μg total RNA was resolved on methyl mercury/1.5% agarose gels as described (Bailey and Davidson, 1976). Gels were subsequently stained with ethidium bromide (1 μg/ml in 0.5 M ammonium carbonate) to visualize RNA for integrity and loading consistency. RNA was electro transferred to nylon membranes as described for the primer extension protocol. Membranes were hybridized with digoxigenin-labeled RNA probes using the Genius Non-Radioactive Nucleic Acid Labeling and Detection System for filter hybridizations as described

(Boehringer Mannheim). Full-length digoxigenin-labeled antisense probes corresponding to human insulin, rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (corresponding to bases 21 to 1162 of published sequence, Fort *et al.*, 1985) and the neomycin resistance gene (control template supplied in Genius 4 Kit) were made using Genius 4 RNA Labeling Kit (Boehringer Mannheim) using either T7 or T3 Polymerase. Exposures of chemiluminescent detected membranes were performed using Xomat-AR autoradiography film (Kodak). In some cases, blots were hybridized with a ^{32}P-labeled cRNA probe for human insulin.

Stimulated insulin secretion assay. Four million RIN cells were seeded in 9 ml media in 25 cm^2 flasks (butyrate-treated cells were seeded at 10^6 cells). Cells were then cultured with daily media changes for one week with or without 1 mM butyrate until cells reached 70-80% confluency. Prior to assay, cells were incubated 2 times for twenty minutes at 37°C in RPMI media lacking glucose and supplemented with 0.1% BSA and 20 mM HEPES, pH 7.2. The basal incubation of cells was for 1 h at 37°C in 4 ml RPMI containing 0 mM glucose, 0.1% BSA, 20 mM HEPES and 100 µM diazoxide (Sigma Chemical Co.). Basal secretion samples were collected and aliquotted for insulin immunoassays and HPLC analysis of insulin species. This was followed by the stimulated incubation of cells for 1 hour at 37°C in 4 ml RPMI with 5 mM glucose, 0.1% BSA, 20 mM HEPES, 10 mM each leucine, arginine and glutamine, 100 µM carbachol (Sigma Chemical Co.) and 100 µM IBMX. Stimulated secretion samples were then collected and aliquotted. Cells were returned to a basal incubation for 1 hour at 37°C in 4 ml RPMI containing 0 mM glucose, 0.1% BSA, 20 mM HEPES and 100 µM diazoxide.

Cells were then collected for determination of insulin content and cell number by addition of EDTA to the media to a final concentration of 2 mM and pipetting up and down to remove cells. Twenty percent of the cell suspension was taken for determination of DNA content. The remainder of the sample was centrifuged at 220 x g for 5 minutes to pellet the cells. The cell pellet was resuspended in 0.5 ml cold 0.1 M acetic acid/0.1% BSA and sonicated on ice (Setting 2, Sonic Dismembranator 50, Fisher Scientific, Pittsburgh, PA). The sonicate was aliquotted for insulin immunoassays and HPLC analysis of insulin species.

Determination of DNA content and cell number. RIN cells are pelleted and PBS removed. 0.5 ml of DNA extraction buffer (2.0 M NaCl, 2.0 mM EDTA, 40 mM Phosphate buffer, pH 7.4) is added to RIN samples and the RIN cells are sonicated, on ice, for 30 seconds at ~30% power (Fisher 50 watt sonicator). Four microliters of sonicate are then diluted into 1 ml fresh DNA assay dye solution (TNE - 10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 7.4, containing 0.1 μg/ml Hoechst dye 33258 (Polysciences or Molecular Probes), with calf thymus DNA as a standard (Clontech Inc.). Samples are read using a DNA fluorimeter (Hoeffer Scientific Instruments, Model TKO100). 6 μg genomic DNA per 10^6 cells was used for the conversion from DNA content to cell number values.

HPLC analysis of insulin processing intermediates. Acid/ethanol extracts of whole cells or conditioned media was prepared and analyzed by high performance liquid chromatography as described (Halban *et al.*, 1986, Sizonenko and Halban, 1991). Immunoreactive insulin (IRI) species were quantitated by radioimmunoassay as described (Halban *et al.*, 1986).

Tumor formation of transfected RIN cell lines in nude rats. Six to 8 week old athymic Fisher nude rats (Strain F344/Ncr-rnu from National Cancer Institute, Fredrick, MD) were housed in a sterile isolation facility with free access to sterile standard laboratory chow and water. Three million cells were injected subcutaneously at two different locations in 100 μl PBS using a 1.0 cc U-100 insulin syringe (Becton Dickenson). Tumors were excised once they were palpable, excess fat and associated tissue dissected away. Samples frozen prior to processing. Body weight and bleeds for blood glucose determination were taken prior to injecting cells and throughout the course of the experiment. Blood glucose was measured using an IBI Biolyzer (Kodak, Eastman Chemical Co.).

Results:

Rat insulinoma cells have been engineered to produce high levels of human insulin. The RIN cell line was derived from a radiation-induced tumor (Gazdar *et al.*, 1980). The insulin secretory characteristics of the parental cells used in these studies, RIN 1046-38, have been

described and shown to exhibit abnormal sensitivity to glucose (Clark *et al.*, 1990 and Ferber *et al.*, 1994). These cells secrete insulin at glucose concentrations of 50 μM, secreting 2-10 ng rat insulin/million cells/hour. This level of insulin is well below levels produced by primary rat or human islets (Rhodes and Halban, 1988 and Marchetti *et al.*, 1994) or other reported rodent insulinoma lines (Asafari *et al.*, 1992, Knaack *et al.*, 1994). RIN 1046-38 cells were stably transfected with an expression plasmid containing a human insulin cDNA driven by the human cytomegalovirus promoter (pCB6/INS). One clone, R5C.I-17, was selected based on high insulin secretion and further characterized. FIG. 4A. shows the total immunoreactive insulin content as measured by RIA of R5C.I-17 versus the parental RIN cell line. R5C.I-17 has a total insulin content of 450 ng per million cells, 3-fold above parental RIN.

Chronic culture of rat insulinoma cells in sodium butyrate has been shown to increase endogenous insulin message, content and secretion (Swarovsky *et al.*, 1994). To determine if similar increases would result from a human insulin transgene in rat insulinoma cells, R5C.I-17 cells were cultured for 7 days in 1.0 mM sodium butyrate. Cell growth was retarded but continued over the course of the week. Insulin content was determined at the end of the week and showed a 3-fold increase per cell above the untreated cells (FIG. 4A), consistent with data on the increase in content of endogenous insulin (Swarovsky *et al.*, 1994). This higher level of human insulin content suggests that the RIN 1046-38 cells are capable of synthesizing and storing more human insulin. Sodium butyrate treatment is also used to transiently induce insulin production in the large scale bioreactor.

To increase the level of production of human insulin, R5C.I-17 cells were stably transfected a second time with pRIP7/INS. This expression plasmid utilizes the rat insulin 1 promoter driving expression of human insulin. EP11.3E9 was identified based on an increased insulin production above R5C.I-17 and characterized further. The insulin content of EP11.3E9 is 1400 ng per million cells, four times higher than its parent, R5C.I-17 or RIN (FIG. 4A).

Human insulin, like endogenous rat insulin, is secreted via the regulated pathway in the engineered RIN cell lines. Insulin secretion values for a one hour pre-incubation in buffer alone

(basal values) followed by a one hour incubation in a cocktail of IBMX, glucose and amino acids (stimulated levels) are shown in FIG. 4B. Low basal insulin secretion is seen from R5C.I-17 and EP11/3E9, even with the human insulin transgene constitutively expressed by the CMV promoter. A higher basal secretion is seen from the butyrate-treated R5C.I-17 cells. However, in all lines, insulin release was significantly increased following stimulation to levels of 150, 425 and 450 ng per million cells per hour from R5C.I-17, butyrate-treated R5C.I-17 and EP11/3E9, respectively. Stimulated insulin release per hour ranges from 25 to 35% of the intracellular stores for all four RIN lines, a value consistent with primary islet data (Curry, 1986 and Li *et al.*, 1994). R5C.I-17 has maintained its insulin output through more than 100 population doublings without drug selection (approximately one year in culture).

Results from transgeneic animals (Schnetzler *et al.*, 1994) and from cell lines (Halban and Wollheim, 1980) have supported the idea of a physiological set point for insulin production in β-cells. However, a threshold or upper limit on insulin production in the current engineered RIN cells has not been observed.

Human proinsulin is efficiently processed to mature insulin by rat insulinoma cells. Intracellular insulin species were isolated from parental RIN, R5C.I-17 and EP11/3E9 cells by acid extraction. Separation by HPLC of the insulin species produced by these cells was done as described (Halban *et al.*, 1986, Sizonenko and Halban, 1991). The analysis indicates that human insulin produced by the rat insulinoma is efficiently processed to mature insulin with very low detectable levels of pro-insulin or other processing intermediates (FIG. 5A, FIG. 5B, FIG. 5C). Processing of human insulin is slightly less efficient compared to the processing of rat insulin at these levels of production. While the percentage of intracellular rat proinsulin and intermediates is 3 to 4% of total rat insulin in all cell lines examined, the percentage of intracellular human proinsulin and intermediates is 8% in R5C.I-17 and approaches 18% in EP11/3E9. The ability of RIN cell lines to efficiently process proinsulin to mature insulin in these engineered lines demonstrates the maintenance of the high levels of expression of the endoproteases PC2 and PC3, known to be responsible for insulin processing (Vollenweider *et al.*, 1995). This is an important feature of the RIN cell lines being developed.

Expression of human insulin transgene is stable *in vivo*. RIN cells injected subcutaneously into a nude rat will form tumors. These tumors can then be excised and analyzed for gene expression. As has been seen previously, the majority of the tumor mass is RIN cell in origin with only small numbers of cells being host-derived in the form of endothelium, fibroblasts, *etc.* (Schnedl *et al.*, 1994). This allows for a convenient model for analysis of both endogenous and transgene expression in RIN cells *in vivo*. In the absence of any restraint of tumor growth, time points are restricted to one to two months because RIN cells secrete increasing amounts of rat, and in our studies human insulin, leading to hypoglycemia. Blood glucose levels were monitored throughout the course of the experiment.

One and a half million R5C.I-17 cells were injected subcutaneously at two sites per animal. The animals quickly become hypoglycemic in 8 to 10 days following this dose of cells (FIG. 6). Following the onset of hypoglycemia, animals are maintained on glucose in their drinking water as well as IP glucose injections prior to surgery. One animal demonstrated a blood glucose rebound following removal of both tumors. This rebound after two days of exogenous insulin-induced hypoglycemia is followed by the rapid removal of the exogenous insulin source.

Nine tumor masses in all were isolated from four animals between days 13 and 31. These tumors ranged in size from 40 to 200 mg in wet weight. RNA was isolated from tumors and expression of several gene products was analyzed and compared to uninjected cells maintained in tissue culture (*in vitro*). Primer extension analysis was used to compare the rat and human insulin signals in the same samples. The same primer hybridizes efficiently to both messages, but upon primer extension gives two different size products which can easily be resolved and quantitated. The results of this analysis are shown in FIG. 7. The same amount of starting RNA was used in each reaction from either cells maintained *in vitro* or from tumors, but contamination of non-RIN cells may cause the rat and human insulin signals to be underrepresented in the tumor samples. No attempt was made to correct for this. The signal for the human insulin transgene driven by the CMV promoter was very constant throughout the time points examined. No

diminution of signal was apparent, suggesting the *in vivo* environment had no deleterious effects over the course of this experiment. Subsequent experiments have analyzed tumors at time points of 48 days (versus 31 days here) with no diminution in CMV driven/human insulin transgene expression.

To further test the stability of the CMV driven transgenes *in vivo*, engineered RIN cells were implanted into nude rats and transgene expression assessed with time. Several independent cell lines were implanted into multiple animals, expressing at least three different transgenes. The use of independent cell lines with different integration sites should give an unbiased answer to the issue of CMV promoter stability in RIN cells in this particular model. Longer time points of 48 days have been analyzed with no reduction in CMV driven expression. The *in vivo* model of implanting RIN cells into nude rats is limited by the uncontrolled growth of the RIN cells as a tumor. All the RIN lines used here make endogenous rat insulin, with some also making human insulin so that the animals quickly become hypoglycemic. Efforts were made to maintain the animals blood glucose by administering glucose in the drinking water or by i.p. injections, allowing analysis of tumors at longer time points. Alternatively, lower doses of cells can be injected initially (3×10^5 rather than 3×10^6) which is how a 48-day tumor was generated and analyzed for maintenance of gene expression. Unfortunately, this lower dose of injected cells leads to a more sporadic tumor growth, making it harder to generate samples for analysis.

Surprisingly, endogenous insulin expression increased in all nine *in vivo* samples examined. This was unexpected since all nine tumors were excised following periods of extreme hypoglycemia, conditions known to down regulate pancreatic β-cell insulin message (Giddings *et al.*, 1982 and Brunstedt and Chan, 1982). Comparison of the ratio of rat to human insulin signals changed from 0.73 +/- 0.6 for several *in vitro* samples to 1.87 +/- 0.17 for the nine tumor samples. The 0.73 *in vitro* ratio correlates very well with the ratio of rat to human insulin (1 part rat to 1.5 parts human) observed for the R5C.I-17 cell line (see FIG. 5 for mass ratios). The increased rat to human message signal in tumors is paralleled by an increased rat to human insulin content in tumors subjected to acid extraction and HPLC separation of the insulin species.

A similar result was obtained following injection of the high human insulin producing cell line, EP11/3E9, into nude rats. Animals became hypoglycemic while maintaining body weight over the course of the experiment (FIG. 6). Nine tumors were isolated between days 15 and 28 following injection. Primer extension analysis of RNA isolated from the tumors allows for separation of three insulin messages in the EP11/3E9 line. Extension of the endogenous rat insulin message and the human insulin message driven by the CMV promoter produced the identical pattern as seen in R5C.I-17, the parent cell line to EP11/3E9. A third extension product results from expression of the human insulin transgene message by the rat insulin 1 promoter. Primer extension analysis on the tumor samples as well as the cell lines maintained *in vitro* show human insulin driven by the CMV promoter is stable throughout the course of the *in vivo* experiment. Again, endogenous rat insulin is upregulated in the *in vivo* environment, even in the face of hypoglycemia. The human insulin transgene driven by the rat insulin promoter is expressed throughout the course of the *in vivo* experiment.

In vivo potency of engineered RIN cell lines. When the engineered RIN lines are growing as tumors in the nude rats, their secreted insulin eventually impacts on the blood glucose levels of the healthy animals causing hypoglycemia (FIG. 6). While parental RIN cells have endogenous rat insulin outputs that eventually lead to hypoglycemia, RIN cells engineered to overexpress human insulin should induce hypoglycemia either faster with the same number of cells or require a smaller tumor mass. The inventors measured the tumor mass needed to induce the initial hypoglycemia in a nude rat as an indicator of *in vivo* potency of the engineered RIN cells.

Tumors were removed from the nude rats injected with either the R5C.I-17, EP11/3E9 or parental RIN 1046-38 at the first sign of hypoglycemia. The time between injection of a constant number of cells to hypoglycemia varied from 12 to 13 days for R5C.I-17 and EP11/3E9 (FIG. 6) to 28 days for the parental cells. All of the lines grow at the same rates *in vitro*. A plot of the tumor mass *versus* the *in vitro* stimulated insulin secretion values for these lines (FIG. 4B) is shown in FIG. 8. The higher the *in vitro* insulin output (both rat and human insulin), the smaller the tumor mass needed to cause hypoglycemia.

Endogenous GLUT-2 expression in RIN cells is lost *in vivo*. The expression of several other genes in the tumor samples was analyzed and compared to RIN cells maintained *in vitro*. The results of the analysis of both endogenous genes and introduced transgenes is shown in FIG. 9. RNA from two independent tumor samples from day 24 and day 25 were combined (*in vivo* sample) and compared to RNA from R5C.I-17 cells maintained in tissue culture (*in vitro* sample). Message levels of endogenous glucokinase, hexokinase I, amylin, GAPDH, sulfonylurea receptor and IPF1, as well as message levels of human insulin and the neomycin resistance transgenes, were relatively unchanged following 24 to 25 days of *in vivo* passage of R5C.I-17 cells. In contrast, the message level of endogenous GLUT-2 detected in R5C.I-17 cells maintained *in vitro* is completely absent in tumors at the 24/25 day time point. This result was duplicated on a separate analysis for confirmation (GLUT-2 a and b, FIG. 9). Analysis of individual tumors from day 13 through day 31 demonstrated expression of GLUT-2 was already absent at the earliest time point analyzed and remained absent throughout the remainder of the experiment.

Loss of *in vivo* GLUT-2 expression could have serious consequences for the performance of cell lines designed for insulin delivery to animals or patients with diabetes. Stable transfection of insulin producing cell lines with the GLUT-2 cDNA has been shown to confer glucose-stimulated insulin secretion (GSIS), while transfection of the same cells with a related transporter, GLUT-1, has no such effect (Hughes *et al.*, 1992, 1993; Ferber *et al.*, 1994; U.S. Patent 5,427,940). Furthermore, loss of GLUT-2 expression has been documented in a large number of rodent models of type II diabetes (NIDDM) in which β-cell failure involving loss of GSIS is a cause of hyperglycemia (Johnson *et al.*, 1990; Orci *et al.*, 1990; Thorens *et al.*, 1990; Unger, 1991). Endogenous GLUT-2 expression is apparently down-regulated or extinguished under diverse physiological conditions. In addition to the studies cited above, in which animals were hypoglycemic, implantation of normal islets from db/- mice into diabetic, hyperinsulinemic db/db mice or db/- mice rendered diabetic and hypoinsulinemic by injection of the β-cell cytotoxin, streptozotocin, resulted in loss of GLUT-2 expression in the transplanted islets (Thorens, 1992b). These results suggest that loss of GLUT-2 may also be responsible for impaired glucose responsive insulin release in human islets transplanted into patients with Type I

or II diabetes (Scharp *et al.*, 1994). Reduced GLUT-2 suppression in the face of hyperglycemia is surprising in light of recent studies demonstrating that the GLUT-2 promoter is activated by glucose in hepatocytes or the insulinoma cell line INS-1 (Waeber *et al.*, 1994). Overall, these studies strongly imply that the GLUT-2 promoter is adversely effected by various metabolic perturbations *in vivo*, and that this promoter element is not appropriate for use in directing stable expression of heterologous genes in cell lines.

FIG. 10 illustrates that GLUT-2 expression can, in fact, be maintained in RIN cells implanted into nude rats for relatively prolonged periods of time if the gene is stably transfected under the control of a viral promoter such as CMV. A RIN 1046-38 clone expressing high levels of rat GLUT-2 driven by the CMV promoter was generated using pCB7/GLUT-2 (clone EP49/206) as previously described (Ferber *et al.*, 1994). Animals injected with RIN EP49/206 form solid tumors and become hypoglycemic, much as reported for animals receiving cells containing only the endogenous GLUT-2 gene. Unlike the untransfected cells, however, GLUT-2 mRNA levels are maintained at a high, constant level over the two time points sampled, 16 and 34 days (FIG. 10A). These particular cells also were stably transfected with plasmids containing the human insulin and glucokinase cDNAs under control of CMV, and transcript levels for these other transgenes were maintained in a stable fashion, analogous to GLUT-2. These results indicate that cell lines transfected with multiple genes under control of a strong viral promoter like CMV are able to maintain stable expression of all transgenes for prolonged periods of time when transplanted into animals. These results are surprising and would not have been expected in light of previous studies from other groups who have reported that strong viral promoters such as CMV or RSV are often down-regulated in the *in vivo* environment (Palmer *et al.*, 1989 and 1991, Scharfmann *et al.*, 1991, Challita and Kohn, 1994).

Increased insulin production using expression plasmids containing an internal ribosome binding site. A new insulin expression plasmid was designed that links the expression of the drug selection marker to the expression of insulin. The plasmid, pCMV8/INS/IRES/NEO, utilizes the CMV promoter to drive a bicistronic message containing the human insulin open reading frame upstream of the neomycin resistance open reading frame. Placed between the two

reading frames is a portion of the 5′-transcribed leader of the gene encoding human Glucose Regulated Protein 78 (GRP78; Ting and Lee, 1988). The 5′-leader of human GRP 78 has been shown to direct internal initiation of translation (Internal Ribosome Entry Site, IRES) allowing for construction of functional polycistronic genes in mammalian cells (Macejak and Sarnow, 1991). In order to generate Neo-resistant cells with this plasmid, the human insulin message must also be present, increasing the number of RIN clones that express human insulin protein. Since internal initiation of translation by IRES elements is less efficient than normal 5′ cap-dependent initiation (Macejak and Sarnow, 1991), cells must express high levels of the bicistronic transgene in order to survive drug selection. In this way, it should be possible to directly select with G418 clones expressing high levels of human insulin.

Twenty-nine independent G418 resistant clones from an electroporation of parental RIN 1046-38 cells with pCMV8/INS/IRES/NEO were screened for insulin content following acid extraction as described. The results are shown in FIG. 11 with the insulin content of R5C.I-17 (450 ng/million cells, FIG. 4A) used for comparison. Twenty-nine out of 29 clones expressed detectable levels of human insulin with at least 10 out of 29 of the clones (34%) expressing levels of human insulin more than 2-times that of R5C.I-17. RNA was isolated from the 5 highest insulin producing clones and human insulin message analyzed using primer extension. Starting inputs of 10 and 3 μg of RNA from these 5 clones, as well as from a polyclone from this electroporation, were compared to 10 μg of RNA from R5C.I-17. In the 5 monoclones as well as the polyclone, high levels of human insulin message were detected at the expected size of 280 base pairs with three other minor premature termination signals of approximately 190, 130 and 115 bases. Even with 3 μg of input RNA, the human insulin signal is still comparable to the signal from 10 μg of RNA from R5C.I-17, a level of human insulin message in these clones in line with the higher levels of insulin protein.

One clone, EP18/3E1 has been further characterized. The insulin content of EP18/3E1 is 1300 ng per million cells with a stimulated insulin secretion rate of 500 ng/million cells/hour. These levels of insulin are comparable to those achieved in EP11/3E9, our highest insulin

producing clone to date (FIG. 4). However, in contrast to previous insulin producing clones, EP18/3E1 and other high insulin producing clones (FIG. 11) were generated from one round of electroporation using a single expression plasmid. The utility of the expression plasmid pCMV8/INS/IRES/NEO is in both the high numbers of positive clones and the higher insulin outputs of individual clones. Also, only one drug selection marker was used as opposed to two in generating EP11/3E9.

Introduction of a second human insulin transgene into R5C.I-17 cells produced 11/3E9, a cell line with higher insulin production. Similarly, a second insulin construct was expressed in 18/3E1 cells to produce clones with increased insulin output. The construct consists of the human insulin gene linked to the puromycin resistance gene and the transcription of the bicistronic message produced is controlled by the CMV promoter. Colonies of cells that grew after selection in 2 ug/ml of puromycin were screened for increased insulin output. FIG. 12A demonstrates the expression of human insulin RNA of both bicistronic transgenes, and the increased insulin content for 5 selected clones. The cell line EP111/220 exhibited the highest cellular insulin content (FIG. 12B) and secreted the most insulin. The EP111/220 clone when incubated with the stimulation cocktail of mixed nutrients and secretagogues (as for FIG. 5B) secreted 0.99 μg insulin/10^6 cells-hour. Currently, EP111/220 represents the highest documented insulin secretion of our cells engineered with human insulin.

The insulin content and secretory output of human islets may be estimated from reports in the literature . The average human pancreas contains about 0.9 g of islets (K. Saito *et al.*, 1978) which equals 9 x 10^8 cells (Finegood *et al.*, 1995), and the average human pancreas contains 200 U of insulin (with a 3-fold range; Wrenshall *et al.*, 1952). Thus, when *in situ*, the insulin content of the average human islets approximately 0.22 U/10^6 cells, or 8 μg/10^6 cells. Freshly isolated human islets are reported (Eizirik *et al.*, 1992 and 1994) to contain 8-10 μg/6 μg DNA (6 μg =10^6 cells). The same authors report that after one week of culture human islets contain 4-5 μg/6 μg DNA and with stimulation secrete 459 ng/6 μg DNA/h. Freshly isolated rat islets, for comparison, are reported to contain 4-8 μg insulin/6 μg DNA, and with stimulation secrete 0.2 to

2 μg insulin/6 μg DNA (Tokuyama *et al.*, 1995; Rhodes and Halban, 1988; Nielsen, 1985). The functionally-normal mouse β-cell lines secrete 400-800 ng insulin/h upon stimulation, and contain 3-10 μg insulin/10^6 cells (Miyazaki *et al.*, 1990; Radvanyi *et al.*, 1993; Knaack *et al.*, 1994). The values presented for human islet insulin content and secretion are expected to represent the higher end of the range, because human islets are known to be less potent than rodent islets, both *in vitro* (Smith and Wilson, 1991) and *in vivo* (Jansson *et al.*, 1995). The cell line EP111/220 has an insulin content that appears to be 60-75% of the value presented for cultured human islets, while insulin secretion appears to surpass that of cultured human islets.

The humanized β-cell lines generated in these studies exhibit a number of unique characteristics. First they express only one of the two rodent insulin genes (Fiedorek *et al.*, 1990 and the inventors' data), which will be advantageous in knockout development of complete insulin-humanized β-cell lines. Second, the present engineered lines have the capability to increase insulin secretion 10- to 20-fold in response to stimuli. This characteristic is similar to that of β-cell lines derived from SV40-T antigen transgenic mice such as MIN6 (Miyazaki *et al.*, 1990), and βHC (Radvanyi *et al.*, 1993) cell lines as well as normal β-cells (Curry, 1986). Third, these cells maintain essentially normal processing of human proinsulin, even though the exogenous protein is in excess of endogenous rat protein. Normal processing is not present in INS-1 (Neerman-Arbez *et al.*, 1993) and βTC cells (Nagamatsu and Steiner, 1992) two β-cell lines that have been examined for this property. Finally, the present cell lines demonstrate that iterative introduction of the insulin gene provides an approach whereby human insulin output can be stably achieved which (at minimum) matches that of cultured human islets.

Analysis of other promoter/enhancer elements for driving insulin expression. Several other enhancer/promoters were compared to the CMV enhancer/promoter for their ability to direct transcription of the same bicistronic message (5′-intron/hINScDNA/IRES/NEO/hGH/3′-polyA) in stably transfected RIN38 cells. These promoters include the rat insulin 1 gene promoter (RIP), modified RIP (FFE/RIP), RIP linked with the rat insulin 1 gene intron (RIP/RIPi) in place of the hybrid adenovirus/immunoglobulin 5′-intron, the Rous Sarcoma Virus

Long Terminal Repeat (RSV), the human glyceraldehyde-3-phosphate dehydrogenase promoter (GAPDH), and the mouse metallothionein promoter (MT). Expression plasmids were constructed by removing the CMV promoter found in pCMV8/INS/IRES/NEO and replacing it with the promoter to be tested. In this way, message levels and insulin outputs from the RIN clones constructed with the various promoters can be compared directly.

RIP activity is approximately 30- to 50-fold lower than that of the CMV promoter in transiently transfected RIN38 cells. However, in stably transfected RIN38 cells, RIP activity is much closer to the activity of the CMV promoter. The level of human insulin (hINS) mRNA derived from pRIP8/hINS/IRES/NEO is, on average, approximately only 3- to 5-fold lower than levels obtained from stable RIN38 lines containing pCMV8/hINS/IRES/NEO. The Northern blot depicted in FIG. 13 demonstrates this result as the level of hINS mRNA from two pRIP8/hINS/IRES/NEO RIN lines, 2.18 and 2.38, is only 3-fold lower than the level of hINS mRNA from the pCMV8/hINS/IRES/NEO RIN line, EP18/3E1. As stated earlier, the EP18/3E1 line has a very high insulin content, approximately equivalent to that of a normal human β-cell. Therefore, in addition to the CMV promoter, RIP offers another choice as a strong transcriptional activator.

RIP also was modified in an attempt to make it an even stronger transcriptional activator. The principal modification made to RIP was the attachment of Far-FLAT mini-enhancers (FF mini-enhancer). The FF mini-enhancer is located between -247 and -198 of RIP and contains several cis-acting regulatory elements crucial for RIP activity in b cells (Karlsson *et al.*, 1987; Karlsson *et al.*, 1989). The FF mini-enhancer region contains both the Far box (-239 to -230) and the FLAT element (-222 to -208) which further consists of two adjacent regulatory motifs, FLAT F and FLAT E. When isolated from the rat insulin 1 gene promoter and multimerized to yield 5 linked copies, the FF mini-enhancer is almost as active as an intact RIP in transiently transfected β-cells (German *et al.*, 1992). Three base changes in the FLAT E motif at positions -209, -211, and -213 can further increase the activity of the FF minienhancer (now called FFE minienhancer) approximately 3-fold in transiently transfected β-cells (German *et al.*, 1992). A transient transfection system with RIN38 cells was set up for initial screening of modified RIP

promoter/enhancers. Results from the transient transfections utilizing a human growth hormone (hGH) reporter gene demonstrated that two modified RIP enhancer/promoters were 5-fold more active than RIP. The two modified RIP enhancer/promoters consisted of an intact RIP (-415 to +1) to which either three or six copies of FFE minienhancers had been attached just upstream of -415 of RIP (the FFE six-mer is in the reverse orientation with respect to RIP). Coexpression of the RIP transcription factor, IPF-1, along with either pFFE3/RIP/hGH or pFFE6/RIP/hGH produced an 8-fold increase in activity over that of RIP alone.

To test whether or not the FFE-modified RIP enhancer/promoters would increase RIP activity in stably transfected RIN38 cells to the same extent as was demonstrated in transiently transfected RIN38 cells, FFE3/RIP was placed into the 5'-intron/hINScDNA/IRES/NEO/hGH/3'-polyA stable-transfection vector. A large number of RIN38 cell lines containing pFFE3/RIP8/INS/IRES/NEO were analyzed for FFE3/RIP activity. A number of clonal lines expressed higher human insulin mRNA than was observed for the best pRIP8/INS/IRES/NEO lines. Phosphoimager analysis of the Northern blot shown in FIG. 13 demonstrated that FFE3/RIP clones 4.17 and 4.32 produced approximately 2-fold more hINS than the highest-producing RIP lines 2.18 and 2.38. Therefore, these data demonstrate that RIP activity was enhanced in stable RIN lines by the addition of 3 FFE mini-enhancers, although not to the same extent as was shown in the transient transfection system. pFFE6/RIP8/INS/IRES/NEO is currently being introduced stably into RIN38 cells. Attempts to stably coexpress IPF-1 are also underway and are discussed below.

A second modification to RIP occurred by placing the rat insulin 1 gene intron (RIPi) immediately downstream of the transcriptional start site. It was previously noted that RIP activity was significantly increased in transgenic mice and, to a lesser extent, in cultured β-cells when combined with RIPi. A large number of stable RIN38 lines transfected with pRIP8/RIPi/INS/IRES/NEO were established and examined for hINS mRNA levels. As was observed for the FFE3 minienhancer, on average, the addition of RIPi to RIP yielded a modest but significant increase in hINS mRNA levels. The RIP/RIPi line, 2.65, expressed a level of hINS mRNA equivalent to the CMV promoter line, 18/3E1, and three times more hINS mRNA

than the 2.18 and 2.38 RIP lines (FIG. 13). Since the addition of either RIPi or the FFE mini-enhancers enhances RIP activity, then combining both RIPi and FFE mini-enhancers with RIP could result in an additive increase of overall RIP strength. To test this idea, pFFE6/RIP8/RIPi/INS/IRES/NEO has been constructed and stably transfected into RIN38 cells. p(RIE)$_3$/-85RIP/RIPi/INS/IRES/NEO, a plasmid which contains both RIPi and three full-length rat insulin 1 gene enhancers instead of mini-enhancers, has also been constructed and transfected into RIN38 cells. FFE6/RIP/RIPi did act as a strong transcriptional activator but was only slightly stronger than either FFE6/RIP or RIP/RIPi alone. Interestingly, the tandemly linked full-length RIP enhancers were very weak transcriptional activators when p(RIE)$_3$/-85RIP/RIPi/INS/IRES/NEO was stably integrated into RIN38 cells. This had not been the case in transiently transfected RIN38 cells in which the three linked RIP enhancers produced high-level expression of a linked reporter gene.

Another RIP derivative, pILPR/RIP8/INS/IRES/NEO has also been constructed in an attempt to generate a more potent insulin promoter. The human Class III insulin-linked polymorphic region (ILPR) is composed of 139 tandemly-repeated 14 bp sequences and lies immediately upstream of the human insulin gene promoter/enhancer (Owerbach and Aagaard, 1984). It has recently been demonstrated that the presence of the Class III ILPR significantly increases the transcriptional activity of the human insulin promoter/enhancer (Kennedy *et al.*, 1995). Likewise, fusing the Class III ILPR to RIP may also increase RIP activity. pILPR/RIP8/INS/NEO has been constructed and stably introduced into RIN38 cells. Analysis of polyclonal and monoclonal lines containing pILR/RIP8/INS/IRES/NEO demonstrate that the human Class III ILPR had no significant effect on RIP activity.

pRIP8(O_2)7 is a modified RIP that has been altered by inserting seven copies of the operator site [(O_2)7] from the *E. coli* tetracycline (*tet*)-resistance operon between the RIP enhancer and promoter at position -85. The tetracycline-resistance operon regulatory system (Gossen and Bujard, 1992) is a binary system in which a transactivator protein is also required. The transactivator is a combination of the tet repressor (tetR), which binds very tightly to tet operator sites, fused to the transcriptional activation domain of virion protein 16 (VP16) from

herpes simplex virus. Both pRIP8(O2)7/RIPi/INS/IRES/NEO and an expression plasmid containing the tetR-VP16 transactivator will be stably transfected into RIN38 cells. Precedence for this type of scheme was recently demonstrated when the activity of the already potent CMV promoter was increased another 10-fold by inserting seven tet operator sites between the enhancer and promoter followed by cotransfection with the tetR-VP16 transactivator (Liang *et al.*, 1995).

The transcriptional activity of promoters other than CMV, RIP, and RIP derivatives also has been analyzed. Stable RIN38 lines were established which contained the promoter from the Rous Sarcoma Virus Long Terminal Repeat (RSV) driving the standard hINS/IRES/NEO stable transfection vector. In general, the RSV promoter produced hINS mRNA levels roughly equivalent to those produced by RIP. Therefore, the RSV promoter, like the CMV promoter, RIP, and RIP derivatives, acts as a strong transcriptional activator in RIN cells in culture. The human glyceraldehyde-3-phosphate dehydrogenase promoter (GAPDH) was also tested in stably transfected RIN38 cell lines and found to be a weak transcriptional activator. In most GAPDH promoter lines, hINS mRNA was either barely or not detectable by Northern blot analysis.

Promoter stability *in vivo*. As described earlier for the CMV and RIP promoters, the activity for some of the RIP derivatives, RSV, and GAPDH promoters was analyzed *in vivo* by subcutaneous injection of engineered RIN 1046-38 lines into athymic Fisher nude rats. *In vivo* activity of RIP was also reanalyzed, but this time without the presence of a CMV driven transgene as was the case for RIN line EP11/3E9. Time points were again restricted to one to two months as most of the animals developed hypoglycemia by two weeks after injection. The data from these experiments is summarized below.

In vivo RIP activity was examined for two independent RIN lines containing the pRIP8/INS/IRES/NEO transgene. Each line was injected into two individual nude rats. Animals containing either line became hypoglycemic between one to two weeks after injection. Tumors were excised at different intervals, homogenized, and analyzed for hINS mRNA levels by Northern blotting. The amount of hINS mRNA remained constant out to the longest examined

time points, 31 days for line 2.18 and 36 days for line 2.38. Therefore, RIP activity remained stable throughout the length of the experiment. The same results were obtained for the modified RIP promoter/enhancers, RIP/RIPi and FFE3/RIP. Both RIP/RIPi and FFE3/RIP produced constant levels of hINS mRNA out to the longest time point of 49 days.

The activity of the RSV promoter appears to be attenuated *in vivo*. Despite the formation of medium to large tumors, neither animal injected with the 3.4 line became hypoglycemic even after 36 days. Presumably, if analyzed at later time points, these animals would become hypoglycemic due to the endogenous expression of rat insulin from the engineered RIN lines. Both animals injected with the 3.34 line eventually did become hypoglycemic but it took much longer (20 to 30 days) than it did for the RIP and modified RIP lines (10 to 15 days). These data suggest that although the RSV enhancer/promoter is a strong transcriptional activator in cultured RIN cells, it may be unsuitable to direct the expression of a linked transgene in RIN cells in an *in vivo* situation. Further *in vivo* testing of RSV promoter activity utilizing a transgene other than the human insulin cDNA is presently underway.

GAPDH promoter activity remained stable *in vivo* out to the longest time point of 22 days. Both animals injected with the 4.5 line (the GAPDH line that produced the highest level of insulin mRNA) started to become hypoglycemic by 13 to 15 days. This result was somewhat surprising based on the relatively low abundance of hINS mRNA expressed in this line.

A concern with the use of the viral promoters is their long-term stability of expression *in vivo*. There are numerous reports concerning loss of transgene expression *in vivo*, either following introduction of genes *in vivo* with recombinant viruses or introduction of genes into cells *ex vivo* followed by implantation of the cells *in vivo* (Palmer *et al.*, 1989 and 1991, Scharfmann *et al.*, 1991, Challita and Kohn, 1994). This second scenario is analogous to the proposed use of the cell lines being developed here for therapeutic use.

Interestingly, the RSV promoter driving transgenes in RIN clones appear to be attenuated *in vivo*. The mechanism for this attenuation is not clear. Evidence suggests that some of the

problems with long term stability of expression of transgenes driven by viral promoters is due to immune recognition and ultimately rejection of the engineered cells (Dai *et al.*, 1995, Yang *et al.*, 1994). Immune recognition could be directed against the transgene product itself or against other antigens expressed following introduction of the transgenes (*i.e.*, low level viral protein expression from recombinant viral transductions). However, in these studies using nude rats, there is no immune rejection of the implanted cells.

Cell growth, insulin content, and processing in bioreactors. The oxygen gas controller output is monitored throughout the run. It is an indirect indication of the cells' oxygen consumption rate. It rises steadily from around -40 at 0 hours to around 60 at approximately 500 hours where it stabilizes for the rest of the run. The rate of increase of the controller output correlates with an expected growth rate of the culture, and maximum level of 60 is consistent with achieving a cell density of 1.1-2.3 x 10^8 cells per ml of bed volume. The cell densities are confirmed at the end of the culture. With a surface-to-volume ratio of 120 cm^2/cm^3, the polyester disc bed yields a surface cell density comparable to that obtainable in two dimensional T flask culture. It is important to note that the growth and the sustained densities in the reactor are achieved using a serum free media. High density cultures have been maintained problem free for up to 2000 hours in serum free medium. This observation is novel and very useful in the design of a bulk process for production of biological pharmaceuticals.

Cells harvested from the reactor at the end of culture by trypsinization, plated onto T75 culture flasks, and assayed for insulin secretion performance after 24 hours of culture, show no significant difference relative to sister cells maintained in T75 flask culture, suggesting that the bioreactor milieu is not changing the cells' phenotype in any detectable fashion and that the cells quickly readapt to culture in tissue culture flasks.

HPLC separation of samples collected mid-run at around 550 hours of culture showed effective insulin processing. The ratio of mature human insulin to human proinsulin was 92:8. This efficient processing is obtained from a culture that has reached a steady state of oxygen uptake, indicating no overall growth, and that is sustained in a serum free medium.

The bioreactor data indicates that the steady state environment in the reactor allows for growth of up to approximately 2 x 10^8 cells per ml bed, while maintaining pathways crucial for complete processing and storage of insulin.

EXAMPLE 4

Human insulin disulfide mutant production

Methods:

Human insulin disulfide mutant expression plasmid. The human insulin open reading frame was amplified with the polymerase chain reaction from a human insulin cDNA using oligos 1 and 2 (CCGGGGATCCTTCTGCCATGGCCC, SEQ ID NO:38 and GGGCTAGATCTAGTTGCTGTAGTTCTCCAGCTGGTAGAGGGAGCAGATGCTAGTACT GCATTGTTCCAC, SEQ ID NO:39) generating a 359 base product (SEQ ID NO:3). Oligo 1 introduces a *Bam*HI site 7 bases upstream of the initiator methionine of insulin. Oligo 2 introduces a *Bgl*II site just downstream of the insulin stop codon and introduces two point mutations into the insulin coding region. These mutations change cysteine at position 96 and cysteine at position 109 to serines (SEQ ID NO:4). Both of these amino acid substitutions are in the insulin A chain and disrupt the two disulfide bonds normally formed between the A and B chains. The mutated insulin protein should be expressed, targeted to the regulated secretory pathway and proteolytically processed to human insulin A, B and C chain. Upon stimulated secretion, the three peptide chains would be released by the cell without the normal disulfide bonds between the A and B chain. As a control, the wild-type human insulin open reading frame was amplified with the polymerase chain reaction from a human insulin cDNA using oligos 1 and 3 (CCGGGGATCCTTCTGCCATGGCCC, SEQ ID NO:38 and GGGCTAGATCTAGTTGCAGTAGTTCTC, SEQ ID NO:40). Again, Oligo 1 introduces a *Bam*HI site 7 bases upstream of the initiator methionine of insulin. Oligo 2 introduces a *Bgl*II site just downstream of the insulin stop codon without introducing any changes into the insulin coding sequence. The resulting 358 base pair PCR™ products were cloned directly into pNoTA/T7 (Prime PCR™ Cloner Cloning System, 5 Prime to 3 Prime, INC.) generating pNoTA/T7/mutINS and pNoTA/T7/wtINS. These plasmids were subsequently restricted with

*Bam*HI and *Bgl*II endonucleases and ligated into *Bam*HI digested pCMV8/IRES/NEO/hGH PolyA, generating pCMV8/mutINS/IRES/NEO and pCMV8/wtINS/IRES/NEO, respectively.

A variation of pCMV8/mutINS/IRES/NEO was created by restoring the normal 3′-untranslated region of the insulin cDNA to its correct position following the insulin disulfide mutant open reading frame. An *Hga*I cleavage site is located 9 bases 3′ of the insulin stop codon, base 364 of SEQ ID NO:1. pBS/INS was digested with *Hga*I, treated with Klenow fragment, and then digested with HindIII. The resulting 198 base pair fragment was ligated into pNoTA/T7/mutINS that had been digested with *Bgl*II, treated with Klenow fragment, and then digested with *Hin*DIII. The resulting plasmid, pNoTA/T7/mutINS+INS3′, contains an essentially restored human insulin cDNA except for the two point mutations introduced into the coding region and a 5 base deletion at the *Bgl*II/*Hga*I cloning junction. This 198 base pair fragment contains 64 bases of the insulin 3′-untranslated region, a 41 base pair poly A tract, a 16 base pair poly C tract and 77 base pairs of polylinker sequence from the subcloning vectors. pNoTA/T7/mutINS+INS3′ was digested with *Bam*HI, generating a 512 base fragment containing the mutant insulin and reconstructed insulin 3′ sequence, which was ligated into the *Bam*HI site of pCMV8/IRES/NEO/hGHPolyA, generating pCMV8/mutINS+3′/IRES/NEO.

Cell culture and stable transfection of cell lines. As described above for insulin producing cells.

Immunohistochemical staining for human insulin C-peptide. Individual G418-resistant RIN clones generated by electroporation using pCMV8/mutINS+3'/IRES/NEO were screened by immunostaining for human C-peptide. Cells were plated on multiwell slides one or more days before staining. Slides with spread cells were rinsed with PBS, then fixed 15-30 minutes in 4% paraformaldehyde. Fixation was followed by a PBS rinse and permeabilization by passage through an ethanol series of 50%-70%-50% (5 minutes each). Permeabilization was followed by a PBS rinse and a 30 minute incubation in 50 mM Tris, pH 7.4, with 1% goat serum, 0.05% Triton and 0.1% azide. Slides were incubated with 1:10,000 dilution of rabbit anti-human C-

peptide (Linco Inc.) for 24 hours. Excess primary antibody was removed with sequential washes (3 minutes each) with PBS-Triton (0.05%), PBS alone, and 50 mM Tris, pH 8.0. The slides were then incubated with an alkaline phosphatase-labeled second antibody (goat anti-rabbit IgG, Sigma Chemicals) in 50 mM Tris with 1% BSA and 1 mM magnesium chloride (Tris-BSA-Mg) for 30 minutes. Excess second antibody was removed with 3 washes of Tris-BSA-Mg. Alkaline phosphatase activity was then visualized by incubating 5 minutes in an alkaline phosphatase substrate solution (BCIP/NBT).

Northern analysis. Northern analysis of mutant insulin transcripts in cell lines was performed as described above for human insulin message detection using a full-length digoxigenin-labeled antisense probe corresponding to the neomycin resistance gene (control template supplied in Genius 4 Kit).

Results:

Recent reports suggest that immunomodulatory treatments with insulin can delay or prevent the onset of hyperglycemia in NOD mice (Shehadeh *et al.*, 1994; Sadelain *et al.*, 1990; Muir *et al.*, 1993). Clinical trials evaluating the prophylactic nature of insulin in humans at high risk for the development of type I diabetes are underway (Keller *et al.*, 1993). Recently, immunization with metabolically inactive insulin B-chain also prevented the onset of hypoglycemia in NOD mice, suggesting an active induction of immunoregulation by insulin. Development of an *in vivo* cell-based delivery system of insulin or metabolically inactive forms of insulin could be used prophylactically in humans at high risk of developing type I diabetes. Cell lines producing and secreting high levels of mature human insulin have already been described here. This would be done in the context of the expression of reduced endogenous rat insulin. Neuroendocrine cells producing an inactive, mutant human insulin, in the context of reduced endogenous rat insulin production, would offer a safer, and possibly more efficacious approach. The use of metabolically inactive insulin would negate the possibility of insulin induced hypoglycemia. Higher amounts of a metabolically inactive insulin could therefore be safely administered *in vivo*, possibly increasing the efficacy of the treatment.

To this end, RIN cells have been engineered to produce a mutant form of human insulin. Insulin is initially produced in the cell as proinsulin, a larger peptide precursor consisting of the linear arrangement of insulin B-chain C-chain A-chain. The maturation of proinsulin to mature insulin is well understood (Halban, 1991) with three major steps in the process. The first is folding of the proinsulin into a native conformation in the immature secretory granules. The second step involves the formation of three disulfide bonds, one intramolecular in the A-chain and two intramolecular between the A-chain and the B-chain. The final step is the endoproteolytic processing by PC2 and PC3 followed by carboxypeptidase processing in the mature secretory granule. The mature granules contain an equimolar mix of C-chain (C-peptide) and mature insulin consisting of a A-chain/B-chain heterodimer covalently linked by the two intramolecular disulfide bonds. A mutant form of insulin was constructed from the human insulin cDNA in which the two codons encoding cysteins in the insulin A-chain have been mutated to codons encoding serines (SEQ ID NO:3). Expression of this mutant open reading frame should produce a mutant insulin peptide (SEQ ID NO:4) that still folds normally, the intrachain disulfide bond in the A-chain can still form, and endoproteolytic processing and carboxypeptidase cleavage can still occur. The mature granules should now contain an equimolar mix of C-chain (C-peptide) and free B-chain and A-chain. The B-chain is identical in sequence to the wild-type human insulin B-chain used in studies showing the prevention of the onset of hypoglycemia in NOD mice (Muir *et al.*, 1995). Stimulated release of the contents of the secretory granules would release all three peptides. Engineering of these RIN cells in the context of reduced rat insulin production would ensure no insulin biologic activity.

EXAMPLE 5

Human Growth Hormone Production

Methods:

Human growth hormone production plasmid. The gene encoding human growth hormone was isolated on a 2086 base *Bam*HI/*Age*I restriction endonuclease fragment from pOGH (Nichols Institute Diagnostics, Inc., San Capistrano, CA). This fragment corresponds to bases 498 to 2579 of the published gene sequence (SEQ ID NO:9, Seeburg, 1982). The *Bam*HI site is located at the

normal site of transcription of the message, 61 bases 5′ of the initiator methionine. The *Age*I site is located 3′ of the transcribed sequences of the growth hormone gene. This fragment was ligated into pCB6 (Brewer, 1994) that had been digested with *Bgl*II and *Age*I, generating pCB6/hGH. The *Bgl*II site places the hGH gene just downstream of the CMV promoter. The *Age*I site in pCB6 is located in the human growth hormone polyadenylation element contained in that plasmid. The polyadenylation element is restored by cloning the entire human growth hormone gene into pCB6. Stable transformants of pCB6/hGH are selected in G418.

Cell culture and stable transfection of cell lines. These studies were performed as described above for insulin producing cells.

Screening and characterization of human growth hormone producing clones. Individual G418 resistant clones generated by electroporation using pCB6/hGH were screened for hGH in the conditioned media using an hGH radioisotopic assay kit (Nichols Institute Diagnostics).

Stimulated growth hormone secretion assay and determination of DNA content and cell number. Done as described for insulin secretion assay and cell number determination.

Results:

Mammalian cell production of human growth hormone. Growth hormone has been shown to be the major regulator of growth in children as well as maintaining or restoring various metabolic functions which can decrease with age (Isaksson *et al.*, 1985 and Arimura, 1994). Purified recombinant human growth hormone is now being produced from mammalian cells in bioreactors for clinical use (Eshkol, 1992). Constitutive cell-based delivery of growth hormone from *ex vivo* engineered primary fibroblasts (Selden *et al.*, 1987 and Heartlein *et al.*, 1994) or primary myoblasts (Dhawan *et al.*, 1991 and Barr and Leiden, 1991) is also being attempted. Fully processed, bioactive growth hormone is produced in all of these systems. Our attempts to engineer neuroendocrine cells to produce recombinant human growth hormone offers two advantages. The first is the ability to engineer high levels of growth hormone into a stable cell line with the various methods outlined here to maximize production levels. This engineering is

being done in a cell line in which production of an endogenous secreted protein has been blocked. The second advantage is that the growth hormone produced in these cells is packaged into secretory granules where regulated release of growth hormone is possible. Normally, growth hormone is not secreted constitutively, but is secreted in a pulsatile manner as regulated by Growth Hormone Releasing Factor and Somatostatin (Arimura, 1994). Growth hormone produced recombinantly in neuroendocrine cells is known to be secreted through the regulated secretory pathway where its release from the cells can be regulated (Moore and Kelly, 1985). In β-cells, growth hormone produced from a transgene is also secreted via the regulated secretory pathway and secretion can be costimulated along with the endogenous insulin (Welsh *et al.*, 1986).

RIN 1046-38 clones produce high levels of recombinant human growth hormone. Seventeen independent clones derived from electroporation of RIN 1046-38 cells with pCB6/hGH were screened for secretion of human growth hormone (hGH). No detectable hGH was detectable from conditioned media from parental RIN 1046-38. Fourteen of the 17 clones expressed significant levels of hGH. Six clones were expanded and characterized further.

hGH is expected to be secreted via the regulated secretory pathway in these clones. Cells were cultured for 24 hours in fresh tissue culture media containing 11 mM glucose and 5% fetal calf serum. This conditioned media was collected and immunoreactive hGH was determined (6 independent samples/clone were analyzed, 24 hour collection). Cells were washed and either incubated for one hour in media lacking glucose and containing 100 μM diazoxide (basal, 2 samples per clone) or incubated for one hour in media containing 5 mM glucose, 100 μM carbachol, 100 μM IBMX and amino acids (stimulated, 4 samples per clone). Cell numbers for each sample was determined and all hGH values are normalized to μg of secreted product per million cells. The values are reported in FIG. 14.

Over a 24 hour collection, the six clones secreted between 25 and 229 μg hGH per million cells per 24 hours. Clone EP111/31 has consistently been the highest hGH producing

clone in both the initial screens and in these studies. 229 µg hGH per million cells per 24 hours is higher than any value of hGH production by a mammalian cell. Previous reported values are in the range of 7-20 µg/million cells/24 hours (Pavlakis and Hamer, 1983) and the highest value reported is 40 µg/million cells/24 hours (Heartlein *et al.*, 1994).

hGH secretion by these six clones is also exquisitely regulated. Basal secretion values were all less than 100 ng/million cells/hour, easily detected in the assay, but barely visible in FIG. 14. Basal values are in the range of 0.1% to 1.0% of the stimulated values for each clone. Stimulated secretion ranged from 6 to 40 µg hGH/million cells/hour. The one hour output of EP111/31 of 40 µg/million cells is equivalent to the best 24 hour output reported to date (Heartlein *et al.*, 1994).

The absolute outputs of hGH by RIN clones, as well as the fact that it is secreted via the regulated secretory pathway, are important for both *in vitro* production and *in vivo* cell-based delivery. For *in vitro* production, these cells are producing more hGH in normal tissue culture per 24 hours than previously described cells. Cyclical stimulation of these cells in a bioreactor setting, as previously described for insulin production, cab be used for bioreactor production. *In vivo* cell-based delivery of hGH could use the cells in their present form where secretion of hGH would be fairly constant. Alternatively, further engineering of the cells could produce a more physiological pulsatile release of hGH *in vivo* by conferring regulation of growth hormone secretion to growth hormone-releasing factor and/or somatostatin, or other regulators of somatotropes (Arimura, 1994).

EXAMPLE 6

A. Rat Insulin Promoter Factor 1

Methods:

Rat IPF1 expression plasmids. A plasmid containing the rat IPF1 cDNA was obtained from Chris Wright (XB-pdx1). This plasmid contains the open reading frame of rat IPF1 (SEQ ID NO:5, bases 7 to 861) cloned into pXBm (Krieg and Melton, 1984), placing *Xenopus* β globin

5′- and 3′-transcribed but untranslated sequences 5′ and 3′ of the rat IPF1 sequence. This construct was made to help stabilize the IPF1 message, allowing for higher steady-state message levels and protein production. A *Hin*DIII/*Bam*HI fragment containing the IPF1 and globin sequences was ligated into the *Hin*DIII and *Bam*HI sites of pCB6 (Brewer, 1994), generating pCB6/IPF1. Alternatively, the IPF1 and globin sequences of pCB6/IPF1 was removed by digestion with *Bgl*II and *Bam*HI and cloned into the *Bam*HI site of pCMV8/IRES/NEO/hGHPolyA, generating pCMV8/IPF1/IRES/NEO. Stable transfectants of both of these expression plasmids are selected using G418.

It was not clear that the *Xenopus* β globin sequences would stabilize the IPF1 transgene in RIN cells. For this reason, the IPF1 open reading frame was amplified with the polymerase chain reaction from pCB6/IPF1 using two oligos (GGATCCATGAACAGTGAGGAGCAG, SEQ ID NO:41 and AGATCTTCACCGGGGTTCCTGCGG, SEQ ID NO:42). The resulting 867 base product (SEQ ID NO:5) was cloned into pNoTA/T7 (5 Prime to 3 Prime, Inc., Boulder, CO) generating pNoTA/T7/IPF1. The IPF1 open reading frame was removed from pNoTA/T7/IPF1 by digestion with *Bam*HI and was ligated into *Bam*HI digested pCB6, generating pCB6/IPF1(-Bg). Alternatively, the same IPF1 *Bam*HI fragment was ligated into *Bam*HI digested pCMV8/IRES/NEO/hGHPolyA, generating pCMV8/IPF1(-Bg)/IRES/NEO. A final expression plasmid was made, ligating the IPF1 *Bam*HI fragment into *Bam*HI digested pCMV8/Ins3′/IRES/NEO, generating pCMV8/IPF19-Bg)/Ins3′/IRES/NEO. The Ins3′ nontranslated region in these plasmids was described earlier for the insulin disulfide mutant example and is contained on a 198 base pair *HgaI*/*Hin*DIII fragment. This fragment was ligated into pCMV8/IRES/NEO/hGHPolyA generating pCMV8/Ins3′/IRES/NEO. Stable transfectants of all of these expression plasmids are selected using G418.

Cell culture and stable transfection of cell lines. These studies were performed as described above for insulin producing cells.

Screening and characterization of IPF1 producing clones. Northern analysis of individual G418 resistant clones generated from the various IPF1 expression plasmids was done as

described above for the human insulin northern analysis. Blots were hybridized with a ^{32}P-labeled cRNA probe corresponding to the rat IPF1 open reading frame (SEQ ID NO:5).

Results:

Overexpression of IPF-1 in RIN 1046-38 cells. IPF-1 functions both in the specification of a region of the primitive gut to form pancreas in the maturation of the pancreatic β cells. Because RIN 1046-38 cells retain only some of the differentiated features of a normal b cell, overexpression of IPF-1 in these cells could cause them to function more like mature b cells. Thus redifferentiated RIN cells may serve as a more effective bioreactor for the production of biologically relevant secreted proteins.

In initial experiments, stable transfection of RIN 1046-38 cells with either pCMV8/IPF-1/IRES/NEO or pCB6/IPF-1 resulted in a low number of NEO-resistant colonies. None of these colonies expressed the IPF-1 transgene as demonstrated by Northern blot analysis. A second round of stable transfections were performed with IPF-1 constructs in which the *Xenopus* 5' and 3' betaglobin untranslated sequences (UTR) were removed [IPF-1(-Bg)]. Also, in some constructs, the potentially stabilizing Ins3' UTR was fused immediately downstream of the IPF-1 cDNA. A moderate number of NEO-resistant colonies were obtained from RIN cells transfected with either PCMV8/IPF-1(-Bg)/IRES/NEO or pCMV8/IPF-1(-Bg)/Ins3'/IRES/NEO. Northern analysis of RNA from a mixed population of colonies containing either construct demonstrated that the IPF-1 transgene mRNA was indeed overexpressed related to endogenous IPF-1 (FIG. 16, lanes labeled polyclone #1 and #2). The addition of the 3'Ins UTR to the IPF-1 cDNA did not appear to have a significant effect on IPF-1 transgene expression.

Also shown in FIG. 16 are several clonal RIN lines overexpressing IPF-1 mRNA. As would be expected, some of the clonal lines express more IPF-1 mRNA than the polyclone and some less since the polyclone represents an average IPF-1 expression level from many drug-resistant colonies. Although not shown here, the polyclonal cells were analyzed for the presence of IPF-1 protein by Western blotting. A slight overexpression of IPF-1 protein was detected over and above endogenously expressed IPF-1 protein in untransfected RIN 1046-38 cells. Clonal

lines containing IPF-1 transgenes are currently being analyzed for increased levels of IPF-1 protein.

The IPF-1 containing polyclonal lines were also checked for increased levels of endogenous insulin, glucokinase, and GLUT-2. Increased levels of any one or all three of these proteins could potentially be indicative of more differentiated RIN cells. Northern analysis revealed that neither endogenous insulin nor GLUT-2 mRNA was effected by slight overexpression of IPF-1 protein in the polyclonal RIN lines. However, glucokinase mRNA was slightly elevated in the IPF-1 transgene containing lines. This might be expected since it has been recently demonstrated that IPF-1 interacts with the β-cells glucokinase promoter to play a role in the glucokinase gene regulation (Watada *et al.*, 1996). It is also well proven that IPF-1 is important in insulin gene regulation (Peers *et al.*, 1994), but as stated above, there was not an elevated level of insulin mRNA in the IPF-1 polyclones. Whether or not slight elevation in glucokinase has any physiological significance is currently under investigation. Additionally, some of the clonal lines demonstrating a higher level of IPF-1 mRNA (FIG. 16) than the polyclonal lines are being analyzed in the same manner as the polyclonal RIN lines.

B. Alternative Drug Selection Markers

Methods:

Expression plasmids with alternative selection markers. To facilitate engineering of multiple genes into the same cell line or to optimize expression of a given gene, alternative expression plasmids containing other drug selection markers were designed. The drug selection markers utilized include the hygromycin resistance gene (HYGRO), the puromycin resistance gene (PURO), the dihydrofolate reductase gene (DHFR) conferring resistance to methotrexate, the xanthine-guanine phosphoribosyltransferase gene (GPT) conferring resistance to mycophenolic acid, the Zeocin resistance gene (ZEO), and the histidinol selection gene (HISD). All of the drug selection genes were tested for their ability to confer drug resistance to RIN cells in two contexts. The first was by substituting the new drug selection gene for the neomycin resistance gene in pCMV8/IRES/NEO. In this context, the drug resistance gene is transcribed off of the CMV promoter as the downstream open reading frame of a bicistronic message. The

second is by substituting the new drug selection gene for the neomycin resistance gene in pCB6 (Brewer, 1994) such that the new drug selection gene is driven by the SV40 promoter. pCB7 (Brewer *et al.*, 1994) was constructed this way with the hygromycin resistance gene replacing the neomycin resistance gene.

The open reading frame of the hygromycin resistance gene was amplified using the polymerase chain reaction from pCB7 using oligos (GGGGATCCGATATGAAAAAGCCTG, SEQ ID NO:43 and CGAGATCTACTCTATTCCTTTGC, SEQ ID NO:44). The resulting 1048 base product was digested with *Bam*HI and *Bgl*II and ligated into the *Bam*HI site of pCMV8 generating pCMV8/HYGRO. In a second step, the IRES element (SEQ ID NO:11) contained on a 235 base *Bam*HI/*Bgl*II fragment, was ligated into the *Bam*HI site of pCMV8/HYGRO generating pCMV8/IRES/HYGRO. Stable transformants of pCB7 and pCMV8/IRES/HYGRO are selected using 300 µg/ml hygromycin (Boehringer Mannheim) for 14 days without media changes.

The *E. coli* open reading frame encoding XGPRT was amplified with the polymerase chain reaction from pSV3/GPT (ATCC#37144, Mulligan and Berg, 1980 and 1981) using oligos (CCGGATCCCATGAGCGAAAAAT, SEQ ID NO:45 and GGAGATCTTTAGCGACCGGAGAT, SEQ ID NO:46). The resulting 476 base pair amplified product was restricted with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pCMV8, generating pCMV8/GPT. Next, the IRES element (SEQ ID NO:11) was ligated into the *Bam*HI site of pCMV8/GPT, generating pCMV8/IRES/GPT. The GPT open reading frame was isolated from pCMV8/GPT by digestion with *Bam*HI and *Sma*I and the resulting 482 base pair fragment was ligated into pCB6/intron (see above) that had previously been digested with *Nar*I, treated with Klenow fragment and then digested with *Bcl*I, generating pCB8. Stable transformants of pCMV8/IRES/GPT and pCB8 are selected using 2.5 to 3.0 µg/ml mycophenolic acid (Sigma Chemical Co.) in media without exogenous xanthine added for 14 days. Media was changed every 3 to 4 days.

The open reading frame of the mouse dihydrofolate reductase cDNA was amplified with the polymerase chain reaction from pSV3-dhfr (ATCC#37147, Subramani *et al.*, 1981) using oligos (CCGGATCCATGGTTCGACCATTG, SEQ ID NO:47 and GGAGATCTGTTAGTCTTTCTTC, SEQ ID NO:48). The resulting 581 base pair amplified product was restricted with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pCMV8, generating pCMV8/DHFR. Next, the IRES element (SEQ ID NO:11) was ligated into the *Bam*HI site of pCMV8/DHFR, generating pCMV8/IRES/DHFR. The DHFR open reading frame was isolated from pCMV8/DHFR by digestion with *Bam*HI and *Sma*I and the resulting 582 base pair fragment was ligated into pCB6/intron (see above) that had previously been digested with *Nar*I, treated with Klenow fragment and then digested with *Bcl*I, generating pCB9. Stable transformants of pCMV8/IRES/DHFR and pCB9 are selected using 1 to 10 μg/ml methotrexate (Amethopterin, Sigma Chemical Co.) for 14 days with media changes every 3 to 4 days.

The open reading frame of the HisD gene was amplified with the polymerase chain reaction from pREP8 (Invitrogen, Inc.) using oligos (CCGGATCCATGAGCTTCAATAC, SEQ ID NO:49 and CCAGATCTGCTCATGCTTGCTCC, SEQ ID NO:50). The resulting 1063 base pair amplified product was restricted with BamHI and BglII and subcloned into the *Bam*HI site of pCMV8, generating pCMV8/HISD. Next, the IRES element (SEQ ID NO:11) was ligated into the *Bam*HI site of pCMV8/HISD, generating pCMV8/IRES/HISD. Stable transformants of pCMV8/IRES/HISD are selected in media with 0.8 to 1.0 mg/ml histidinol for 14 days. Media was changed every 3-4 days.

The puromycin resistance gene was isolated from pPUR (Clonetech, Inc.) by digestion with *Pst*I and *Xba*I. The resulting 792 base pair fragment was treated with Klenow fragment and ligated into the *Sma*I site of pCMV8, generating pCMV8/PURO. Next, the IRES element (SEQ ID NO:11) was ligated into the *Bam*HI site of pCMV8/PURO, generating pCMV8/IRES/PURO. The PURO open reading frame was isolated from pCMV8/PURO by digestion with *Nco*I, treated with Klenow fragment, and then digested with *Bam*HI. The resulting 723 base fragment was ligated into pCB6/intron (see above) that had previously been digested with *Nar*I, treated with Klenow fragment, and then digested with *Bcl*I, generating pCB10. Stable transformants of

pCMV8/IRES/PURO and pCB10 are selected using 1.75 to 2.0 µg/ml puromycin (Sigma Chemical Co.) for 10 days with media changes every 3 to 4 days.

The zeocin resistance gene was isolated from pZeoSV (Invitrogen, Inc.) by digestion with *Nco*I and *Acc*I. The resulting 430 base fragment was treated with Klenow Fragment and ligated into the *Sma*I site of pCMV8, generating pCMV8/ZEO. Next, the IRES element (SEQ ID NO:11) was ligated into the BamHI site of pCMV8/ZEO, generating pCMV8/IRES/ZEO. The ZEO open reading frame was isolated from pCMV8/ZEO by digestion with *Rsr*II, treated with Klenow fragment, and then digested with *Bam*HI. The resulting 406 base fragment was ligated into pCB6/intron (see above) that had previously been digested with *Nar*I, treated with Klenow fragment, and then digested with *Bcl*I, generating pCB11. Stable transformants of pCMV8/IRES/ZEO and pCB11 are selected using 400 µg/ml Zeocin (Invitrogen, Inc.) for 14 days with media changes every 3 to 4 days.

EXAMPLE 7

Rat Amylin Production

Methods:

Amylin expression plasmids. A *Hin*DIII/ *Xba*1 fragment corresponding to bases -66 to 611 of the published rat amylin cDNA sequence (SEQ ID NO:7, (Leffert, *et al.*, 1989)) encoding rat preproamylin (SEQ ID NO:8) was treated with Klenow Fragment to blunt the ends. This blunt ended fragment was ligated into the Klenow treated Xba1 site of pCMV8/IRES/NEO/hGH PolyA generating pCMV8/r.Amylin/IRES/NEO. The CMV promoter drives transcription of a bicystronic messenger RNA with rat amylin encoded in the upstream open reading frame and the neomycin resistance gene encoded in the downstream open reading frame. Stable transfectants from this plasmid are selected in G418.

The human amylin coding region was isolated by use of the polymerase chain reaction utilizing two oligos (TTTGCTGATATTGCTGAC (SEQ ID NO:62) and TGGGACCTTAGTTAGTAC (SEQ ID NO:63)) and human pancreatic cDNA as a template

(Human Pancrease QUICK-Clone cDNA, 7115-1). The resulting 491 base pair fragment (SEQ ID NO:52) encoding human preproamylin (SEQ ID NO:53) was ligated into the PCR cloning vector, pNoTA/T7 (5 Prime to 3 Prime Inc., Boulder Co.), generating pNoTAT7/h.Amylin. pNoTAT7/h.Amylin was restricted with Xba1 and the resulting 523 base fragment containing the human amylin open reading frame was ligated into the Xba1 site of pCB7/intron generating pCB7/intron/h.Amylin. The CMV promoter drives transcription of the human amylin coding sequence while the hygromycin resistance gene is transcribed using the SV40 promoter from an independent transcriptional unit encoded on the plasmid (Brewer 1994).

A final expression plasmid capable of coexpressing human insulin, rat amylin and the neomycin resistance marker was constructed. The human GRP 78 sequence encoding the internal ribosome entry site (IRES, SEQ ID NO:11) on a BamH1/ BglII fragment was ligated into the BamH1 site of pBS/INS, a plasmid containing the human insulin cDNA (SEQ ID NO:1), generating pBS/INS/IRES. pBS/INS/IRES was digested with *Xho*1 and *Xba*1, treated with Klenow and the resulting fragment containing INS and the IRES sequence was ligated into pCMV8/r.Amylin/IRES/NEO that had been digested with Xba1 and Klenow treated, generating pCMV8/INS/IRES/r.Amylin/IRES/NEO. The CMV promoter drives expression of a tricistronic message encoding human insulin, rat amylin and the neomycin resistance marker. Stable transfectants from this plasmid are selected in G418.

Cell culture and stable transfection of cell lines. RIN 1046-38 cells were cultured and transfected as described above for insulin producing cells.

Immunohistochemical Staining for Rat Amylin. Individual RIN clones generated by electroporation using amylin expression plasmids were screened using an anti-rat amylin polyclonal antibody (Peninsula Labs, IHC 7323). Cells were plated on coverslips or in "chamber slides" one or more days before immunostaining. Medium is removed from cells, rinse cells with PBS, then fix cells with 4% paraformaldehyde, 15-30 minutes. Rinse with PBS, then successive washes in 50%, 70% and 50% ethanol for 5 minutes each. Rinse with PBS and then block free aldehydes and non-specific sites using 1% serum (same species as second antibody), 0.05%

Triton, 0.1% azide in 50 mM Tris, pH 7.4 for 15 to 30 minutes. Fixed cells were incubated with anti-rat amylin primary antibody at 1:2,000 and 1:600 dilutions for 80 minutes at room temperature. Wash in PBS-Triton (0.05%) wash, 3 minutes, PBS alone for 3 minutes, then rinse in 50 mM Tris, pH 8.0. Incubate cells with Alkaline Phosphatase labeled second antibody for 0.5 hr in 0.5%BSA, 1.0 mM $MgCl_2$, 50 mM Tris, pH 8.0. Cells are washed 2-3 times in 50 mM Tris, pH 8.0, 1 mM MgCl. Finally, incubate cells with fresh BCIP/NBT solution (0.3 mg/ml nitroblue terazolium, 0.15 mg/ml bromo-chloryl-indoyl phosphate, disodium salt (both from Sigma Chemicals) in 50 mM Tris, pH 8.0, 1 mM MgCl). Reaction is stopped by washing in water and then air drying.

Northern analysis. Northern analysis of rat amylin transcripts in cell lines was done as described above for human insulin message detection. Filters were hybridized with a full-length digoxigenin-labeled antisense probe corresponding to the rat amylin cDNA (SEQ ID NO:7) made using Genius 4 RNA Labeling Kit (Boehringer Mannheim) and T7 polymerase. Northern analysis of rat peptidylglycine alpha-amidating monooxygenase (PAM) in cell lines was done as described using a digoxigenin-labeled antisense probe corresponding to the bases 240 to 829 of the rat PAM cDNA (Stoffers *et al.*, 1989) made using Genius 4 RNA Labeling Kit (Boehringer Mannheim) and T7 polymerase.

Insulin and Amylin Assays. Immuno reactive insulin (IRI) species were detected by radioimmuno-assay as described (Halban, *et al.*, 1986) or using a commercially available insulin radioimmuno-assay (Coat-a-count, Diagnostic Products Corp., Los Angeles). Imunoreactive amylin species were detected by radioimmuno-assay as described (Pieber, *et al.*, 1994) or using a commercially available rat amylin immunoassay (Peninsula Laboratories, EIAH-7323).

Alternatively, immunoreactive amylin was detected with the following modifications. The same anti-amylin polyclonal serum (T-486-6, Pieber, *et al.*, 1993) was used at a final dilution of 1:20,000 in RIA buffer (Peninsula Labs, Cat# RIK-BUF). ^{125}I labeled rat amylin and rat amylin peptide standard for the RIA were purchased from Peninsula Labs (Cat#Y-7323 and #7323 respectively). Human amylin species were quantitated using the rat amylin immunoassays

read against a human amylin standard (Bachem, Inc., PCPE60). Standard dilution's ranged from 0.19 to 25 ng/ml with 0.0125 μCi trace labeled rat amylin per assay tube. Bound and free amylin were separated by shaking at 200 rpm overnight at room temperature using a goat anti-rabbit IgG/biotin conjugate (1:10,000 final dilution, Sigma Chemical Co., St. Louis, Cat.# B-8895) and an avidin coated bead (Nichols Institute Diagnostics, San Juan Capistrano, Ca., Cat#30-0591). Samples were counted on a Gamma C-12 gamma counter (Diagnostic Products Corp., Los, Angeles) and values determined using the enclosed Log-Logit software.

Results:

Peptidylglycine alpha-amidating monooxygenase expression in cell lines. Alpha-amidation is now appreciated as a critical determinant for biological activity of a large number of peptide hormones. Table 4 represents a sample of human peptide hormones that are known to be amidated *in vivo*. The enzyme involved in alpha-amidation, peptidylglycine alpha-amidating monooxygenase (PAM), has been well characterized at the molecular level (reviewed in Eiper *et al.*, 1992a). Although there is only one gene in mammals encoding PAM (Ouafik *et al.*, 1992), there are several forms of PAM due to alternative splicing and endoproteolytic processing (Stoffers *et al.*, 1989 and 1991, Eiper *et al.*, 1992b) leading to both membrane-bound and secreted forms of PAM. PAM is also known to be developmentally regulated and differentially expressed *in vivo* (Ouafik *et al.*, 1989). The importance of alpha-amidation of peptide hormones is such that the presence of the consensus glycine followed by two basic amino acids (lysine and/or arginine) in a novel amino acid sequence can be predictive of its being a precursor to a bioactive polypeptide (Cuttita, 1993).

Amylin and GLP-1 are two peptide hormones that are amidated *in vivo*. A more complete list of amidated human polypeptide hormones is found in Table 4. Attempts at mammalian cell production of any of these hormones requires endoproteolytic cleavage of larger precursors, carboxypeptidase trimming and alpha-amidation. For instance, Glucagon-Like Peptide 1 (GLP-1) is a peptide hormone with powerful insulinotropic effects secreted from the intestinal L cells in response to meals (Kreymann and Williams, 1987). It processed from a larger polypeptide precursor through steps that are very similar to the processing of amylin. Processing of GLP-1

involves the action of the endoproteases PC2 and PC3 and carboxypeptidase on the same precursor that glucagon (Mojsov *et al.*, 1986 and Rouille *et al.*, 1995). The final biologically active peptide is a mixture of GLP-1 7-37 and GLP-1 7-36 amide, a difference resulting from the alternative processing of the glycine at position 37 to an alpha-amidated form by peptidylglycine alpha-amidating monooxygenase (PAM) (Orskov *et al.*, 1989 and Mojsov *et al.*, 1990). Both GLP-1 7-37 and GLP-1 7-36 amide are both biologically active in humans (Orskov *et al.*, 1993). The rat insulinoma cell line used here, RIN 1046-38 has already been shown to express sufficient levels of PC2, PC3 and carboxypeptidase for complete processing of highly expressed human insulin.

Amylin is a 37 amino acid polypeptide hormone again processed from a larger precursor polypeptide by the proteolytic processing (Sanke *et al.*, 1988). Amylin is normally co-produced and co-secreted with insulin by b-cells, acting as a hormone to regulate carbohydrate metabolism (Hoppener *et al.*, 1994). However, unlike insulin, amylin is alpha-amidated by PAM in the b-cells (Sanke *et al.*, 1988). Overexpression of amylin in RIN 1046-38 cells will serve as a demonstration of the ability of these cells to produce amidated peptide hormones.

Northern analysis was used to address the endogenous levels of PAM in various cell lines. Expression of PAM in RIN 1046-38 is compared to AtT-20 and two related RIN lines, RIN 1027-B2 and RIN 1046-44 (Philippe *et al.*, 1987). Endogenous expression of a single PAM message of approximately 3.5 kB is easily detected in all three RIN lines (FIG. 15A, Lanes 1, 3 and 4). Lower expression of two PAM messages of approximately 4.0 and 3.5 kB is found in AtT-20 cells (FIG. 15A, Lane 2). PAM message sizes of 3.5 to 4.0 kB is consistent with the larger spliced variants of PAM message known to encode active PAM protein (Stoffers *et al.*, 1989). Expression of endogenous PAM was compared with expression of endogenous amylin in these same cell lines. The three RIN lines with high levels of PAM also showed high levels of endogenous amylin expression (FIG. 15A, Lanes 1, 3 and 4). AtT20 cells, a pituitary cell line does not have any endogenous amylin expression. Interestingly, two RIN 1046-38 derived clones (EP18/3G8 expressing large amounts of human insulin (FIG. 11) and EP53/114 overexpressing rat glucokinase) that no longer express endogenous amylin show lower levels of expression of

endogenous PAM (FIG. 15A, Lanes 5 and 6). The majority of RIN 1046-38 derived clones continue to express both endogenous amylin and PAM, suggesting that RIN 1046-38 derived clones will maintain the ability to efficiently amidate peptide hormones.

The high level of PAM expression in RIN 1046-38 compared to AtT-20 is very encouraging. Comparison of PAM expression in other cell types has shown that AtT-20 cells express very high enzyme levels (Takeuchi *et al.*, 1990). This includes higher levels than PC12 cells and RIN5-f cells, a rat insulinoma line that is fairly dedifferentiated when compared to RIN 1046-38. Maintaining high PAM activity in RIN 1046-38, similar to maintaining high levels of PC2 and PC3 activity, suggests overexpression of transgenes for amidated peptide hormones such as amylin will result in their efficient production.

The rat amylin expression plasmid, pCMV8/r.AMYLIN/IRES/NEO was electroporated into RIN 1046-38 cells and stable clones selected in G418. Analysis of polyclones by Northern analysis demonstrates efficient expression of the AMYLIN/IRES/NEO bicystronic message (FIG. 15B). Individual stable clones were screened for amylin expression using an in situ immunostaining protocol using two dilutions of the primary amylin antibody. At the lower dilution (1:200) all the cells are positive due to the levels of endogenous amylin. At the higher dilution (1:1000), only a subset of clones continued to show staining, presumably due to overexpression of the amylin transgene. Five such clones from this series (BG97 clones) were picked and secreted amylin immunoreactive species determined, using cells in the basal state or stimulated for secretion. Regulated secretion of amylin is demonstrated in FIG. 17.

The human amylin expression plasmid, pCB7/intron/h.AMYLIN was electroporated into RIN 1046-38 cells and stable clones selected in hygromycin. Clones from this series (BG182 clones) were screened using the amylin immuno assay. Once individual clones had been established in 24 or 6 well tissue culture plates, a 24 hour media sample was collected and assayed. Cell number was estimated from visual inspection of the culture plates. BG182 clones with obvious increased amylin immunoreactivity per million cells were identified and characterized further.

Several independent approaches for coengineer amylin and insulin overproduction in RIN 1046-38 cells were attempted. These included engineering human insulin expression into a rat amylin producing RIN clone (pCMV8/INS/IRES/PURO into BG97/134, generating the EP183 and EP196 series), engineering human amylin into a human insulin producing RIN clone (pCB7/intron/h.AMYLIN into BG18/3E1, generating the EP184 and EP197 series), and finally engineering human insulin and rat amylin co-expression into RIN 1046-38 using a single plasmid construct (pCMV8/INS/IRES/r.Amylin/IRES/NEO into RIN 1046-38, generating the EP181 series). In all cases, individual drug resistant clones were screened by amylin and/ or insulin immunoassay of a 24 hour sample collected from cells cultured in fresh tissue culture media with an estimate of cell number. Individual clones from each series were identified and characterized further.

Table 9 gives examples of several RIN cell lines overexpressing insulin and /or amylin at ratios varying over three orders of magnitude. In addition to the ratios, absolute amounts of insulin and amylin are given in ng per million cells per hour during a stimulated secretion experiment. Levels of human amylin were determined using the commercially available rat amylin assay and running both a rat amylin and human amylin standard curve. Differences in the two curves were used for a correction factor for crossreactivity in the assay.

Table 9

Ratios of Amylin to Insulin in various transfected cells.

Cell line	(Transgene)	Amylin (ng/e6/hr)	Insulin (ng/e6/hr)	Ratio
EP18/3E1 (h.Ins into RIN 1046-38)		1.4	750	0.002
RIN 1046-38 (none)		1.4	50	0.028
EP97 series (Rat Amylin into RIN 1046-38)				
/134	"	17.4	100	0.17
/138	"	6.4	nd	
/117	"	5.4	nd	
/137	"	4.9	40	0.12
/109	"	3.6	nd	
EP182 series (Human amylin into RIN 1046-38)				
/1	"	23	50	0.46
/5	"	46	50	0.92
/7	"	30	50	0.6
/11	"	6	50	0.12
/12	"	40	50	0.8
/13	"	37	50	0.74

Several independent approaches for coengineering amylin and insulin in RIN 1046-38 cells were used. These included engineering human insulin expression into a rat amylin producing RIN clone (pCMV8/INS/IRES/PURO into BG97/134, generating the EP183 and EP196 series), engineering human amylin in human insulin producing RIN clone (pCB7/intron/h.AMYLIN into BG18/3EI, generating the EP184 and 197 series) and finally engineering human insulin and rat amylin co-expression into RIN 1046-38 using a single plasmid construct (pCMV8/INS/IRES/r.AMYLIN/IRES/NEO into RIN 1046-38, generating EP181 series). In all cases individual drug resistant clones were screened by amylin and/or insulin immunoassay of a 24 hour sample collected from cells cultured in fresh tissue culture media with an estimate of cell number. Conditioned media samples from the various clones were collected and amylin and insulin immunoreactive species quantitated on the same samples. Ratios of amylin to insulin for these samples are shown in Table 10).

Table 10

Cell line (Transgene)	Amylin (ng/ml)	Insulin (ng/ml)	Ratio
EP181 series (h.Ins+r.Amy into RIN 1046-38)			
/3	1.66	73.5	0.23
/57	2.37	120.3	0.020
/58	2.18	91.0	0.024
/59	4.2	171.9	0.024
/65	4.39	155.9	0.028
/70	3.13	130.2	0.024
Parental RIN 1046-38			**0.028**
EP183 series (h.INS into EP97/134)			
/18	1.89	9.3	0.20
/20	"	20.3	0.09
/21	"	10.0	0.19
/22	"	14.1	0.134
/24	"	9.2	0.20
/25	"	12.3	0.15
/26	"	14.5	0.13
/27	"	10.2	0.19
/32	"	15.61	0.12
/33	"	15.56	0.12
/34	"	15.8	0.12
/35	"	14.39	0.13
/36	"	13.8	0.14
/37	"	8.1	0.23
Parental EP97/134			**0.17**
EP197 series(h.AMY into EP18/3E1)			
/4	2.03	228	0.009
/7	7.05	620	0.011
/9	1.15	97	0.012
/10	1.05	110	0.010
/16	1.63	210	0.008
/17	2.40	125	0.019
/18	3.95	596	0.007
/19	4.70	970	0.005
/21	5.66	615	0.009
/22	4.55	725	0.006
/23	4.90	500	0.010
/24	4.22	650	0.006
/25	4.85	535	0.009

/26	4.48	645	0.007
/27	5.02	640	0.008
/29	6.10	610	0.010
Parental EP18/3E1			**0.002**

EXAMPLE 8

Bioreactor production of amylin and amylin-related species from engineered RIN cells.

Bioreactor Inoculation and Culture. BG97/134 cells were grown, split, and maintained in RPMI 1640 medium with 2 mM glutamine (JRH Bioscience) supplemented with 4% fetal calf serum (JRH) and 0.125 µg/ml G418 (Gibco BRL) in T75 culture flasks as described previously in this document. A pilot scale bioreactor (Celigen Plus, New Brunswick Scientific (NBS), Edison, NJ) with dissolved oxygen electrode, pH electrode (both Ingold), and 4-gas PID controller is set up for perfusion culture with a packed bed of polyester discs (FibraCell, NBS). The reactor has a working volume of 1.25 liters and a packed bed volume of 0.7 liters containing 70 grams of polyester discs.

Cells are trypsinized and seeded into the reactor containing the same media composition as the maintenance media at a density of approx. 2.5 X 10^5 cells per ml of working volume. After transfer, the cells allowed to seed onto the bed material with an impeller speed of 75 rpm and no media perfusion.

After seeding, the impeller speed is kept at 75 rpm and the culture is maintained with no perfusion for approx. 90 hours. Media perfusion is started and the flow rate is linearly brought from 0 working volumes per day (WV/d) to 3.54 WV/d over the course of the following 500 hours. The perfusion rate is thereafter maintained constant at 3.54 WV/d. The perfusion media is RPMI 1640 with 2 mM glutamine supplemented with 2 g/l glucose (final concentration of 4 g/l), 0.10% fraction V bovine serum albumin, 10 µg/ml human apo-transferrin, 50 uM each of ethanolamine and o-phosphorylethanolamine, and 0.10% cholesterol rich lipids from adult bovine serum (all Sigma Chemicals, Saint Louis, MO) (a modification from Clark, *et al.*, 1990). The perfusion media contains no fetal calf serum.

The culture temperature is maintained at 37°C, the dissolved oxygen level at 60% (indexed relative to saturation of air in phosphate buffered saline), and the pH at 7.4. Glucose

levels in the reactor is maintained at 2-3 g/l by adjusting the perfusion rate and the glucose concentration in the fresh perfusion media. Similar RIN bioreactor cultures have been maintained successfully for as long as 2000 hours in the bioreactor under similar conditions.

Media samples were collected once daily and quantitatively analyzed for insulin and amylin secreted into the media by ELISA as previously described. Ammonia and lactate levels are monitored in the daily samples and analyzed using an automated analyzer (IBI Biolyzer, Johnson & Johnson, New Brunswick, NJ).

Bioreactor production of rat amylin *(Cell growth and density).* The PID controller's oxygen gas controller output is monitored throughout the run. It rises steadily from around -40 at 0 hours to around 60 at approx. 400 hours where it stabilizes for the rest of the run. The rate of increase of the controller output is consistent with an empirically expected growth rate, and the maximum level of 60 is consistent with achieving a cell density of 1.1-2.3 x 10^8 cells per ml of bed volume. It should be noted that this growth and sustained density is achieved using a serum free media. This observation is unexpected.

Under normal culture, ammonia levels are maintained in the range of 2-5 mM which doesn't influence cell viability of most cell types. However, in that range ammonia has an inhibitory effect on insulin secretion from RIN cells. Lactate are normally maintained between 2 and 10 mM, but have in some runs built up to concentration close to 30 mM. In the normally observed range of lactate, both cell viability and secretion are not significantly affected. However, at the higher concentrations lactate can have an effect on both functions.

Peak levels of amylin measured in the perfused media varies from 25 to 30 ng/ml, corresponding to 61.5 to 1.8pg/10^6 cells/hour. Compared to secretion of cultures on two dimensional tissue culture flask surfaces of approx. 17.4 ng/10^6 cells/hour, the bioreactor secretion numbers represent a dramatic drop in performance. Several factors can be influencing the secretion potential of the cells in the bioreactor environment: Cell-cell contact inhibition brought about by the high densities achieved in a 3-dimensional culture bed; media availability

(fresh media available per cell per day); built-up of inhibitory levels of ammonia, lactate, and other metabolites or factor.

EXAMPLE 9

RIN Cell Lines Expressing Insulin And Amylin Demonstrate Efficient Proteolytic Processing Of The Prehormones To Hormones. Demonstration Of Purification Of Processed Amylin By HPLC.

Methods

HPLC Separation of Insulin, Amylin, and Amylin Related Peptides. Acid/ethanol extracts of whole cells or conditioned media was prepared and analyzed by high performance liquid chromatography as described (Halban, *et al.*, 1986, Sizonenko and Halban, 1991). Alternatively, extracts of whole cells were prepared by sonication in 1% Triflouroacetic acid/ 50% acetonitrile, followed by high speed centrifugation. The HPLC system used was a Beckman System Gold with Gold Noveau Software equipped with a model 126 solvent module and a model 166 ultraviolet absorption detector (Beckman Instruments, Inc.) and a FC204 fraction collector (Gilson Instruments, Inc.). A Merck LiChroCART 250-4 with Lichospere 100 RP-18 column (25 cm x 4.6 mm) in combination with a Lichospher 100 RP-18 guard column was used for chromatographic separation. Alternatively, a Vydac 214 TP C4 column (25 cm x 4.6 mm) (Alltech, Inc., Deerfield, IL) in combination with a Macrosphere 300 C4 guard column (Alltech, Inc.) was used for chromatographic separation. Solvent systems, gradients and flow rates were used as described (Halban, *et al.*, 1986) as was processing of individual fractions including neutralization and lyopholization. Standards used included human proinsulin and insulin, rat amylin (Penninsula Laboratories, Inc., Cat. #7323), and human amylin (Bachem, Inc., PCPE60).

Immunoprecipitation of Amylin and Amylin-Related Peptides. RIN cell lines (parental RIN 1046-38, rat amylin producing BG97/134 and human amylin producing BG182/12) were metabolically labeled in 6-well tissue culture dishes using ^{35}S by culturing cells initially for 20 minutes in DMEM lacking methionine and cystine (ICN Biomedicals, Inc., Costa Mesa, Ca.) followed by a two to four hour incubation in the same media with 17 uCi/ ml. Trans-Label (ICN

Pharmaceuticals, Inc., Irvine, Ca.). For pulse chase/ experiments, cells were metabolically labeled for 3 hours followed by a one hour chase by incubating the cells at 37 C in RPMI with 5 mM glucose, 0.1 % BSA, 20 mM HEPES, 10 mM each leucine, arginine and glutamine, 100 uM carbachol and 100 uM IBMX. Media was collected after the 1 hour incubation, Triton X 100 added to a final concentration of 2% and immunoprecipitations done as described below for the cell lysates without dilution into the antibody binding buffer. Clarified cell lysates were prepared as described using two different lysis buffers, NP-40 and RIPA lysis buffer (from "Antibodies: A Laboratory Manual", E. Harlow and D. Lane, Eds., Cold Spring Harbor Laboratories, 1988, pg.447). Lysates were diluted 10-fold into antibody binding buffer (2% Triton-X, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 8.0) followed by the addition of 5 µg amylin-specific IgG and incubation overnight at 4 C. Amylin antisera used included rabbit anti-amylin amide (rat) IgG, rabbit anti-amylin 25-37 amide (human) IgG (both from Peninsula Laboratories, Inc., Belmont, Ca.), and rabbit anti-amylin amide (rat) IgG (Pieber, *et al.*, 1994). Immuno complexes were isolated by binding for one hour at 4 C to 20 microliters of a 20% slurry of Protein A Sepharose beads (Sigma Chemicals, Inc.) rehydrated in 10 mM NaBorate, pH 8.0. Protein A Sepharose beads were pelleted by centrifugation and washed sequentially in wash buffer #1 (150 mM NaCl, 0.5%SDS, 10 mM Na Borate, pH 8.0) and wash buffer #2 (150 mM NaCl, 0.1%SDS, 10 mM Na Borate, pH 8.0). Immuno complexes were removed from the Protein A Sepharose beads by heating for 30 minutes at 42 C in 30 microliters sample buffer (2% SDS, 12 % glycerol, 2% beta-mercaptoethanol, .01% bromphenol blue (all w/v), 50 mM Tris, pH 6.8).

Immunoprecipitates were separated using a glycerol containing separating gel (16.5 %T, 6 %C) as described (Schagger and Von Jagow, 1987). Five micrograms of synthetic human amidated amylin (BACHEM California, Inc., Torrance, Ca.), rat amidated amylin (Peninsula Laboratories, Inc.) or 30 microliters SeeBlue pre-stained molecular weight markers (Novex, Inc., San Diego) were run as standards in parallel lanes. Following electrophoresis, gels were divided and processed. Lanes containing immunoprecipitates and one molecular weight marker lane were processed for fluorography using Entensify autoradiography enhancer as described (NEN Research Products, Boston) and exposed to Xomat-AR autoradiography film (Kodak, Inc.). Lanes containing molecular weight markers and the amylin standards were stained Brilliant Blue

R (Sigma Chemicals, 25 milligrams per 100 mls. 10 % glacial acetic acid) for one hour followed by destaining in 10 % glacial acetic acid to visualize relative migration of the amylin standards.

Results:

Novel posttranslational processing has been achieved including dibasic amino acid protease processing from proamylin, amidation, and glycosylation. Naturally occurring human amylin extracted from blood or human pancreas has been shown be very heterogeneous (Pittner, *et al.*, 1994 and Percy, *et al.*, 1996). This heterogeneity is largely due to O-linked glycosylation of amylin in one of two possible sites in the protein (Percy, *et al.*, 1996 and Rittenhouse, *et al.*, 1996). The degree of heterogeneity of RIN produced rat and human amylin is being determined at BetaGene by HPLC in combination with a sensitive ELISA for amylin as well as amylin immunoprecipitations and SDS/PAGE analysis.

Human Proinsulin Is Efficiently Processed To Mature Insulin By Rat Insulinoma Cells. Intracellular insulin species were isolated from parental RIN 1046-38 and EP 18/3E1 cells by acid/ethanol extraction. Separation by HPLC of the insulin species produced by these cells was done as described (Halban, *et al.*, 1986, Sizonenko and Halban, 1991). The analysis indicates that human insulin produced by the rat insulinoma is efficiently processed to mature insulin with very low detectable levels of pro-insulin or other processing intermediates (FIG. 18). Processing of human insulin is slightly less efficient compared to the processing of rat insulin at these levels of production. While the percentage of intracellular rat proinsulin and intermediates is 3 to 4% of total rat insulin in all cell lines examined, the percentage of intracellular human proinsulin and intermediates is 18% in EP18/3E1.

The ability of RIN cell lines to efficiently process proinsulin to mature insulin in these engineered lines demonstrates the maintenance of the high levels of expression of the endoproteases PC2 and PC3, known to be responsible for insulin processing (Vollenweider, *et al.*, 1995). This is a critical feature of the RIN cell lines being developed. HPLC analysis of secreted forms of insulin following stimulation demonstrates the human insulin released is predominantly mature insulin with very low detectable pro-insulin or processing intermediates

appearing in the media. These values are lower than measured in vivo circulating levels of proinsulin (Rhodes and Alarcon, 1994).

Stimulated secretion samples of parental RIN 1046-38 cells, the rat amylin producing clone EP97/134, and the human amylin producing clone EP182/12 were prepared as described in Example 7. Following this incubation, cell extracts were prepared of the cells to determine the intracellular contents of the three cell lines. Extracts were prepared by two methods. The first was an extraction of the contents into 1.0 M acetic acid as performed for insulin (Halban, *et al.*, 1986). Alternatively, extracts were prepared using 1% triflouroacetic acid/ 50% acetonitrile (TFA/ACN extract). This alternative extraction was attempted to minimize amyloid formation of amylin species which would be lost from the extracts by centrifugation. Immunoreactive amylin species in ng per million cells from the above samples are shown in FIG. 19. Assays were done using the rat amylin assay as described in Example 7.

As expected, parental RIN 1046-38 cells secrete a low amount of material with low amounts of extractable intracellular amylin species. The rat and human amylin engineered lines both demonstrate higher amounts of secreted and intracellular amylin species. The rat amylin producing clone secretes about ten times more rat amylin than the parental cells and has intracellular amounts between 20 to 28 times more than parental cells. The fraction of immunoreactive amylin secreted in one hour is 14 to 20% of the intracellular content. Approximately 40% more immunoreactive material was extracted using the TFA/ACN procedure. The human amylin producing clone had lower values overall than expected.

Even though the samples were read against a human amylin standard curve, the crossreactivity of the assay may still be a problem and give lower values. The amylin assay utilizes rat amylin as the competing species. The human amylin producing clones had approximately three times more immunoreactive secreted material compared to parental cells. Intracellular content was two to six times higher than parental cells. Interestingly, the acetic acid extract gave the higher values as compared to the TFA/ACN extract. The ratio of secreted to intracellular material was 17%, a value very similar to the rat amylin producing clone.

Rat Amylin HPLC Demonstrating Processing And Purification (FIG. 20A). Extracts of a rat amylin producing RIN EP183/20 (see Table 10) were prepared and separated by the same HPLC system and conditions as used for separation of the insulin species. Synthetic amidated, rat amylin was also run to determine its retention time. Plots of the UV absorbances versus time for the HPLC runs of the RIN extract are shown in FIG. 20A. A major UV peak at app. 21.5 minutes was easily detected as well as a minor UV peak at app. 20 minutes. Individual fractions assayed for amylin immunoreactive species across these UV peaks demonstrated a peak of immunoreactivity that again coincided with the major UV peak at 21.5 minutes (FIG. 20A). Again the minor peak at 20 minutes from the RIN cell extract showed no immunoreactivity. Only the indicated fractions were assayed for amylin immunoreactivity. There is a strong possibility that other UV peaks could consist of amylin-related species. There are several UV peaks, such as those at 7.5, 16.3, 17.6, 26, and 31 minutes, that are as yet unidentified. Unidentified amylin-related species are expected to be present in a RIN cell extract, including O-linked glycosylated amylin species. It is presently not known whether the rat amylin immuno assay being used is capable of recognizing alternatively processed forms of amylin such as glycosylated species, or any other amylin related species. The UV peak at 26 minutes does not contain any amylin immunoreactive material, but this UV peak does coincide in retention time to processed human insulin (FIG. 18).

HPLC Fractionation of Extracts from Human Amylin Producing RIN Cells. Extracts of a human amylin producing RIN BG 182/12 cells and parental RIN 38 cells were prepared using the TFA/ACN extraction protocol and fractionated using a C4 column as described in Materials and Methods (FIG. 20B). This extraction protocol and HPLC column are identical to those used in Pittner, *et al.*, 1994, where the heterogeneous nature of naturally occurring human amylin was first described. HPLC fractions were assayed using the anti-amylin polyclonal serum (T-486-6, Pieber, *et al.*, 1993) as described in Materials and Methods. The BG 182/12 samples were read against a human amylin standard in the RIA while the RIN 38 samples were read against a rat amylin standard. The positions of the fractionated human and rat standards are also indicated.

The Rin 38 extract shows very little amylin immunoreactivity in any of the fractions (FIG. 20B). A minor peak in the initial void volume fractions (fraction 4) and in the fractions where the rat amylin standard fractionates (fractions 11-13) are all that is detected. This most likely represents the low endogenous levels of rat amylin found in parental RIN 38 cells. In contrast, several large peaks of immunoreactive material are detected in fractions from the human amylin producing cells. The largest peaks are found in fractions 27 through 32, with possibly two major species resolved. Human amidated amylin standard runs very close to these peaks (fraction 33). Other minor peaks are found in the initial void volume (fraction 4) and fractions 11 through 17, again close to where the rat standard fractionates.

The identity of all the fractionated immunoreactive species in the human amylin producing cells is presently not known. Based on the lack of immunoreactive material found in the RIN 38 extract, all of these peaks are likely to be derived from various processing intermediates of human amylin. It is interesting that the heterogeneous nature of human amylin produced by RIN cells is similar to that described for human amylin extracted from plasma (Pittner, *et al.*, 1994). This report shows three major species of human amylin, one identical in HPLC fractionation to the synthetic human amylin peptide, with two others fractionating just prior to the standard. The extraction and HPLC column used here were the same as reported in Pittner, et al. with the only difference being the acetonitrile gradient conditions used. It is interesting to speculate that the heterogeneous human amylin produced in RIN cells is at least similar to naturally occurring human amylin. Of note is that there is no evidence that rat amylin produced in RIN cells is heterogeneous (FIG. 20A). This agrees with the results of Pieber, *et al.* (1993), where rat amylin extracted from plasma and fractionated runs as a single species at the identical retention time as synthetic rat amylin.

Recombinant Human Amylin is Heterogeneous with Apparent Molecular Weights of 4 to 8 Kd. RIN 1046-38 cells or human amylin producing BG 182/12 cells were metabolically labeled using $^{35}35$ and clarified lysates prepared using two different lysis buffers. Immunoprecipitation of amylin species from these extracts were attempted using three amylin antibodies. Two commercially available antibodies raised against either amidated rat amylin or a

fragment of amidated human amylin failed to immunoprecipitate any labeled species. A third antibody raised against amidated rat amylin (Pieber, *et al.*, 1994) immunoprecipitated labeled species specifically from the both extracts prepared from the human amylin producing RIN clone as compared to parental RIN 1046-38 cells (FIG. 21A). One major species running at an apparent molecular weight of 5.5 kD and a minor species running at 5 and 7 to 8 kD are seen. There is a very faint band at the apparent molecular weight of 4.0 kD where the human amidated amylin standard migrates (FIG. 21A). However, this is also close to where the rat amidated standard migrates, so this 4.0 kD band could represent either human or endogenous rat amylin.

A pulse chase experiment with a long pulse followed by a chase in media that stimulates secretion of amylin immunoreactive material was performed. Results of immunoprecipitations and SDS PAGE fractionation from the cell lysate without a chase, cell lysates following the chase and from the collected media are shown in FIG. 21B. The four immunoprecipitated species of with apparent molecular weights of 4.0, 5.0, 5.5 and 7.0 to 8.0 are easily seen in the cell lysate without a chase. All four species are reduced in intensity in the cell lysate following the one hour chase with a corresponding increase in the four species in the media. This strongly suggests that the heterogeneous immunoreactive amylin material produced by the human amylin engineered RIN cells is efficiently secreted and remains heterogeneous. The ratio of the 5.0 versus the 5.5 kD band is different between the intracellular material from cell lysates is compared to the secreted material, possibly representing further processing events.

Recombinant human amylin species of an apparent molecular weight of 5.5 and 7 to 8 kD are identical to amylin species extracted from human plasma (Percy, *et al.*, 1996). It is now understood that the heterogeneous nature of naturally occurring human amylin is due to O-linked glycosylation (Rittenhouse, *et al.*, 1996). Experiments to analyze the heterogeneous recombinant human amylin produced from RIN cells is underway, specifically looking for O-linked glycosylated species.

EXAMPLE 10

RIN Cell Lines Expressing Preproglucagon Demonstrate Efficient Amidation Of A Secreted, Processed Polypeptide.

Methods:

Preproglucagon cDNA isolation and expression plasmid. Total rat pancreatic RNA was reverse transcribed into total cDNA using AMV Reverse Transcriptase as recommended by the supplier (Promega, Inc.). A rat glucagon cDNA corresponding to bases 10 to 904 of the published sequence (Heinrich, Gros *et al.*, 1984) was amplified with the polymerase chain reaction from the pancreatic cDNA using oligos (CCACCTGTCTACACCTCCTCTC (SEQ ID NO:36)

and GTAATCCAGGTGTCGTGACTGC (SEQ ID NO:37)). The resulting 895 base PCR product (SEQ ID NO:55) encoding rat preproglucagon (SEQ ID NO:56) was ligated into pNoTA/T7 as recommended by supplier (5 Prime to 3 Prime, Inc.), generating pNoTAT7/r.Glucagon.

A human preproglucagon cDNA was generated by the polymerase chain reaction using oligos (CAGCACACTACCAGAAGACAGC (SEQ ID NO:64) and CACCACTGTGGCTACCAGTTCT (SEQ ID NO:65)) and human pancreatic cDNA as a template (Human Pancrease QUICK-Clone cDNA, 7115-1). The resulting 955 base pair fragment (SEQ ID NO:57) encoding human preproglucagon (SEQ ID NO:58) was ligated into the PCR cloning vector, pNoTA/T7 (5 Prime to 3 Prime Inc., Boulder Co.), generating pNoTAT7/h.glucagon. pNoTAT7/h.glucagon was restricted with BamH1 and the resulting 984 base fragment containing the human amylin open reading frame was ligated into the BamH1 site of pCMV8/IRES/NEO/hGHPolyA, generating pCMV8/hGlucagon/IRES/NEO. The CMV promoter drives transcription of a bicystronic messenger RNA with human glucagon encoded in the upstream open reading frame and the neomycin resistance gene encoded in the downstream open reading frame. Stable transfectants from this plasmid are selected in G418.

Construction Of Mutated Human Preproglucagon cDNA And Expression Plasmid. A mutation was introduced into the human preproglucagon cDNA by the polymerase chain reaction using sets of oligos for site directed mutagenesis with a plasmid containing the human preproglucagon cDNA as a template (pNoTAT7/h.glucagon). The first set of oligos (CAGCACACTACCAGAAGACGC (SEQ ID NO:66 and CTGTAGTCACTAGTGAATGTGCCCTGTGAATGGGCCTTGTGGTCG (SEQ ID NO:67)) generated a 215 base pair fragment with a single base pair change of G to C at position 181. This base change results in an amino acid substitution of arginine to alanine at amino acid residue 52 of preproglucagon. This fragment was ligated into the PCR cloning vector, pNoTA/T7 (5 Prime to 3 Prime Inc. Boulder Co.), generating pNoTAT7/5' mut.gluc.

A second set of oligos (CAGCACACTACCAGAAGACAGC (SEQ ID NO:64) and ACTAGTGAATGTGCCCTG (SEQ ID NO:68)) was used to introduce a second mutation using pNoTAT7/5'mut.glu. as a template. The resulting 207 base pair fragment, in addition to having the original G to c base pair change at position 181, had a second change of C to T at position 204. This resulted in the creation of a unique SpeI restriction endonuclease recognition site, with no change to the deduced amino acid sequence. This 207 base pair fragment was ligated into PCR cloning vector, pNoTA/T7, generating pNoTAT7/5'mut.gluc.Spe. A final set of oligos (ACTAGTGACTACAGCAAG (SEQ ID NO:69) and CACCACTGTGGCTACCAGTTCT (SEQ ID NO:65)) was used to introduce the corresponding C to T base pair change and the creation of a unique SpeI restriction endonuclease recognition site in the 3' end of preproglucagon. The resulting 755 base pair fragment was ligated into the PCR cloning vector, pNoTA/T7, generating pNoTAT/3'mut.gluc.Spe. pNoTAT7/5'mut.gluc.Spe was digested with *Spe*1 and *Hin*DIII and the resulting fragment ligated into pNoTAT7/3'mutgluc.Spe that had been also digested with *Spe*1 and *Hin*DIII. The resulting plasmid pNiTAT7/mut.glucagon, contains a reconstructed preproglucagon cDNA (SEQ ID NO:60) with 2 base pair changes in the sequence at position 181 (G to C) and 204 (C to T) relative to SEQ ID NO:57. The resulting amino acid sequence (SEQ ID NO:61 has a single amino acid substitution of arginine to alanine at amino acid residue 52 relative to SEQ ID NO:58. pNoTAT7/mut.glucagon was restricted with Xba1 and BamH1 and the resulting 976 base fragment containing the mutant preproglucagon open reading frame was

ligated into the Xba1 and BamH1 site of pCMV8/IRES/NEO/hGHPolyA, generating pCMV8/mut.glucagon/IRES/NEO. The CMV promoter drives transcription of a biscystonic messenger RNA with the mutant preproglucagon encoded in the upstream open reading frame and the neomycin resistance gene encoded in the downstream open reading frame. Stable transfectants from this plasmid are selected in G418.

Cell Culture- RIN 1046-38 (Gazdar, Chick *et al.*, 1980; Clark, Burnham *et al.*, 1990), Rin 1027-B2 and Rin 1046-44 (Philippe, Chick *et al.*, 1987) were grown in Medium 199 with Earle's salts, containing 11 mM glucose and supplemented with 5% fetal calf serum (Mediatech, Washington, D.C.), 100 milliunits/ ml penicillin and 100 µg/ ml streptomycin. AtT-20 derived cell lines were cultured as described (Hughes, Johnson *et al.*, 1992). Cells were passaged once a week using 0.05% trypsin- EDTA solution and kept under an atmosphere of 95% air and 5% CO_2 at 37 C.

Northern analysis- Total RNA from RIN cell lines grown in vitro was isolated using RNAzol B RNA Isolation Reagent (Cinna/ Biotex Laboratories Int.). 10 µg total RNA was resolved on methyl mercury/ 1.5% agarose gels as described (Bailey and Davidson 1976). Gels were subsequently stained with ethidium bromide (1µg/ml in 0.5 M $NH_4CH_3CO_2$) to visualize RNA for integrity and loading consistency. RNA was electro transferred to Qiabrane Uncharged Nylon Membrane (Qiagen, Inc.) using a Transphor Unit, TE50X (Hoefer, Inc.). Membranes were hybridized with digoxigenin-labeled RNA probes using the Genius Non radioactive Nucleic Acid Labeling and Detection System for filter hybridizations as described (Boehringer Mannheim). Full-length digoxigenin-labeled antisense probes corresponding to rat insulin (SEQ ID NO:59), rat amylin (SEQ ID NO:7), and human glucagon (SEQ ID NO:55) were made using Genius 4 RNA Labeling Kit (Boehringer Mannheim) using either T7 or T3 Polymerase. Northern analysis of rat peptidylglycine alpha-amidating monooxygenase (PAM) in cell lines was done as described using a digoxigenin-labeled antisense probe corresponding to the bases 240 to 829 of the rat PAM cDNA (Stoffers, *et al.*, 1989) made using Genius 4 RNA Labeling Kit (Boehringer Mannheim) and T7 polymerase. Exposures of chemiluminescent detected membranes was done using Xomat-AR autoradiography film (Kodak).

Immunoassays For Glucagon, GLP-1 (Non-Amidated) and GLP-1 (Amidated).
Immunoreactive species of glucagon, glucagon-like peptide 1 (7-37, non amidated) and glucagon-like peptide (7-36, amide) were determined as described by the suppliers of the respective commercial kits (all purchased from Peninsula Laboratories Inc. Cat #s RIK-7165, RIK-7123 and RIK-7168, respectively)

Results:

Northern analysis of several of the rat amylin producing clones (BG97 series) was done probing for amylin messages (endogenous and transgene), PAM and endogenous insulin as shown in FIG. 22. Amylin transgene messages and endogenous insulin messages varied between clones with message levels being consistent with detected immunoreactive hormone levels (Table 9 shows insulin and amylin hormone levels for these clones). Endogenous PAM message in the amylin producing clones compared to parental RIN 1046-38 is very stable. In fact, the majority of RIN 1046-38 derived clones continue to express both endogenous amylin and PAM, suggesting that RIN 1046-38 derived clones will maintain the ability to efficiently amidate peptide hormones.

The high level of PAM expression in RIN 1046-38 compared to AtT-20 is very encouraging. Comparison of PAM expression in other cell types has shown that AtT-20 cells express very high enzyme levels (Takeuchi, *et al.*, 1991). This includes higher levels than PC12 cells and RIN5-f cells, a rat insulinoma line that is fairly dedifferentiated when compared to RIN 1046-38. Maintaining high PAM activity in RIN 1046-38, similar to maintaining high levels of PC2 and PC3 activity, suggests overexpression of transgenes for amidated peptide hormones such as amylin will result in their efficient production.

Post translational processing of preproglucagon into glucagon is dependent upon the specialized functions found in cells with a regulated secretory pathway. This is true in the endogenous cells that normally make glucagon (pancreatic alpha cells) and, as demonstrated in this patent, is true for RIN 1046-38 cells. Expression of preproglucagon transgenes in a variety

of cell lines has demonstrated cell-specific differences in processing (Drucker, Mojsov *et al.*, 1986). RIN 1046-38 cells have the capacity to produce, process, store and secrete human insulin as demonstrated in the above example. This includes high endogenous expression of PC2 and PC3, endoproteases involved in processing both insulin and glucagon (Vollenweider and Kaufmann *et al.*, 1995) (Rouille, *et al.*, 1995). RIN 1046-38 clones overexpressing preproglucagon transgenes should efficiently process the precursor into mature glucagon, store it in secretory granules and release glucagon upon stimulation to the media. This would of course be useful for both large scale production of glucagon as well as in vivo cell based delivery of glucagon.

RIN 1046-38 cells should also process preproglucagon into glucagon, GLP-I and II. Final maturation of GLP-1 involves C-terminal amidation by peptidylglycine alpha-amidating monooxygenase (PAM). Engineering RIN cells to predominantly produce glucagon or GLP-1 is possible by molecular engineering. Processing of preproglucagon to glucagon is predominantly by the action of PC2 while processing to GLP-1 is predominantly by PC3 (Rouille, *et al.*, 1995). Overexpression of either a PC2 or PC3 transgene could result in predominant expression one peptide hormone over another. Alternatively, the glucagon transgene could be mutated such that the dibasic amino acid residues recognized by PC2 and PC3 are altered such that only glucagon or GLP-1 is capable of being processed to the mature, biologically active polypeptide.

Human Preproglucagon Expression in RIN 1046-38 Cell Lines. Rat insulinoma cells have been shown to express the glucagon message (Philippe, *et al.*, 1987). A series of RIN cell derivatives all originating from the same original insulinoma (Gazdar, Chick *et al.*, 1980) were screened for expression of endogenous glucagon message. While RIN 1046-38 cells do not express endogenous glucagon, other RIN cells do (Philippe *et al.*, 1987), suggesting expression of preproglucagon in RIN 1046-38 cells is possible.

pCMV8/h.Glucagon/IRES/NEO was electroporated into RIN 1046-38 cells and stable clones selected in G418. Individual colonies were screened using the non-amidated GLP-1 immunoassay. Once individual clones had been established in 24 or 6 well tissue culture plates, a

24 hour media sample was collected and assayed. Cell number was estimated from visual inspection of the culture plates. Clones with obvious increased GLP-1 immunoreactivity were identified and characterized further.

Three such individual clones (BG175/5, 174/19 and 174/45) were examined for regulated secretion of immunoreactive species of GLP-1 and glucagon. Culturing of cells under either a one hour basal condition (B), a one hour stimulated condition (S) and a 24 hour condition (24) were collected and the same sample assayed for non-amidated GLP-1, amidated GLP-1 and glucagon. Each immunoassay is specific with no crossreactivity according to the specifications supplied with the kits. Immunoreactive hormone levels for these three wild type human preproglucagon expressing clones as compared to unengineered RIN 1046-38 cells are given in Table 11. Parental RIN 1046-38 cells have undetectable or very low levels of GLP-1 or glucagon species. All three wild type preproglucagon expressing clones had easily detectable levels of all three hormones. Comparison of stimulated to basal samples demonstrated a good fold induction in secretion for all hormones. Of interest is the percentage of non-amidated GLP-1 to total GLP-1 (non-amidated and amidated). This percentage under a range of culturing conditions in all three clones is 49% $\pm$9%. This percentage of RIN secreted GLP-1 species in vitro is very similar to the percentage of GLP-1 species secreted in vivo by the endogenous L cells of the intestine (Mojsov, *et al.*, 1990).

Table 11

CELL	CULTURE	NON*	FOLD(S/B)*	AMIDE *	FOLD (S/B)	NON (%TOTAL)	GLUCAGON
WILD TYPE							
175/5	B	0.57		0.51		53	0.18
	S	3.12	5.44	3.85	7.55	45	0.39
	24	26.76		18.3		59	43.65
174/19	B	0.4		0.38		51	not detected
	S	1.63	4.13	3.65	9.73	31	4.0
	24	8.69		9.23		49	7.3
174/45	B	0.91		0.52		64	not detected
	S	5.02	5.51	5.8	11.15	46	17.8
	24	11.94		15.38		44	14.33
AVERAGE			5.03±0.6		9.48±1.5	49±9	
MUTANT							
191/18	B	2.35		0.91		72	2.14
	S	5.41	2.3	7.4	8.17	42	5.05
	24	31.02		16.45		65	58.48
191/17	B	1.47		0.76		66	0.64
	S	3.72	2.52	5.7	7.54	39	3.8
CELL	CULTURE	NON*	FOLD(S/B)*	AMIDE *	FOLD (S/B)*	NON (%TOTAL)	GLUCAGON
	24	18.46		11.16		62	52.71

191/26	B	1.74		0.58		75	1.45
	S	10.37	5.96	6.25	10.87	62	16.5
	24	31.33		16.64		65	0.54
191/30	B	0.6		0.28		68	0.54
	S	2.59	4.28	4.95	17.67	34	6.1
	24	16.45		13.99		54	51.49
AVERAGE			3.76±1.5		11.06±4.0	59±13	
RIN 1046-38	B	0.08		not detected		na	not detected
	S	0.09	1.09	0.23	NA	27	not detected
	24	0.08		not detected		NA	0.19

* values are in ng/ml

Mutated Preproglucagon Expression in RIN Cell Lines. pCMV8/mutant.Glucagon/IRES/NEO was electroporated into RIN 1046-38 cells and stable clones selected in G418. Individual colonies were screened using the non-amidated GLP-1 immunoassay as done before for the wild type preproglucagon expressing clones. Clones with obvious increased GLP-1 were again identified and characterized further.

Four such individual clones (BG 191 series, Table 9) were examined for regulated secretion of immunoreactive species of GLP-1 and glucagon. Culturing and assays for non-amidated GLP-1, amidated GLP-1 and glucagon were done as before. All four mutant preproglucagon expressing clones had easily detectable levels of all three hormones. Comparison of stimulated to basal samples again demonstrated a good fold induction in secretion for all hormones. The percentage of non-amidated GLP-1 to total GLP-1 (non-amidated and amidated is 59% ±13%, again very similar to the wild type expressing cells or in vivo levels. Glucagon immunoreactive species were easily detected, with levels comparable to wild type expressing cells. Whether the glucagon immunoassay discriminates between fully processed glucagon and the major unprocessed form of glucagon, glicentin, is presently not known. Conformation that the mutated preproglucagon can only produce non-biologically active glicentin and GLP-1 will be needed.

EXAMPLE 11

Novel In vivo Activity From Tumors Expressing Amylin And Insulin

Methods

Tumor Formation Of Transfected RIN Cell Lines In Nude Rats. 6 to 8 week old athymic Fisher nude rats (200-300 grams, Strain F344/Ncr-rnu from National Cancer Institute, Fredrick, MD.) were housed in a sterile isolation facility with free access to sterile standard laboratory chow and water. Blood glucose was measured using an IBI Biolyzer (Kodak, Eastman Chemical Co.) or a chemstrip glucometer (Beta-Glucose Photometer, Hemocue, Inc., Sweden). A pre-transplant body weight and blood glucose profile was determined 3 days before transplant with

daily tail vein bleeds between 8:00 and 9:00 AM. Post-transplant body weight was recorded and blood glucose profile was determined throughout the course of the experiment by daily tail vein bleeds between 8:00 and 9:00 AM. Pre-transplant serum insulin and amylin levels were determined on the morning of bleed (0.5 ml bleed). Post-transplant insulin and amylin levels were determined 7 days after transplant and again at various times as blood glucose levels begins to decline.

Amylin and Insulin Blood Serum Sample Preparation - 500 blood samples are transferred to an Eppendorf tube containing 5 ul of a 100x protease inhibitor cocktail (100x stock:; 320 mM EDTA,1 mg/ml aprotinin). Samples are stored on ice until clot formation, then microfuged for 5 min. Serum is transferred to fresh tube and stored at -20 C until assayed. Serum insulin levels were determined as described by the supplier of the insulin radioimmuno-assay (Coat-a-count, Diagnostic Products Corp., Los Angeles). Serum amylin levels were determined using a rat amylin radio-immunoassay as described by the supplier (Peninsula Laboratories, Inc., Cat. # RIK-7323).

RIN cell preparation and injection. 24 h before implantation, media was changed to serum free media to minimize transplantation of foreign antigens in serum. Cells were washed with PBS-CMF, then trypsinized. 5 volumes of serum free media was added and cells pelleted by low speed centrifuge. Remove media and wash cells 2 X PBS, (calcium and magnesium free). Add freezing solution (5mM KH_2PO_4, 25 mM KOH, 30 mM NaCl, 0.5 mM $MgCl_2$, 20 mM L-lactic acid, 30 mM L-glutamine, 5 mM glucose, 167 mM myo-inositol, and 200 mM sorbitol (pH 7.2-7.4; filter sterilize) to pellet, adjusting cell concentration to 4 million cells/ml. Aliquot 4 million cells to a 1.5 ml. Eppendorf tube and compare packed cell volumes after microfuging at low speed, as a check on cell number, then resuspend cells in 2400 ul freezing solution. Nude rats are anesthetized with i.p. injection of 0.1 ml Nembutol (50 mg/ml) per 100 g body weight. Swab dorsal area at kidney level -with 70% EtOH, then inject 20,000 cells per gm. body weight subcutaneously above animals right kidneys.

Nude Rat dissection and analysis. Nude rats with palpable tumors were anesthetized as before. A dorsal midsagital incision through skin of back was made, the skin pealed back to reveal the tumor laterally at level of the kidney. Photographs were taken and then the tumor was removed using forceps and iris scissors. The tumor was blotted on Whatman paper to remove excess liquid and then weighed. Tumors were dissected in half with one half frozen in liquid nitrogen and stored at -80 C while the other half was fixed in Bouin's Fixative for histology (85 parts saturated picric acid solution, 10 parts 37% formaldehyde, 5 parts glacial acetic acid). Tumors were placed in tissue cassette and fixed in a beaker containing 3 X volume of Bouin's for 24 hours at room temperature. Next day, wash in cold running tap water for 24 h. Transfer tumor for storage to a beaker containing 3 X volume of 10% formalin.

In addition to isolation of palpable tumors, pancreases were isolated from the animals. A midsagital laperotomy was performed to expose abdominal contents, exposing the pancreas in area of spleen. A portion of the pancreas in juxtaposition to spleen was isolated, dissected in half, and one half frozen in liquid nitrogen and stored at -80 C while the other half fixed in Bouin's Fixative for histology.

Streptozotocin-induced Diabetic Nude Rats. Diabetes was induced in nude rats by a one time IV administration of 65mg/kg body weight streptozotocin.

Results:

Three RIN lines were injected into rats, RIN 1046-38, rat amylin engineered EP97/134, and human amylin engineered EP182/7. These three cell lines produced identical amounts of insulin but exhibited a 12 fold (EP97/134) and 36 fold (EP182/7) difference in amylin output (Table 9). RIN 1046-38 cells do secrete amylin and insulin at a physiologic ratio of .028, EP97/134 secretes amylin and insulin at a ratio of 0.17, and EP182/7 secrete human (and low levels of rat amylin) at an amylin/insulin ratio of 0.6.

Transplantation into nude rats of parental, rat amylin and, to a lesser extent, human amylin engineered RIN lines shows that there is an additive effect of RIN produced amylin with insulin in helping lower blood glucose. Daily blood glucose levels of five nude rats injected with rat amylin producing cells and five injected with human amylin producing cells are compared to three nude rats injected with parental RIN 1046-38 cells and an uninjected control. As shown in FIG. 22, the rat amylin producing cells induced hypoglycemia rapidly with an average onset time of 8.4 days ±1.2 days. Human amylin producing cells had an average onset time of 14.8 ±1.8 days. Parental RIN 1046-38 cells had an average onset time of 17.3 ±3.4 days. The simplest explanation is amylin is doing exactly what it has been described to do, slowing gastric emptying which results in lower glucose levels in the blood (Young, *et al.*, 1995; Brown, *et al.*, 1994). Rat amylin in rats is very effective at this while human amylin in rats has may have an attenuated effect. Animals with human amylin producing cells did in fact have diarrhea, so something gastric was going on. With a constitutive source of insulin present (endogenous insulin from tumor cells), blood glucose falls as amylin increases.

Confirmation that amylin expression is maintained in vivo in the engineered RIN cells comes from Northern analysis of RNA isolated from tumors at the end of the experiment. RNA was isolated from rat tumors arising from either parental or rat amylin producing RIN cells following in vivo passage and was compared to the same RIN cells maintained in vitro. Northern analysis using a rat amylin specific probe demonstrates no difference in either the low levels of endogenous amylin expression in either RIN 1046-38 or BG97/134 cells or high levels of the rat amylin transgene in engineered BG97/134 cells (FIG. 24).

A second animal model, the streptozotocin-induced diabetic nude rats, was also used. Diabetes was induced in rats two weeks prior to injection of either RIN 38, EP97/134, or EP111/228 (our highest insulin producing RIN cell with only low endogenous amylin production). As shown in FIG. 25, all strep-injected animals were diabetic with blood glucose's

of 400 mg/dl. Once again, EP 97/134 cells induced hypoglycemia more rapidly than RIN 38 cells. EP111/228, with a 15 fold elevated insulin production compared to RIN 38 and EP97/134, induced hypoglycemia more rapidly than any other cell line but only twice as rapidly as the amylin overproducing line, BG97/134.

To directly test the blood glucose lowering effects of a RIN line that coproduces elevated levels of amylin and insulin, four independent RIN lines were injected into nude rats. These lines were parental RIN 1046-38 cells, EP18/3E1 cells overexpressing human insulin, EP97/134 cells overexpressing rat amylin, and EP 181/59 cells overexpressing human insulin and rat amylin. Secreted amylin/insulin ratios as measured in vitro are .03, .002, .17 and .024 as shown in Tables 9 and 10. EP181/59 cells were chosen because of the high overexpression of both rat amylin and human insulin, resulting in an almost normal amylin/insulin ratio. The absolute production of rat amylin and human amylin are both elevated five fold compared to the parental RIN 1046-38. These absolute outputs, on a per cell basis, is actually about half the output of either human insulin from EP18/3E1 cells or rat amylin from EP97/134 cells.

Cells were injected into nude rats, with four animals used per group injection group. Three uninjected rats were used as normal controls. Blood glucose was monitored throughout the experiment. The average blood glucose values of each group are shown in FIG. 26. The normal rats maintained blood glucose levels of 110 to 120 mg/dl throughout the experiment. All four injected animal groups demonstrated a drop in blood glucose levels as the tumors grew. As in the previous experiment, nude rats bearing the rat amylin producing clone showed a quicker drop in blood glucose than the unengineered parental cells. The drop in blood glucose in the rat amylin producing clones was actually earlier than the human insulin producing clone, EP 18/3E1. Surprisingly, the amylin/insulin coexpressing cells, EP181/59, were very effective at lowering blood glucose, even with lower absolute outputs of either amylin or insulin than EP18/3E1 or EP 97/134.

Serum amylin and insulin levels are given in FIG. 27 and FIG. 28 with the molar ratio of amylin to insulin given in FIG. 29. It should be noted that insulin values were determined using a human insulin standards. This underestimates the rat insulin levels by about three fold as seen in the normal rat control serum values of between 1.5 and 2 ng/ml rather than 4 to 6 ng/ml. This also skews the amylin/insulin ratios reported in FIG. 29, such that normal ratios are between .08 and .1 rather than the expected ratio of .02 to .03. As expected, serum amylin levels rose dramatically in the rats bearing the rat amylin overexpressing cells.

Serum amylin rose moderately in the insulin/amylin coexpressing lines, perhaps 3 to 4 fold above normal physiologic levels. Rats bearing the insulin overproducing or unengineered parental cells only had a moderate increase in serum amylin. Serum insulin values rose fairly high in the insulin overproducing line and moderately high in the insulin/amylin producing line. Rats bearing the amylin overproducing cells or unengineered parental cells had moderate increases in serum insulin. As shown in FIG. 29, the ratios of amylin to insulin throughout the experiment in the various animal groups is informative. Animals bearing the insulin overproducing clones result in lower amylin/insulin ratios than normal animals. Animals bearing the amylin overproducing clones result in higher amylin/insulin ratios than normal animals. Animals bearing the unengineered parental cells or the amylin/insulin coengineered cells, both having in vitro amylin ratios in the normal range, maintain a near normal ratio throughout the course of the experiment.

This result demonstrates a beneficial effect of lowering blood glucose by coengineering amylin and insulin production in RIN cells. Lower blood glucose is achieved with comparatively lower outputs of either amylin or insulin overproduced alone. Also, serum levels of both amylin and insulin needed for this effect are only moderately increased above normal animals by about four fold while maintaining a near normal ratio of the two.

The inventors studies show that amylin related species act additively with insulin in lowering blood glucose. A cell overexpressing insulin and amylin will lower blood glucose more effectively than a cell overexpressing either one by itself. RIN clones coengineered for amylin and insulin over-production have already been isolated and characterized.

The inventors have further demonstrated that the rat amylin producing RIN cell line used lowers blood glucose quicker than human amylin producing RIN cell line or parental RIN cell. This effect could be clone specific, or it demonstrates an advantage that rat amylin confers the ability of a given tumor to deliver hormones to the serum. A very possible advantage is increased vascularization stimulated by rat amylin in rats. Experiments designed to directly check this are being planned. Increased vascularization in the context of devices is an obvious benefit.

EXAMPLE 12

Increased Expression of either Human Amylin or Mutated Proglucagon by Generation of Fusion Proteins with Human Growth Hormone

Methods:

Fusion protein expression plasmids. pCB6/hGH was digested with TthIIIi, treated with Klenow, then digested with Spe1 generating a 2060 base pair fragment containing the majority of the human growth hormone 5' coding sequence. pNoTAT7/h.Amylin was digested with BsiHKA1, treated with Klenow, then digested with Xba1 generating a 418 base pair fragment containing the human proamylin coding sequence. These two fragments were ligated into pCB6 that had been digested with Spe1 and Xba1. The resulting plasmid, pCB6/hGH.hAMYfusion contains the insert SEQ ID NO:70 where the first 1466 base pairs are derived from the human growth hormone gene (bases 498 to 1964 of published sequence, Seeburg, 1982) fused to 418 base pairs derived from the human amylin cDNA. The resulting fusion protein, SEQ ID NO:71,

consists of the first 198 amino acids (out of 217 amino acids) of human growth hormone fused in frame to the final 76 amino acids (amino acids 14 through 89) of human amylin.

pNoTAT7/mutGlucagon was digested with PvuII and XbaI generating a 891 base pair fragment containing the mutated proglucagon coding sequence. This fragment was ligated with the above mentioned TthIIIi, Klenow treated, SpeI fragment of pCB6/hGH into pCB6 that had been digested with SpeI and XbaI. The resulting plasmid, pCB6/hGH.mutGlucagonfusion contains the insert SEQ ID NO:72, where the first 1466 base pairs are derived from the human growth hormone gene (bases 498 to 1964 of published sequence, Seeburg, 1982) fused to 891 base pairs derived from the mutated proglucagon cDNA. The resulting fusion protein, SEQ ID NO:73, consists of the first 198 amino acids (out of 217 amino acids) of human growth hormone fused in frame to the final 162 amino acids (amino acids 19 to 180) of mutated proglucagon.

Results:

pCB6/hGH.hAMYfusion and pCB6/hGHmutGlucagonfusion were electroporated into RIN1046-38 cells as well as RIN1046-44 cells. The latter cell line was generated from the same original rat insulinoma as the RIN 1046-38 line, but expresses very low levels of endogenous insulin (Philippe, *et al.*, 1987). Stable clones of each were selected using G418.

The expectation is that both fusion proteins will be efficiently targeted to the regulated secretory pathway, as has been described for similar growth hormone fusion constructs (Moore and Kelly, 1985). Once targeted to the secretory granules, post-translational processing events including proteolytic cleavage at dibasic amino acid residues and amidation should occur. The 1000 fold difference in expression of wild type growth hormone (app. 100 ug/million cells/24 hours) versus either amylin or GLP-1 (app. 100ng/million cells/24 hours) appears inherent to the growth hormone sequences.

EXAMPLE 13

Genomic Site-Directed Mutagenesis with Oligonucleotides

The inventors have previously demonstrated that derivative cell lines of the RIN 1046-38 cell line are capable of performing homologous recombination by disrupting an allele of the hexokinase I gene. Feasibility studies are currently underway to determine if RDOs or DNA oligonucleotides can be used for the purpose of targeted gene disruption in RIN and other cell lines. Two test systems have been designed for testing oligonucleotides: the disruption of the neomycin phosphotransferase transgene, and the disruption of the glucose transporter, type 2 (GLUT-2). As a preliminary experiment to testing RDOs or DNA oligonucleotides, protocols for efficient delivery of DNA into RIN cell lines by electroporation have been optimized.

A. Optimization of transfection of RIN cell lines.

A number of transfection protocols were tested on RIN 1046-38 cell lines including a variety of electroporation conditions and multiple kinds of liposome-mediated transfection. All protocols, except one set of electroporation conditions, failed to produce transfection efficiencies of greater than 5%. Protocols were optimized for delivery of exogenous DNA to RIN cells by electroporation using two types of DNA: a plasmid vector encoding beta–galactosidase (β–gal) that is transcribed from the CMV promoter, and a DNA oligonucleotide (62mer) that had been radiolabeled with ^{32}P-dCTP. One set of electroporation conditions resulted in 25 - 40% transfection of the total cell population as determined by colorometric, cytochemical assays for β–gal activity (Bassel-Duby *et al.*, 1992). Cells were grown to about 80% confluence in Medium 199/ 5% fetal calf serum/ 11 mM glucose (Growth Medium) and were re-fed with fresh Growth Medium one day prior to electroporation. Cells were harvested by trypsinization, counted, pelleted by centrifugation at 1000 rpm for 5 minuets, and resuspended in Growth Medium at a density of 2 x 10^7 cells/ml. 0.5 ml of cell suspension was mixed with 60 µl of the following DNAs: either 10 µg of β–gal plasmid or 40 nM of oligonucleotide and 110 µg of sonicated salmon sperm DNA. The cells plus DNA were mixed gently, transferred to a 0.4 mM

cuvette, and electroporated at 600 μF, 250 volts using and Electro Cell Manipulator 600, BTX Electroporation System. The electroporated cells were removed from the cuvette and diluted into 25 - 30 mls of 37°C Growth Medium containing 5 mM butyrate. Following incubation for 12-16 hours at 37°C, 5% CO_2 in the growth medium with butyrate, cells were washed once with growth medium, and maintained in growth medium. In the case of cells transfected with β–gal, cells were maintained 48-72 hours following transfection and fixed with 0.5% glutaraldehyde for 10-15 minutes for cytochemical detection of β–gal using the 5-bromo-4-chloro-3-indoyl-β–D-galactopyranoside (x-gal) as a substrate (Bassel-Duby *et al.*, 1992).

To determine if conditions optimized for plasmid DNA would translate to efficient uptake of oligonucleotides, the above electroporation protocol was applied to a 62mer that have been radiolabeled with ^{32}P using the Redi-prime Random Primer labeling Kit (Amersham Life Sciences). Oligonucleotide (40 nM) was electroporated into cells. Cells were analyzed post-transfection at 0, 3, 6, and 24 hours in two ways. First, total radioactivity in the media, cytoplasmic cellular fractions, and nuclear cellular fractions was determined by scintillation counting,. And second nucleic acids were harvested from cellular fractions by phenol/chloroform/isoamyl extraction and fractionated through denaturing polyacrylamide (PAG) gels (Ebbinghaus *et al.*, 1996).

There was a marked enhancement in nuclear radioactivity in the presence of electroporation as compared to control cells that were mixed with oligonucleotide but not electroporated. In the presence of electroporation, about 29, 55, and 66% of total intracellular counts segregated to the nuclear fraction at 0 , 3, 6, and 24 hours, respectively. In contrast, only 1 - 24% of total intracellular radioactivity was detected in the nuclear fraction through the 24 hour time point. It was also observed that intact, apparently full-length oligonucleotide could be extracted from cells which had been electroporated, as evidenced by fractionation on denaturing PAG gels and autoradiography. Extracts from cells that had been mixed with the oligonucleotide but not electroporated did not yield detectable oligonucleotide by this method of analysis

suggesting that radioactivity that was detected in the non--electroporated cellular fractions was derived from the exchange of radiolabel, not from the oligonucleotide.

From these studies it has been concluded that the electroporation protocol described above is a preferred method for transfecting both plasmid DNA and oligonucleotides into RIN cells.

B. Disruption of the neomycin phosphotransferase (NPT) transgene by RDOs.

Multiple RIN cell lines are available that have been engineered to contain an integrated copy of the NPT gene. An RDO for disruption of transgenic NPT has been designed that is complementary to nucleotides to 54 to 78 of NPT counting the "A" of the first methionine as 1. Further, the RDO contains a single base change relative to the wild-type NPT (A to C at position 66). If gene conversion by the RDO is successful, a T will converted to a G, Tyr22 will be converted to a stop codon, resistance to G418 will be lost, and a unique *Mae* I restriction site will be introduced. The RDO also contains features previously described such as self-annealing hairpin loops at each end, and 2'-O-methylation of the ribose sugars. The sequence of the RDO with these features is (5' to 3' and referred hereafter as AT142):
GCTATTCGGCTAGGACTGGGCACAATTTT**uugugcccag**TCCTA**gccgaauagc**GCGCGTTTT CGCGC (SEQ ID NO:74), where large caps represent DNA residues and small, bold letters indicate RNA residues.

RIN cell lines with a single integrated copy of NPT will be electroporated, as described in materials and methods, with varying concentrations of RDO AT142. 4 to 6 hours following transfection genomic DNA from pools of transfectants will be analyzed for detection of a T to G conversion at position 66 of the NPT transgene. Following isolation of genomic DNA, the first about 200 base pairs of the NPT transgene will be amplified by the polymerase chain reaction (PCR) using oligonucleotides that flank position 66. Following amplification, PCR products

will be digested with *Mae* I to determine if any gene conversions have occurred. If the case of successful gene inactivation by the RDO, the PCR product will be digested into two bands. The wild-type NPT transgene PCR product will be resistant to *Mae* I digestion. If NPT gene disruption is detectable by PCR/*Mae* I digestion, small pools of clones will be analyzed for loss of resistance to G418. Following electroporation, cells will be plated into 96 well plates at densities of 3 to 5 cells/well. 3 days following electroporation, cells will be exposed to G418, and each well will be scored for the presence of cell death.

C. Disruption of transgenic GLUT-2 in RIN and 293 cell lines.

RIN cell lines and 293 cell lines have been engineered to express high levels of a transgenic GLUT-2 transporter as detailed herein above. The presence of this transporter confers sensitivity to the cytotoxin streptozotocin (STZ), and thereby provides a means of negative selection (Schnedl *et al.*, 1994). Both RIN and 293 cell lines that express high levels of a GLUT-2 transporter will be transfected with RDOs designed to target and disrupt transgenic GLUT-2, and 4 - 6 hours later cells will be exposed to cytotoxic levels of STZ. Surviving clones will be analyzed for the presence of an inactivated GLUT-2 transgene by analysis of genomic DNA. In the case of the targeted inactivation of transgenic GLUT-2, leucine at position 10 will converted to a stop codon as a result of a T to A conversion, and a unique *Avr* II restriction site will be created in the transgenic GLUT-2. This unique site can be detected by the amplification of genomic DNA that flanks the site by PCR, followed by digestion of the amplified DNA with *Avr* II. One such RDO that potentially accomplishes the targeted disruption as described above is the following sequence:

TCACCGGAACCTAGGCTTTCACTGTTTTT**acagugaaag**CCTAG**guuccgguuga**GCGCGTTT TCGCGC (SEQ ID NO:75), where large capitals represent DNA residues and small bold letters represent RNA residues.

Attempts to disrupt transgenic GLUT-2 will also be made with non-chimeric DNA oligonucleotides that contain phosphorothioate modified backbones to enhance stability. It has been reported that inclusion of phosphorothioate derivatives within the DNA backbone decreases sensitivity to nucleases (Vosberg and Eckstein, 1982; Monia et al., 1996). Oligonucleotides have been designed that should selectively target the transgenic GLUT-2 by spanning an area of homology that is interrupted in the endogenous GLUT-2 gene by an intron. If targeting and modification of the GLUT-2 transgene are successful, glutamine at position 35 will be converted to a stop codon, and a new *Afl* II site will be introduced into the DNA at this position. DNA oligonucleotides will be examined for the ability to target and disrupt the transgenic GLUT-2:

oligo name: AT157 (5'to3')

GsGTTCCTTCCAGTTCGGATATGACATCGGTGTGATCAATGCACCTTAAGAGGTAAT AATATCCCATTATCGACATGTTTTGGGTGTTCCTsC (SEQ ID NO:76),

oligo name: AT158 (5' to 3')

GsAGGAACACCCAAAACATGTCGATAATGGGATATTATTACCTCTTAAGGTGCATTG ATCACACCGATGTCATATCCGAACTGGAAGGAACsC (SEQ ID NO:77),

oligo name: AT159 (5' to 3')

GsGATATGACATCGGTGTGATCAATGCACCTTAAGAGGTAATAATATCCCATTATCG ACATsG (SEQ ID NO:78),

and oligo name: AT160

CsATGTCGATAATGGGATATTATTACCTCTTAAGGTGCATTGATCACACCGATGTCAT ATCsC (SEQ ID NO:79).

Each of above the 4 oligonucleotides have phosphorothioate modifications in the backbone near the 3' and 5' ends as indicated by "s" in the sequence. Oligonucletides will be introduced into cells both as single-stranded molecules and as double-stranded complexes. The following oligonucletide pairs contain complementary sequences and will form duplexes: AT157-AT158, AT157-AT160, AT158-AT159, and AT159-AT160. Cell lines that express high levels of transgenic GLUT-2 will be electroporated with oligonucleotides as described above, and exposed to levels of STZ that are lethal to cells expressing non-disrupted transgenic GLUT-2. Genomic

DNA of surviving cells will be analyzed for the presence of disrupted transgenic GLUT-2 by amplification of DNA containing the putative mutation by PCR, followed by digestion with *Afl* II.

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

V. REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

Adams *et al.*, "Porin interaction with hexokinase and glycerol kinase: Metabolic microcompartmentation at the outer mitochondrial membrane," *Bioch. Med. Met. Biol.*, 45:271-291, 1991.

Aguilar-Bryan *et al.*, "Cloning of the Beta cell high-affinity sulfonylurea receptor: A regulator of insulin secretion," *Science*, 268:423-426, 1995.

Altman *et al.*, *Diabetes* 35:625-633, 1986.

Anderson *et al.*, "Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme," *J.B.C.*, 264:8222-8229, 1989.

Arimura *et al.*, "Regulation of growth hormone secretion." The Pituitary Gland, 2nd Edition, Chap.9:217-259. H. Imura, Ed., Raven Press, N.Y., 1994.

Arora, *et al. J.B.C.* 268:18259-18266, 1993.

Asfari *et al.*, "Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines." *Endocrinology*, 130:167-178, 1992.

Bahnemann *et al.* "Animal cell, viral antigens and vaccines," *Abs. Pap. ACS*, 180:5. 1980.

Baichwal *et al.*, "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," *In:* Kucherlapati R, ed. Gene transfer. New York: Plenum Press, pp. 117-148, 1986.

Baijal, *et al., J.E. Arch. Bioch. Biophys.* 298:271-278, 1992.

Bailey *et al.*, "Methylmercury as a reversible denaturing agent for agarose gel electrophoresis," *Anal. Biochem.*, 70:75-85, 1976.

Barr *et al.*, "Systemic delivery of recombinant proteins by genetically modified myoblasts," *Science*, 254:1507-1509, 1991.

Bassel-Duby, R., Hernandez, M.D., Gonzalez, M.A., Krueger, J.K., Williams, R.S., 1992. A 40 kilodalton protein binds specifically to an upstream sequence element essential for muscle-specific transcription of the human myglobin promoter. *Mol. Cell. Biol.* 12: 5024-5033.

Bauchwitz, R. and Holloman, W.K. 1990. Isolation of the REC2 gene controlling recombination in *Ustilago maydis. Gene* 96:285-288.

Beaumont, K., Kennery, M.A., Young, A.A. and Rink, T.J., "High affinity and amylin binding sites in rat brain," *Molecular Pharmacology* 44:493-497, 1993.

Becker *et al.*, "Differential effects of overexpressed glucokinase and hexokinase I in isolated islets: Evidence for functional segregation of the high and low K_m enzymes," *J. Biol. Chem.*, 271:390-394, 1996.

Becker *et al.*, "Use of recombinant adenovirus for metabolic engineering of mammalian cells," *Methods in Cell Biology*, 43:161-189, Roth, M., Ed. New York, Academic Press, 1994.

Becker *et al.*, "Overexpression of hexokinase 1 in isolated islets of langerhans via recombinant adenovirus," *J.B.C.*, 269:21234-21238, 1994.

Bell, *et al.*, "Characterization of the 56-kDa subunit of yeast trehalose-6-phosphate synthase and cloning of its gene reveal its identity with the product of CIF1, a regulator of carbon catabolite inactivation," *Eur. J. Biochem.*, 209:951-959, 1992.

Bell *et al.*, "Nucleotide sequence of a cDNA clone encoding human preproinsulin." *Nature* 282:525-527, 1979.

Benjannet *et al.*, "Comparative biosynthesis, covalent post-translational modifications and efficiency of prosegmant cleavage of the prohormone convertases PC1 and PC2: Glycosylation, sulphation and identification of the intracellular site of prosegment cleavage of PC1 and PC2," *Biochem. J.*, 294:735-743, 1993.

Benvenisty and Neshif, "Direction introduction of genes into rats and expression of the genes," *Proc. Nat'l Acad. Sci. USA*, 83:9551-9555, 1986.

Berzal-Herranz, A. *et al.*, *Genes and Devel.*, 6:129-134, 1992.

Bhogal, R., Smith, D.E., and Bloom, S.R., "Investigation and characterization of binding sites for islet amyloid polypeptide in rat membranes, *Endrocrinology*, 130,2:906-913, 1992.

Blasquez *et al.*, "Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases," *FEBS Lett.* 329:51-54, 1993.

Brewer, "Cytomegalovirus plasmid vectors for permanent lines of polarized epithelial cells," *In: Methods in Cell Biology*, 43:233-245, Roth, M., Ed. New York, Academic Press, 1992.

Brewer, "Cytomegalovirus plasmid vectors for permanent lines of polarized epithelial cells," In: Methods in Cell Biology, 43:233-245, Roth, M., Ed. New york, Academic Press, 1994.

Brunstedt *et al.*, "Direct effect of glucose on the preproinsulin mRNA level in isolated pancreatic islets," *B.B.R.C.*, 106:1383-1389, 1982.

Burch, *et al.*, "Adaptation of glycolytic enzymes. Glucose use and insulin release during fasting and refeeding." *Diabetes* 30:923-928.

Burgess *et al.*, "Constitutive and regulated secretion of proteins," *Ann. Rev. Cell Biology*, 3:243-293, 1987.

Cech *et al.*, "*In vitro* splicing of the ribosomal RNA precursor of *Tetrahymena*: involvement of a guanosine nucleotide in the excision of the intervening sequence," *Cell*, 27:487-496, 1981.

Challita *et al.*, "Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation *in vivo*," *Proc. Natl. Acad. Sci. USA*, 91:2567-2571, 1994.

Chang *et al.*, "Foreign gene delivery and expression in hepatocytes using a hepatitis B virus vector," *Hepatology*, 14:134A, 1991.

Chavez *et al.*, "Expression of exogenous proteins in cells with regulated secretory pathways," *In*: *Methods in Cell Biology*, 43:263-288, Roth, M., Ed. New York, Academic Press, 1994.

Chen and Okayama, "High-efficiency transfection of mammalian cells by plasmid DNA," *Mol. Cell Biol.*, 7:2745-2752, 1987.

Chen *et al.*, "Regulated Secretion of Prolactin by the mouse cell line Beta TC-3," *Biotechnology*, 13:1191-1197, 1995.

Chen *et al.*, *Proc. Nat'l Acad. Sci. USA* 93:14795 (1996).

Chowrira, B.H. *et al.*, *Biochemistry*, 32, 1088-1095.

Chowrira, B.H. *et al.*, "In vitro and in vivo comparison of hammerhead, hairpin, and hepatitis delta virus self-processing ribozyme cassettes." *J.B.C.*, 269:25856-25864, 1994.

Clark *et al.*, "Modulation of glucose-induced insulin secretion from a rat clonal beta-cell line," *Endocrinology*, 127:2779-2788, 1990.

Clark *et al.*, "Islet cell culture in defined serum-free medium," *Endocrinology*, 126:1895-1903, 1990.

Coffin, "Retroviridae and their replication," *In*: *Virology*, Fields *et al.* (eds.), New York: Raven Press, pp. 1437-1500, 1990.

Cole-Strauss, A., Yoon, K., Wiang, Y. Byrne, B.C., Rice. M.C. Gryn, J., Holloman, W.K., and Kmiec, E.B. 1996. Correction of the mutation responsible for sickle cell anemia by and RNA-DNA oligonucleotide. *Science* 273: 1386-1389.

Cone *et al.*, *Proc. Nat'l Acad. Sci. U.S.A.* 81:6349-6353, 1984

Cordell *et al.*, "Isolation and characterization of a cloned rat insulin gene." Cell, 18:533-543, 1979.

Couch *et al.*, "Immunization with types 4 and 7 adenovirus by selective infection of the intestinal tract," *Am. Rev. Resp. Dis.*, 88:394-403, 1963.

Coupar *et al.*, "A general method for the construction of recombinant vaccinia virus expressing multiple foreign genes," *Gene*, 68:1-10, 1988.

Curry, "Insulin content and insulinogenesis by the perfused rat pancreas: Effects of long term glucose stimulation," *Endocrinology*, 118;170-175, 1986.

Cuttitta, "Peptide amidation: Signature of bioactivity," *The Anatomical Record*, 236:87-93, 1993.

D'Santos, C.S., Gatti, A., Poyner, D.R., and Hanley, M.R., "Stimulation of Adenylate Cyclase by Amylin in CHO-K1 Cells," *Molecular Pharmacology* 41:894-899, 1992.

Dai *et al.*, "Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: Tolerization of factor IX and vector antigens allows for long-term expression," *P.N.A.S. USA*, 92:1401-1405, 1995.

Danos *et al.*, *Proc. Nat'l Acad. Sci. U.S.A.* 85:6460-6464, 1988.

Day *et al.*, "Distribution and regulation of the prohormone convertases PC1 and PC2 in the rat pituitary" *Molecular Endocrinology*, 6:485-497, 1992.

Dhawan *et al.*, "Systemic delivery of human growth hormone by injection of genetically engineered myoblasts," *Science*, 254:1509-1512, 1991.

Dickerson *et al.*, "Transfected human Neuropeptide Y cDNA expression in mouse pituitary cells," *J.B.C.*, 262:13646-13653, 1987.

Drucker *et al.*, "Cell-specific post-translational processing of preproglucagon expressed from a metallothionein-glucagon fusion gene," *J.B.C.*, 261:9637-9643, 1986.

Dubensky *et al.*, "Direct transfection of viral and plasmid DNA into the liver or spleen of mice," *Proc. Nat. Acad. Sci. USA*, 81:7529-7533, 1984.

Ebbinghaus, S.W., Vigneswaran, N., Miller, C.R., Chee-Awai, R.A., Mayfield, C.A., Curiel, D.T., and Miller, D.M. 1996. Efficient delivery of triplex forming oligonucleotides to tumor cells by adenovirus-polylysine complexes. *Gene Therapy* 3: 287-297.

Efrat, *et al.*, "Ribozyme-mediated attenuation of pancreatic Beta-cell glucokinase expression in transgenic mice results in impaired glucose-induced insulin secretion," *Proc. Natl. Acad. Sci. USA*, 91:2051-2055, 1994.

Efrat *et al.*, "Murine insulinoma cell line with normal glucose-regulated insulin secretion," *Diabetes*, 42:901-907, 1993.

Efrat *et al.*, "Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene," *P.N.A.S.*, 85:9037-9041, 1988.

Efrat *et al.*, "Conditional transfromation of a pancreatic beta cell line derived from transgenic mice expressing a tetracycline-regulated oncogene," *Proc. Natl. Acad. Sci., USA*, 92: 3576-3580, 1995.

Eipper *et al.*, "Alternative splicing and endoproteolytic processing generate tissue-specific forms of pituitary peptidylglycine alpha-amidating monooxygenase (PAM)," *J.B.C.*, 267:4008-4015, 1992b.

Eipper *et al.*, "The biosynthesis of neuropeptides: Peptide alpha-amidation," *Annu. Rev. Neuroscience*, 15:57-85, 1992a.

Eizirik, D.L., Korbutt, G.S., Hellerstrom, C., (1992), "Prolonged exposure of human pancreatic islets to high glucose concentrations in vitro impairs the beta-cell function.", *J. Clin. Invest.*, 90(4):1263-1268.

Epstein, *et al.*, "Expression of yeast hexokinase in pancreatic beta-cells of transgenic mice reduces blood glucose, enhances insulin secretion, and decreases diabetes," *Proc. Natl. Acad. Sci. USA*, 89:12038-12042, 1992.

Ercolani *et al.*, "Isolation and complete sequence of a functional human glyceraldehyde-3-phosphate dehydrogenase gene," *J. Biol. Chem.*, 263;15335-15341, 1988.

Eshkol, "Mammalian cell-derived recombinant human growth hormone: Pharmacology, metabolism and clinical results," *Hormone Research*, 37:1-3, 1992.

Fanciulli *et al.*, "Glycolysis and growth rate in normal and in hexokinase-transfected NIH-3T3 cells," *Oncology Res.*, 6(9): 405-409, 1994.

Fechheimer *et al.*, "Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading," *Proc. Nat'l Acad. Sci. USA*, 84:8463-8467, 1987.

Felgner, *et al.*, *Arch. Bioch. Biophys.* 182:282-94, 1977.

Ferber, *et al.*, "GLUT-2 Gene Transfer into Insulinoma Cells Confers Both Low and High Affinity Glucose-stimulated insulin Release", *The Journal of Biological Chemistry*, 269:12523-12529, 1994.

Ferkol *et al.*, *FASEB J.*, 7:1081-1091, 1993.

Fiedorek *et al.*, "Selective expression of the insulin I gene in rat insulinoma-derived cell lines," *Mol. Endocrin.*, 4:990-999, 1990.

Fiek *et al.*, "Evidence for identity between hexokinase-binding protein and the mitochondrial porin in the outer membrane of rat liver mitochondria," *Biochem. Biophys. Acta*, 688:429-440, 1982.

Finegood, D.T., Scaglia, L., Bonner-Weir, S., Elliot, P.,"A simple mathemathical model," *Diabetes*, 44(3):249-256, 1995.

Forster & Symons, "Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites," *Cell*, 49:211-220, 1987.

Fort *et al.*, "Various rat adult tissues express only one major species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family," *Nucleic Acids Research*, 13:1431-1442, 1985.

Fraley *et al.*, "Entrapment of a bacterial plasmid in phospholipid vesicles: Potential for gene transfer," *Proc. Nat'l Acad. Sci. USA*, 76:3348-3352, 1979.

Fricker, "Carboxypeptidase E," *Ann. Rev. Physiology*, 50:309-321, 1988.

Friedmann, "Progress toward human gene therapy," *Science*, 244:1275-1281, 1989.

Fritschy *et al.*, *Diabetes*, 40:37, 1991.

Frougel *et al.*, "Familial hyperglycemia due to mutations in glucokinase," *N. Engl. J. Med.*, 328:105-112, 1993.

Gazdar *et al.* "Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor," *Proc. Natl. Acad. Sci. USA*, 77:3519-3523, 1980.

Gedulin, B., E. Larson, *et al.*, (1993) "The selective amylin antagonist, AC187, enhances the insulin response during intravenous glucose tolerance tests in anesthetized rats." *Diabetes* 42(Suppl.1):229A.

Gelb *et al.*, "Targeting of hexokinase 1 to liver the hepatoma mitochondria," *Proc. Natl. Acad. Sci. USA*, 89:202-206, January, 1992.

Gerlach *et al.*, "Construction of a plant disease resistance gene from the satellite RNA of tobacco rinspot virus," *Nature (London)*, 328:802-805, 1987.

German *et al.*, "The insulin and islet amyloid polypeptide genes contain similar cell-specific promoter elements that bind identical β-cell nuclear complexes," *Mol. Cell. Biol.*, 12:1777-1788, 1992.

Ghosh and Bachhawat, "Targeting of liposomes to hepatocytes," *In:* Wu G. and C. Wu ed. Liver diseases, targeted diagnosis and therapy using specific receptors and ligands. New York: Marcel Dekker, pp. 87-104, 1991.

Ghosh-Choudhury *et al.*, "Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full-length genomes," *EMBO J.*, 6:1733-1739, 1987.

Giddings *et al.*, "Effects of glucose on proinsulin messenger RNA in rats *in vivo*," *Diabetes*, 31:624-629, 1982.

Goding, In: Monoclonal Antibodies: Principles and Practice, 2d ed., Academic Press, Orlando, FL, pp. 60-61, 65-66, 71-74, 1986.

Gomez-Foix *et al.*, "Adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes confers altered regulation of glycogen," *J. Biol. Chem.*, 267:25129-25134, 1992.

Gopal, "Gene transfer method for transient gene expression, stable transfection, and cotransfection of suspension cell cultures," *Mol. Cell Biol.*, 5:1188-1190, 1985.

Gossen *et al.*, "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters," *Proc. Natl. Acad. Sci. USA*, 89:5547-5551, 1992.

Graham & Prevec, "Adenovirus-based expression vectors and recombinant vaccines," *Biotechnology*, 20:363-390, 1992.

Graham and Prevec, "Manipulation of adenovirus vector," *In:* E.J. Murray (ed.), Methods in Molecular Biology: Gene Transfer and Expression Protocol, Clifton, NJ: Humana Press, 7:109-128, 1991.

Graham and van der Eb, "A new technique for the assay of infectivity of human adenovirus 5 DNA", *Virology*, 52:456-467, 1973.

Graham *et al.*, "Characteristics of a human cell line transformed by DNA from human adenovirus type 5", *J. Gen. Virol.*, 36:59-72, 1977.

Grampp *et al.*, "Use of regulated secretion in protein production from animal cells: An overview," *Advances in Biochemical Engineering*, 46:35-62, 1992.

Gross *et al.*, "Partial diversion of a mutant proinsulin (B10 aspartic acid) from the regulated to the constitutive secretory pathway in transfected AtT-20 cells," *P.N.A.S.*, 86:4107-4111, 1989.

Grunhaus and Horwitz, "Adenovirus as cloning vector," *Seminar in Virology*, 3:237-252, 1992.

Hakes *et al.*, "Isolation of two complementary deoxyribonucleic acid clones from a rat insulinoma cell line based on similarities to Kex2 and furin sequences and the specific localization of each transcript to endocrine and neuroendocrine tissues in rat," *Endocrinology*, 129:3053-3063, 1991.

Halban, "Structural domains and molecular lifestyles of insulin and its precursors in the pancreatic beta cell," *Diabetologia*, 34:767-778, 1991.

Halban *et al.*, "Abnormal glucose metabolism accompanies failure of glucose to stimulate insulin release from a pancreatic cell line (RINm5f)," *Biochem. J.*, 212:439-443, 1983.

Halban *et al.*, "High-performance liquid chromatography (HPLC): a rapid, flexible and sensitive method for separating islet proinsulin and insulin," *Diabetologia*, 29:893-896, 1986.

Halban *et al.*, "Intracellular degradation of insulin stores by rat pancreatic islets *in vitro*: An alternative pathway for homeostasis of pancreatic insulin content," *J.B.C.*, 255:6003-6006, 1980.

Harland & Weintraub, "Translation of mammalian mRNA injected into Xenopus oocytes is specifically inhibited by antisense RNA," *J. Cell Biol.*, 101:1094-1099, 1985.

Haseloff, T. and Gerlach, W.L. "Simple RNA enzymes with new and highly specific endoribonuclease activities." *Nature*, 334:585-591, 1988.

Heartlein *et al.*, "Long-term production and delivery of human growth hormone *in vivo*," *P.N.A.S.*, 91:10967-10971, 1994.

Heinrich *et al.*, "Pre-glucagon messenger ribonucleic acid: Nucleotide and encoded amino acid sequences of the rat pancreatic complementary deoxyribonucleic acid., *Endocrinology*, 115:2176-2181, 1984.

Hermonat and Muzycska, "Use of adenoassociated virus as a mammalian DNA cloning vector: Transduction of neomycin resistance into mammalian tissue culture cells," *Proc. Nat'l Acad. Sci. USA*, 81:6466-6470, 1984.

Hersdorffer *et al.*, "Efficient gene transfer in live mice using a unique retroviral packaging line," *DNA Cell Biol.*, 9:713-723, 1990.

Herz and Gerard, "Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice," *Proc. Nat'l Acad. Sci. USA* 90:2812-2816, 1993.

Hoppener *et al.*, "Molecular physiology of the islet amyloid polypeptide (IAAP)/ amylin gene in man, rat, and transgenic mice," *J.Cell. Biochem.*, 55S:39-53, 1994.

Horwich *et al.* "Synthesis of hepadenovirus particles that contain replication-defective duck hepatitis B virus genomes in cultured HuH7 cells," *J. Virol.*, 64:642-650, 1990.

Hosokawa, *et al.*, "Upregulated hexokinase activity in isolated islets from diabetic 90% pancreatectomized rats." *Diabetes* 44:1328-1333, 1995.

Hughes *et al.*, "Transfection of AtT-20_{ins} Cells with GLUT-2 but Not GLUT-1 Confers Glucose-stimulated Insulin Secretion", *The Journal of Biological Chemistry*, 268:15205-15212, 1993.

Hughes *et al.*, "Engineering of Glucose-stimulated Insulin Secretion and Biosynthesis in Non-islet Cells", *Proc. Natl. Acad. Sci. USA*, 89:688-692, 1992.

Hughes *et al.*, "Expression of normal and novel glucokinase mRNA's in anterior pituitary and islet cells," *J. Biol. Chem.*, 266:4521-4530, 1991.

Hughes *et al.*, "Transfection of AtT-20ins cells with GLUT-2 but not GLUT-1 confers glucose-stimulated insulin secretion: relationship to glucose metabolism," *J.B.C.*, 268:15205-15212, 1993.

Inagaki *et al.*, "Reconstitution of IKATP: An inward rectifier subunit plus the sulfonylurea receptor," *Science*, 270:1166-1170, 1995.

Isaksson *et al.*, "Mode of action of pituitary growth hormone on target cells," *Ann. Rev. Physiol.*, 47:483-499, 1985.

Ishibashi *et al.*, "Hypercholesterolemia in Low Density Lipoprotein Receptor Knockout Mice and its Reversal by Adenovirus-mediated Gene Delivery," *J. Clin. Invest*, 92:883-893, 1993.

Jenkins *et al.*, "Getting the glycosylation right: Implications for the biotechnology industry" *Nature Biotechnol.* 14: 975-976, 1996

Johnson *et al.*, "Underexpression of Beta cell high K_m glucose transporters in noninsulin-dependent diabetes," *Science*, 250:546-549, 1990.

Jones and Shenk, "Isolation of deletion and substitution mutants of adenovirus type 5," *Cell*, 13:181-188, 1978.

Jonsson *et al.*, "Insulin-promoter-factor 1 is required for pancreas development in mice," *Nature*, 371:606-609, 1994.

Joyce, "RNA evolution and the origins of life," *Nature*, 338:217-244, 1989.

Kaneda *et al.*, "Increased expression of DNA cointroduced with nuclear protein in adult rat liver," *Science*, 243:375-378, 1989.

Karlsson *et al.*, *EMBO J.*, 5:2377-2385, 1986.

Karlsson *et al.*, "A mutational analysis of the insulin gene transcription control region: expression in β-cells is dependent on two related sequences within the enhancer," *Proc. Natl. Acad. Sci. USA*, 84:8819-8823, 1987.

Karlsson *et al.*, "Individual protein-binding domains of the insulin gene enhancer positively activate β-cell-specific transcription," *Mol. Cell. Biol.*, 9:823-827, 1989.

Kato *et al.*, "Expression of hepatitis β virus surface antigen in adult rat liver," *J. Biol. Chem.*, 266:3361-3364, 1991.

Kaufman and Sharp, "Construction of a modular dihydrofolate reductase cDNA gene: Analysis of signals utilized for efficient expression," *Mol. Cell. Biology*, 2:1304-1319, 1982.

Keller *et al.*, "Insulin prophylaxis in individuals at high risk for the development of type I diabetes mellitus," *Lancet.*, 341:927-928, 1993.

Kennedy *et al.*, "The minisatellite in the diabetes susceptibility locus IDDM2 regulates insulin transcription," *Nature Genetics*, 9:293-298, 1995.

Kim & Cech, "Three dimensional model of the active site of the self-splicing rRNA precursor or Tetrahymena," *Proc. Natl. Acad. Sci. USA*, 84:8788-8792, 1987.

Klein *et al.*, "High-velocity microprojectiles for delivering nucleic acids into living cells," *Nature*, 327:70-73, 1987.

Kmiec, E.B., Cole, A., and Holloman, W.K. 1994. The REC2 gene encodes the homologous pairing protein of *Ustilago maydis*. *Mol. Cell. Biol.* 14: 7163-7172.

Knaack *et al.*, "Clonal Insulinoma Cell Line That Stably Maintains Correct Glucose Responsiveness," *Diabetes*, 43:1413-1417, December, 1994.

Kohler & Milstein, *Nature*, 256:495-497, 1975.

Kohler & Milstein, *Eur. J. Immunol.*, 6:511-519, 1976.

Kreymann and Williams, "Glucagon-like peptide-1 7-36: a physiological incretion in man," *Lancet*, 2:1300-1303, 1987.

Kruse *et al.*, "An endocrine-specific element is an integral component of an exocrine-specific pancreatic enhancer," *Genes and Dev.*, 7:774-786, 1993.

Kyte and Doolittle, *J.Mol.Biol.*, 157:105-132, 1982.

Lacy *et al.*, *Science*, 254:1782-1784, 1991.

Larsson and Litwin, "The growth of polio virus in human diploid fibroblast grown with cellulose microcarriers in suspension culture," *Dev. Biol. Standard.*, 66:385-390, 1987.

Le Gal La Salle *et al.*, "An adenovirus vector for gene transfer into neurons and glia in the brain," *Science*, 259:988-990, 1993.

Leffert *et al.*, "Rat amylin: Cloning and tissue-specific expression in pancreatic islets," *Proc. Natl. Acad. Sci. USA*, 86: 3127-3130, 1989.

Leonard *et al.*, "Characterization of Somatostatin Transactivating Factor-1, a Novel Homeobox Factor That Stimulates Somatostatin Expression in Pancreatic Islet Cells," *Mol Endocrinol*, 7:1275-1283, 1993.

Levrero *et al.*, "Defective and nondefective adenovirus vectors for expressing foreign genes *in vitro* and *in vivo*," *Gene*, 101:195-202, 1991.

Li *et al.*, "Effect of disruption of actin filaments by Clostridium botulinum C2 toxin on insulin secretion in HIT-T15 cells and pancreatic islets," *M.B.C.*, 5:1199-1213, 1994.

Liang *et al.*, "Effects of alternate RNA splicing on glucokinase isoform activities in the pancreatic islet, liver, and anterior pituitary," *J.B.C.*, 266:6999-7007, 1991.

Liang *et al.*, "Enhanced and Switchable Expression Systems for Gene-Transfer," *J. Cell. Biochem.* Supplement 21A:379, 1995.

Liang, *et al.* "Glucose regulates glucokinase activity in cultured islets from rat pancreas." *J. Biol. Chem.* 265:16863-16866, 1990.

Lieber, A. and Strauss, M. "Selection of efficient cleavage sites in target RNAs by using a ribozyme expression library." *Mol. Cell. Biol.*, 15: 540-551, 1995.

Lim, "Encapsulation of biological materials," US Patent 4,352,883, October 5, 1982.

Lin *et al.*, "Pit-1-dependent pituitary cell proliferation involves activation of the growth hormone releasing factor receptor gene," *Nature*, 360:765-768, 1992.

Linden *et al.*, "Pore protein and the hexokinase-binding protein from the outer membrane of rat liver mitochondria are identical," *FEBS Lett.*, 141:189-192, 1982.

McGarry, D.J., "Disordered metabolism in diabetes: Have we underemphasized the fat component?", *Journal of Cellular Biochemistry* 55, supp:29-38, 1994.

Macejak and Sarnow, "Internal initiation of translation mediated by the 5′ leader of a cellular mRNA," *Nature*, 353:90-94, 1991.

Madsen, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, *85*:6652-6656, 1988.

Mann *et al.*, "Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus," *Cell*, 33:153-159, 1983.

Marchetti *et al.*, "Pulsatile insulin secretion from isolated human pancreatic islets," *Diabetes*, 43:827-830, 1994.

Marie *et al.*, "The pyruvate kinase gene is a model for studies of glucose-dependent regulation of gene expression in the endocrine pancreatic Beta-cell type," *J.B.C.*, 268:23881-23890, 1993.

Markowitz *et al.*, "A safe packaging line for gene transfer: Separating viral genes on two different plasmids," *J. Virol.*, 62:1120-1124, 1988.

Matchinsky, "Glucokinase as as sensor and metabolic signal generator in pancreatic Beta-Cells and hepatocytes," *Diabetes*, 39:647-652, 1990.

Mayo, "Molecular cloning and expression of a pituitary-specific receptor for growth hormone-releasing hormone," *Mol. Endocrinol.*, 1734-1744, 1992.

Meglasson and Matschinsky, "Pancreatic islet glucose metabolism and regulation of insulin secretion," *Diabetes Metab. Rev.*, 2:163-214, 1986.

Michel & Westhof, "Modeling of the three-dimensional architecture of group I catalytic introns based on comparative sequence analysis," *J. Mol. Biol.*, 216:585-610, 1990.

Milgram *et al.*, "Expression of individual forms of peptidylglycine alpha-amidating monooxygenase in AtT-20 cells: Endoproteolytic processing and routing to secretory granules," *J.C.B.*, 117:717-728, 1992.

Miller *et al.*, "IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene," *EMBO J.*, 13:1145-1156, 1994.

Miyazaki *et al.*, "Establishment of a pancreatic Beta-cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms,' *Endocrinology*, 127:126-132, 1990.

Mizrahi, "Production of human interferons: An overview," *Process Biochem.*, (August):9-12, 1983.

Mojsov *et al.*, "Both amidated and nonamidated forms of glucagon-like peptide 1 are synthesized in the rat intestine and the pancreas," *J.B.C.*, 265:8001-8008, 1990.

Mojsov *et al.*, "Preglucagon gene expression in the pancreas and intestine diversifies at the level of post-translational processing," *J.B.C.*, 261:11880-11889, 1986.

Monia, B.P, Johnston, J.F., Sasmor, H., and Cummins, L.L. 1996. Nuclease resistance and antisense activity of modified oligonucleotides targeted to Ha-ras. J. Biol. Chem. 271: 14533-14540.

Moore *et al.*, "Expressing a human proinsulin cDNA in a mouse ACTH-secreting cell. Intracellular storage, proteolytic processing, and secretion on stimulation," *Cell*, 35:531-538, 1983.

Moore *et al.*, "Secretory protein targeting in a pituitary cell line: Differential transport of foreign secretory proteins to distinct secretory pathways," *J.C.B.*, 101:1773-1781, 1985.

Muir *et al.*, "Antigen specific immunotherapy. Oral tolerance and subcutaneous immunization in the treatment of insulin-dependent diabetes," *Diabetes. Metab. Rev.*, 9:279-287, 1993.

Muir *et al.*, "Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intraislet interferon-gamma transcription," *J. Clin. Invest.*, 95:628-634, 1995.

Mulligan, "The Basic Science of Gene Therapy," *Science*, 260:926-932, 1993.

Mulligan *et al.*, "Selection for animal cells that express the Escherichia coli gene coding xanthine-guanine phosphoribosyltransferase," *Proc. Natl. Acad. Sci. USA*, 78:2072-2076, 1981.

Mulligan *et al.*, "Expression of a bacterial gene in mammalian cells," *Science*, 209:1422-1427, 1980.

Murakami, M., S. Suzuki, *et al.* (1990) "Effects of amylin on insulin secretion from RINm5F cells." *Diabetes* 39(Suppl 1):266A.

Nagamatsu S and Steiner DF *Endocrinology* 130: (2) p748-54, 1992

Neerman-Arbez M *et al.*, *J Biol Chem*, 268 (22) p16098-100, 1993

Newgard, and McGarry, "Metabolic coupling factors in pancreatic Beta-cell signal transduction," *Ann. Rev. Biochem.*, 64:689-719, 1995

Newgard *et al.*, "Glucokinase and glucose transporter expression in liver and islets: implications for control of glucose homeostasis," *Biochem. Soc. Trans.*, 18:851-853, 1990.

Nicolas and Rubinstein, "Retroviral vectors," *In: Vectors: A survey of molecular cloning vectors and their uses*, Rodriguez and Denhardt (eds.), Stoneham: Butterworth, pp. 494-513, 1988.

Nicolau and Sene, "Liposome-mediated DNA transfer in eukaryotic cells," *Biochem. Biophys. Acta*, 721:185-190, 1982.

Nicolau *et al.*, "Liposomes as carriers for *in vivo* gene transfer and expression," *Methods Enzymol.*, 149:157-176, 1987.

Nilsson and Mosbach, "Immobilized animal cells," *Dev. Biol. Standard.*, 66:183-193, 1987

Nielsen *Acta Endocrinol*: 266 p1-39, 1985

O'Shea and Sun, *Diabetes* 35:943-946, 1986.

Ogawa *et al.*, "Loss of glucose-induced insulin secretion and GLUT-2 expression in transplanted Beta-cells," *Diabetes*, 44:75-79, 1995.

Ohagi *et al.*, "Identification and analysis of the gene encoding PC2, a prohormone convertase expressed in neuroendocrine tissues," *P.N.A.S.*, 89:4977-4981, 1992.

Ohlsson *et al.*, "IPF1, a homeodomain-containing transactivator of the insulin gene," *EMBO J.*, 12:4251-4259, 1993.

Ohsawa, H., A. Kanatsuka, *et al.*, (1989) "Islet amyloid polypeptide inhibits glucose-stimulated insulin secretion from isolated rat pancreatic islets." *Biochem.Biophys Res. Comm.* 160:961-967.

Orci *et al.*, "Evidence that down-regulation of Beta-cell glucose transporters in non-insulin-dependent diabetes may be the cause of diabetic hyperglycemia," *P.N.A.S.*, 87:9953-9957, 1990.

Orskov *et al.*, "Biological effects and metabolic rates of glucagonlike peptide-1 7-36 amide and glucagonlike peptide-1 7-37 in healthy subjects are indistinguishable," *Diabetes*, 42:658-661, 1993.

Orskov *et al.*, "Complete sequence of glucagon-like peptide-1 from human and pig small intestine," *J.B.C.*, 264:12826-12829, 1989.

Ouafik *et al.*, "Developmental regulation of peptidylglycine alpha-amidating monooxygenase (PAM) in rat heart atrium and ventricle," *J.B.C.*, 264:5839-5845, 1989.

Ouafik *et al.*, "The multifunctional peptidylglycine alpha-amidating monooxygenase gene: Exon/intron organization of catalytic, processing, and routing domains," *Molecular Endocrinology*, 6:1571-1584, 1992.

Owerbach and Aagaard, "Analysis of a 1963-bp polymorphic region flanking the human insulin gene," *GENE*, 32:475-479, 1984.

Palmer *et al.*, "Production of human Factor IX in animals by genetically modified skin fibroblasts: Potential therapy for hemophilia B," *Blood*, 73:438-445, 1989.

Palmer *et al.*, "Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes," *Proc. Natl. Acad. Sci. USA*, 88:1330-1334, 1991.

Palukaitis, P. *et al.*, "Characterization of a viroid associated with avacado sunblotch disease." *Virology*, 99:145-151, 1979.

Paskind *et al.*, "Dependence of moloney murine leukemia virus production on cell growth," *Virology*, 67:242-248, 1975.

Pavlakis and Hamer, "Regulation of a metallothionein-growth hormone hybrid gene in bovine papilloma virus," *P.N.A.S.* 80:397-401, 1983.

Peers *et al.*, "Insulin Expression in Pancreatic Islet Cells Relies on Cooperative Interactions between the Helix Loop Helix Factor E47 and the Homeobox Factor STF-1," *Mol Endocrin*, 8:1798-1806, 1994.

Pelletier and Sonenberg, "Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA," *Nature*, 334:320-325, 1988.

Perales *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:4086-4090, 1994.

Percy, A.J., Trainor, D.A., *et al.*, (1996) "Development of sensitive immunoassays to detect amylin and amylin-like peptides in unextracted plasma." *Clin. Chem.* 42:576-585.

Perriman, R. *et al.*, "Extended target-site specificity for a hammerhead ribozyme." *Gene*, 113:157-163, 1992.

Perrotta and Been, *Biochem.* 31:16, 1990.

Petricciani, "Should continuous cell lines be used as substrates for biological products?," *Dev. Biol. Standard.*, 66:3-12, 1985.

Philippe *et al.*, "Multipotential phenotypic expression of genes encoding peptide hormones in rat insulinoma lines," *J. Clin. Invest.*, 79:351-358, 1987.

Phillips *et al.*, *In*: *Large Scale Mammalian Cell Culture* (Feder, J. and Tolbert, W. R., eds.), Academic Press, Orlando, FL, U.S.A., 1985.

Pieber *et al.*, "Direct plasma radioimmunoassay for rat amylin-(1-37): concentrations with acquired and genetic obesity," *Am. J. Physiol.*, 267:E156-E164, 1994.

Pittner, R.A., Albrandt, K., *et al.*, (1994) "Molecular physiology of amylin." *J.Cell Biochem.* 55:19-28.

Polakis and Wilson, "An Intact Hydrophobic N-Terminal Sequence Is Critical for Binding of Rat Brain Hexokinase to Mitochondria," *Arch. Biochem. Biophys.*, 236(1):328-337, January, 1985.

Potter *et al.*, "Enhancer-dependent expression of human k immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation," *Proc. Nat'l Acad. Sci. USA*, 81:7161-7165, 1984.

Powell *et al.*, "Efficient targeting to storage granules of human proinsulins with altered propeptide domain," *J.C.B.*, 106:1843-1851, 1988.

Prody, G.A. *et al.*, "Autolytic processing of dimeric plant virus satellite RNA." *Science*, 231, 1577-1580, 1986.

Quaade *et al.*, "Analysis of the protein products encoded by variant glucokinase transcripts via expression in bacteria," *FEBS Lett*, 280:47-52, 1991.

Racher *et al.*, *Biotechnology Techniques*, 9:169-174, 1995.

Radvanyi *et al.*, "Pancreatic beta bells cultured from individual preneoplastic foci in a multistage tumorigenesis pathway: a potentially general technique for isolating physiologically representative cell lines," *Molec. and Cell. Biol.*,13(7): 4223-4232, 1993.

Ragot *et al.*, "Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice," *Nature*, 361:647-650, 1993.

Reinhold-Hurek & Shub, "Self-splicing introns in tRNA genes of widely divergent bacteria," *Nature*, 357:173-176, 1992.

Renan, "Cancer genes: current status, future prospects, and applicants in radiotherapy/oncology," *Radiother. Oncol.*, 19:197-218, 1990.

Rhodes and Alarcon, "What beta-cell defect could lead to hyperproinsulinemia in NIDDM?," *Diabetes*, 43:511-517, 1994.

Rhodes and Halban, "The intracellular handling of insulin-related peptides in isolated pancreatic islets," *Biochem. J.*, 251:23-30, 1988.

Rich *et al.*, "Development and analysis of recombinant adenoviruses for gene therapy of cystic fibrosis," *Hum. Gene Ther.*, 4:461-476, 1993.

Ridgeway, "Mammalian expression vectors," *In:* Rodriguez RL, Denhardt DT, ed. Vectors: A survey of molecular cloning vectors and their uses. Stoneham: Butterworth, pp. 467-492, 1988.

Rippe *et al.*, "DNA-mediated gene transfer into adult rat hepatocytes in primary culture," *Mol. Cell Biol.*, 10:689-695, 1990.

Rittenhouse, J., Chait, B.T., *et al.* (1996) "Heterogeneity of naturally-occurring human amylin due to glycosylation." *Diabetes* 45 (Sup.2):234A.

Rosenfeld *et al.*, "Adenovirus-mediated transfer of a recombinant a1-antitrypsin gene to the lung epithelium *in vivo*," *Science*, 252:431-434, 1991.

Rosenfeld *et al.*, "*In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium," *Cell*, 68:143-155, 1992.

Rouille *et al.*, "Differential processing of proglucagon by the subtilisin-like prohormone convertases PC2 and PC3 to generate either glucagon or glucagon-like peptide," *J.B.C.*, 270:26488-26496, 1995.

Rouille *et al.*, "Proglucagon is processed to glucagon by prohormone convertase PC2 in alphaTC1-6 cells," *P.N.A.S.*, 91:3242-3246, 1994.

Roux *et al.*, "A versatile and potentially general approach to the targeting of specific cell types by retroviruses: Application to the infection of human cells by means of major histocompatibility complex class I and class II antigens by mouse ecotropic murine leukemia virus-derived viruses," *Proc. Natl. Acad. Sci. USA*, 86:9079-9083, 1989.

Rubin, B.P., Ferguson, D.O., and Holloman, W.K. 1994. Structure of *REC2*, a recombinational repair gene of *Ustilago maydis*, and its function in homologous recombination between plasmid and chromosomal sequences. *Mol. Cell. Biol.*, 14: 6287-6296.

Sadelain *et al.*, "Prevention of type I diabetes in NOD mice by adjuvant immunotherapy," *Diabetes*, 39:583-589, 1990.

Sambanis *et al.*, "Use of regulated secretion in protein production from animal cells: An evaluation with the AtT-20 model cell line," *Biotechnology and Bioengineering*, 35:771-780, 1990.

Sambanis *et al.*, "A model of secretory protein trafficking in recombinant AtT-20 cells," *Biotechnology and Bioengineering*, 36:280-295, 1991.

Sanke *et al.*, "An islet amyloid peptide is derived from an 89-amino acid precursor by proteolytic processing," *J.B.C.*, 263:17243-17246, 1988.

Santerre *et al.*, "Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic beta-cells," *P.N.A.S.*, 78:4339-4343, 1981.

Sarver, et al, "Ribozymes as potential anti-HIV-1 therapeutic agents," *Science*, 247:1222-1225, 1990.

Sato *et al.*, *Proc. Nat'l Acad. Sci. U.S.A.* 48:1184-1190, 1962.

Sauer, "Manipulation of transgenes by site-specific recombination: Use of Cre recombinase," *Methods in Enzymology*, 225:890-900, 1993.

Scanlon *et al.*, "Ribozyme-mediated cleavages of c-fos mRNA reduce gene expression of DNA synthesis enzymes and metallothionein," *Proc. Nat'l Acad. Sci. USA*, 88:10591-10595, 1991.

Scharfmann *et al.*, "Long-term *in vivo* expression of retrovirus-mediated gene transfer in mouse fibroblast implants," *P.N.A.S. USA*, 88:4626-4630, 1991.

Scharp *et al.*, "Protection of encapsulated human islets implanted without immunosuppression in patients with Type I or Type II diabetes and in nondiabetic control subjects," *Diabetes*, 43:1167-1170, 1994.

Schmidt *et al.*, "Synthesis and targeting of insulin-like growth factor-1 to the hormone storage granules in an endocrine cell line," *J.B.C.*, 269:27115-27124, 1994.

Schnedl *et al.*, "STZ transport and cytotoxicity: Specific enhancement in GLUT-2-expressing cells," *Diabetes*, 43:1326-1333, 1994.

Schnetzler *et al.*, "Adaptation to supraphysiological levels of insulin gene expression in transgenic mice: Evidence for the importance of posttranscriptional regulation," *J. Clin. Invest.*, 92:272-280, 1994.

Schwab *et al.*, "Complete amino acid sequence of rat brain hexokinase, deduced from the cloned cDNA, and proposed structure of a mammalian hexokinase," *Proc. Natl. Acad. Sci. USA*, 86:2563-2567, 1989.

Seeburg, "The human growth hormone gene family: Nucleotide sequences show recent divergence and predict a new polypeptide hormone," *DNA*, 1;239-249, 1982.

Selden *et al.*, "Implantation of genetically engineered fibroblasts into mice: Implications for gene therapy," *Science*, 236:714-718, 1987.

Sevarino *et al.*, "Cell-specific processing of preprosomatostatin in cultured neuroendocrine cells," *J.B.C.*, 262:4987-4993, 1987.

Sevarino *et al.*, "Thyrotropin releasing hormone (TRH) precursor processing," *J.B.C.*, 264:21529-21535, 1989.

Shehadeh *et al.*, "Effect of adjuvant therapy on development of diabetes in mouse and man," *Lancet.*, 343:706-707, 1994.

Sioud *et al.*, "Preformed ribozyme destroys tumour necrosis factor mRNA in human cells," *.J Mol. Biol.*, 223:831-835, 1992.

Sizonenko and Halban, "Differential rates of conversion of rat proinsulins I and II: Evidence for slow cleavage at the B-chain/C-chain junction of proinsulin II," *Biochemistry J.*, 278:621-625, 1991.

Smeekens and Steiner, "Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease *Kex2*," *J.B.C.*, 265:2997-3000, 1990.

Smith and Wilson, *Arch. Bioch. Biophys.* 287:359-366, 1991.

Steiner *et al.*, "The new enzymology of precursor processing endoproteases," *J.B.C.*, 267:23435-23438, 1992.

Stoffers *et al.*, "Alternative mRNA splicing generates multiple forms of peptidyl-glycine alpha-amidating monooxygenase in rat atrium," *P.N.A.S.*, 86:735-739, 1989.

Stoffers *et al.*, "Characterization of novel mRNA's encoding enzymes involved in peptide alpha-amidation," *J.B.C.*, 266:1701-1707, 1991.

Stossel, T., "The Molecular Basis of Blood Diseases," Chapter 14, pp. 499-533, W.B. Saunders Co., Philadelphia, PA, 1987.

Stratford-Perricaudet and Perricaudet, "Gene transfer into animals: the promise of adenovirus," p. 51-61, *In: Human Gene Transfer*, Eds, O. Cohen-Haguenauer and M. Boiron, Editions John Libbey Eurotext, France, 1991.

Stratford-Perricaudet *et al.*, "Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector," *Hum. Gene Ther.*, 1:241-256, 1990.

Subramani *et al.*, "Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors," *Mol. Cell. Biol.*, 1:854-864, 1981.

Sullivan *et al.*, *Science* 252:718-721, 1991.

Swarovsky *et al.*, "Sodium butyrate induces neuroendocrine cytodifferentiation in the insulinoma cell line RINm5F," *Pancreas*, 9:460-468, 1994.

Symons, R.H., "Avacado sunblotch viroid: primary sequence and proposed secondary structure." *N.A.R.*, 9:6527-6537, 1981.

Symons, R.H. "Small catalytic RNAs." *Annu. Rev. Biochem.*, 61:641-671, 1992.

Takeda *et al.*, "Organization of the human GLUT-2 (Pancreatic Beta-cell and hepatocyte) glucose transporter gene," *Diabetes*, 42:773-777, 1993.

Takeuchi *et al.*, "Expression of human pancreatic peptide in heterologous cell lines," *J.B.C.*, 266:17409-17415, 1991.

Temin, "Retrovirus vectors for gene transfer: Efficient integration into and expression of exogenous DNA in vertebrate cell genome," *In*: *Gene Transfer*, Kucherlapati (ed.), New York: Plenum Press, pp. 149-188, 1986.

Thompson, J.D. *et al.*, "Ribozymes in gene therapy." *Nature Medicine*, 1:277-278, 1995.

Thorens, "Expression cloning of the pancreatic betacell receptor for the gluco-incretion hormone glucagon-like peptide 1," *P.N.A.S.*, 89:8641-8646, 1992.

Thorens *et al.*, "Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney and beta-pancreatic islet cells." Cell, 55:281-290, 1988.

Thorens *et al.*, "The loss of GLUT-2 expression by glucose-unresponsive beta cells of *db/db* mice is reversible and is induced by the diabetic environment," *J. Clin. Invest.*, 90:77-85, 1992b.

Thorens *et al.*, "Reduced expression of the liver/beta-cell glucose transporter isoform in glucose-insensitive pancreatic beta-cells of diabetic rats," *Proc. Natl. Acad. Sci. USA*, 87:6492-6496, 1990.

Thorne *et al.*, "Expression of mouse proopiomelanocortin in an insulinoma cell line," *J.B.C.*, 264:3545-3552, 1989.

Ting and Lee, "Human gene encoding the 78,000-Dalton glucose-regulated protein and its pseudo gene: Structure, conservation, and regulation," *DNA*, 7:275-286, 1988.

Tokuyama *et al.*, "Evolution of beta-cell dysfunction in the male Zucker diabetic fatty rat," *Diabetes*, 44(12):1447-1457, 1995.

Top *et al.*, "Immunization with live types 7 and 4 adenovirus vaccines. II. Antibody response and protective effect against acute respiratory disease due to adenovirus type 7," *J. Infect. Dis.*, 124:155-160, 1971.

Tsukuda, T., Bauchwitz, R., and Holloman, W.K. 1989. Isolation of the *REC*1 gene controlling recombination in *Ustilago maydis*. *Gene* 85:335-341.

Tur-Kaspa *et al.*, "Use of electroporation to introduce biologically active foreign genes into primary rat hepatocytes," *Mol. Cell Biol.*, 6:716-718, 1986.

Unger, "Diabetic hyperglycemia: Link to impaired glucose transport in pancreatic Beta cells," *Science*, 251:1200-1205, 1991.

Unger and Orci, "Glucagon and the Alpha cell," *N.E.J.M.*, 304:1518-1524, 1981.

Usdin *et al.*, "Gastric inhibitory peptide receptor, a member of the secretin-vasoactive intestinal peptide receptor family, is widely distributed in peripheral organs and the brain," *Endocrinology*, 133:2861-2870, 1993.

van Wezel, "Growth of cell-strains and primary cells on microcarriers in homogeneous culture," *Nature*, 216:64-65, 1967.

Varmus *et al.*, "Retroviruses as mutagens: Insertion and excision of a nontransforming provirus alter the expression of a resident transforming provirus," *Cell*, 25:23-36, 1981.

Vollenweider *et al.*, "Processing of proinsulin by furin, PC2 and PC3 in (co)transfected COS (monkey Kidney) cells," *Diabetes*, 44:1075-1080, 1995.

Vosberg, H.T. and Eckstein, F. 1982. Effect of deoxynucleoside phosphorothioates incorporated in DNA on cleavage by restriction enzymes. J. Biol. Chem. 257:6595-6599.

Voss-McGowan, M.E., Xu, B., and Epstein, P.N., "Insulin synthesis, secretory competence, and glucose utilization are sensitized by transgenic yeast hexokinase," *J. Biol. Chem.* 269:15814-15818, 1994.

Waeber *et al.*, "Characterization of the murine high K_m glucose transporter GLUT-2 gene and its transcriptional regulation by glucose in a differentiated insulin-secreting cell line," *J. Biological Chemistry*, 43:26912-26919, 1994.

Wagner *et al.*, *Science*, 260:1510-1513, 1990.

Wagoner, P.K., Chen, C., *et al.*, (1993) "Amylin modulates Beta-cell glucose sensing via effects on stimulus-secretion coupling." *Proc.Natl.Acad.Sci., USA*, 90:9145-9149.

Wang *et al.*, "Design and performance of a packed bed bioreactor for the production of recombinant protein using serum-free medium," Proceeding of the Japanese Society for Animal Cell Technology, 1994.

Wang *et al.*, "High density perfusion culture of hybridoma cells for production of monoclonal antibodies in the CelliGen packed bed reactor, *In*: *Animal Cell Technology: Basic & Applied Aspects*, S. Kaminogawa *et al.*, (eds), vol. 5, pp463-469, Kluwer Academic Publishers, Netherlands, 1993.

Wang *et al.*, "Modified CelliGen-packed bed bioreactor for hybridoma cell cultures," *Cytotechnology*, 9:41-49, 1992.

Watada *et al.*, "The human glucokinase gene β-cell-type promoter," *Diabetes*, 15:1478-1488, 1996

Welsh *et al.*, "Stimulation of growth hormone synthesis by glucose in islets of Langerhans isolated from transgenic mice," *J.B.C.*, 261:12915-12917, 1986.

White and Wilson, *Arch. Bioch. Biophys.* 259:402-411, 1987.

White and Wilson, *Arch. Bioch. Biophys.* 277:26-34, 1990.

Whitesell *et al.*, "Transport and metabolism of glucose in an insulin-secreting cell line, BetaTC-1," *Biochemistry*, 30:11560-11566, 1991.

Willnow and Herz, "Homologous Recombination for Gene replacement in Mouse Cell Lines," *Methods in Cell Biology*, 43 pt A:305-334, 1994.

Wilson, "Brain hexokinase: A proposed relation between soluble-particulate distribution and activity *in vivo*," *J. Biol. Chem.*, 243:3640-3647, 1968.

Wilson, "Hexokinases," *In: Reviews of Physiology, Biochemistry and Pharmacology*, Pette, D. (Ed.), 126:65-174, 1994.

Wilson, "Ligand induced confirmations of rat brain hexokinase: Effects of glucose-6-phosphate and inorganic phosphate," *Arch. Biochem. Biophys.*, 159:543-549, 1973.

Wilson, "Regulation of mammalian hexokinase activity," *In: Regulation of Carbohydrate Metabolism*, Beitner, R. (Ed.) Vol. 1, CRC, Boca Raton, 45-81, 1985.

Wong *et al.*, "Appearance of β-lactamase activity in animal cells upon liposome mediated gene transfer," *Gene*, 10:87-94, 1980.

Wu and Wu, "Evidence for targeted gene delivery to HepG2 hepatoma cells *in vitro*," *Biochemistry*, 27:887-892, 1988.

Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.

Wu and Wu, "Receptor-mediated *in vitro* gene transfections by a soluble DNA carrier system," *J. Biol. Chem.*, 262:4429-4432, 1987.

Xie and Wilson, "Rat brain hexokinase: the hydrophobic N-terminus of the mitochondrially bound enzyme is inserted in the lipid bilayer," *Arch. Biochem. Biophys.*, 267:803-810, 1988.

Yang *et al.*, "*In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment," *Proc. Nat'l Acad. Sci. USA*, 87:9568-9572, 1990.

Yang *et al.*, "Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy," *P.N.A.S. USA*, 91:4407-4411, 1994.

Yoon, K. Cole-Strauss, A., and Kmiec, E.B. 1996. Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA-DNA oligonucleotide. *Proc. Natl. Acad. Sci.* USA 93: 2071-1076.

Young, A.A., P. Carlo, et al., (1992). "$^{8-37}$hCGRP, an amylin receptor antagonist, enhances the insulin response and perturbs the glucose response to infused arginine in anesthetized rats." *Mol. Cell. Endocrin.* 84: R1-R5.

Yuan, Y. and Altman, S. "Selection of guide sequences that direct efficient cleavage of mRNA by human ribonuclease P." *Science*, 263:1269-1273, 1994.

Yuan, Y. *et al.*, "Targeted cleavage of mRNA by human RNase P." *P.N.A.S.*, 89:8006-8010, 1992.

Yun and Eipper, "Addition of an endoplasmic reticulum retention/retrieval signal does not block maturation of enzymatically active peptidylglycine alpha-amidating monooxygenase," *J.B.C.*, 270:15412-15416, 1995.

Zelenin *et al.*, "High-velocity mechanical DNA transfer of the chloramphenicol acetyltransferase gene into rodent liver, kidney and mammary gland cells in organ explants and *in vivo*," *FEBS Lett.*, 280:94-96, 1991.

U.S. Patent 4,892,538

U.S. Patent 5,399,346

U.S. Patent 5,011,472

WO 89/01967

WO 89/01967

WO 90/02580

WO 90/02580

WO 90/15637

WO 90/15637

WO 91/10425

WO 91/10425

WO 91/10470

WO 91/10470

EPO 0273085

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Newgard, Christopher B.
Halban, Philippe
Normington, Karl D.
Clark, Samuel A.
Thigpen, Anice E.
Quaade, Christian
Kruse, Fred

(ii) TITLE OF INVENTION: Recombinant Expression of Proteins From Secretory Cell Lines

(iii) NUMBER OF SEQUENCES: 69

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Arnold, White & Durkee
(B) STREET: P. O. Box 4433
(C) CITY: Houston
(D) STATE: TX
(E) COUNTRY: USA
(F) ZIP: 77210-4433

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US Unknown
(B) FILING DATE: Concurrently Herewith
(C) CLASSIFICATION: Unknown

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Highlander, Steven L.
(B) REGISTRATION NUMBER: 47,642
(C) REFERENCE/DOCKET NUMBER: UTSD:426\HYL

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (512) 418-3000
(B) TELEFAX: (512) 474-7577

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 515 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCCGGG GGTCCTTCTG CCATGGCCCT GTGGATGCGC CTCCTGCCCC TGCTGGCGCT 60

GCTGGCCCTC TGGGGACCTG ACCCAGCCGC AGCCTTTGTG AACCAACACC TGTGCGGCTC 120

ACACCTGGTG GAAGCTCTCT ACCTAGTGTG CGGGGAACGA GGCTTCTTCT ACACACCCAA 180

GACCCGCCGG GAGGCAGAGG ACCTGCAGGT GGGGCAGGTG GAGCTGGGCG GGGGCCCTGG 240

TGCAGGCAGC CTGCAGCCCT TGGCCCTGGA GGGGTCCCTG CAGAAGCGTG GCATTGTGGA 300

ACAATGCTGT ACCAGCATCT GCTCCCTCTA CCAGCTGGAG AACTACTGCA ACTAGACGCA 360

GCCCGCAGGC AGCCCCCCAC CCGCCGCCTC CTGCACCGAG AGAGATGGAA TAAAGCCCTT 420

GAACCAGCAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAC CCCCCCCCCC 480

CCCCCTGCAG CAATGGCAAC AACGTTGCGG AATTC 515

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Leu Trp Met Arg Leu Leu Pro Leu Leu Ala Leu Leu Ala Leu
1 5 10 15

Trp Gly Pro Asp Pro Ala Ala Ala Phe Val Asn Gln His Leu Cys Gly
20 25 30

Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe
35 40 45

Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly
50 55 60

Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu

65 70 75 80

Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys
85 90 95

Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
100 105 110

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 359 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCGGGGATCC TTCTGCCATG GCCCTGTGGA TGCGCCTCCT GCCCCTGCTG GCGCTGCTGG 60

CCCTCTGGGG ACCTGACCCA GCCGCAGCCT TTGTGAACCA ACACCTGTGC GGCTCACACC 120

TGGTGGAAGC TCTCTACCTA GTGTGCGGGG AACGAGGCTT CTTCTACACA CCCAAGACCC 180

GCCGGGAGGC AGAGGACCTG CAGGTGGGGC AGGTGGAGCT GGGCGGGGGC CCTGGTGCAG 240

GCAGCCTGCA GCCCTTGGCC CTGGAGGGGT CCCTGCAGAA GCGTGGCATT GTGGAACAAT 300

GCAGTACTAG CATCTGCTCC CTCTACCAGC TGGAGAACTA CAGCAACTAG ATCTAGCCC 359

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 110 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Leu Trp Met Arg Leu Leu Pro Leu Leu Ala Leu Leu Ala Leu
1 5 10 15

Trp Gly Pro Asp Pro Ala Ala Ala Phe Val Asn Gln His Leu Cys Gly
20 25 30

Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe
35 40 45

Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly
50 55 60

Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu
65 70 75 80

Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Ser
85 90 95

Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Ser Asn
100 105 110

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 867 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGATCCATGA ACAGTGAGGA GCAGTACTAC GCGGCCACAC AGCTCTACAA GGACCCGTGC 60

GCATTCCAGA GGGGCCCGGT GCCAGAGTTC AGCGCTAACC CCCCTGCGTG CCTGTACATG 120

GGCCGCCAGC CCCCACCTCC GCCGCCACCC CAGTTTACAA GCTCGCTGGG ATCACTGGAG 180

CAGGGAAGTC CTCCGGACAT CTCCCCATAC GAAGTGCCCC CGCTCGCCTC CGACGACCCG 240

GCTGGCGCTC ACCTCCACCA CCACCTTCCA GCTCAGCTCG GGCTCGCCCA TCCACCTCCC 300

GGACCTTTCC CGAATGGAAC CGAGCCTGGG GGCCTGGAAG AGCCCAACCG CGTCCAGCTC 360

CCTTTCCCGT GGATGAAATC CACCAAAGCT CACGCGTGGA AAGGCCAGTG GGCAGGAGGT 420

GCTTACACAG CGGAACCCGA GGAAAACAAG AGGACCCGTA CTGCCTACAC CCGGGCGCAG 480

CTGCTGGAGC TGGAGAAGGA ATTCTTATTT AACAAATACA TCTCCCGGCC CCGCCGGGTG 540

GAGCTGGCAG TGATGTTGAA CTTGACCGAG AGACACATCA AAATCTGGTT CCAAAACCGT 600

CGCATGAAGT GGAAAAAAGA GGAAGATAAG AAACGTAGTA GCGGGACCCC GAGTGGGGGC 660

GGTGGGGGCG AAGAGCCGGA GCAAGATTGT GCGGTGACCT CGGGCGAGGA GCTGCTGGCA 720

GTGCCACCGC TGCCACCTCC CGGAGGTGCC GTGCCCCCAG GCGTCCCAGC TGCAGTCCGG 780

GAGGGCCTAC TGCCTTCGGG CCTTAGCGTG TCGCCACAGC CCTCCAGCAT CGCGCCACTG 840

CGACCGCAGG AACCCCGGTG AAGATCT 867

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 284 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Asn Ser Glu Glu Gln Tyr Tyr Ala Ala Thr Gln Leu Tyr Lys Asp
1 5 10 15

Pro Cys Ala Phe Gln Arg Gly Pro Val Pro Glu Phe Ser Ala Asn Pro
20 25 30

Pro Ala Cys Leu Tyr Met Gly Arg Gln Pro Pro Pro Pro Pro Pro Pro
35 40 45

Gln Phe Thr Ser Ser Leu Gly Ser Leu Glu Gln Gly Ser Pro Pro Asp
50 55 60

Ile Ser Pro Tyr Glu Val Pro Pro Leu Ala Ser Asp Asp Pro Ala Gly
65 70 75 80

Ala His Leu His His His Leu Pro Ala Gln Leu Gly Leu Ala His Pro
85 90 95

Pro Pro Gly Pro Phe Pro Asn Gly Thr Glu Pro Gly Gly Leu Glu Glu
100 105 110

Pro Asn Arg Val Gln Leu Pro Phe Pro Trp Met Lys Ser Thr Lys Ala
115 120 125

His Ala Trp Lys Gly Gln Trp Ala Gly Gly Ala Tyr Thr Ala Glu Pro
130 135 140

Glu Glu Asn Lys Arg Thr Arg Thr Ala Tyr Thr Arg Ala Gln Leu Leu
145 150 155 160

Glu Leu Glu Lys Glu Phe Leu Phe Asn Lys Tyr Ile Ser Arg Pro Arg
165 170 175

Arg Val Glu Leu Ala Val Met Leu Asn Leu Thr Glu Arg His Ile Lys
180 185 190

```
Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys Glu Glu Asp Lys
            195                 200                 205

     Lys Arg Ser Ser Gly Thr Pro Ser Gly Gly Gly Gly Gly Glu Glu Pro
         210                 215                 220

     Glu Gln Asp Cys Ala Val Thr Ser Gly Glu Glu Leu Leu Ala Val Pro
     225                 230                 235                 240

     Pro Leu Pro Pro Pro Gly Gly Ala Val Pro Pro Gly Val Pro Ala Ala
                     245                 250                 255

     Val Arg Glu Gly Leu Leu Pro Ser Gly Leu Ser Val Ser Pro Gln Pro
                 260                 265                 270

     Ser Ser Ile Ala Pro Leu Arg Pro Gln Glu Pro Arg
             275                 280
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 677 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
AAGCTTCAGG CTGCCAGCAC ACTATCTGTT ATTGCTGCCA CTGCCCACTG AAAGGGATCT      60

TGAGACATGA GGTGCATCTC CAGGCTGCCA GCTGTTCTCC TCATCCTCTC GGTGGCACTC     120

GGCCACTTGA GAGCTACACC TGTCGGAAGT GGTACCAACC CTCAGGTGGA CAAACGGAAG     180

TGCAACACAG CCACATGTGC CACACAACGT CTGGCAAACT TCTTGGTTCG CTCCAGCAAC     240

AACCTTGGTC CAGTCCTCCC ACCAACCAAT GTGGGATCCA ATACATATGG GAAGAGGAAT     300

GTGGCAGAGG ATCCAAATAG GGAATCCCTG GATTTCTTAC TCCTGTAAAG TCAATGTACT     360

CCCGTATCTC TTATTACTTC CTGTGTAAAT GCTCTGATGA TTTCCTGAAT AATGTAACAG     420

TGCCTTCAAC GTGCCTGTGC TTGCTGTGTT TGTAAATTCT TATTCTAAGA CGTGCTTTAA     480

ACTGAGTGTT GATAAAGGTC AGGGTGAATA CCTCTCTAAT CACAACATGT TCTTGGCTGT     540

ACATCGATAT CGTAGGAACA CTTAAAATTT CTGTTTTTAC CTTGTAACTC TATGACTCAA     600
```

GTTTAACAAT AAAGGAGGGC GTGGGATGGT GGACTTGAAA AGTCATTAAC AGCTCATAGT 660

AAATTTCTGA TTCTAGA 677

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 93 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Arg Cys Ile Ser Arg Leu Pro Ala Val Leu Leu Ile Leu Ser Val
1 5 10 15

Ala Leu Gly His Leu Arg Ala Thr Pro Val Gly Ser Gly Thr Asn Pro
20 25 30

Gln Val Asp Lys Arg Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg
35 40 45

Leu Ala Asn Phe Leu Val Arg Ser Ser Asn Asn Leu Gly Pro Val Leu
50 55 60

Pro Pro Thr Asn Val Gly Ser Asn Thr Tyr Gly Lys Arg Asn Val Ala
65 70 75 80

Glu Asp Pro Asn Arg Glu Ser Leu Asp Phe Leu Leu Leu
85 90

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2086 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGATCCAAGG CCCAACTCCC CGAACCACTC AGGGTCCTGT GGACAGCTCA CCTAGCTGCA 60

ATGGCTACAG GTAAGCGCCC CTAAAATCCC TTTGGCACAA TGTGTCCTGA GGGGAGAGGC 120

AGCGACCTGT AGATGGGACG GGGGCACTAA CCCTCAGGGT TTGGGGTTCT GAATGTGAGT 180

ATCGCCATCT AAGCCCAGTA TTTGGCCAAT CTCAGAAAGC TCCTGGCTCC CTGGAGGATG 240

GAGAGAGAAA AACAAACAGC TCCTGGAGCA GGGAGAGTGT TGGCCTCTTG CTCTCCGGCT 300

CCCTCTGTTG CCCTCTGGTT TCTCCCCAGG CTCCCGGACG TCCCTGCTCC TGGCTTTTGG 360

CCTGCTCTGC CTGCCCTGGC TTCAAGAGGG CAGTGCCTTC CCAACCATTC CCTTATCCAG 420

GCTTTTTGAC AACGCTATGC TCCGCGCCCA TCGTCTGCAC CAGCTGGCCT TTGACACCTA 480

CCAGGAGTTT GTAAGCTCTT GGGGAATGGG TGCGCATCAG GGGTGGCAGG AAGGGGTGAC 540

TTTCCCCCGC TGGAAATAAG AGGAGGAGAC TAAGGAGCTC AGGGTTTTTC CCGACCGCGA 600

AAATGCAGGC AGATGAGCAC ACGCTGAGCT AGGTTCCCAG AAAAGTAAAA TGGGAGCAGG 660

TCTCAGCTCA GACCTTGGTG GGCGGTCCTT CTCCTAGGAA GAAGCCTATA TCCCAAAGGA 720

ACAGAAGTAT TCATTCCTGC AGAACCCCCA GACCTCCCTC TGTTTCTCAG AGTCTATTCC 780

GACACCCTCC AACAGGGAGG AAACACAACA GAAATCCGTG AGTGGATGCC TTCTCCCCAG 840

GCGGGGATGG GGGAGACCTG TAGTCAGAGC CCCCGGGCAG CACAGCCAAT GCCCGTCCTT 900

GCCCCTGCAG AACCTAGAGC TGCTCCGCAT CTCCCTGCTG CTCATCCAGT CGTGGCTGGA 960

GCCCGTGCAG TTCCTCAGGA GTGTCTTCGC CAACAGCCTG GTGTACGGCG CCTCTGACAG 1020

CAACGTCTAT GACCTCCTAA AGGACCTAGA GGAAGGCATC CAAACGCTGA TGGGGGTGAG 1080

GGTGGCGCCA GGGGTCCCCA ATCCTGGAGC CCCACTGACT TTGAGAGACT GTGTTAGAGA 1140

AACACTGGCT GCCCTCTTTT TAGCAGTCAG GCCCTGACCC AAGAGAACTC ACCTTATTCT 1200

TCATTTCCCC TCGTGAATCC TCCAGGCCTT TCTCTACACT GAAGGGGAGG GAGGAAAATG 1260

AATGAATGAG AAAGGGAGGG AACAGTACCC AAGCGCTTGG CCTCTCCTTC TCTTCCTTCA 1320

CTTTGCAGAG GCTGGAAGAT GGCAGCCCCC GGACTGGGCA GATCTTCAAG CAGACCTACA 1380

GCAAGTTCGA CACAAACTCA CACAACGATG ACGCACTACT CAAGAACTAC GGGCTGCTCT 1440

ACTGCTTCAG GAAGGACATG GACAAGGTCG AGACATTCCT GCGCATCGTG CAGTGCCGCT 1500

CTGTGGAGGG CAGCTGTGGC TTCTAGCTGC CCGGGTGGCA TCCCTGTGAC CCCTCCCCAG 1560

TGCCTCTCCT GGCCCTGGAA GTTGCCACTC CAGTGCCCAC CAGCCTTGTC CTAATAAAAT 1620

TAAGTTGCAT CATTTTGTCT GACTAGGTGT CCTTCTATAA TATTATGGGG TGGAGGGGGG 1680

TGGTATGGAG CAAGGGGCCC AAGTTGGGAA GACAACCTGT AGGGCCTGCG GGGTCTATTC 1740

GGGAACCAAG CTGGAGTGCA GTGGCACAAT CTTGGCTCAC TGCAATCTCC GCCTCCTGGG 1800

TTCAAGCGAT TCTCCTGCCT CAGCCTCCCG AGTTGTTGGG ATTCCAGGCA TGCATGACCA 1860

GGCTCAGCTA ATTTTTGTTT TTTTGGTAGA GACGGGGTTT CACCATATTG GCCAGGCTGG 1920

TCTCCAACTC CTAATCTCAG GTGATCTACC CACCTTGGCC TCCCAAATTG CTGGGATTAC 1980

AGGCGTGAAC CACTGCTCCC TTCCCTGTCC TTCTGATTTT AAAATAACTA TACCAGCAGG 2040

AGGACGTCCA GACACAGCAT AGGCTACCTG CCATGGCCCA ACCGGT 2086

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 217 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1 5 10 15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu
20 25 30

Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln
35 40 45

Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys
50 55 60

```
Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe
65                  70                  75                  80

Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys
                85                  90                  95

Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp
            100                 105                 110

Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val
        115                 120                 125

Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu
    130                 135                 140

Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg
145                 150                 155                 160

Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
                165                 170                 175

His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe
            180                 185                 190

Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys
        195                 200                 205

Arg Ser Val Glu Gly Ser Cys Gly Phe
    210                 215
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 235 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
CCGGATCCAG GTCGACGCCG GCCAAGACAG CACAGACAGA TTGACCTATT GGGGTGTTTC      60

GCGAGTGTGA GAGGGAAGCG CCGCGGCCTG TATTTCTAGA CCTGCCCTTC GCCTGGTTCG     120

TGGCGCCTTG TGACCCCGGG CCCCTGCCGC CTGCAAGTCG AAATTGCGCT GTGCTCCTGT     180

GCTACGGCCT GTGGCTGGAC TGCCTGCTGC TGCCCAACTG GCTGGCAAGA TCTCG          235
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 441 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTACCGGGC CCCCCCTCGA GGTCCTGAGC TAAGAATCCA GCTATCAATA GAAACTATGA 60

AACAGTTCCA GGGACAAAGA TACCAGGTCC CCAACAACTG CAACTTTCTG GGAAATGAGG 120

TGGAAAATGC TCAGCCAAGG AAAAAGAGGG CCTTACCCTC TCTGGGACAA TGATTGTGCT 180

GTGAACTGCT TCATCACGGC ATCTGGCCCC TTGTTAATAA TCTAATTACC CTAGGTCTAA 240

GTAGAGTTGT TGACGTCCAA TGAGCGCTTT CTGCAGACTT AGCACTAGGC AAGTGTTTGG 300

AAATTACAGC TTCAGCCCCT CTCGCCATCT GCCTACCTAC CCCTCCTAGA GCCCTTAATG 360

GGCCAAACGG CAAAGTCCAG GGGGCAGAGA GGAGGTGCTT TGGACTATAA AGCTAGTGGA 420

GACCCAGTAA CTCCCAAGCT T 441

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1252 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGTATCTTT GCCCCGATGG ATGCTGGGCC TAGCCAGGGG CTCCCAGTCC CCAGGCGTGG 60

GGTAGAAGTT GGACTCTATA GTCACCTAAG GGCCTATGTT GCAGTCCTGG TCTCAGGCAC 120

GCGGCCTGCA GGAGAGGCTT AAAAGAAGAG AAACTGCACA CAATGGCTAG GTCACCGGCG 180

TTAAAGCTAA GCAAACCCAG CGCTACTCCT GGGCAGCAAC TGCAAAGCGT TTCTTCAGGT 240

CCCTCACCTG TAGAATCAGA GCGGTAGTCG CCTCTGCATG TCTGAGTTCT TACATGTCGT 300

AATGTACAAA CACGATTTCC CCTCAATCAC CGCCCGGAAC AGTACCTCCA ACTTCCCAGA 360

CCCGGATGCC CCAAGAGCCA GAGTAGGGTG GGAAAATCGG GACAGGCCCC CAAATTCCAC 420

TCGGGGGCCT TGAGCTCTTA CATGGTGTCA CGGGGGCAGG TAGTTTGGGT TTAGCAATGT 480

GAACTCTGAC AATTTGGGAT GTAGAGCTGG TGGGCCATCG TGGGACGCCA AGCATCATCC 540

TTAGAGTTTG GATCCTTTAG GGCAGGCAGG CACAGGGACC CAGTGCGAGA TCAGTGAAGC 600

CGCCCAGTTT CGGCTTCCGC TCTTTTTCCA CGCCCACTTG CGTGCTTCTC CAACAGTGTG 660

GATGGGAGGG GTGGGGGACG AGCCCTAATC TCCGAGGAAG GGGTGTGGCC CCGTTCGTGT 720

TCTCCAGTTT GTGGCGTCCT GGATCTGTCC TCTGGTCCCC TCCAGATCGT GTCCCACACC 780

CACCCGTTCA GGCATGGCAC TGTGCCGCCA CGCGTGACCG TGCGCTCCTT ACGTGGGGGA 840

CGTGCAGGGT GCTGCCTCCT TTCCGGTGCG GGAGGGAGCG GCCGTCTTTC TCCTGCTCTG 900

GCTGGGAAGC CCCAGCCATT GCGCTGCAGA GGAGACTTGC AGCCAATGGG GACTGAGGAA 960

GTGGGCCGGC TGGCGGTTGT CACCCTCCCG GGGACCGGAG CTCCGAGGTC TGGAGAGCGC 1020

AGGCAGACGC CCGCCCCGCC CGGGGACTGA GGGGGAGGAG CGAAGGGAGG AGGAGGTGGA 1080

GTCTCCGATC TGCCGCTGGA GGACCACTGC TCACCAGGCT ACTGAGGAGC CACTGGCCCC 1140

ACACCTGCTT TTCCGCATCC CCCACCGTCA GCATGATCGC CGCGCAACTA CTGGCCTATT 1200

ACTCACCGAG CTGAAGGATG ACCAAGTCAA AAAGGTGAGC CCCGCCGGCG CC 1252

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 410 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAATTCTGTT GGGCTCGCGG TTGACCACAA ACTCTTCGCG GTCTTTCCAG TACTCTTGGA 60

TCGGAAACCC GTCGGCCTCC GAACGGTACT CCGCCACCGA GGGACCTGAG CGAGTCCGCA 120

TCGACCGGAT CGGAAAACCT CTCGACTGTT GGGGTGAGTA CTCCCTCTCA AAAGCGGGCA 180

TGACTTCTGC GCTAAGATTG TCAGTTTCCA AAAACGAGGA GGATTTGATA TTCACCTGGC 240

CCGCGGTGAT GCCTTTGAGG GTGGCCGCGT CCATCTGGTC AGAAAAGACA ATCTTTTTGT 300

TGTCAAGCTT GAGGTGTGGC AGGCTTGAGA TCTGGCCATA CACTTGAGTG ACAATGACAT 360

CCACTTTGCC TTTCTCTCAC ACAGGTGTCC ACTCCCAGGT CCAACTGCAG 410

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 175 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GATCCCTTCA TCAGGCCATC TGGCCCCTTG TTAATAATCG ACTGACCCTA GGTCTAAGAT 60

CCCTTCATCA GGCCATCTGG CCCCTTGTTA ATAATCGACT GACCCTAGGT CTAAGATCCC 120

TTCATCAGGC CATCTGGCCC CTTGTTAATA ATCGACTGAC CCTAGGTCTA AGATC 175

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTTCCCCTCG AGCACCGCCC GGAACAGTAC C 31

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTTGCGCCTC GAGCATGCTG ACGGTGGGGG 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTTGGACTCG AGAGTCACCT AAGGGCCTAT G 31

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GATTGGGAAG ACAATAGCAG GCATGC 26

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGTCGCCTCT GCATGTCTGA GTTC 24

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTTGAGCTCT TACATGGTGT CACG 24

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TCCCCAGGCG TGGGGTAGAA G 21

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CAACCGGTGG GACATTTGAG TTGC 24

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCAAGTCATT ATAGAATCAT AGTC 24

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCGGATCCCA TGATTGAACA AGAT 24

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCAAGATCTC GCTCAGAAGA ACTC 24

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCGGATCCAG GTCGACGCCG GCCAA 25

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGAGATCTTG CCAGCCAGTT GG 22

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GATCCCTTCA TCAGGCCATC TGGCCCCTTG TTAATAATCG ACTGACCCTA GGTCTAA 57

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GATCTTAGAC CTAGGGTCAG TCGATTATTA ACAAGGGGCC AGATGGCCTG ATGAAGG 57

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTCCCAAGCT TAAGTGACCA GCTACAA 27

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GGGCAACCTA GGTACTGGAC CTTCTATC 28

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGGTCTAGAG GACCTGTTCC CACCG 25

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCCGAATTCG AGGAGCAGAG AGCGAAGC 28

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GCCAGCAGGG GCAGGAGGCG CATCCACAGG GCCAT 35

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCACCTGTCT ACACCTCCTC TC 22

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GTAATCCAGG TGTCGTGACT GC 22

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCGGGGATCC TTCTGCCATG GCCC 24

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GGGCTAGATC TAGTTGCTGT AGTTCTCCAG CTGGTAGAGG GAGCAGATGC TAGTACTGCA 60

TTGTTCCAC 69

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GGGCTAGATC TAGTTGCAGT AGTTCTC 27

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GGATCCATGA ACAGTGAGGA GCAG 24

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

AGATCTTCAC CGGGGTTCCT GCGG 24

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GGGGATCCGA TATGAAAAAG CCTG 24

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CGAGATCTAC TCTATTCCTT TGC 23

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCGGATCCCA TGAGCGAAAA AT 22

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GGAGATCTTT AGCGACCGGA GAT 23

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCGGATCCAT GGTTCGACCA TTG 23

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GGAGATCTGT TAGTCTTTCT TC 22

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CCGGATCCAT GAGCTTCAAT AC 22

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CCAGATCTGC TCATGCTTGC TCC 23

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
1 5 10 15

Val Arg Ser Ser Asn Asn Leu Gly Pro Val Leu Pro Pro Thr Asn Val
20 25 30

Gly Ser Asn Thr Tyr
35

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 491 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

TTTGCTGATA TTGCTGACAT TGAAACATTA AAAGAAAATT TGAGAAGCAA TGGGCATCCT 60

GAAGCTGCAA GTATTTCTCA TTGTGCTCTC TGTTGCATTG AACCATCTGA AAGCTACACC 120

CATTGAAAGT CATCAGGTGG AAAAGCGGAA ATGCAACACT GCCACATGTG CAACGCAGCG 180

CCTGGCAAAT TTTTTAGTTC ATTCCAGCAA CAACTTTGGT GCCATTCTCT CATCTACCAA 240

CGTGGGATCC AATACATATG GCAAGAGGAA TGCAGTAGAG GTTTTAAAGA GAGAGCCACT 300

GAATTACTTG CCCCTTTAGA GGACAATGTA ACTCTATAGT TATTGTTTTA TGTTCTAGTG 360

ATTTCCTGTA TAATTTAACA GTGCCCTTTT CATCTCCAGT GTGAATATAT GGTCTGTGTG 420

TCTGATGTTT GTTGCTAGGA CATATACCTT CTCAAAAGAT TGTTTTATAT GTAGTACTAA 480

CTAAGGTCCC A 491

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Met Gly Ile Leu Lys Leu Gln Val Phe Leu Ile Val Leu Ser Val Ala
1 5 10 15

Leu Asn His Leu Lys Ala Thr Pro Ile Glu Ser His Gln Val Glu Lys
20 25 30

Arg Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe
35 40 45

Leu Val His Ser Ser Asn Asn Phe Gly Ala Ile Leu Ser Ser Thr Asn
50 55 60

Val Gly Ser Asn Thr Tyr Gly Lys Arg Asn Ala Val Glu Val Leu Lys
65 70 75 80

Arg Glu Pro Leu Asn Tyr Leu Pro Leu
85

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
1 5 10 15

Val His Ser Ser Asn Asn Phe Gly Ala Ile Leu Ser Ser Thr Asn Val
20 25 30

Gly Ser Asn Thr Tyr
35

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 895 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CCACCTGTCT ACACCTCCTC TCAGCTCAGT CCCACAAGGC AGAATAAAAA AATGAAGACC 60

GTTTACATCG TGGCTGGATT GTTTGTAATG CTGGTACAAG GCAGCTGGCA GCATGCCCCT 120

CAGGACACGG AGGAGAACGC CAGATCATTC CCAGCTTCCC AGACAGAACC ACTTGAAGAC 180

CCTGATCAGA TAAACGAAGA CAAACGCCAT TCACAGGGCA CATTCACCAG TGACTACAGC 240

AAATACCTAG ACTCCCGCCG TGCTCAAGAT TTTGTGCAGT GGTTGATGAA CACCAAGAGG 300

AACCGGAACA ACATTGCCAA ACGTCATGAT GAATTTGAGA GGCATGCTGA AGGGACCTTT 360

ACCAGTGATG TGAGTTCTTA CTTGGAGGGC CAGGCAGCAA AGGAATTCAT TGCTTGGCTG 420

GTGAAAGGCC GAGGAGAACG AGACTTCCCG GAAGAAGTCG CCATAGCTGA GGAACTTGGG 480

CGCAGACATG CTGATGGATC CTTCTCTGAT GAGATGAACA CGATTCTCGA TAACCTTGCC 540

ACCAGAGACT TCATCAACTG GCTGATTCAA ACCAAGATCA CTGACAAGAA ATAGGAATAT 600

TTCACCATTC ACAACCATCT TCACAACATC TCCTGCCAGT CACTTGGGAT GTACATTTGA 660

GAGCATATCC GAAGCTATAC TGCTTTGCAT GCGGACGAAT ACATTTCCCT TTAGCGTTGT 720

GTAACCCAAA GGTTGTAAAT GGAATAAAGT TTTTCCAGGG TGTTGATAAA GTAACAACTT 780

TACAGTATGA AAATGCTGGA TTCTCAAATT GTCTCCTCGT TTTGAAGTTA CCGCCCTGAG 840

ATTACTTTTC TGTGGTATAA ATTGTAAATT ATCGCAGTCA CGACACCTGG ATTAC 895

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 180 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Met Lys Thr Val Tyr Ile Val Ala Gly Leu Phe Val Met Leu Val Gln
1 5 10 15

Gly Ser Trp Gln His Ala Pro Gln Asp Thr Glu Glu Asn Ala Arg Ser
20 25 30

Phe Pro Ala Ser Gln Thr Glu Pro Leu Glu Asp Pro Asp Gln Ile Asn
35 40 45

Glu Asp Lys Arg His Ser Gln Gly Thr Phe Thr Ser Asp Tyr Ser Lys
50 55 60

Tyr Leu Asp Ser Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn
65 70 75 80

Thr Lys Arg Asn Arg Asn Asn Ile Ala Lys Arg His Asp Glu Phe Glu
85 90 95

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu
100 105 110

Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Gly

```
        115                 120                 125

Glu Arg Asp Phe Pro Glu Glu Val Ala Ile Ala Glu Glu Leu Gly Arg
    130                 135                 140

Arg His Ala Asp Gly Ser Phe Ser Asp Glu Met Asn Thr Ile Leu Asp
145                 150                 155                 160

Asn Leu Ala Thr Arg Asp Phe Ile Asn Trp Leu Ile Gln Thr Lys Ile
                165                 170                 175

Thr Asp Lys Lys
            180
```

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 955 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

```
CAGCACACTA CCAGAAGACA GCAGAAATGA AAAGCATTTA CTTTGTGGCT GGGTTATTTG      60

TAATGCTGGT ACAAGGCAGC TGGCAACGTT CCCTTCAAGA CACAGAGGAG AAATCCAGAT     120

CATTCTCAGC TTCCCAGGCA GACCCACTCA GTGATCCTGA TCAGATGAAC GAGGACAAGC     180

GCCATTCACA GGGCACATTC ACCAGTGACT ACAGCAAGTA TCTGGACTCC AGGCGTGCCC     240

AAGATTTTGT GCAGTGGTTG ATGAATACCA AGAGGAACAG GAATAACATT GCCAAACGTC     300

ACGATGAATT TGAGAGACAT GCTGAAGGGA CCTTTACCAG TGATGTAAGT TCTTATTTGG     360

AAGGCCAAGC TGCCAAGGAA TTCATTGCTT GGCTGGTGAA AGGCCGAGGA AGGCGAGATT     420

TCCCAGAAGA GGTCGCCATT GTTGAAGAAC TTGGCCGCAG ACATGCTGAT GGTTCTTTCT     480

CTGATGAGAT GAACACCATT CTTGATAATC TTGCCGCCAG GGACTTTATA AACTGGTTGA     540

TTCAGACCAA AATCACTGAC AGGAAATAAC TATATCACTA TTCAAGATCA TCTTCACAAC     600

ATCACCTGCT AGCCACGTGG GATGTTTGAA ATGTTAAGTC CTGTAAATTT AAGAGGTGTA     660

TTCTGAGGCC ACATTGCTTT GCATGCCAAT AAATAAATTT TCTTTTAGTG TTGTGTAGCC     720

AAAAATTACA AATGGAATAA AGTTTTATCA AAATATTGCT AAAATATCAG CTTTAAAATA     780
```

TGAAAGTGCT AGATTCTGTT ATTTTCTTCT TATTTTGGAT GAAGTACCCC AACCTGTTTA 840

CATTTAGCGA TAAAATTATT TTTCTATGAT ATAATTTGTA AATGTAAATT ATTCCGATCT 900

GACATATCTG CATTATAATA ATAGGAGAAT AGAAGAACTG GTAGCCACAG TGGTG 955

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 180 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Met Lys Ser Ile Tyr Phe Val Ala Gly Leu Phe Val Met Leu Val Gln
1 5 10 15

Gly Ser Trp Gln Arg Ser Leu Gln Asp Thr Glu Glu Lys Ser Arg Ser
20 25 30

Phe Ser Ala Ser Gln Ala Asp Pro Leu Ser Asp Pro Asp Gln Met Asn
35 40 45

Glu Asp Lys Arg His Ser Gln Gly Thr Phe Thr Ser Asp Tyr Ser Lys
50 55 60

Tyr Leu Asp Ser Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn
65 70 75 80

Thr Lys Arg Asn Arg Asn Asn Ile Ala Lys Arg His Asp Glu Phe Glu
85 90 95

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu
100 105 110

Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Gly
115 120 125

Arg Arg Asp Phe Pro Glu Glu Val Ala Ile Val Glu Glu Leu Gly Arg
130 135 140

Arg His Ala Asp Gly Ser Phe Ser Asp Glu Met Asn Thr Ile Leu Asp
145 150 155 160

Asn Leu Ala Ala Arg Asp Phe Ile Asn Trp Leu Ile Gln Thr Lys Ile
165 170 175

Thr Asp Arg Lys
180

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 531 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

AGCTTAAGTG ACCAGCTACA ATCATAGACC ATCAGCAAGC AGGTATGTAC TCTCCTGGGT 60

GAGCCCGGTT CCCCCAGCCA AAACTCTAGG GACTTTAGGA AGGATGTGGG TTCCTCTCTT 120

ACATGGACCT TTTCCTAGCC TCAACCCTGC CTATCTTCCA GGTCATTGTT CCAACATGGC 180

CCTGTGGATG CGCTTCCTGC CCCTGCTGGC CCTGCTCGTC CTCTGGGAGC CCAAGCCTGC 240

CCAGGCTTTT GTCAAACAGC ACCTTTGTGG TCCTCACCTG GTGGAGGCTC TGTACCTGGT 300

GTGTGGGGAA CGTGGTTTCT TCTACACACC CAAGTCCCGT CGTGAAGTGG AGGACCCGCA 360

AGTGCCACAA CTGGAGCTGG GTGGAGGCCC GGAGGCCGGG GATCTTCAGA CCTTGGCACT 420

GGAGGTTGCC CGGCAGAAGC GTGGCATTGT GGATCAGTGC TGCACCAGCA TCTGCTCCCT 480

CTACCAACTG GAGAACTACT GCAACTGAGT CCACCACTCC CCGCCCAGAC G 531

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 955 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

CAGCACACTA CCAGAAGACA GCAGAAATGA AAAGCATTTA CTTTGTGGCT GGATTATTTG 60

TAATGCTGGT ACAAGGCAGC TGGCAACGTT CCCTTCAAGA CACAGAGGAG AAATCCAGAT 120

CATTCTCAGC TTCCCAGGCA GACCCACTCA GTGATCCTGA TCAGATGAAC GAGGACAAGG 180

CCCATTCACA GGGCACATTC ACTAGTGACT ACAGCAAGTA TCTGGACTCC AGGCGTGCCC 240

AAGATTTTGT GCAGTGGTTG ATGAATACCA AGAGGAACAG GAATAACATT GCCAAACGTC 300

ACGATGAATT TGAGAGACAT GCTGAAGGGA CCTTTACCAG TGATGTAAGT TCTTATTTGG 360

AAGGCCAAGC TGCCAAGGAA TTCATTGCTT GGCTGGTGAA AGGCCGAGGA AGGCGAGATT 420

TCCCAGAAGA GGTCGCCATT GTTGAAGAAC TTGGCCGCAG ACATGCTGAT GGTTCTTTCT 480

CTGATGAGAT GAACACCATT CTTGATAATC TTGCCGCCAG GGACTTTATA AACTGGTTGA 540

TTCAGACCAA AATCACTGAC AGGAAATAAC TATATCACTA TTCAAGATCA TCTTCACAAC 600

ATCACCTGCT AGCCACGTGG GATGTTTGAA ATGTTAAGTC CTGTAAATTT AAGAGGTGTA 660

TTCTGAGGCC ACATTGCTTT GCATGCCAAT AAATAAATTT TCTTTTAGTG TTGTGTAGCC 720

AAAAATTACA AATGGAATAA AGTTTTATCA AAATATTGCT AAAATATCAG CTTTAAAATA 780

TGAAAGTGCT AGATTCTGTT ATTTTCTTCT TATTTTGGAT GAAGTACCCC AACCTGTTTA 840

CATTTAGCGA TAAAATTATT TTTCTATGAT ATAATTTGTA AATGTAAATT ATTCCGATCT 900

GACATATCTG CATTATAATA ATAGGAGAAT AGAAGAACTG GTAGCCACAG TGGTG 955

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 180 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Met Lys Ser Ile Tyr Phe Val Ala Gly Leu Phe Val Met Leu Val Gln
1 5 10 15

Gly Ser Trp Gln Arg Ser Leu Gln Asp Thr Glu Glu Lys Ser Arg Ser
20 25 30

Phe Ser Ala Ser Gln Ala Asp Pro Leu Ser Asp Pro Asp Gln Met Asn
35 40 45

Glu Asp Lys Ala His Ser Gln Gly Thr Phe Thr Ser Asp Tyr Ser Lys
50 55 60

Tyr Leu Asp Ser Arg Arg Ala Gln Asp Phe Val Gln Trp Leu Met Asn

65 70 75 80

Thr Lys Arg Asn Arg Asn Asn Ile Ala Lys Arg His Asp Glu Phe Glu
85 90 95

Arg His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu
100 105 110

Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg Gly
115 120 125

Arg Arg Asp Phe Pro Glu Glu Val Ala Ile Val Glu Glu Leu Gly Arg
130 135 140

Arg His Ala Asp Gly Ser Phe Ser Asp Glu Met Asn Thr Ile Leu Asp
145 150 155 160

Asn Leu Ala Ala Arg Asp Phe Ile Asn Trp Leu Ile Gln Thr Lys Ile
165 170 175

Thr Asp Arg Lys
180

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

TTTGCTGATA TTGCTGAC 18

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TGGGACCTTA GTTAGTAC 18

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CAGCACACTA CCAGAAGACA GC 22

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

CACCACTGTG GCTACCAGTT CT 22

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CAGCACACTA CCAGAAGACG C 21

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CTGTAGTCAC TAGTGAATGT GCCCTGTGAA TGGGCCTTGT GGTCG 45

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

ACTAGTGAAT GTGCCCTG 18

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

ACTAGTGACT ACAGCAAG 18

VI. CLAIMS

What is claimed is:

1. A method of engineering a mammalian cell:

a) providing a starting cell;

b) introducing into said starting cell an amylin-encoding gene operatively linked to a first promoter; and

c) selecting a cell from step b that exhibits increased amylin production as compared to said starting cell.

2. The method of claim 1, further comprising introducing into said selected cell an insulin-encoding gene operatively linked to a second promoter.

3. The method of claim 1, wherein said starting cell produces amylin.

4. The method of claim 1, wherein said starting cell does not produce amylin.

5. The method of claim 1, wherein said amylin is proteolytically processed.

6. The method of claim 1, wherein said amylin is amidated.

7. The method of claim 1, wherein said amylin is glycosylated.

8. The method of claim 8, wherein said glycosylated amylin is O-glycosylated.

9. The method of claim 8, wherein said glycosylated amylin is N-glycosylated.

10. The method of claim 8, wherein said glycosylated amylin comprises an oligosaccharide linked to threonine-9 of an amylin of SEQ ID NO:51 or SEQ ID NO:53

11. The method of claim 8, wherein said glycosylated amylin comprises an oligosaccharide linked to threonine-6 of an amylin of SEQ ID NO:51 or SEQ ID NO:53.

12. The method of claim 11, wherein said glycosylated amylin comprises an oligosaccharide structure having between about 3 and about 10 saccharide units.

13. The method of claim 12, wherein said glycosylated amylin comprises an oligosaccharide structure having between about 3 and about 10 saccharide units.

14. The method of claim 8, wherein saccharides are selected from the group consisting of ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, fructose, gulose, idose, galactose, talose, ribulose, sorbose, tagatose, gluconic acid, glucuronic acid, glucaric acididuronic acid, rhamnose, fucose, N-acetyl glucosamine, N-acetyl galactosamine, N-acetyl neuraminic acid, sialic acid, amino glycal and a substituted amino glycal.

15. The method of claim 8, wherein said glycosylated amylin comprises [NeuAc,$HexNAc_2$]Gal(β1-3)GalNac.

16. The method of claim 1, wherein said first promoter is selected from the group consisting of CMV IE, SV40 IE, RSV LTR, RIP, modified RIP, POMC and GH.

17. The method of claim 2, wherein said second promoter is selected from the group consisting of CMV IE, SV40 IE, RSV LTR, RIP, modified RIP, POMC and GH.

18. The method of claim 1, wherein the starting cell is a human cell.

19. The method of claim 1, wherein the starting cell is a non-human cell.

20. The method of claim 1, wherein said starting cell is a secretory cell.

21. The method of claim 1, wherein said starting cell is a neuroendocrine cell.

22. The method of claim 1, wherein said starting cell is a beta cell.

23. The method of claim 1, wherein said starting cell is a pituitary cell.

24. The method of claim 1, wherein said starting cell is secretagogue responsive.

25. The method of claim 1, wherein said starting cell is glucose responsive.

26. The method of claim 1, wherein said starting cell is non-glucose responsive.

27. The method of claim 1, wherein the starting cell is derived from a βTC, RIN, HIT, BHC, CM, TRM, TRM6 AtT20, PC12 or HAP5 cell.

28. The method of claim 1, wherein said amylin encoding gene is a human amylin-encoding gene.

29. The method of claim 1, wherein said amylin-encoding gene is linked to a selectable marker.

30. The method of claim 29, wherein said selectable marker is selected from a group consisting of hygromycin resistance, neomycin resistance, puromycin resistance, zeocin, gpt, DHFR and histadinol.

31. The method of claim 2, wherein said insulin gene is linked to a selectable marker gene.

32. The method of claim 31, wherein said selectable marker is selected from a group consisting of hygromycin resistance, neomycin resistance, puromycin resistance, zeocin, gpt, DHFR and histadinol.

33. The method of claim 1, wherein said amylin is an analog of human amylin.

34. The method of claim 1, wherein said amylin is a non-amyloidogenic analog.

35. The method of claim 1, wherein said amylin-encoding gene is a rat amylin-encoding gene.

36. The method of claim 1, wherein said amylin is a rat amylin analog.

37. A method of providing amylin to a mammal comprising

a) providing a starting cell;

b) introducing into said starting cell an amylin-encoding gene operatively linked to a first promoter;

c) selecting a cell of step b that exhibits increased amylin production as compared to said starting cell; and

d) administering said selected cell to a mammal.

38. The method of claim 37, wherein said mammal exhibits at least one pathologic condition selected from the group consisting of angiogenesis, gastric emptying, anorexia, obesity, hypertension, hypercalcemia, Pagets disease and osteoporosis.

39. The method of claim 37, further comprising introducing into said selected cell an insulin-encoding gene operatively linked to a second promoter.

40. The method of claim 37, further comprising (i) encapsulating said selected cell in a biocompatible coating or (ii) placing said cells into a selectively permeable membrane in a protective housing.

41. The method of claim 37, wherein said first promoter is selected from the group consisting of CMV IE, SV40 IE, RSV LTR, RIP, modified RIP, POMC and GH.

42. The method of claim 39, wherein said second promoter is selected from the group consisting of CMV IE, SV40 IE, RSV LTR, RIP, modified RIP, POMC and GH.

43. The method of claim 38, wherein said cell is administered intraperitoneally, subcutaneously or via the CNS.

44. The method of claim 38, wherein the cell is contained within a selectively semi-permeable device, said device being connected to the vasculature of the mammal.

45. A method of producing mammalian amylin comprising:

a) providing a starting cell;

b) introducing into said starting cell an amylin-encoding gene operatively linked to a first c) promoter;

d) selecting a cell of step b that exhibits increased production of amylin as compared to said starting cell; and

e) culturing said selected cell.

46. The method of claim 45, further comprising the step of purifying said amylin.

47. The method of claim 45, wherein said starting cell is a secretory cell.

48. The method of claim 45, wherein said starting cell produces amylin.

49. The method of claim 45, wherein said starting cell does not produce amylin.

50. The method of claim 45, wherein said amylin is proteolytically processed.

51. The method of claim 45, wherein said amylin is amidated.

52. The method of claim 45, wherein said amylin is glycosylated.

53. The method of claim 52, wherein said glycosylated amylin is O-glycosylated.

54. The method of claim 52, wherein said glycosylated amylin is N-glycosylated.

55. The method of claim 45, wherein said promoter is selected from the group consisting of CMV IE, SV40 IE, RSV LTR, RIP, modified RIP, POMC and GH.

56. The method of claim 45, wherein said starting cell is a human cell.

57. The method of claim 45, wherein said starting cell is a non-human cell.

58. The method of claim 45, wherein said amylin-encoding gene is linked to a selectable marker.

59. The method of claim 58, wherein said selectable marker is selected from the group consisting of hygromycin resistance, neomycin resistance, puromycin resistance, zeocin, gpt, DHFR and histadinol.

60. The method of claim 45, wherein said amylin-encoding gene is a human amylin-encoding gene.

61. The method of claim 45, wherein said amylin is an analog of human amylin.

62. The method of claim 45, wherein said amylin is a non-amyloidogenic analog.

63. The method of claim 45, wherein said amylin-encoding gene is rat amylin-encoding gene.

64. The method of claim 45, wherein said amylin is an analog of rat amylin.

65. Amylin produced according to a process comprising the steps of:

a) providing a starting cell;

b) introducing into said starting cell an amylin-encoding gene operatively linked to a first promoter;

c) selecting a cell of step b that exhibits increased production of amylin as compared to d) said starting cell; and

e) culturing said selected cell.

66. The amylin of claim 65, wherein said amylin is secreted.

67. The process of claim 65, further comprising the step of purifying said amylin.

68. The process of claim 65, further comprising the step of introducing into said cell an insulin-encoding gene operatively linked to a second promoter.

69. The method of claim 65, wherein said amylin is proteolytically processed.

70. The method of claim 65, wherein said amylin is amidated.

71. The method of claim 65, wherein said amylin is glycosylated.

72. The method of claim 71, wherein said glycosylated amylin is O-glycosylated.

73. The method of claim 71, wherein said glycosylated amylin is N-glycosylated.

74. The method of claim 65, wherein said first promoter is selected from the group consisting of CMV IE, SV40 IE, RSV LTR, RIP, modified RIP, POMC and GH.

75. The method of claim 68, wherein said second promoter is selected from the group consisting of CMV IE, SV40 IE, RSV LTR, RIP, modified RIP, POMC and GH.

76. The method of claim 68, wherein the amylin-to-insulin content of said selected cell is between about 0.002 to about 10.0.

77. A method of regulating blood glucose levels in a mammal comprising:

a) providing a starting cell;

b) introducing into said starting cell an amylin-encoding gene operatively linked to a first promoter;

c) selecting a cell of step b that exhibit increased amylin secretion as compared to said starting cell; and

d) administering said selected cell to said mammal,

whereby said secreted amylin regulates blood glucose levels of said mammal.

78. The method of claim 76, further comprising the step of providing said cell with an insulin-encoding gene operatively linked to a second promoter.

79. A method for modulating the circulating levels of insulin in a mammal comprising the steps of:

a) providing a starting cell;

b) introducing into said cell an amylin-encoding gene operatively linked to a promoter;

c) selecting a cell of step b that exhibits increased amylin secretion as compared to said starting cell; and

d) administering said selected cell to said mammal,

whereby said secreted amylin modulates glucose-stimulated insulin secretion in said mammal.

80. A method for decreasing glycogen synthesis in a mammal comprising the steps of:

a) providing a starting cell;

b) introducing into said cell with an amylin-encoding gene operatively linked to a first promoter;

c) selecting a cell of step b that exhibits increased amylin secretion as compared to said starting cell; and

d) administering said selected cell to said mammal,

whereby said secreted amylin reduces glycogen synthesis in said mammal.

81. The method of claim 80, wherein said secretory cell further is transfected with an insulin-encoding gene operatively linked to a promoter.

82. A method of screening for an amylin receptor comprising:

a) obtaining amylin from a recombinant amylin expressing secretory cell;

b) admixing said amylin with a composition comprising a putative amylin receptor; and

c) detecting an amylin receptor bound to said amylin.

83. The method of claim 82, wherein said composition comprising a putative amylin receptor is a composition comprising a population of recombinant cells transfected with portions of a DNA library.

84. The method of claim 82, further comprising obtaining a DNA segment from said DNA library that expresses an amylin receptor.

85. An amylin receptor gene, prepared by the process of:

a) obtaining amylin from a recombinant amylin expressing secretory cell;

b) admixing said amylin with a composition comprising a putative amylin receptor said composition comprising a population of recombinant cells transfected with portions of a DNA library; and

c) obtaining a DNA segment from said DNA library that expresses an amylin receptor.

86. An amylin receptor-like gene, wherein at least a portion of said gene hybridizes to at least a portion of the amylin receptor gene of claim 84 under low stringency hybridization conditions.

87. A purified amylin composition comprising an amidated and glycosylated amylin polypeptide.

88. A method for the production of a polypeptide comprising the steps of:

(a) providing a secretory host cell;

(b) blocking the production of an endogenous, secreted polypeptide;

(c) contacting with said host cell an exogenous polynucleotide comprising a gene encoding an exogenous polypeptide, wherein said gene is under the control of a promoter active in eukaryotic cells; and

(d) culturing said secretory host cell under conditions such that said exogenous polynucleotide expresses said exogenous polypeptide.

89. The method of claim 88, wherein said promoter is selected from the group consisting of CMV, SV40 IE, RSV LTR, GAPHD and RIP1.

90. The method of claim 88, wherein said exogenous polynucleotide further comprises an adenovirus tripartite 5′ leader sequence and intron.

91. The method of claim 90, wherein said intron comprises the 5′ donor site of the adenovirus major late transcript and the 3′ splice site of an immunoglobulin gene.

92. The method of claim 88, wherein said exogenous polynucleotide further comprises a polyadenylation signal.

93. The method of claim 88, wherein said secretory host cell is a neuroendocrine cell.

94. The method of claim 93, wherein said secretory host cell is an insulinoma cell.

95. The method of claim 94, wherein said insulinoma cell is a rat insulinoma cell.

96. The method of claim 94, wherein said insulinoma cell is a human insulinoma cell.

97. The method of claim 88, wherein said exogenous polypeptide is secreted.

98. The method of claim 97, wherein said exogenous polypeptide is fusion protein.

99. The method of claim 97, wherein said exogenous polypeptide is amidated.

100. The method of claim 99, wherein said amidated polypeptide is selected from the group consisting of calcitonin, calcitonin gene related peptide (CGRP), β-calcitonin gene related peptide, hypercalcemia of malignancy factor (1-34) (PTH-rP), parathyroid hormone-related protein (107-128) (PTH-rP), parathyroid hormone-related protein (107-111) (PTH-rP), cholecystokinin (26-32) (CCK), galanin message associated peptide, preprogalanin (64-105), gastrin I, gastrin releasing peptide, glucagon-like peptide (GLP-1), pancreastatin, pancreatic peptide, peptide YY, PHM, secretin, vasoactive intestinal peptide (VIP), oxytocin, vasopressin (AVP), vasotocin, enkephalins, enkephalinamide, metorphinamide (adrenorphin), alpha melanocyte stimulating hormone (alpha-MSH), atrial natriuretic factor (5-27) (ANF), amylin, amyloid P component (SAP-1), corticotropin releasing hormone (CRH), growth hormone

releasing factor (GHRH), luteinizing hormone-releasing hormone (LHRH), neuropeptide Y, substance K (neurokinin A), substance P and thyrotropin releasing hormone (TRH).

101. The method of claim 97, wherein said exogenous polypeptide is a hormone.

102. The method of claim 101, wherein said hormone is selected from the group consisting of growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, thyroid-stimulating hormone, leptin, adrenocorticotropin (ACTH), angiotensin I, angiotensin II, β-endorphin, β-melanocyte stimulating hormone (β-MSH), cholecystokinin, endothelin I, galanin, gastric inhibitory peptide (GIP), glucagon, insulin, lipotropins, neurophysins and somatostatin.

103. The method of claim 97, wherein said exogenous polypeptide is a growth factor.

104. The method of claim 103, wherein said growth factor is selected from the group consisting of epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor and insulin-like growth factor 1.

105. The method of claim 88, wherein said endogenous, secreted polypeptide and said exogenous polypeptide are the same.

106. The method of claim 105, wherein said endogenous, secreted polypeptide and said exogenous polypeptide are insulin.

107. The method of claim 88, wherein said exogenous polypeptide enhances the production and/or secretion of at least one polypeptide produced by said cell.

108. The method of claim 107, wherein said exogenous polypeptides is selected from the group consisting of a protein processing enzyme, a receptor and a transcription factor.

109. The method of claim 107, wherein said exogenous polypeptide is selected from the group consisting of hexokinase, glucokinase, GLUT-2, GLP-1, IPF1, PC2, PC3, PAM, glucagon-like peptide I receptor, glucose-dependent insulinotropic polypeptide receptor, BIR, SUR, GHRFR and GHRHR.

110. The method of claim 88, wherein said step (c) further comprises contacting said secretory host cell with a polynucleotide comprising a gene for a selectable marker and step (d) further comprises culturing under drug selection.

111. The method of claim 110, wherein said selectable marker gene is flanked by *Lox*P sites.

112. The method of claim 111, further comprising

(e) contacting the secretory host cell with a polynucleotide encoding the *Cre* protein, wherein said polynucleotide is under the control of a promoter active in eukaryotic cells; and

(f) replicate culturing said cell with and without drug selection.

113. The method of claim 111, wherein said selectable marker is hygromycin resistance and said drug is hygromycin.

114. The method of claim 111, wherein said selectable marker is neomycin and said drug is G418.

115. The method of claim 111, wherein said selectable marker is GLUT-2 and said drug is streptozotocin.

116. The method of claim 111, wherein the genes for said exogenous polypeptide and said selectable marker are part of the same polynucleotide.

117. The method of claim 116, wherein the genes for said exogenous polypeptide and said selectable marker are separated on the same polynucleotide by an internal ribosome entry site.

118. The method of claim 88, wherein said secretory host cell is glucose-responsive.

119. The method of claim 88, wherein said secretory host cell is not glucose-responsive.

120. A secretory host cell comprising an exogenous polynucleotide comprising a gene encoding a first exogenous polypeptide, wherein said secretory host cell is blocked in the expression of at least one endogenous, secreted polypeptide.

121. The secretory host cell of claim 120, wherein said exogenous polynucleotide is inserted into the coding or regulatory region of said endogenous, secreted polypeptide.

122. The secretory host cell of claim 120, wherein said exogenous polynucleotide further comprises a promoter active in eukaryotic cells.

123. A method of preventing type I diabetes comprising the steps of:

(a) identifying a subject at risk of type I diabetes; and

(b) providing to said subject a polynucleotide comprising a gene for a disulfide mutant of human insulin, wherein said mutant gene is under the control of a promoter active in eukaryotic cells.

124. The method of claim 123, wherein said promoter is selected from the group consisting of CMV, SV34 IE, RSV LTR, GAPHD and RIP1.

125. The method of claim 123, wherein said providing comprises introducing said polynucleotide to a cell of said subject *in vivo*.

126. The method of claim 123, wherein said providing comprises contacting with a secretory host cell *ex vivo* and administering said secretory host cell to said subject.

127. A method for treating a subject afflicted with diabetes comprising the steps of:

(a) identifying a subject afflicted with diabetes; and

(b) providing to said subject a secretory host cell, wherein (i) the production of an endogenous, secreted polypeptide has been blocked and (ii) wherein the secretory host cell comprises an exogenous polynucleotide comprising a gene encoding insulin, wherein said gene is under the control of a promoter active in eukaryotic cells.

128. A method for providing a polypeptide to an animal comprising the step of providing to an animal a secretory host cell, wherein (i) the production of an endogenous, secreted polypeptide in said secretory host cell has been blocked and (ii) wherein the secretory host cell comprises an exogenous polynucleotide comprising a gene encoding said polypeptide wherein said gene is under the control of a promoter active in eukaryotic cells.

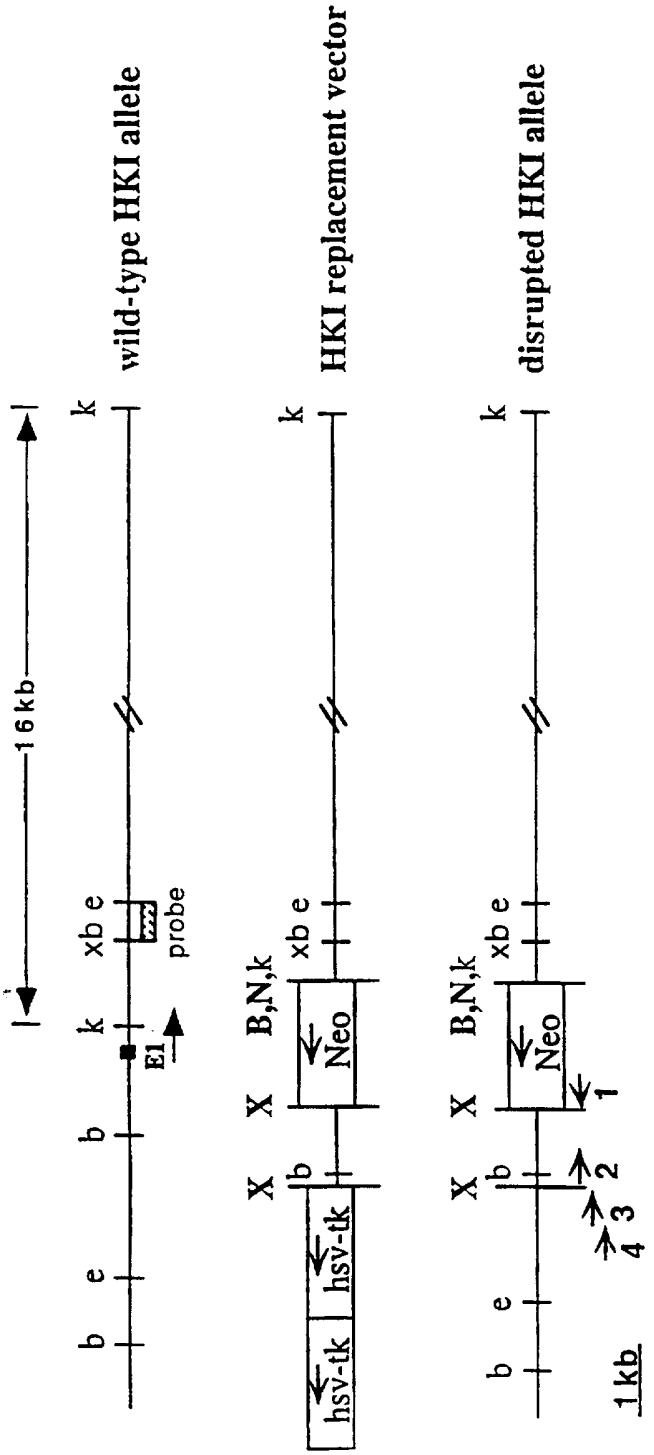

wild-type HKI allele
HKI replacement vector
disrupted HKI allele
16 kb
1 kb
probe
E1
k
xb
e
b
B,N,k
X
Neo
hsv-tk
1
2
3
4

FIG. 1

Genomic Southern Confirming Hexokinase I Gene Disruption

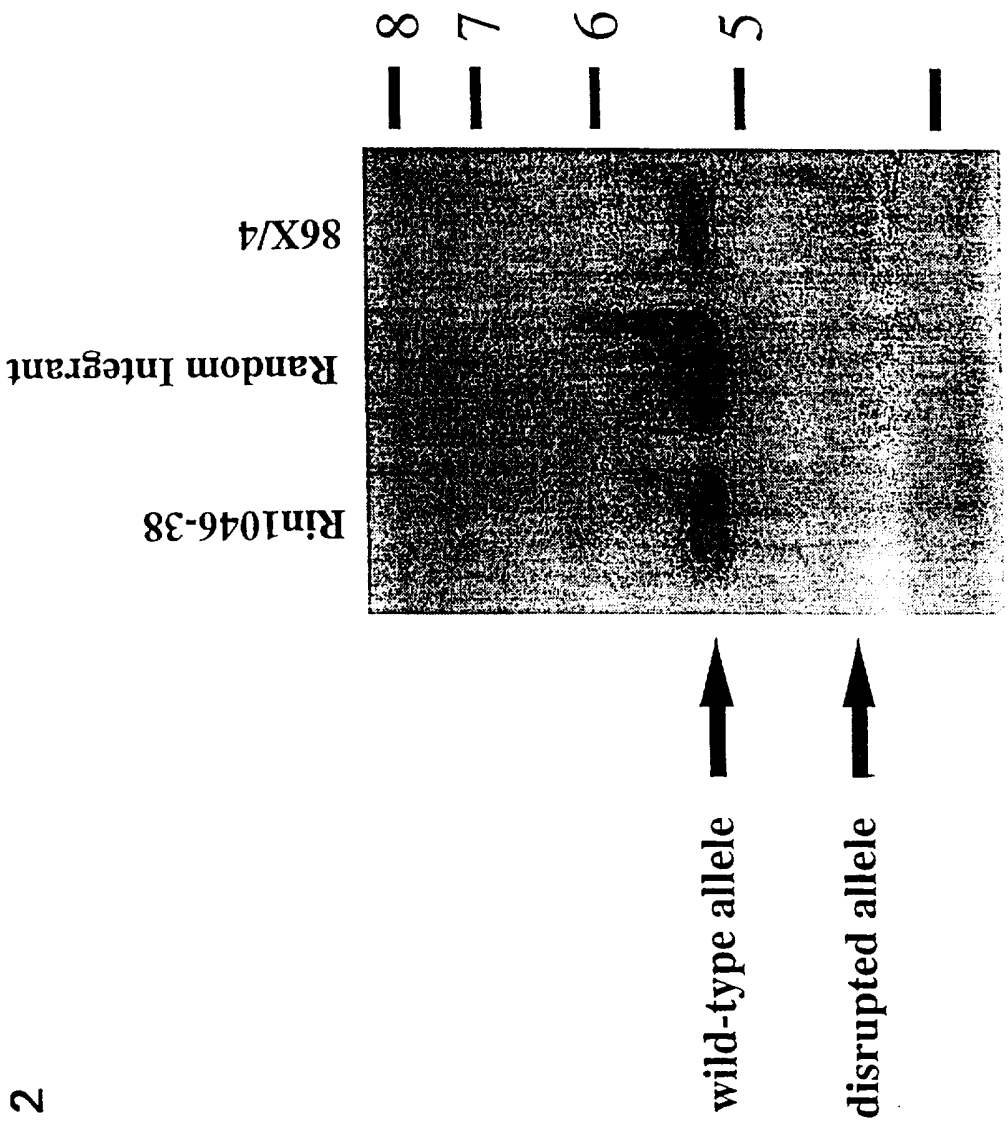


FIG. 2

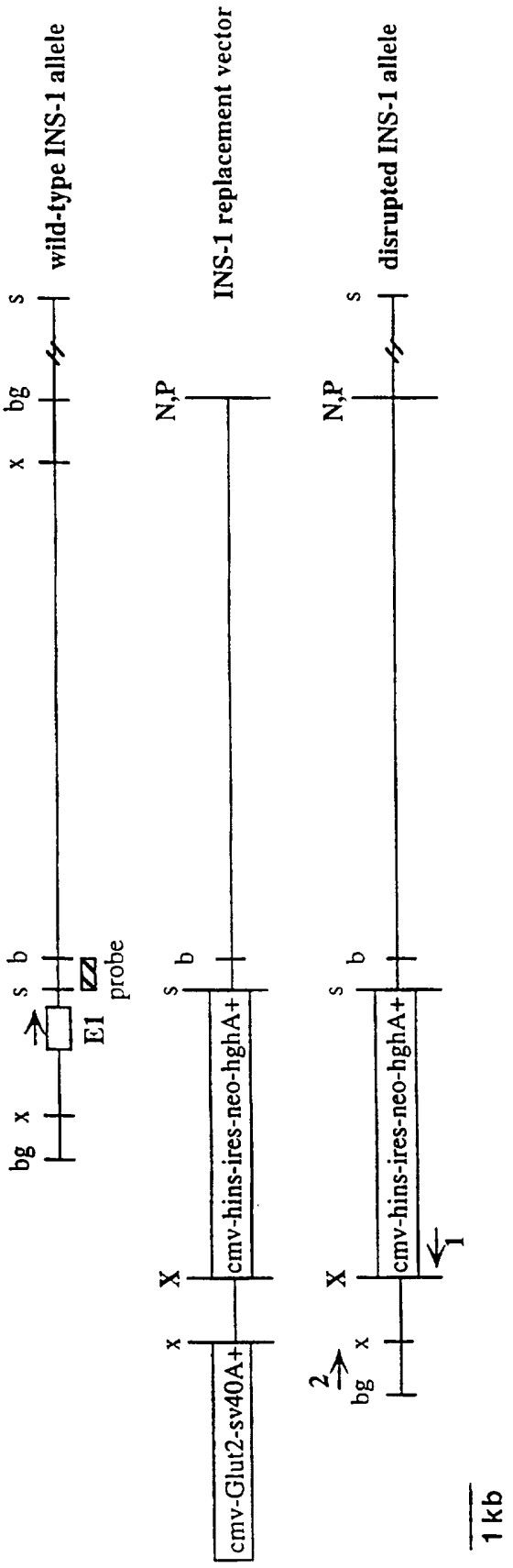

wild-type INS-1 allele
INS-1 replacement vector
disrupted INS-1 allele
s
bg
x
N,P
s
b
E1
probe
bg x
cmv-hins-ires-neo-hghA+
X
x
cmv-Glut2-sv40A+
2
1
1 kb

FIG. 3

Insulin Content of Cell Lines Engineered to Express Human Insulin

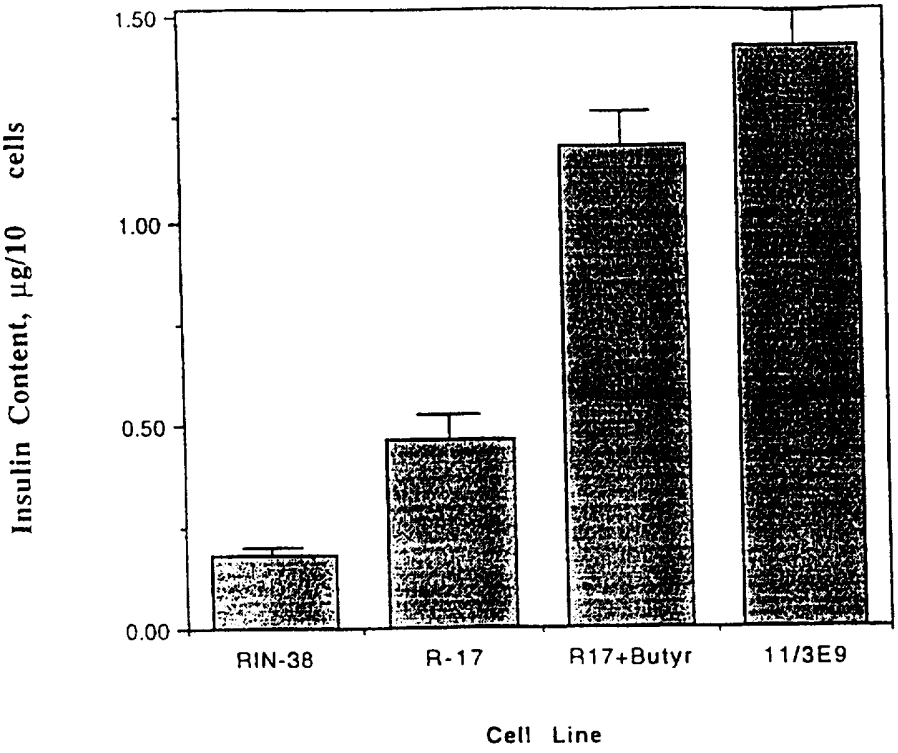


FIG. 4A

Stimulated Insulin Secretion from Cell Lines Engineered to Produce Human Insulin

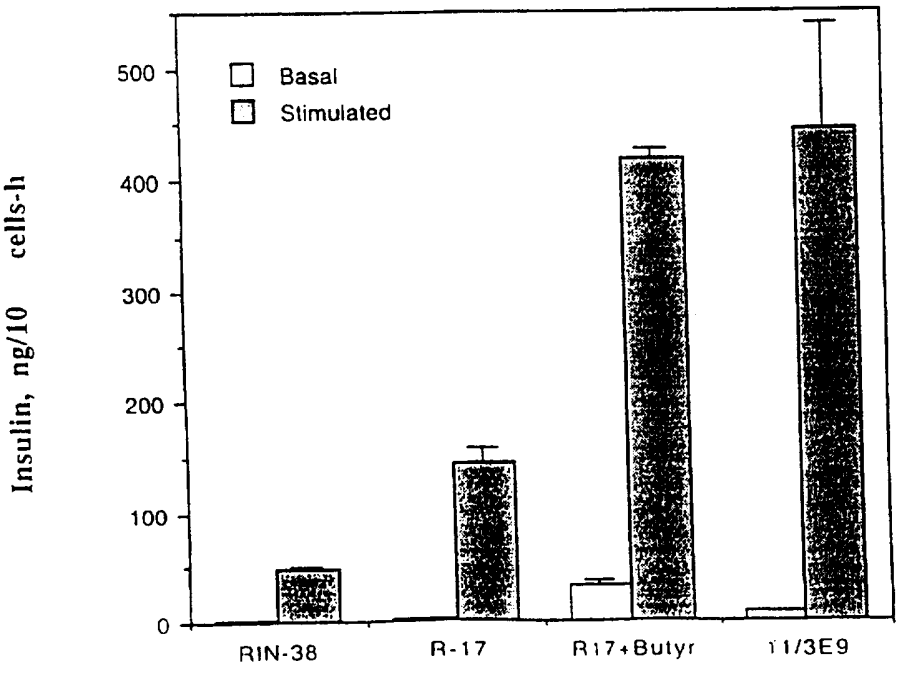


FIG. 4B

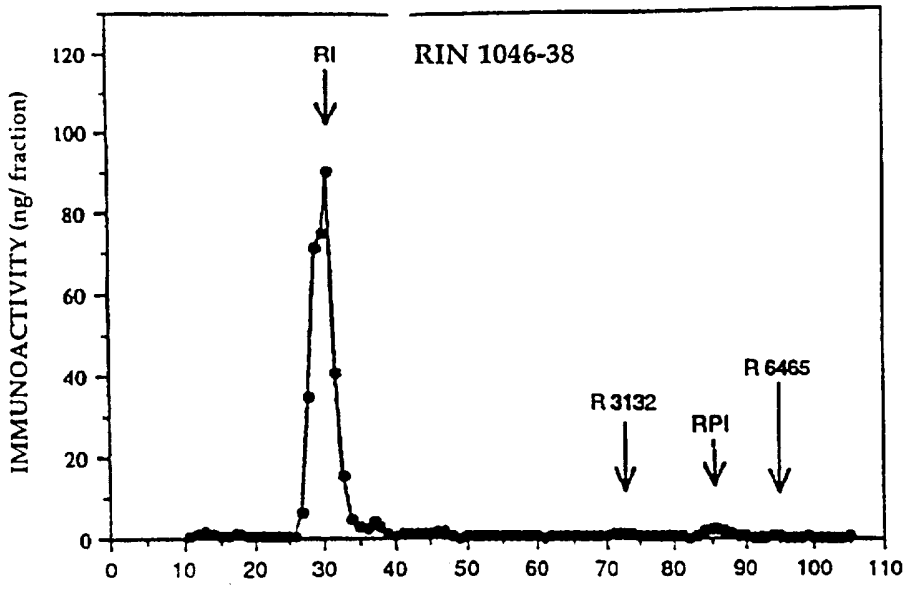


FIG. 5A

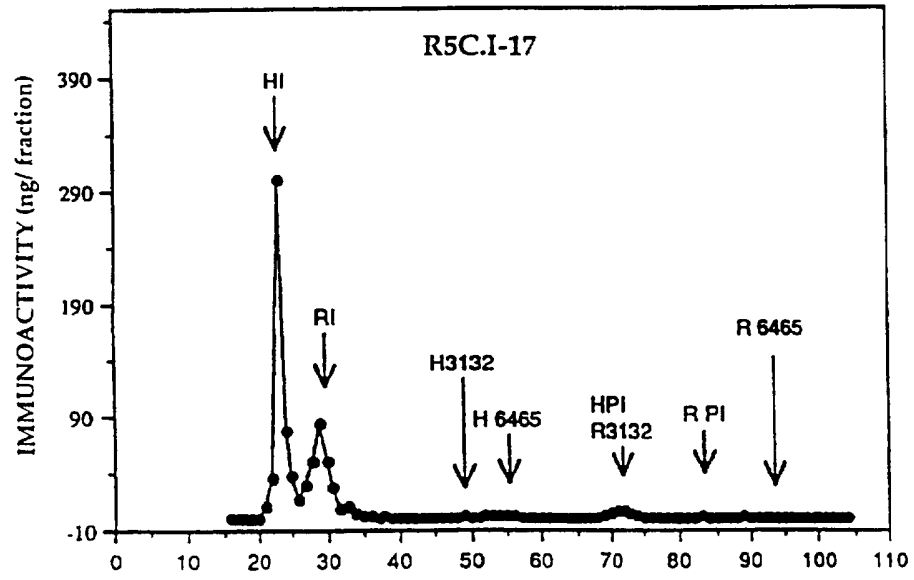


FIG. 5B

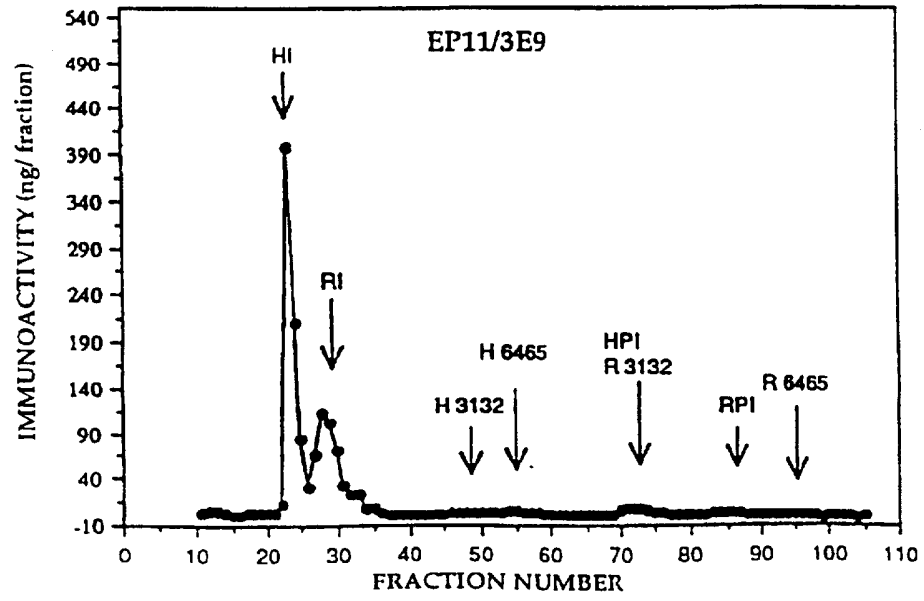
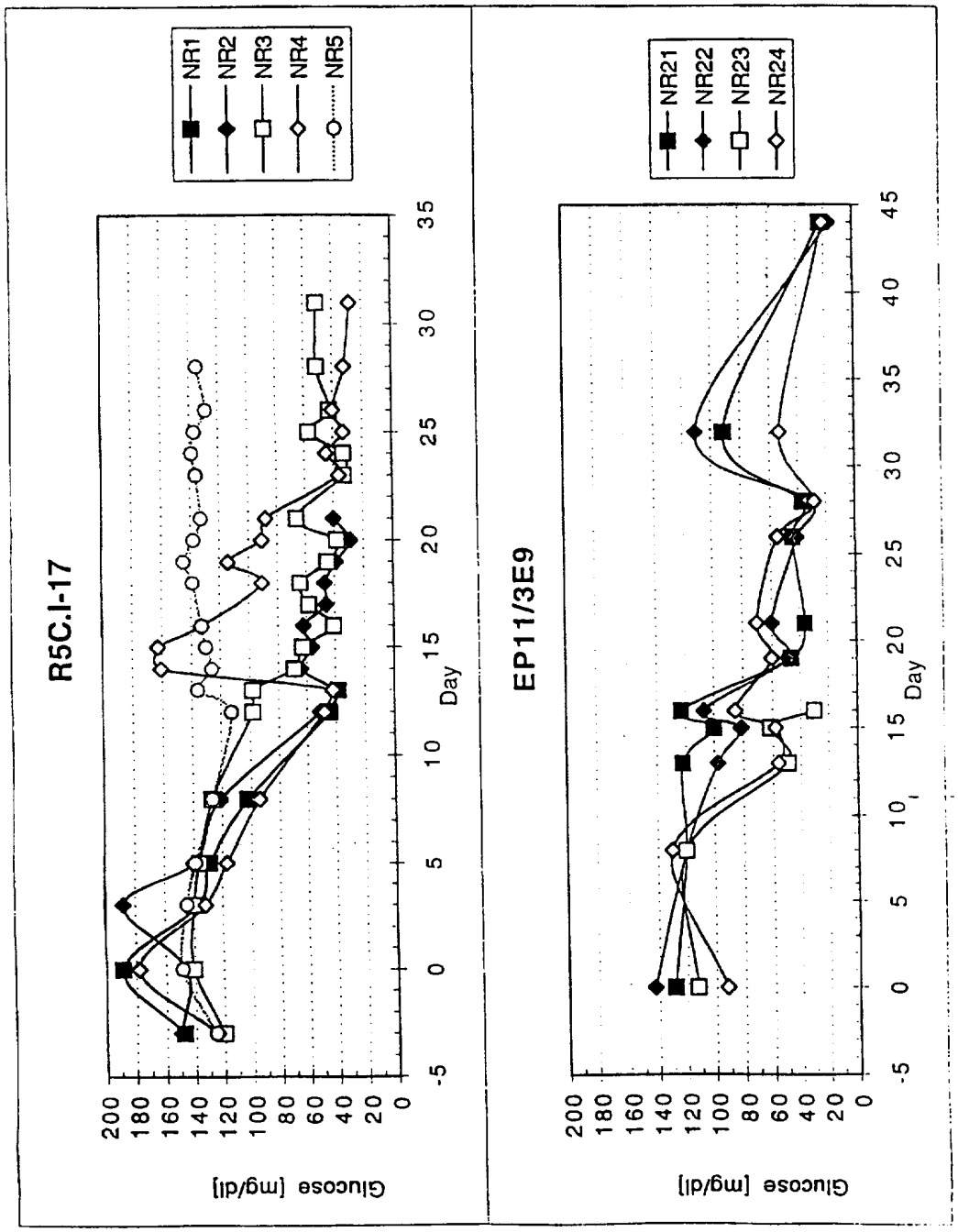


FIG. 5C

FIG. 6A

FIG. 6B


R5C.I-17
NR1
NR2
NR3
NR4
NR5
Glucose [mg/dl]
Day
EP11/3E9
NR21
NR22
NR23
NR24
Glucose [mg/dl]
Day

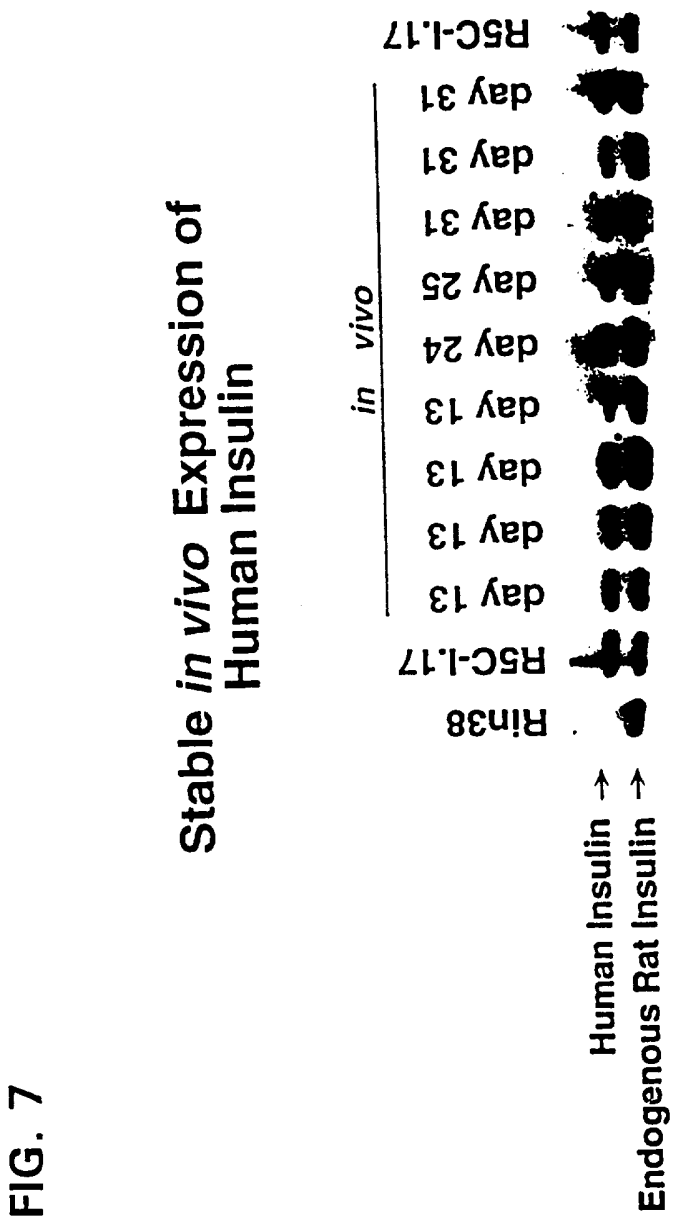

Stable in vivo Expression of Human Insulin
in vivo
Rin38
R5C-I.17
day 13
day 13
day 13
day 13
day 24
day 25
day 31
day 31
day 31
R5C-I.17
Human Insulin
Endogenous Rat Insulin

FIG. 7

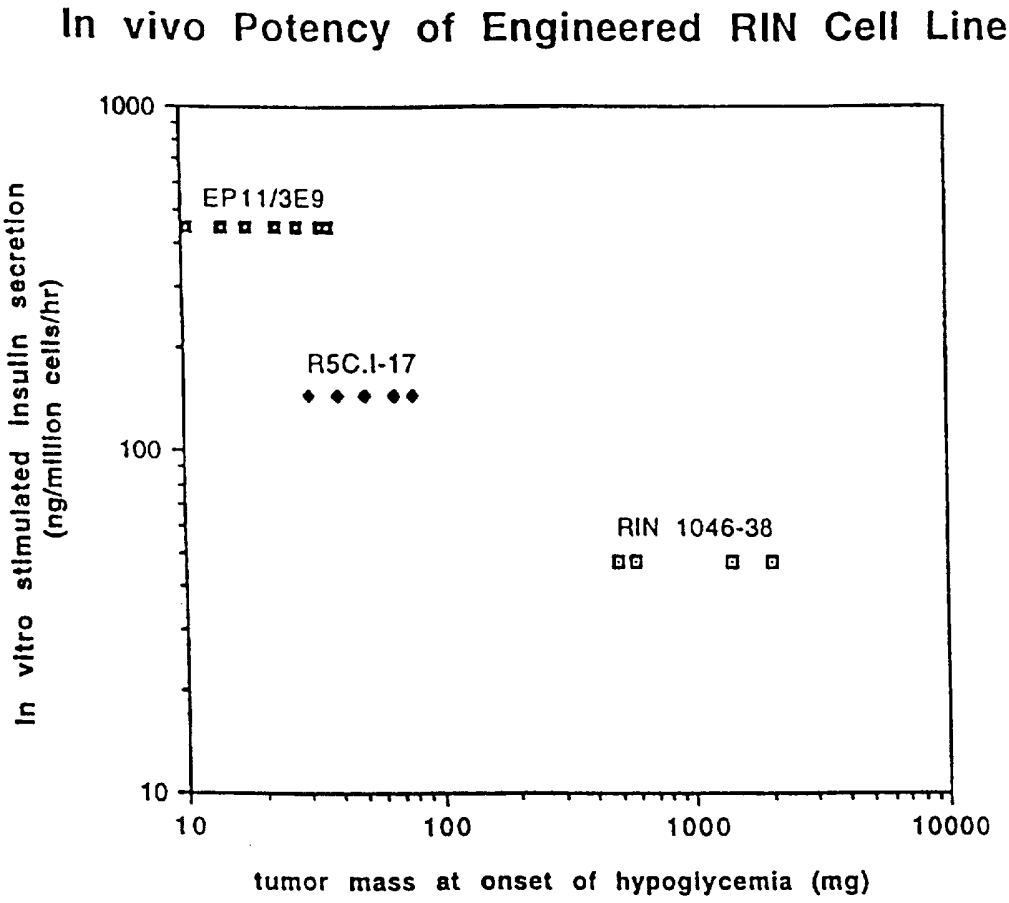

In vivo Potency of Engineered RIN Cell Lines
1000
100
10
EP11/3E9
R5C.I-17
RIN 1046-38
In vitro stimulated insulin secretion (ng/million cells/hr)
10
100
1000
10000
tumor mass at onset of hypoglycemia (mg)

FIG. 8

FIG. 9

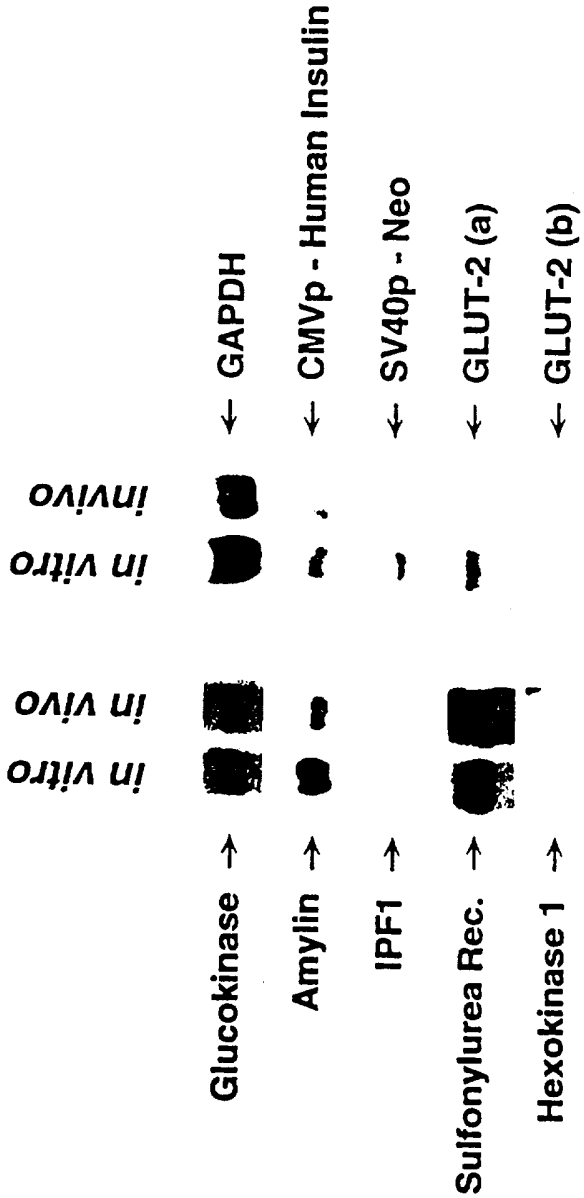

Gene Expression In vitro versus in vivo
in vivo
in vitro
in vivo
in vitro
Glucokinase
Amylin
IPF1
Sulfonylurea Rec.
Hexokinase 1
GAPDH
CMVp - Human Insulin
SV40p - Neo
GLUT-2 (a)
GLUT-2 (b)

FIG. 10A

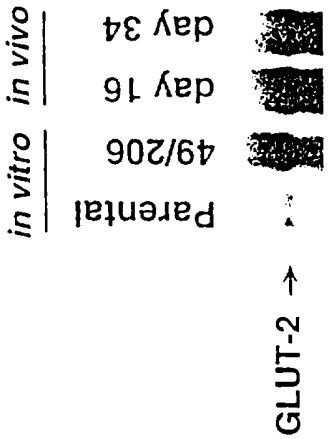
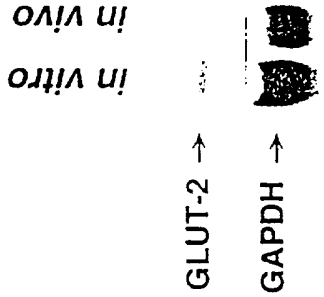


FIG. 10B



Increased Insulin Content using IRES Containing Vectors

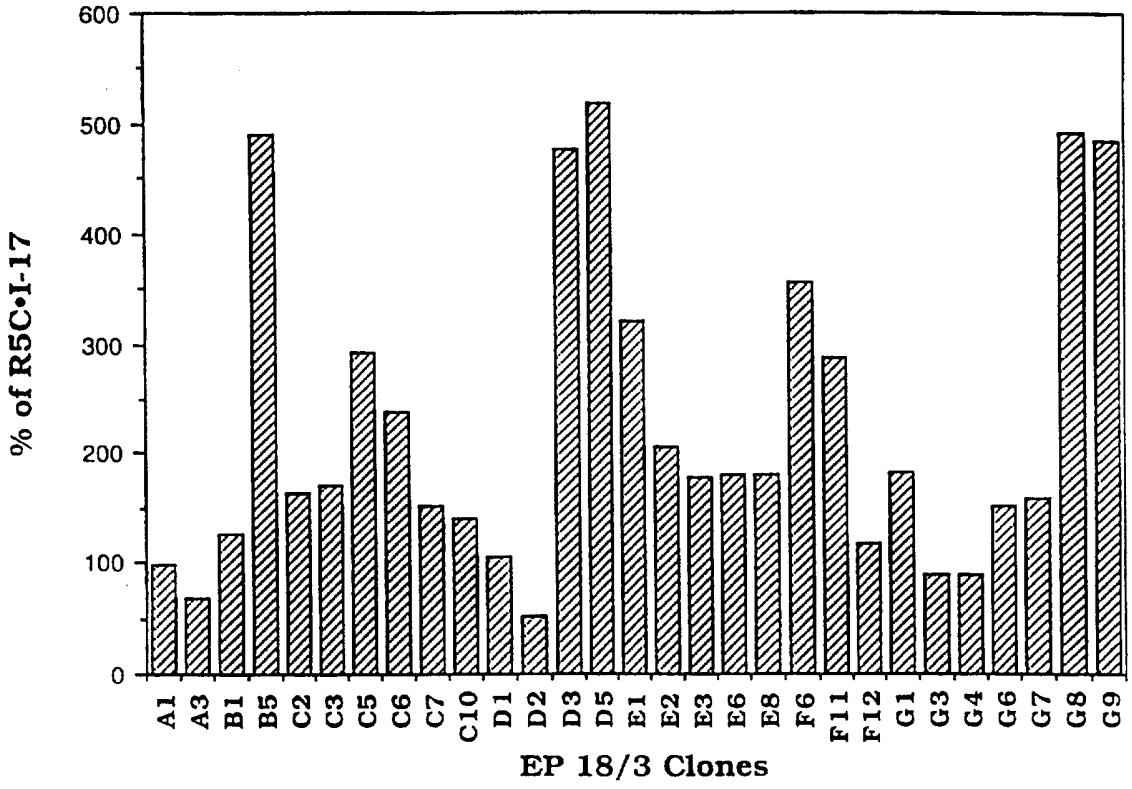


FIG. 11

12

FIG. 12A

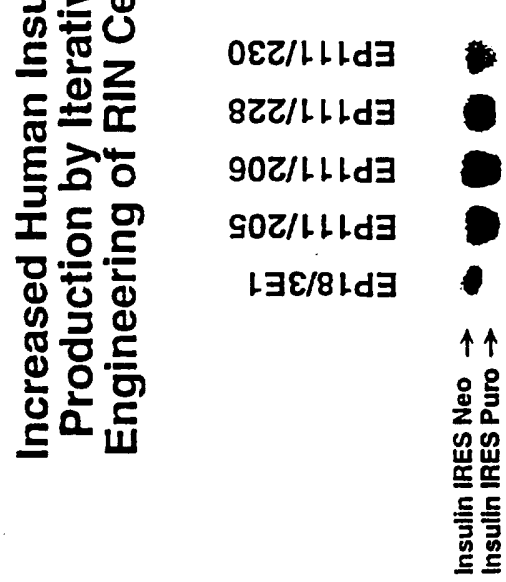


FIG. 12B

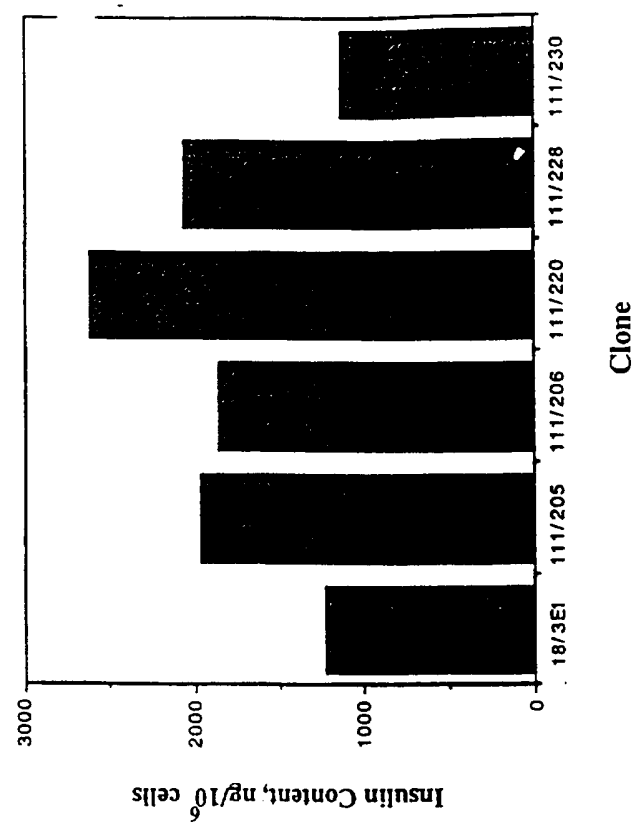
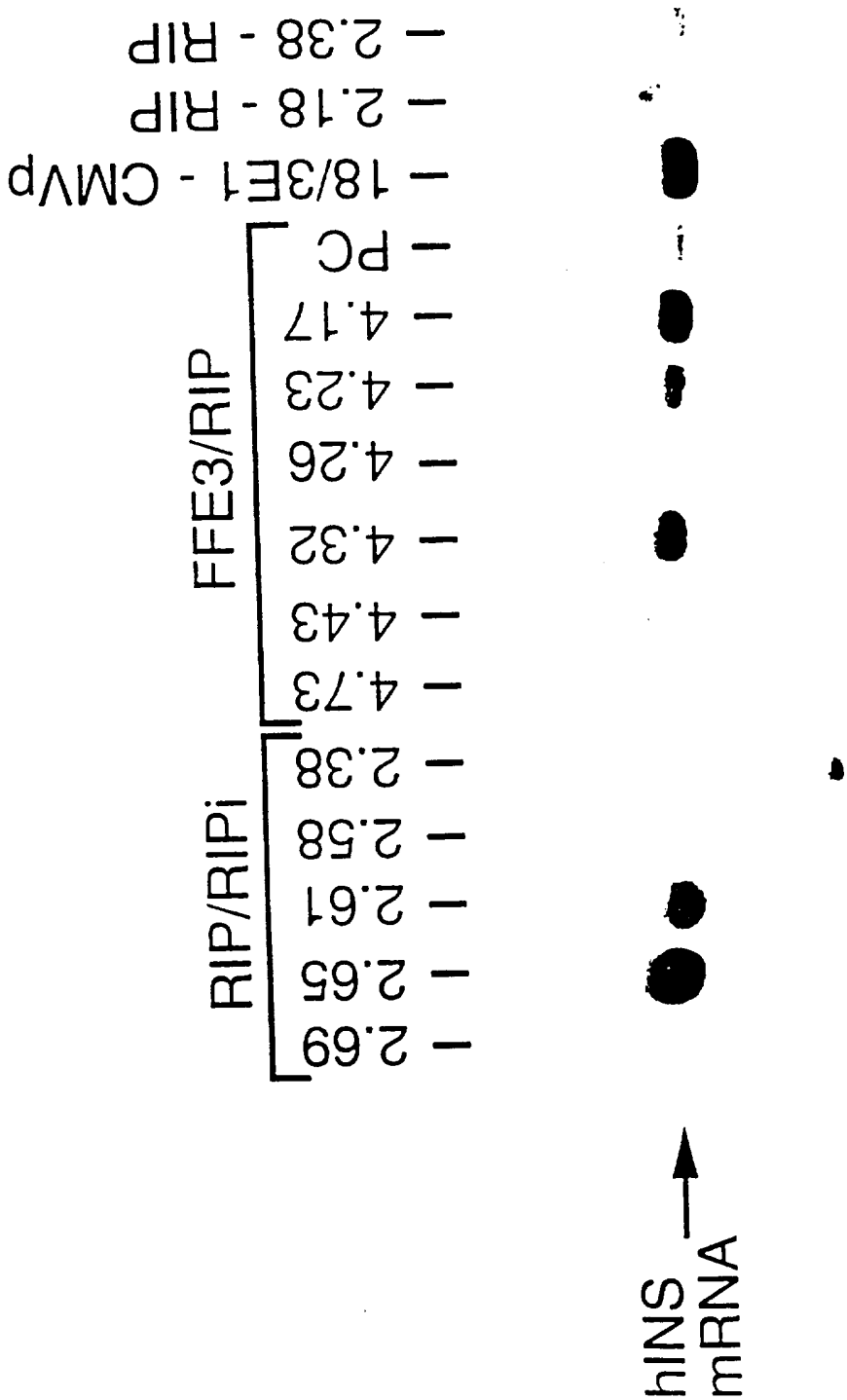


FIG. 13

Comparison of CMV, RIP, FFE3/RIP, and RIP/RIPi Promoter Activity in Stable RIN Lines



hGH Secretion from Engineered RIN Cells

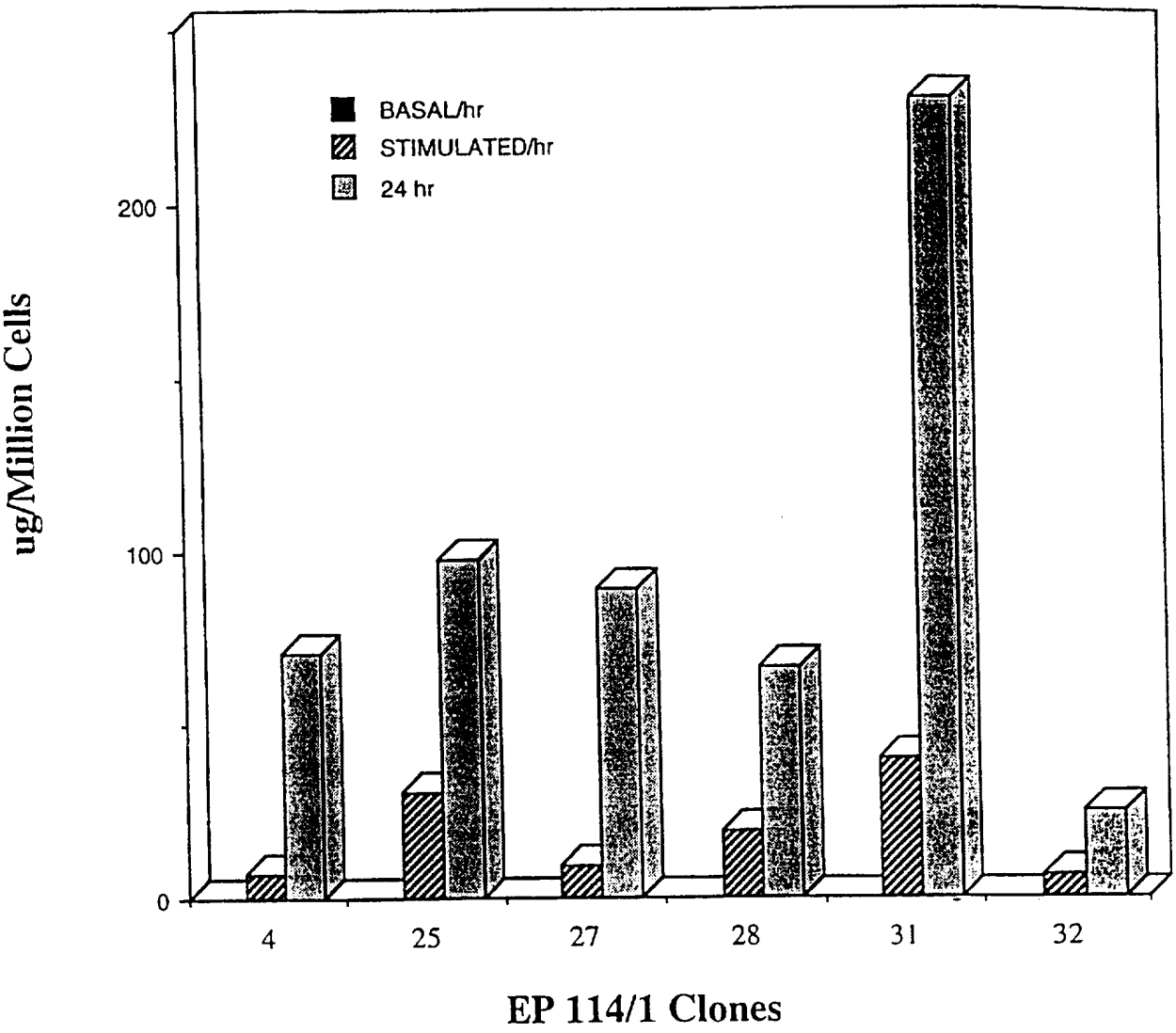


FIG. 14

FIG. 15A

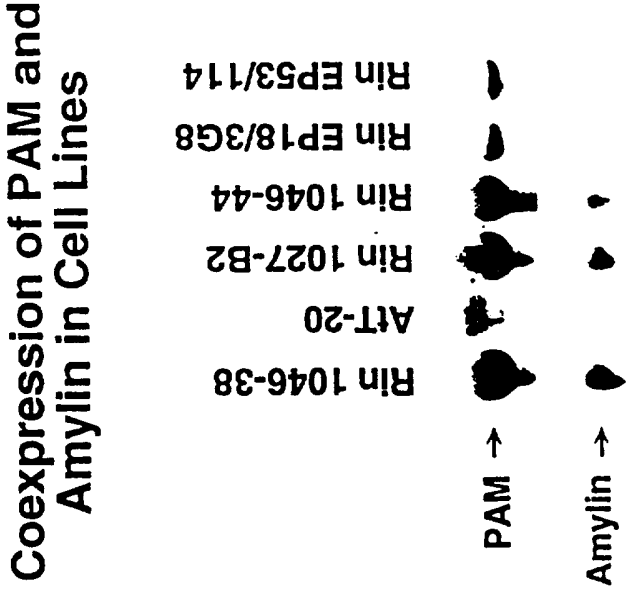


FIG. 15B

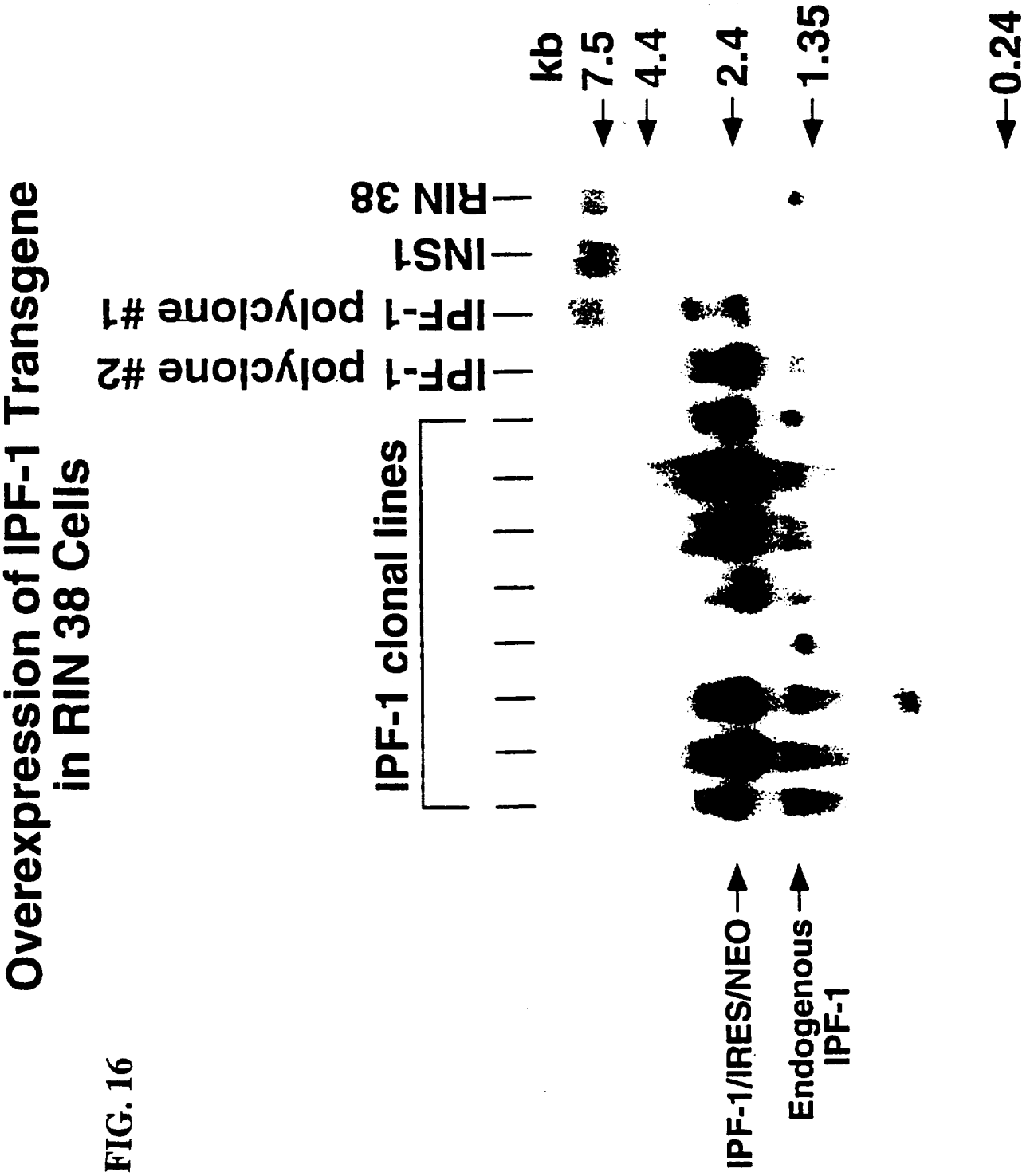

Overexpression of IPF-1 Transgene in RIN 38 Cells
kb
7.5
4.4
2.4
1.35
0.24
RIN 38
INS1
IPF-1 polyclone #1
IPF-1 polyclone #2
IPF-1 clonal lines
IPF-1/IRES/NEO
Endogenous IPF-1

FIG. 16

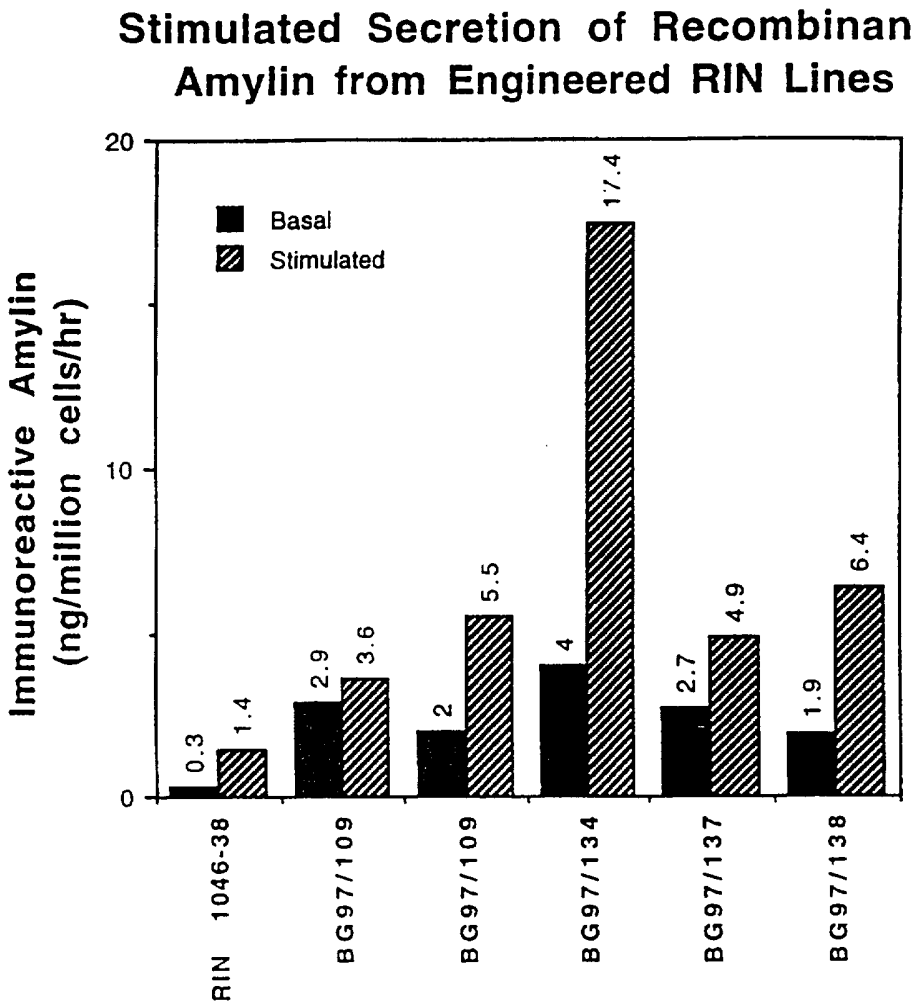

Stimulated Secretion of Recombinant Amylin from Engineered RIN Lines
Immunoreactive Amylin (ng/million cells/hr)
20
10
0
Basal
Stimulated
0.3
1.4
2.9
3.6
2
5.5
4
17.4
2.7
4.9
1.9
6.4
RIN 1046-38
BG97/109
BG97/109
BG97/134
BG97/137
BG97/138

FIG. 17

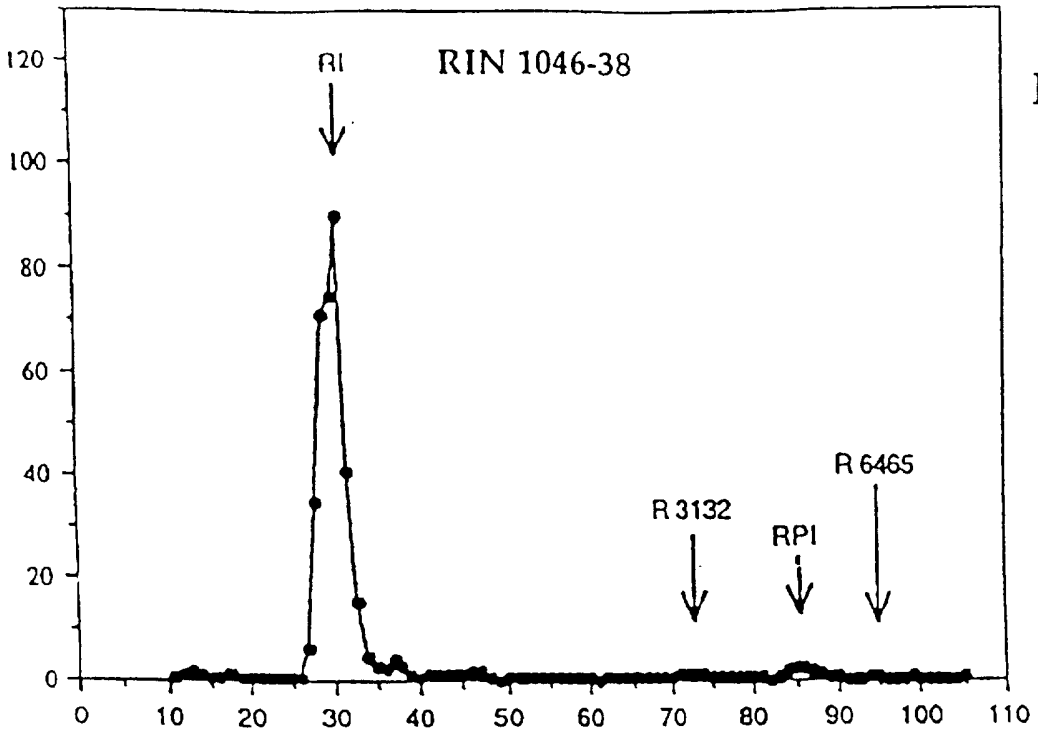


FIG. 18A

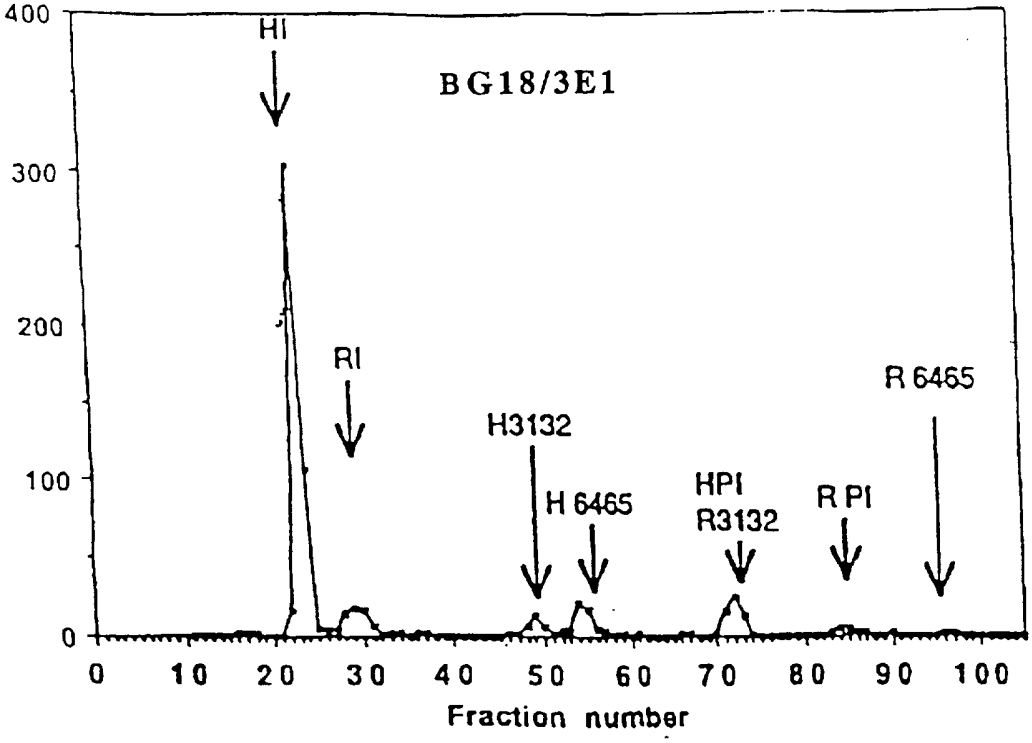


FIG. 18B

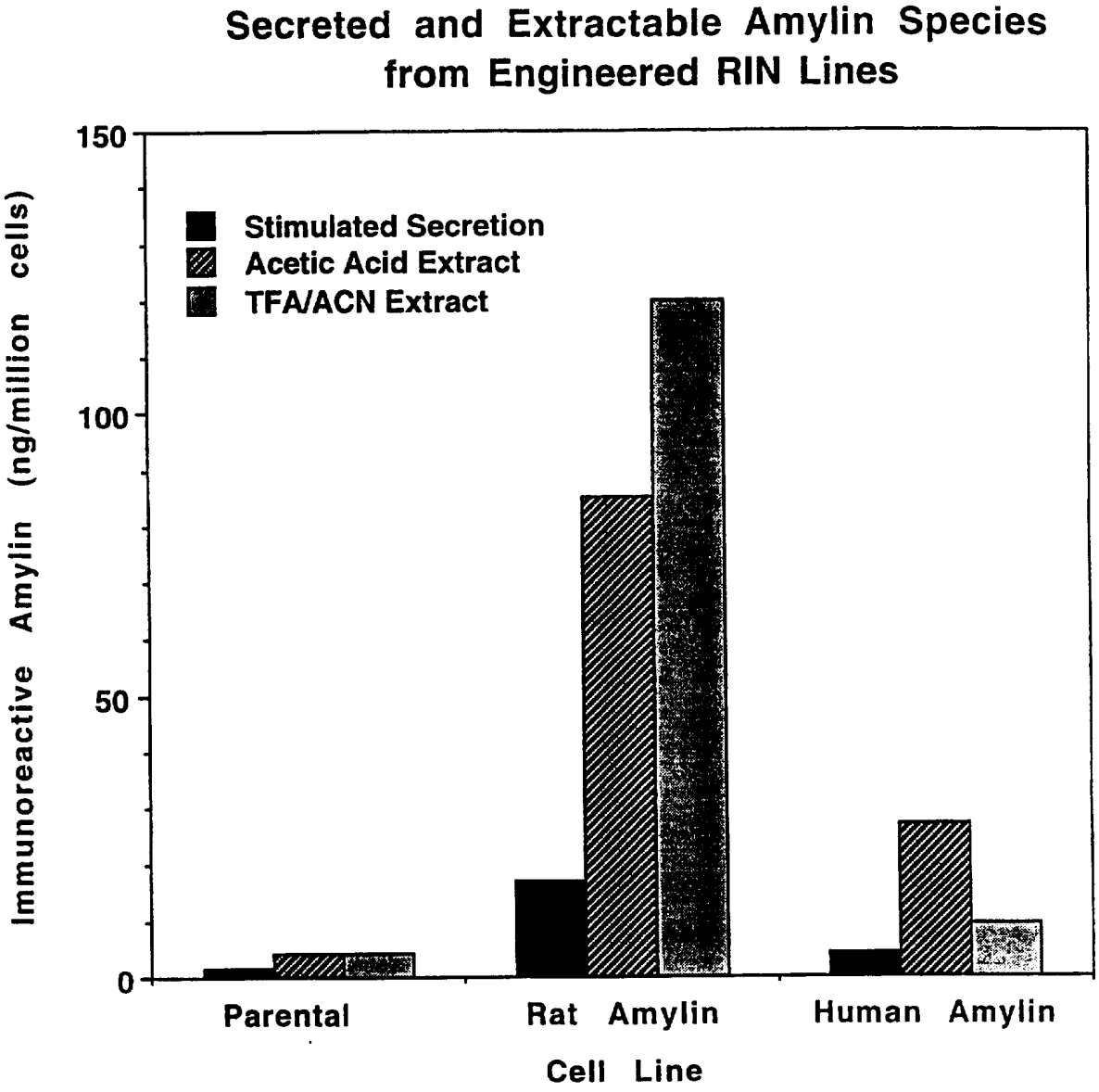

Secreted and Extractable Amylin Species from Engineered RIN Lines
Immunoreactive Amylin (ng/million cells)
150
100
50
0
Stimulated Secretion
Acetic Acid Extract
TFA/ACN Extract
Parental
Rat Amylin
Human Amylin
Cell Line

FIG. 19

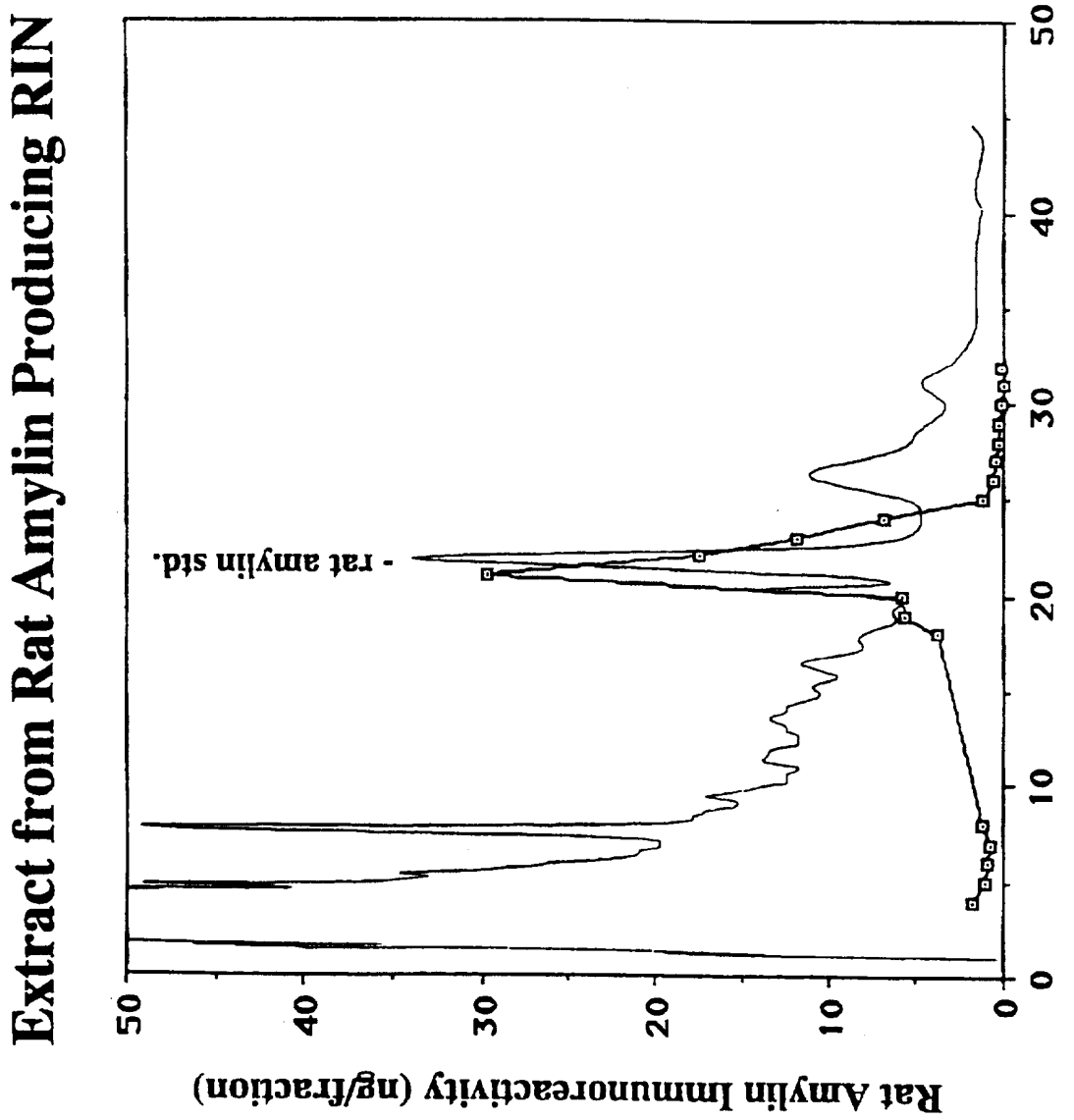

Extract from Rat Amylin Producing RIN
Rat Amylin Immunoreactivity (ng/fraction)
HPLC Fraction
- rat amylin std.
0
10
20
30
40
50

FIG. 20A

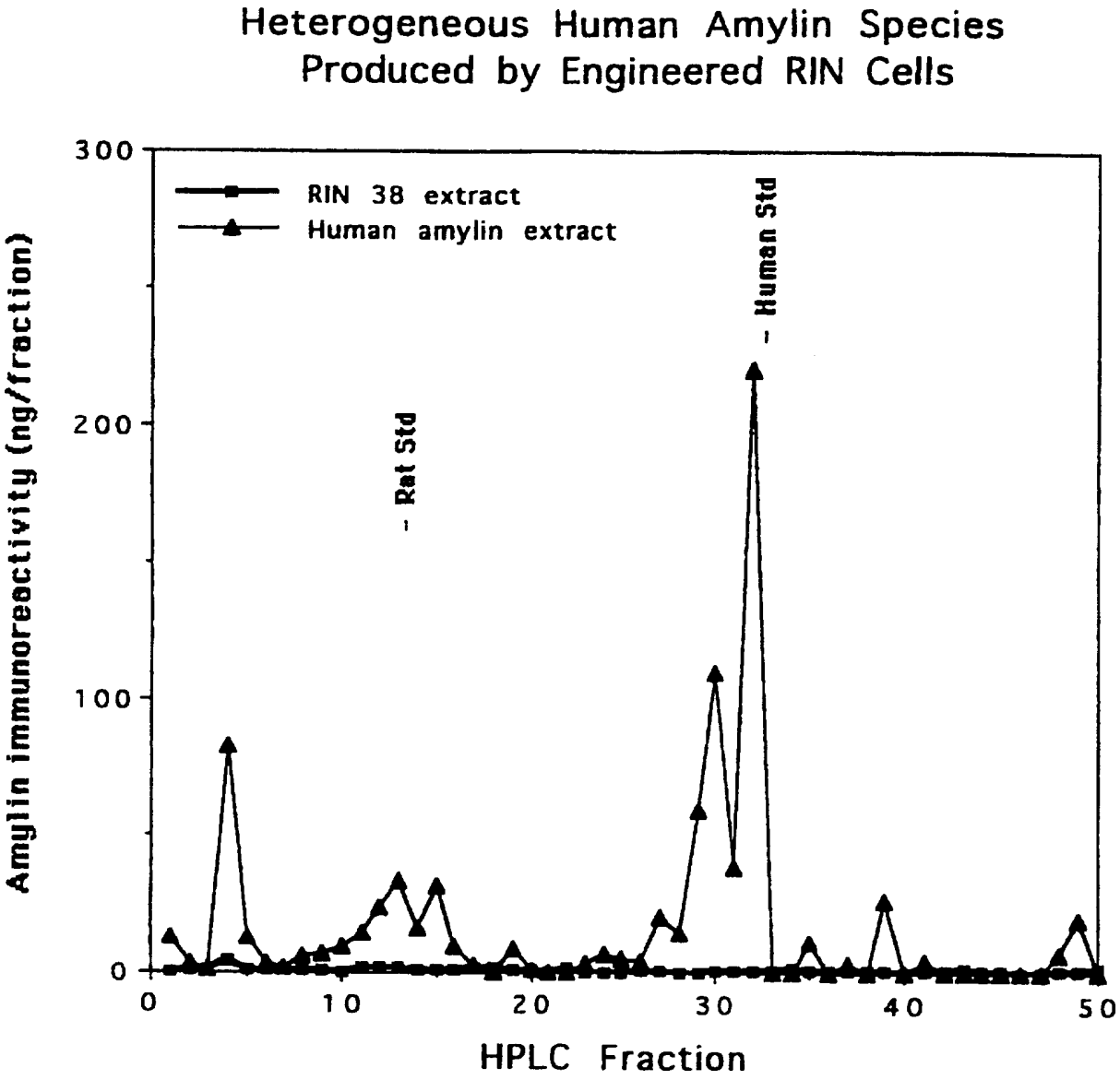

Heterogeneous Human Amylin Species
Produced by Engineered RIN Cells
RIN 38 extract
Human amylin extract
- Rat Std
- Human Std
Amylin immunoreactivity (ng/fraction)
300
200
100
0
0
10
20
30
40
50
HPLC Fraction

FIG. 20B

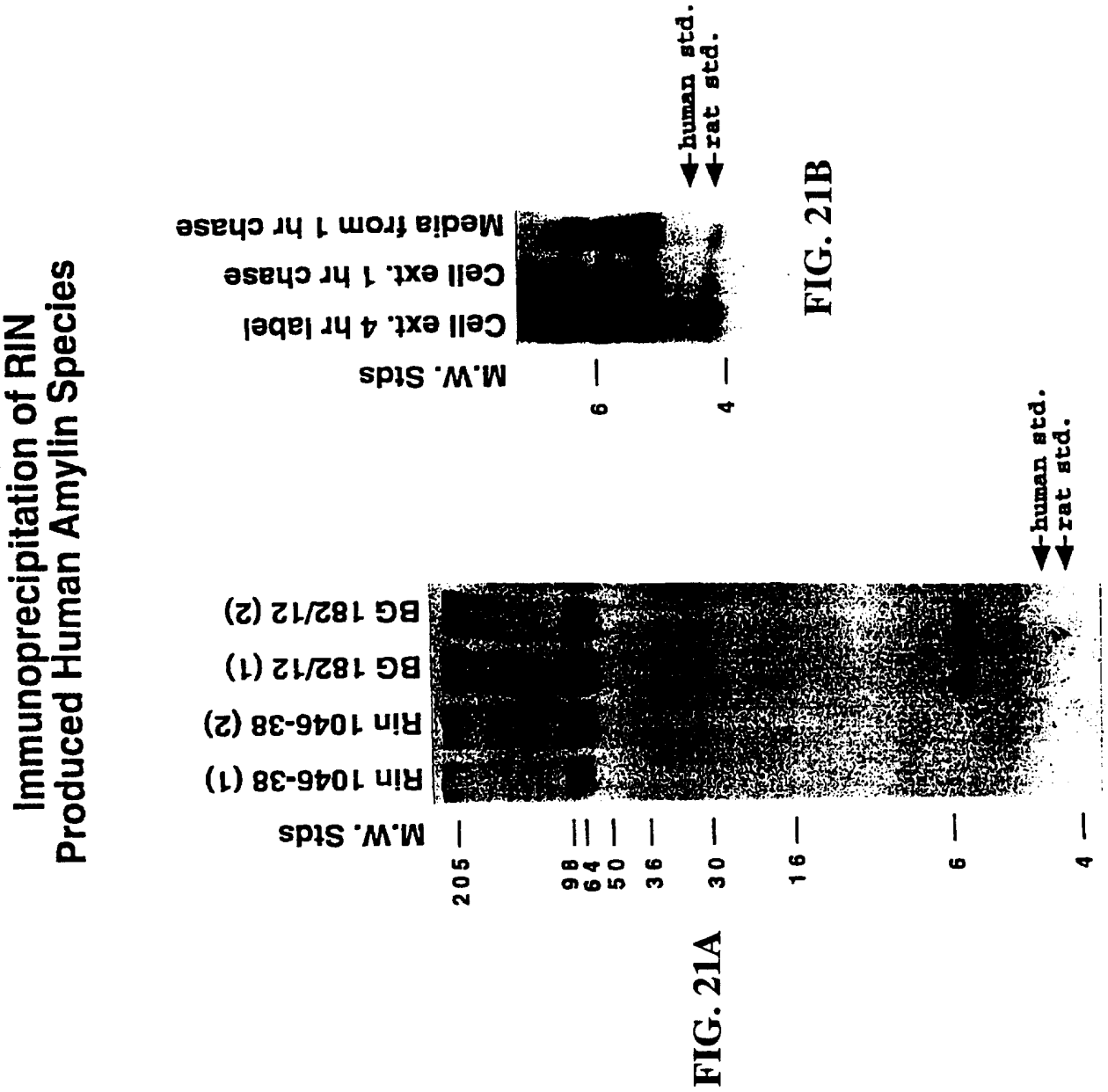
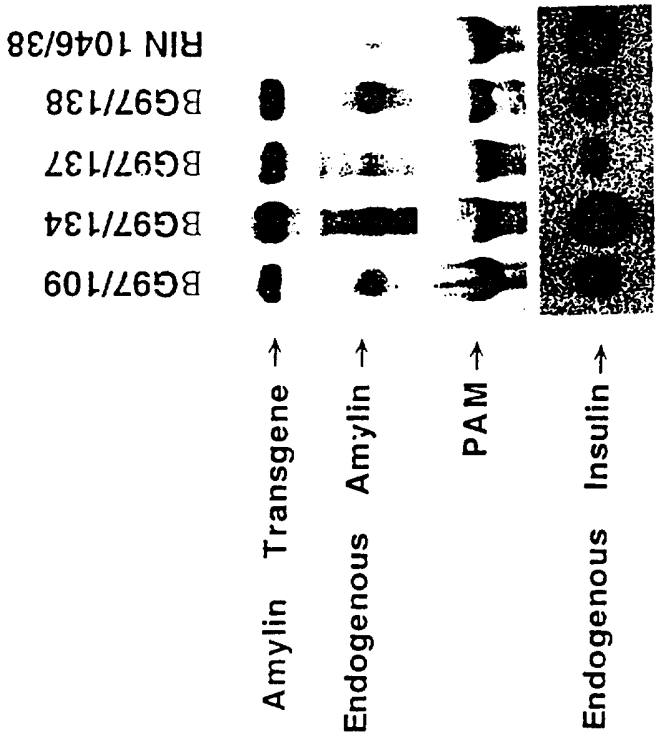

Immunoprecipitation of RIN Produced Human Amylin Species
M.W. Stds
Rin 1046-38 (1)
Rin 1046-38 (2)
BG 182/12 (1)
BG 182/12 (2)
205
98
64
50
36
30
16
6
4
human std.
rat std.
FIG. 21A
M.W. Stds
Cell ext. 4 hr label
Cell ext. 1 hr chase
Media from 1 hr chase
6
4
human std.
rat std.
FIG. 21B

FIG. 22

Coexpression of Amylin, Insulin and PAM in Engineered Cell Lines



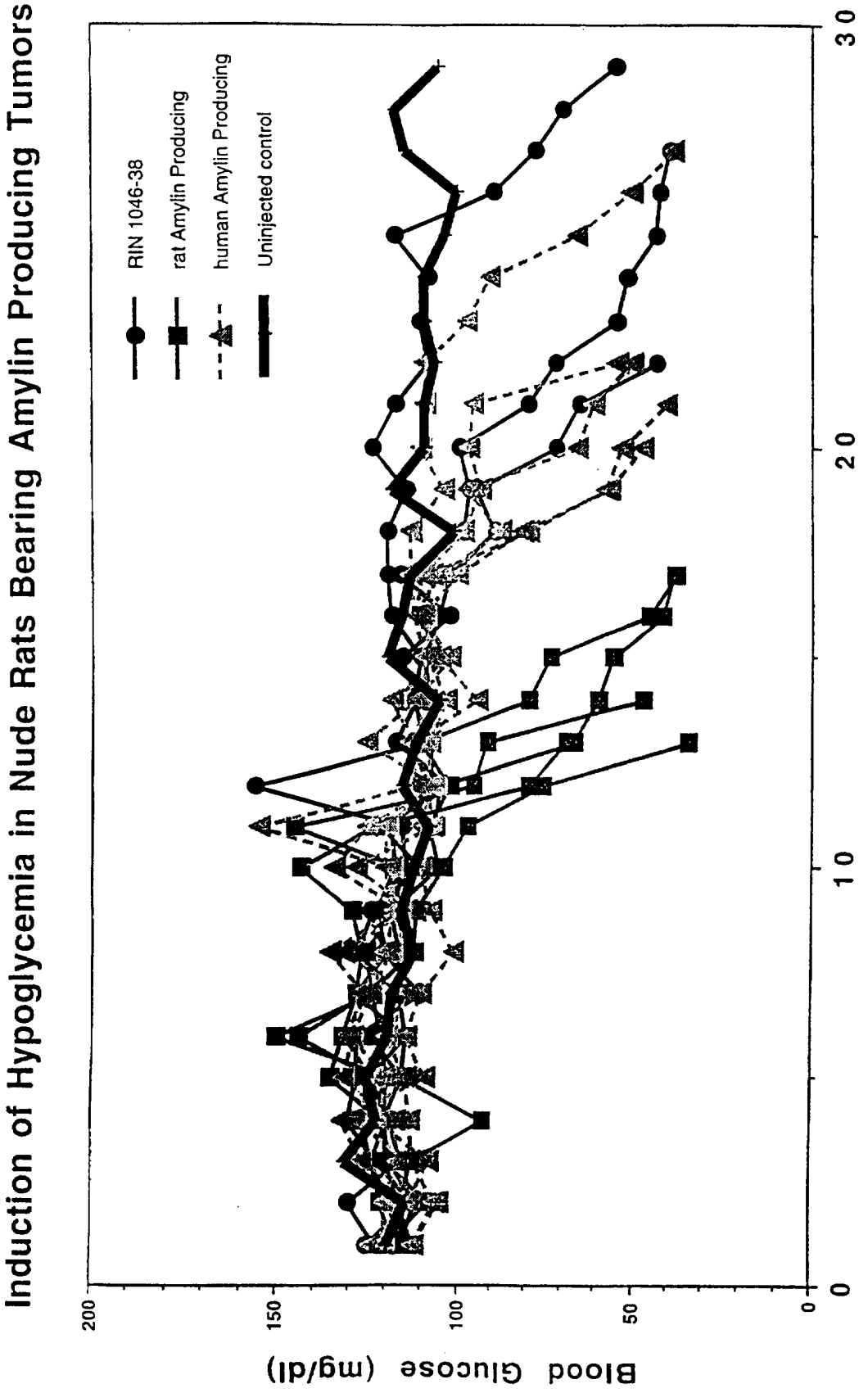

Induction of Hypoglycemia in Nude Rats Bearing Amylin Producing Tumors
RIN 1046-38
rat Amylin Producing
human Amylin Producing
Uninjected control
Blood Glucose (mg/dl)
200
150
100
50
0
0
10
20
30
Days Post Implant

FIG. 23

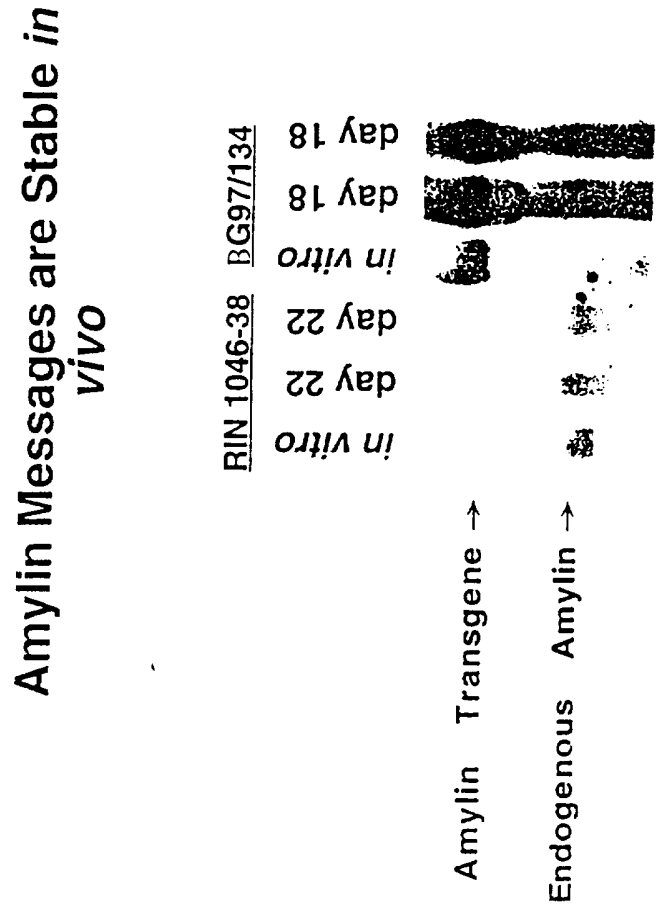

Amylin Messages are Stable in Vivo
RIN 1046-38
BG97/134
in vitro
day 22
day 22
in vitro
day 18
day 18
Endogenous Amylin →
Amylin Transgene →

FIG. 24

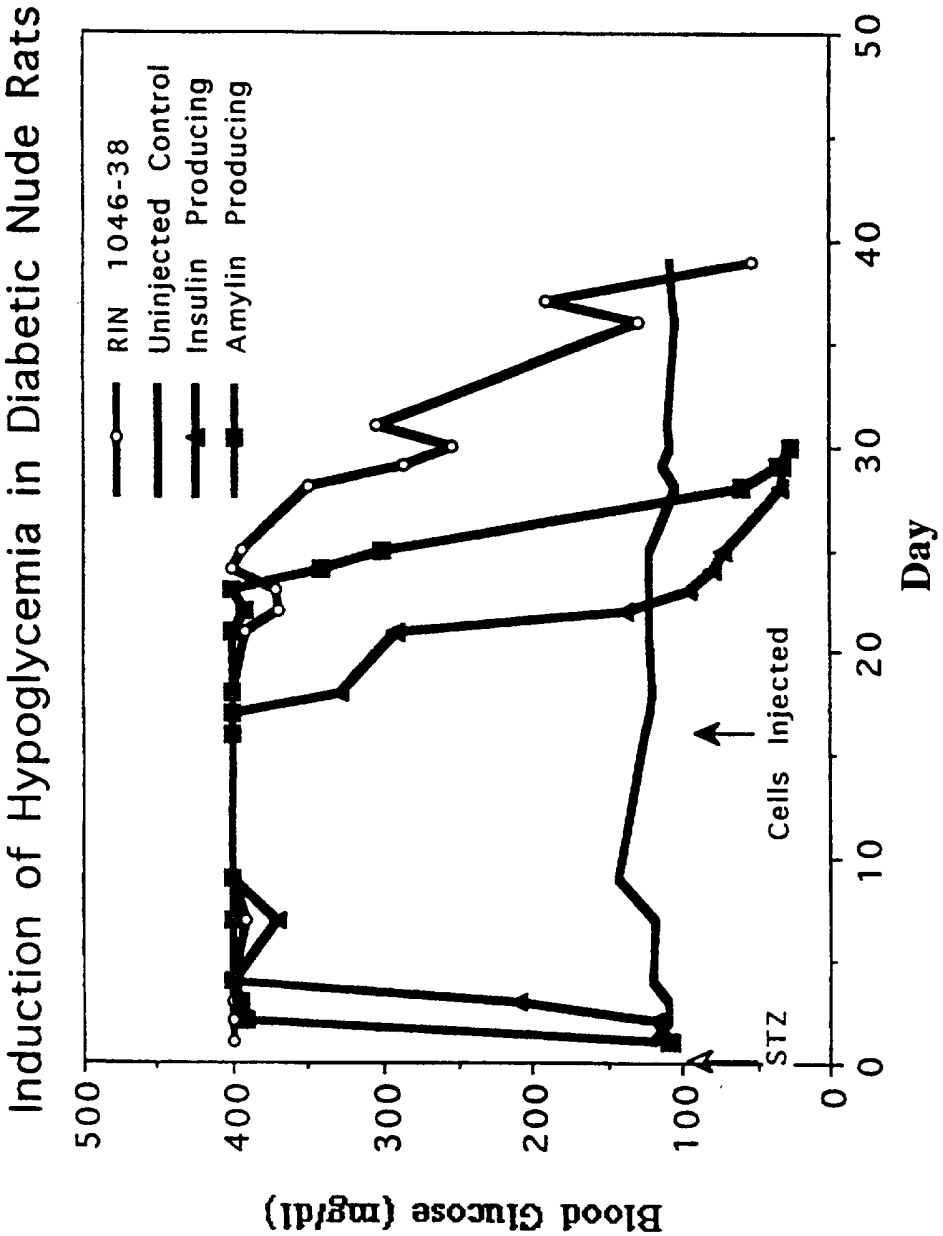

Induction of Hypoglycemia in Diabetic Nude Rats
RIN 1046-38
Uninjected Control
Insulin Producing
Amylin Producing
Blood Glucose (mg/dl)
500
400
300
200
100
0
STZ
Cells Injected
0
10
20
30
40
50
Day

FIG. 25

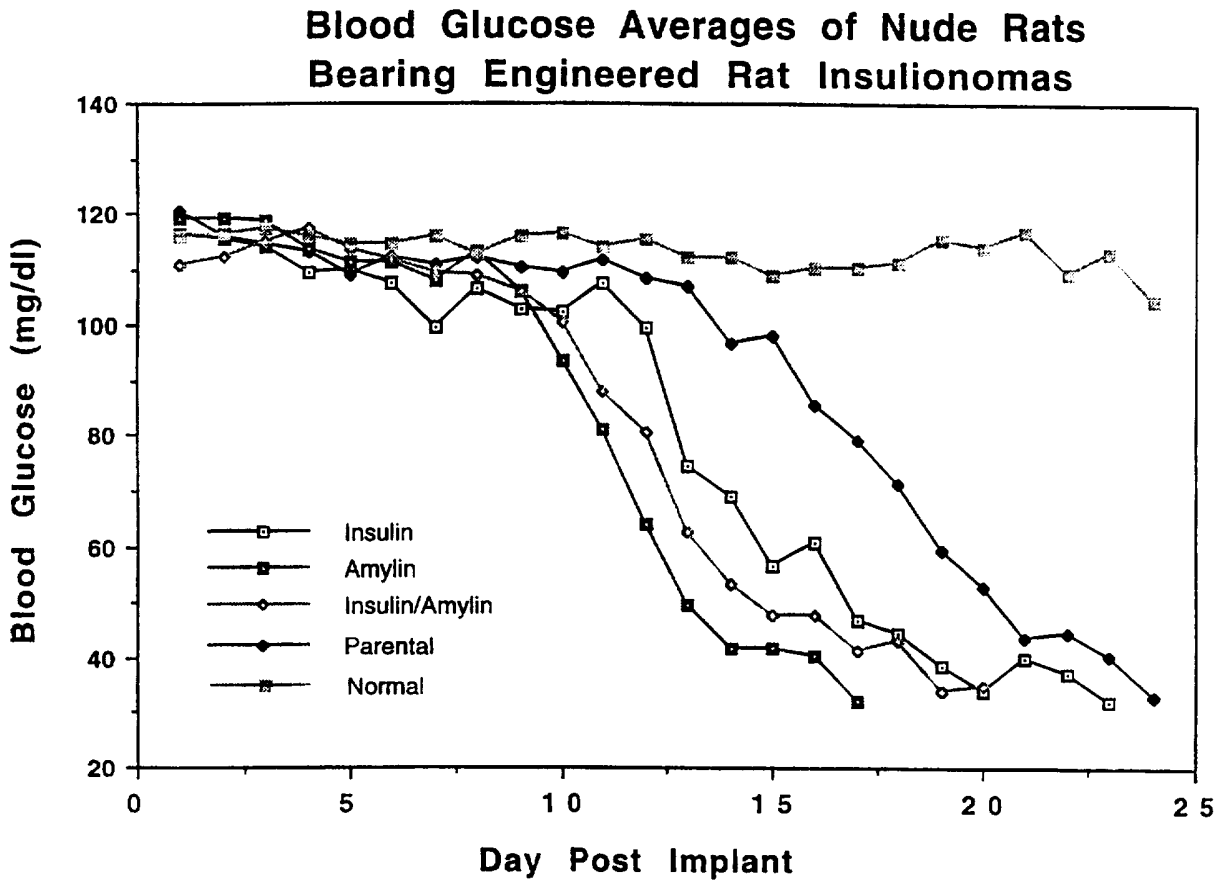

Blood Glucose Averages of Nude Rats
Bearing Engineered Rat Insulionomas
Blood Glucose (mg/dl)
140
120
100
80
60
40
20
0
5
10
15
20
25
Day Post Implant
Insulin
Amylin
Insulin/Amylin
Parental
Normal

FIG. 26

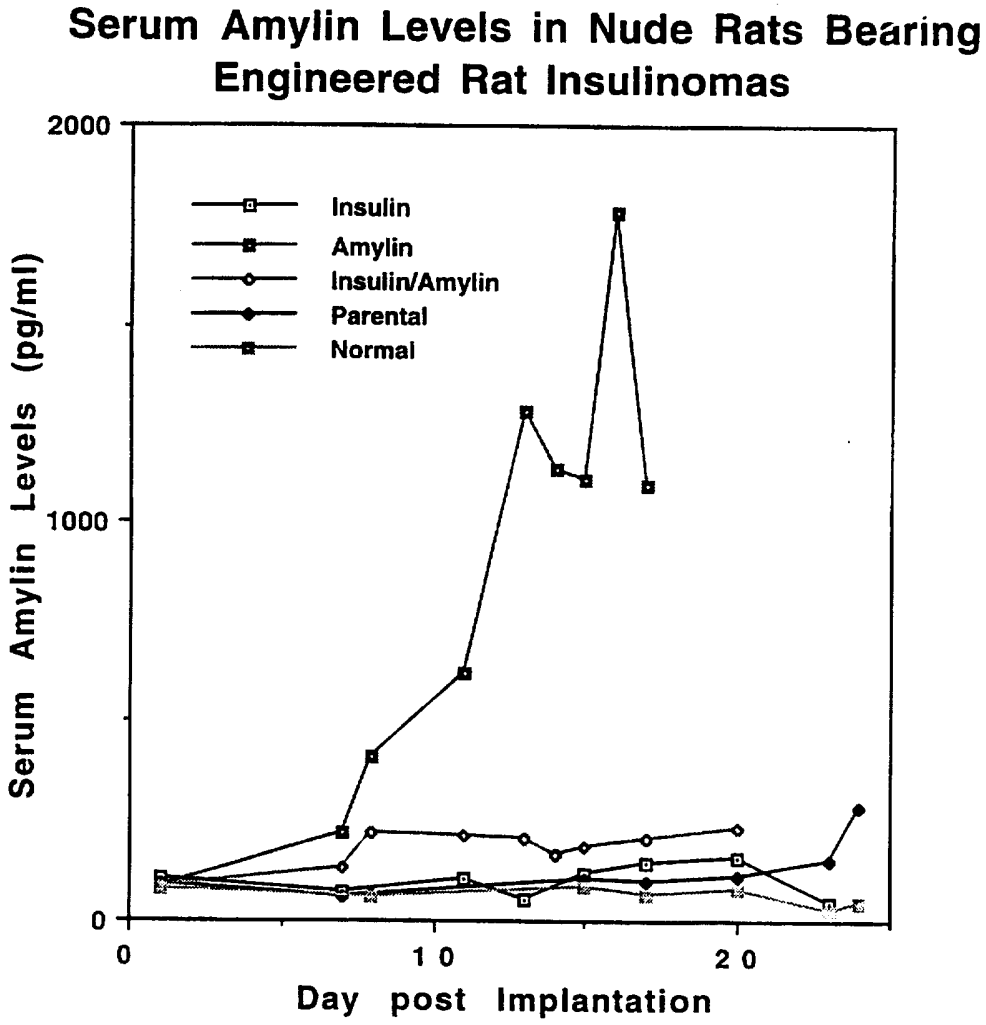

Serum Amylin Levels in Nude Rats Bearing Engineered Rat Insulinomas
2000
1000
0
Serum Amylin Levels (pg/ml)
Insulin
Amylin
Insulin/Amylin
Parental
Normal
0
10
20
Day post Implantation

FIG. 27

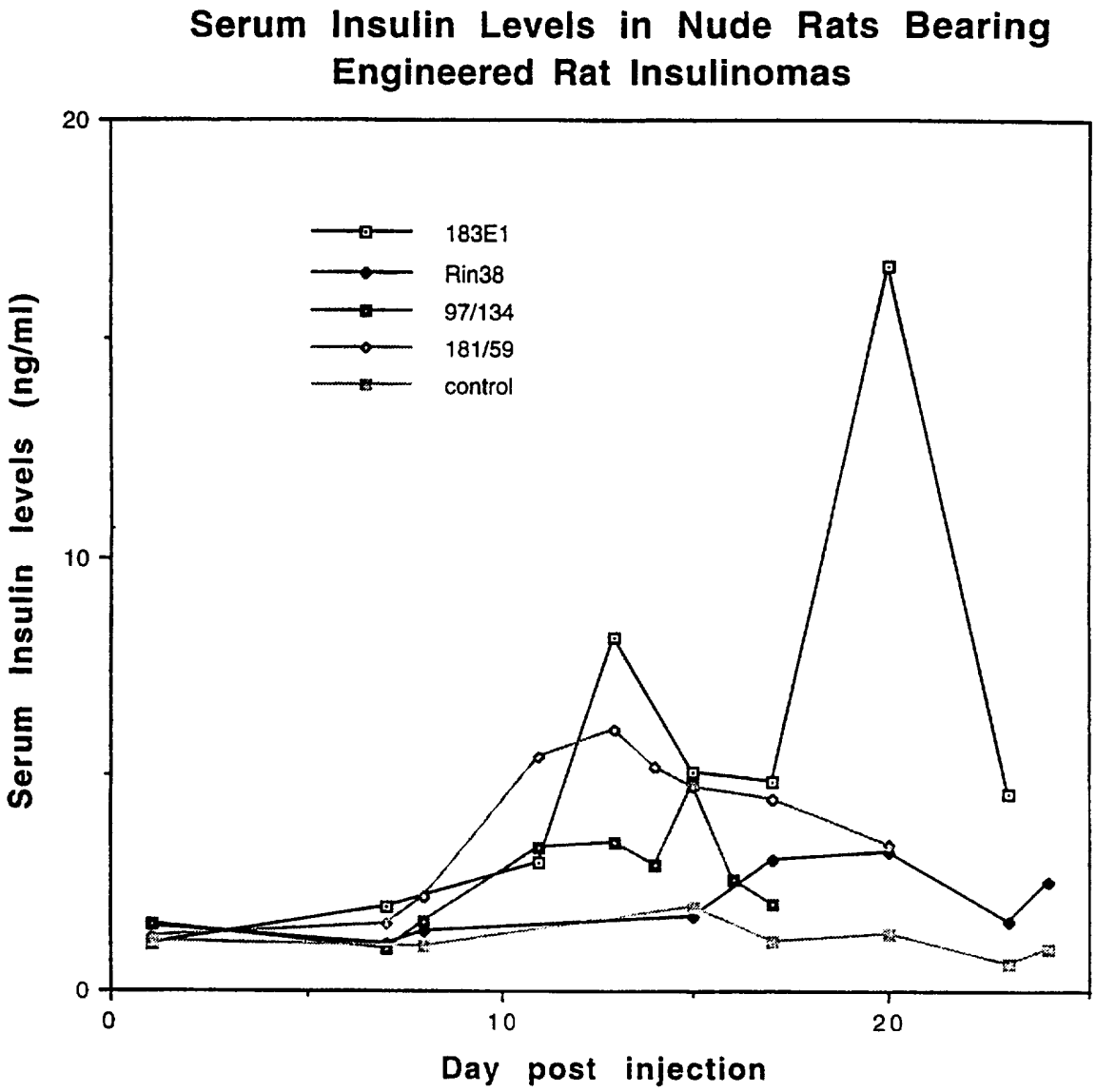

Serum Insulin Levels in Nude Rats Bearing Engineered Rat Insulinomas
Serum Insulin levels (ng/ml)
20
10
0
183E1
Rin38
97/134
181/59
control
0
10
20
Day post injection

FIG. 28

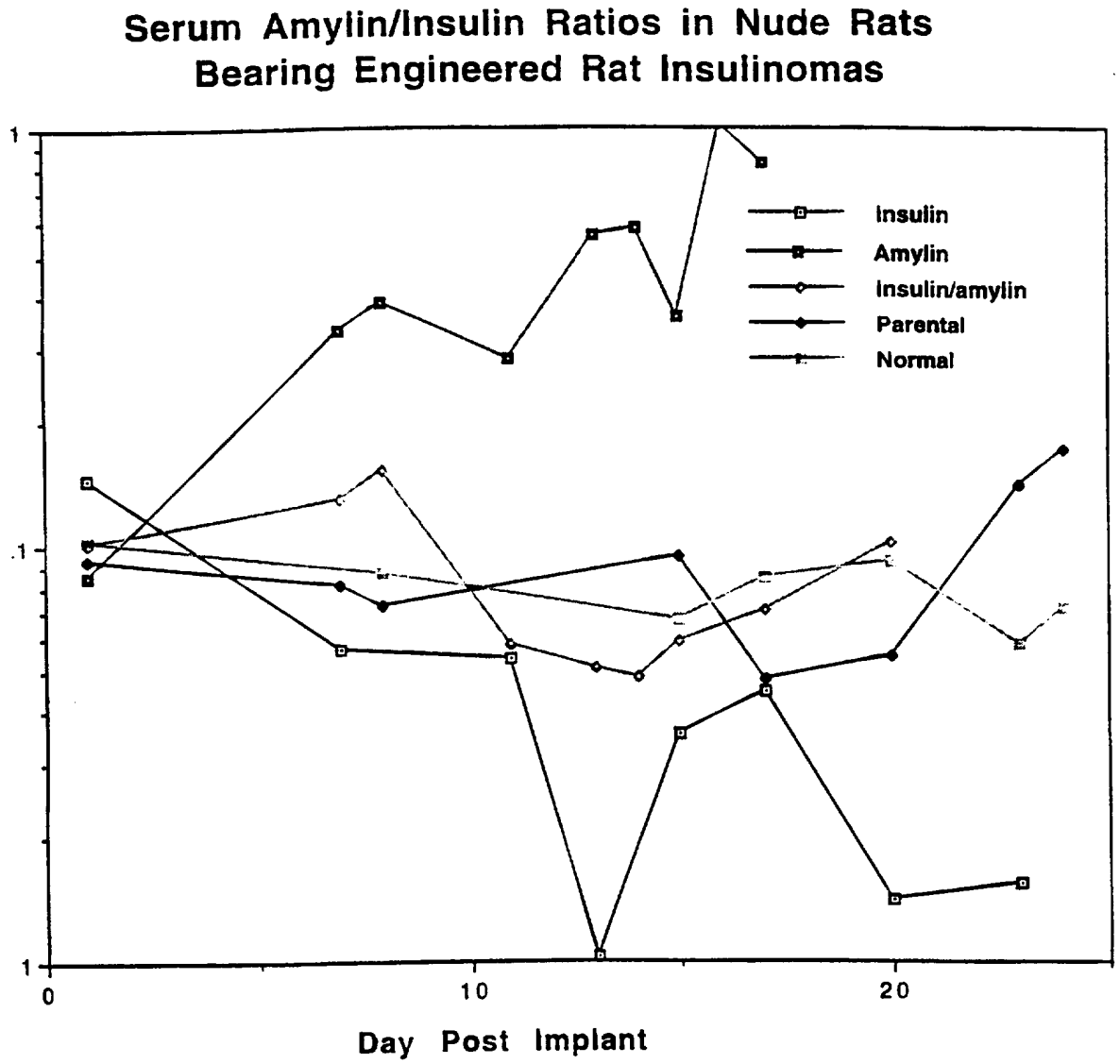

Serum Amylin/Insulin Ratios in Nude Rats
Bearing Engineered Rat Insulinomas
1
.1
1
0
10
20
Day Post Implant
Insulin
Amylin
Insulin/amylin
Parental
Normal

FIG. 29