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| <b>(71) Applicants (for all designated States except US):</b> 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 75207-2107 (US).                                                                                                                                                                                                                                                                                                                      |  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                   |
| <b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only):</b> NEWGARD, Christopher, B. [US/US]; 4415 Ridge Road, Dallas, TX 75229 (US). CLARK, Samuel, A. [US/US]; 1603 South Alamo, Rockwall, TX 75087 (US). THIGPEN, Anice, E. [US/US]; 9014 San Leandro, Dallas, TX 75218 (US). NORMINGTON,                                                                                                                                                                                                                                                                 |  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                   |
| <b>(54) Title:</b> METHODS AND COMPOSITIONS COMPRISING GLUT-2 AND GLUT-2 CHIMERAS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                   |
| <b>(57) Abstract</b><br><p>The present invention provides compositions and various novel uses of the glucose transporter, GLUT-2. Included are methods of using GLUT-2 in cell killing, such as in negative selection and double selection protocols <i>in vitro</i>, in screening methods for identifying genes, promoters and substrates, and in cell killing <i>in vivo</i>, such as may be used in cancer treatment. Also provided are modified functional glucose transporters resistant to diabetic immune attack and methods of making and using such transporters.</p> |  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                   |

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## DESCRIPTION

### METHODS AND COMPOSITIONS COMPRISING GLUT-2 AND GLUT-2 CHIMERAS

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### BACKGROUND OF THE INVENTION

The present application is a continuation-in-part of co-pending U.S. Application, Serial No. 08/546,934, filed October 23, 1995, the entire text and figures of which application are incorporated herein by reference without disclaimer.

10

#### 1. Field of the Invention

The present invention relates generally to the field of glucose transporters and particularly concerns GLUT-2. Provided are various novel methods of using GLUT-2, for example, in negative selection protocols and double selection techniques *in vitro*, in cell killing *in vivo*, and in methods for identifying a variety of genes, promoters and substrates. Also provided are modified functional glucose transporters not subject to diabetic immune attack upon implantation.

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#### 2. Description of the Related Art

Glucose is a polar molecule and is incapable of crossing cellular membranes without the assistance of proteins. Glucose transport proteins exist in two basic classes: the Na/glucose co-transporters, which are capable of active transport of glucose against a concentration gradient; and the facilitated glucose transporters, which are passive, equilibrium transporters (Bell, *et al.*, 1990, Gould and Holman, 1993).

25

The facilitated glucose transporters are known as GLUTs 1 through 7, with the numbering referring to the temporal order in which the family members were identified and cloned. GLUT-6 is now considered to be a pseudogene (Kayano, *et al.*, 1990) and GLUT-7 is thought to be involved in transport of glucose into and out of

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the endoplasmic reticulum (Waddell, *et al.*, 1992). GLUTs 1 through 5 are involved in transport of sugars across the plasma membrane.

The cDNA sequences of human GLUTs 1 through 5 have been determined, and  
5 GLUTs 1 through 4 are found to exhibit a high degree of structural homology. Amino acid sequences predicted from these cDNAs indicate a sequence identity among any two isoforms in the range of 51.6 to 65.3% (Bell, *et al.*, 1990). GLUT-5 is less homologous (38.7-41.7% identity with the other four family members) and appears to transport fructose rather than glucose as its preferential substrate (Burant, *et al.*, 1992).

10

Streptozotocin (glucopyranose, 2-deoxy-2-[3-methyl-5-nitrosourido-D], STZ) was originally isolated as a bacterially produced antibiotic and anti-tumor agent and later found to be a glucose analog (Weiss, 1982). Patients treated with STZ have generally shown symptoms of renal and/or hepatic cytotoxicity (Weiss, 1982). The  
15 use of STZ as an anti-tumor agent was essentially terminated when it was discovered that it caused diabetes in experimental animals, a phenomenon subsequently shown to be due to destruction of insulin-producing  $\beta$ -cells.

The  $\beta$ -cell cytotoxic effect of STZ is thought to be related to its capacity to  
20 produce carbonium ions that cause DNA damage, which in turn activates the nuclear DNA repair enzyme poly (ADP-ribose) synthetase (Okamoto, 1985). Activation of this enzyme results in a dramatic decrease in cellular levels of NAD, the substrate of the poly ADP-ribose synthetase, and consequent decreases in  $\beta$ -cell performance and viability.

25

Streptozotocin has been found to be fully effective in only about 50% of  $\beta$ -cell malignancies (Weiss, 1982) and is reported to be ineffective as a  $\beta$ -cell toxin for human fetal islets (Tuch, *et al.*, 1989) or rat insulinoma cells (Sener, *et al.*, 1986 and LeDoux and Wilson, 1984). It has been reported that the common link between STZ-  
30 resistant populations of  $\beta$ -cells and  $\beta$ -cell derived cell lines is the lack of a normal glucose-stimulated insulin secretion response (Tuch, *et al.*, 1989 and LeDoux and Wilson, 1984).

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Wilson *et al.* have reported differential effects of streptozotocin and its nitrosomethylurea (NMU) moiety in islets and insulinoma cells (Wilson, *et al.*, 1988). Unlike streptozotocin, NMU lacks a  $\beta$ -cell cytotoxic effect. Both compounds are found to alkylate DNA and protein fractions from glucose unresponsive RIN 1046-38  
5 cells, but preferential labeling of the protein fraction by STZ is observed when the drugs are administered to normal islets.

So, it can be seen that although STZ has been known to exert cytotoxic effects on  $\beta$ -cells for a number of years, the mechanism(s) underlying such toxicity are not  
10 fully understood. In particular, the point of entry of STZ into  $\beta$ -cells and the identity of any putative STZ-transport proteins remains to be elucidated.

### SUMMARY OF THE INVENTION

15 In certain embodiments there are provided methods for preparing STZ-sensitive cells which comprise expressing a recombinant GLUT-2 gene in a cell. The "expressing" generally comprises contacting the cell with a recombinant vector comprising a promoter operatively linked to a GLUT-2 gene, wherein the promoter expresses the GLUT-2 gene in the cell.

20

In further embodiments there are provided various methods for use in cell killing. One method of providing an STZ toxin to a cell comprises contacting a cell comprising a recombinant gene that expresses GLUT-2 with a composition comprising STZ. The cell subject to such methods generally comprises a recombinant  
25 vector comprising a promoter operatively linked to a GLUT-2 gene, wherein the promoter expresses the GLUT-2 gene in the cell.

A further method for killing a cell comprises contacting a cell comprising a recombinant gene that expresses GLUT-2 with a composition comprising STZ in an  
30 amount sufficient to kill the cell. Again, the cell preferably comprises a recombinant vector comprising a promoter operatively linked to a GLUT-2 gene, wherein the promoter expresses the GLUT-2 gene in the cell.

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Another method for killing a cell comprises providing to a cell a GLUT-2 glucose transporter and contacting the GLUT-2-containing cell with a composition comprising STZ in an amount sufficient to kill the cell. In such a method, the providing generally comprises the steps of:

5

- (a) obtaining a polynucleotide encoding GLUT-2; and
- (b) expressing the polynucleotide in the cell.

10

The polynucleotide may be comprised in an expression vector, of which an adenoviral vector is a suitable example, and a replication deficient adenoviral vector is one currently preferred example.

15

In the cell killing methods, the cell may be contacted with STZ *in vitro*. These methods often form the basis of selection protocols, wherein the cell can be further transfected with an exogenous gene. The further gene transfection may be prior to, simultaneously with or after providing the GLUT-2.

20

Cell killing methods may also be conducted *in vivo*, where the cell is contacted with STZ by administration of GLUT-2 to an animal or human subject. Such methods are suitable for killing tumor cells, for example, wherein the tumor cell is situated within an animal and is contacted with STZ via intratumoral injection.

25

Also provided are, therefore, methods for treating cancer, which generally comprise expressing a GLUT-2 glucose transporter in cells of a tumor *in vivo* and contacting the tumor with a composition comprising STZ in an amount sufficient to kill the cells of the tumor mass.

30

In particularly surprising aspects of the present invention, the cell killing achieved using GLUT-2 and STZ was discovered to exceed any levels that could have been reasonably predicted. The inventors were thus able to design various selection protocols based upon the surprisingly effective cytotoxic effects of GLUT-2 and STZ.

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The present invention therefore provides methods for killing cells in a cell population, which methods comprise contacting a cell population that includes GLUT-2-expressing cells with a composition comprising STZ in an amount effective and for a period of time sufficient to kill at least about 50% of the GLUT-2-expressing  
5 cells in the cell population.

In each of the cell killing, *in vitro* and *in vivo* selection techniques provided by the present invention, the degree of cell killing may be such that the method kills at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59% or about 60%  
10 of the GLUT-2-expressing cells in the cell population. The method may also kill between about 60%, 65%, 70% and about 75% of the GLUT-2-expressing cells in the cell population.

Preferably, the *in vitro* and *in vivo* cell killing methods of the invention will  
15 be conducted such that the degree of cell killing results in at least about 76%, 80%, 85%, 90% or about 94 or 95% of the GLUT-2-expressing cells in the cell population being killed.

Most preferably, the cell killing and selection methods of the invention will  
20 result in at least about 95%, 96%, 97%, 98% or even about 99% or more of the GLUT-2-expressing cells in the cell population being killed.

The selection methods of the invention may be achieved by providing to cells within the cell population a functional GLUT-2 glucose transporter protein, *i.e.*, the  
25 cells are provided with a functional GLUT-2 glucose transporter protein by the hand of man. Alternatively, the GLUT-2-expressing cells may be cells that naturally express GLUT-2.

The provision of GLUT-2 to create a GLUT-2-expressing cell will generally  
30 be achieved by providing to cells within a cell population a polynucleotide that expresses GLUT-2 in the cells, *e.g.*, using a recombinant vector that comprises a

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promoter operatively linked to a GLUT-2 gene, wherein the promoter expresses the GLUT-2 gene in the cell.

Polynucleotides and vectors may be provided by transfection or by infection  
5 with a recombinant virus that comprises the polynucleotides or recombinant vector. Exemplary recombinant viruses are recombinant retroviruses, adeno-associated viruses (AAV) and adenoviruses that comprise the recombinant vectors. Recombinant adenoviral vectors will often be preferred, with replication deficient recombinant adenoviruses being particularly preferred in certain embodiments.

10

The recombinant vectors for use in the present invention may comprise a constitutive promoter that expresses the GLUT-2 gene. The recombinant vectors may thus comprise a GAPDH, SV40 IE, CMV or an RSV LTR promoter.

15 Alternatively, recombinant vectors may comprise a tissue-specific promoter that expresses the GLUT-2 gene in specific cells of the cell population. Examples including pancreatic  $\beta$  cell-specific promoters that specifically express GLUT-2 in pancreatic  $\beta$  cells within a cell population. Insulin gene promoters such as a rat insulin gene promoter (e.g., RIP1 or RIP2) or a human insulin gene promoter (HIP)  
20 will often be preferred.

The recombinant vectors of the invention may also comprise a modified promoter having increased transcriptional activity, as may be exemplified by a modified promoter that comprises multimerized promoter elements. Recombinant  
25 vectors that comprise a modified promoter that comprises multimerized insulin gene promoter elements form just one example.

The cell killing methods using GLUT-2 may also be supplemented by other negative selection methods in which the cells also express a second negatively  
30 selectable marker gene (the first negatively selectable marker gene being classified as GLUT-2). Such cells are then also contacted with a composition comprising a compound that kills cells expressing the second negatively selectable marker gene.

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The compound is provided in an amount effective and for a period of time sufficient to kill such cells within the cell population.

Exemplary exogenous negatively selectable marker genes include cytosine  
5 deaminase and thymidine kinase, which are to be used functionally with the compounds fluorocytosine and ganciclovir, respectively.

Such methods are envisioned to give effective combined or even synergistic cell killing, particularly when used *in vivo*. This allows for lower doses of each drug  
10 alone to be used, resulting in reduced side effects and toxicity, *e.g.*, in cancer treatment.

All of the methods of the present invention may be used in the context of secretory cells or non-secretory cells. The cells may also be primary GLUT-2-  
15 expressing cells.

The GLUT-2-expressing cells to be targeted by the methods may also be tumor cells, *i.e.*, transformed cells. The tumor cells may naturally express GLUT-2, as is exemplified by insulinoma cells. The tumor cells may also be provided with GLUT-2,  
20 as may be achieved by specific GLUT-2 delivery, including *in vivo* delivery.

Generally, the methods of the present invention will involve STZ incubations of between about 10 minutes and about 3 hours, with about 30 minutes or so being preferred.  
25

Also, in all of the methods the currently preferred amounts of STZ will be between about 0.1 and about 10 mM; more preferably, of between about 0.1 and about 5 mM; between about 0.1 mM and about 2 mM; between about 0.1 mM and about 1 mM; between about 0.1 mM and about 0.5 mM; between about 0.1 and about  
30 0.25 mM; and most preferably, of between about 0.1 mM and about 0.2 mM.

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A further advantage of the invention is that it provides compositions that comprise STZ dissolved in DMSO, which compositions are both effective and stable for long periods of time.

5           The cell killing methods for *in vitro* uses give rise to various selection protocols, as follows. One method is suitable for use in selecting a cell with a site-specific integrated selected gene, i.e., a selected gene integrated into the genome at a site-specific point or in a site-specific manner. Such methods generally comprise the steps of:

10

- (a) obtaining a cell with a GLUT-2 transporter gene incorporated into its genome;
- (b) providing the cell with a polynucleotide comprising the selected gene,  
15           wherein the selected gene is flanked by GLUT-2 sequences; and
- (c) contacting the resultant cell with STZ in an amount and for a period of time sufficient for STZ to kill a GLUT-2 transporter-expressing cell.

20

The method preferably comprises the steps of:

25

- (a) obtaining a cell population, the cells of which comprise a GLUT-2 transporter gene incorporated into their genome;
- (b) providing to the cells of the cell population a polynucleotide comprising the selected gene, wherein the selected gene is flanked by GLUT-2 sequences; and
- (c) contacting the cell population with STZ in an amount effective and for  
30           a period of time sufficient for the STZ to kill at least about 60% or more of the GLUT-2 transporter-expressing cells in the cell population.

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Further selection methods generally comprise the steps of:

- 5
- (a) obtaining a starting cell substantially lacking a functional GLUT-2 transporter gene;
- (b) providing the cell with a polynucleotide comprising (i) a drug selectable marker gene, wherein the drug selectable marker gene is flanked by target gene sequences and (ii) a GLUT-2 transporter gene adjacent thereto;
- 10
- (c) contacting the cell with a drug in an amount and for a period of time sufficient to kill a cell not expressing the drug selectable marker; and
- (d) contacting the cell with a differentially killing effective amount of STZ
- 15 for a period of time sufficient to preferentially kill a cell expressing a functional GLUT-2 transporter.

A "differentially killing effective amount of STZ" and a "period of time sufficient to preferentially kill a cell expressing a functional GLUT-2 transporter" mean that cell killing will be achieved of cells that express a functional GLUT-2 transporter in comparison to a starting cell that substantially lacks a functional GLUT-2 transporter gene, *i.e.*, wherein the "effective amount" is effective to kill a substantial number of cells expressing a functional GLUT-2 transporter but is ineffective in killing a substantial number of cells, or starting cells, that substantially

20

25 lack a functional GLUT-2 transporter gene.

The method may further comprise the steps of:

- (a) obtaining a cell population, the cells of which substantially lack a functional GLUT-2 transporter gene;
- 30

- 10 -

- 5 (b) providing to the cells of the cell population a polynucleotide comprising (i) a drug selectable marker gene, wherein the drug selectable marker gene is flanked by target gene sequences and (ii) a GLUT-2 transporter gene adjacent thereto;
- (c) contacting the cell population with a drug in an amount effective and for a period of time sufficient to kill cells of the cell population that do not express the drug selectable marker, thereby preparing a purged cell population; and
- 10 (d) contacting the purged cell population with STZ in an amount effective and for a period of time sufficient to kill at least about 60% or more of the cells in the purged cell population that express a functional GLUT-2 transporter, and to not kill significant amounts of cells that
- 15 substantially lack a functional GLUT-2 transporter gene.

In such methods, the polynucleotide may further comprise a selected gene flanked by target gene sequences. Again, the currently preferred period of time for use in step (d) is about 30 minutes; and the currently preferred amounts of STZ are

20 between about 0.1 and about 5, 2, 1, 0.5, 0.25 and about 0.2 mM.

As with the other methods described herein, the drug selectable marker gene may be one that confers resistance to neomycin, hygromycin, puromycin, zeocin, mycophenolic acid, histidinol or methotrexate.

25

Further provided are methods for selecting a cell with an integrated selected gene, which generally comprise the steps of:

- 30 (a) providing to a cell a polynucleotide comprising (i) a GLUT-2 transporter gene, (ii) a drug selectable marker gene and (iii) a selected gene, wherein the selected gene is interposed between the transporter gene and the marker gene;

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- (b) contacting the cell with a drug in amount and for a period of time sufficient for the drug to kill a cell not expressing the drug selectable marker gene; and
- 5 (c) contacting the cell with STZ in an amount and for a period of time sufficient for STZ to kill a GLUT-2 transporter-expressing cell.

In preferred embodiments, step (c) will generally further comprise the steps of:

- 10 (i) preparing a first and a second cell culture of the cell;
- (ii) contacting the first cell culture with STZ in an amount and for a period of time sufficient to kill a GLUT-2 transporter-expressing cell; and
- 15 (iii) identifying a cell from the first cell culture that is sensitive to STZ.

Preferred variations comprise the steps of:

- (a) providing to cells of a cell population a polynucleotide comprising (i) 20 a GLUT-2 transporter gene, (ii) a drug selectable marker gene and (iii) a selected gene, wherein the selected gene is interposed between the transporter gene and the marker gene;
- (b) 25 contacting the cell population with a drug in amount effective and for a period of time sufficient for the drug to kill cells of the cell population that do not express the drug selectable marker gene, thereby preparing a purged cell population; and
- (c) 30 contacting the purged cell population with STZ in an amount effective and for a period of time sufficient for the STZ to kill at least about 60% or more of the GLUT-2 transporter-expressing cells of the purged cell population.

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In which case, step (c) may further comprise the steps of:

- (i) preparing a first and a second cell culture of the purged cell population;
- 5 (ii) contacting the first cell culture with STZ in an amount effective and for a period of time sufficient to kill at least about 60% or more of the GLUT-2 transporter-expressing cells of the purged cell population; and
- (iii) identifying a cell from the first cell culture that is sensitive to STZ.

10

Such methods will allow the integrated selected gene to be isolated following the identification of the cell in step (iii), as would be achieved by obtaining the DNA of the integrated selected gene from a cell of the second cell culture that corresponds to the identified cell of the first cell culture.

15

Appropriate drug selectable marker genes, time periods and amounts of STZ are as set forth above, and secretory and non-secretory cells can again be used.

Methods for identifying promoter elements are further provided, which  
20 generally comprise the steps of:

- (a) providing to a cell a polynucleotide comprising a GLUT-2 cDNA that lacks a transcriptional promoter;
- 25 (b) most preferably, preparing a first and a second cell culture of the cell;
- (c) most preferably, contacting the first cell culture with an amount of STZ sufficient to kill a GLUT-2 transporter-expressing cell; and
- 30 (d) identifying a cell from the first cell culture that is sensitive to STZ.

The method may be defined as comprising the steps of:

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- 5
- (a) providing to cells of a cell population a polynucleotide comprising a GLUT-2 cDNA that lacks a transcriptional promoter;
  - (b) preparing a first and a second cell culture of the cell population;
  - (c) contacting the first cell culture with STZ in an amount effective and for a period of time sufficient to kill at least about 60% or more of the GLUT-2 transporter-expressing cells in the first cell culture; and
  - 10 (d) identifying a cell from the first cell culture that is sensitive to STZ.

The methods may further comprise the step of obtaining the promoter element from the corresponding cell of the second cell culture.

- 15
- Still using the *in vitro* cell killing methods, the invention also provides screening methods for identifying polynucleotides capable of conferring STZ resistance to a cell, which methods generally comprise the steps of:

- 20
- (a) providing to a GLUT-2-expressing cell a polynucleotide encoding a putative STZ resistance gene;
  - (b) contacting the cell with STZ in an amount and for a period of time sufficient to kill a GLUT-2-expressing cell; and
  - 25 (c) identifying an STZ resistant cell.

The following steps may also be preferred:

- 30
- (a) providing to a cell within a population of GLUT-2-expressing cells a polynucleotide encoding a putative STZ resistance gene;

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- (b) contacting the population of cells with STZ in an amount effective and for a period of time sufficient to kill at least about 60% or more of the GLUT-2-expressing cells within the population of cells; and
- 5 (c) identifying an STZ resistant cell from the population of cells.

The methods may again further comprise the step of obtaining the STZ resistance gene from the STZ resistant cell.

10 Further methods for identifying a polynucleotide that encodes an STZ transporter protein are provided, comprising:

- (a) obtaining a cell that does not substantially express a functional GLUT-2 transporter;
- 15 (b) providing to the cell a polynucleotide encoding a putative STZ transporter protein;
- (c) contacting the cell with STZ in an amount effective and for a period of time sufficient to kill an STZ- transporting cell; and
- 20 (d) identifying an STZ sensitive cell.

25 Preferred method steps are:

- (a) obtaining a population of cells that do not substantially express functional GLUT-2 transporters;
- 30 (b) providing to a cell of the population of cells a polynucleotide encoding a putative STZ transporter protein;

- 15 -

- (c) contacting the population of cells with STZ in an amount effective and for a period of time sufficient to kill at least about 60% or more of the STZ-transporting cells within the STZ-contacted population of cells; and

5

- (d) identifying an STZ sensitive cell from the STZ-contacted population of cells.

The methods may further comprises the steps of:

10

- (i) preparing a first and a second cell culture of the STZ-contacted population of cells;

15

- (ii) contacting the first cell culture with STZ in an amount effective and for a period of time sufficient to kill at least about 60% or more of the STZ-transporting cells within the STZ-contacted population of cells; and

- (iii) identifying a cell from the first cell culture that is sensitive to STZ.

20

The polynucleotide that encodes the STZ transporter protein may also be obtained from the STZ sensitive cell. For example, by isolating a cell containing a polynucleotide that encodes an STZ transporter protein from the cell of the second cell culture that corresponds to the STZ sensitive cell of the first cell culture.

25

In further embodiments, the present invention provides methods for use in identifying GLUT-2 substrates, which generally comprise determining whether a test substrate competes with STZ in a cell binding, cell transport or cell cytotoxicity assay using a GLUT-2 expressing cell.

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In such embodiments, particularly with cell binding and cell transport assays, the test substrate, STZ, or both, may be labeled. Differential labeling of the test

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substrate and STZ is one contemplated embodiment. Radiolabels are amongst the many possible labels for use in such assays.

5 In these assays, the intracellular level of the test substrate or STZ may be determined. Equally, the extracellular level of the test substrate or STZ may be determined. The amount of the test substrate or STZ may be measured by immunological detection.

10 Preferably, these methods comprise determining whether the test substrate competes with STZ in a cell cytotoxicity assay using a cell population of GLUT-2-expressing cells, wherein the addition of STZ to the cell population results in the killing of at least about 50% or 60% or more of the GLUT-2-expressing cells in the cell population.

15 Kits comprising, in suitable container means, a GLUT-2 glucose transporter gene composition and a negatively selectable marker gene composition are also provided.

20 The GLUT-2 glucose transporter gene and the negatively selectable marker gene may be comprised within distinct expression vectors or may be comprised within a single expression vector.

The kits may further comprise a pharmaceutically acceptable STZ composition. The kits will preferably comprise a cytosine deaminase gene, in which case they may  
25 further comprise a pharmaceutically acceptable fluorocytosine composition.

Thus, the present invention provides GLUT-2 glucose transporter compositions for use in negative selection protocols or treatment regimens, as disclosed herein.

30 One example of a treatment regimen is a method of treating cancer, wherein the method comprises providing a GLUT-2 glucose transporter to cells of a tumor

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within an animal and contacting the tumor with a composition comprising STZ in a therapeutically effective amount sufficient to kill the tumor cells.

5       The method preferably comprises providing a GLUT-2 glucose transporter to a population of tumor cells within an animal and administering to the animal a composition comprising STZ in an amount effective and for a period of time sufficient to kill at least about 50% of the GLUT-2-expressing cells in the tumor cell population.

10       The treatment method may further comprise providing a second negatively selectable marker gene to the tumor cells and administering to the animal a composition comprising a therapeutically effective amount of a compound that kills cells expressing the second negatively selectable marker gene. A preferred example is where the second negatively selectable marker gene is a cytosine deaminase gene and wherein a therapeutically effective amount of fluorocytosine is administered to the  
15       animal.

      In still further embodiments, this invention concerns glucose transporters that confer physiological glucose sensing capacity to cells but that do not render cells subject to diabetic immune destruction, particularly small molecule killing by the  
20       immune system.

      These glucose transporters are exemplified by GLUT-2 transporters that include one or more amino acid mutations and also by GLUT-1/GLUT-2 transporter chimeras. In certain embodiments, the glucose transporter, whether a mutant or chimeric  
25       molecule, will be engineered so that it does not transport STZ.

      Such glucose transporters are preparable by a process that comprises the steps of:

30       (a)     providing a putative glucose transporter to a secretory cell that includes an insulin gene and a hexokinase IV gene but that lacks a GLUT-2 gene; and

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- (b) identifying a cell that has a physiological glucose sensing capacity and that is resistant to diabetic immune destruction.

Resistance to diabetic immune destruction may be assessed, for example, by  
5 resistance to small molecule killing, either *in vitro* or *in vivo*, or by prolonged or increased survival after implantation into a diabetic animal or subject in a semi-permeable device.

In the process of preparing the transporter, preferred steps are currently:

10

- (a) obtaining a polynucleotide encoding the putative glucose transporter;  
and  
  
(b) expressing the polynucleotide in the host cell to be tested for  
15 the physiological glucose sensing and immune resistance.

In certain embodiments, methods for preparing the transporter comprise the steps of:

20

- (a) providing a putative mutant GLUT-2 or GLUT-1/GLUT-2 glucose transporter to a secretory cell that includes an insulin gene and a hexokinase IV gene but that lacks a GLUT-2 gene; and  
  
(b) identifying a cell that has a physiological glucose sensing capacity and  
25 that does not transport STZ.

25

Again, the providing preferably comprises the steps of obtaining a polynucleotide encoding the putative engineered glucose transporter and expressing the polynucleotide in the cell to be tested.

30

Examples of chimeric glucose transporters include GLUT-1/GLUT-2 transporter chimeras that have an extracellular domain that comprises at least a portion

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of an extracellular domain of a GLUT-1 transporter. Further examples are GLUT-1/GLUT-2 transporter chimeras that have an intracellular domain that comprises at least a portion of an intracellular domain of a GLUT-1 transporter. Still further examples are GLUT-1/GLUT-2 transporter chimeras that have a  
5 transmembrane domain that comprises at least a portion of a transmembrane domain of a GLUT-2 transporter.

Examples of mutant glucose transporters include those in which asparagine at position 62 has been replaced with a different amino acid, as further exemplified by  
10 an asparagine to glutamine mutant.

Further provided are polynucleotides encoding glucose transporters that confer physiological glucose sensing capacity to cells but that do not render such cells subject to diabetic immune destruction, e.g., by small molecule killing.  
15

The polynucleotides may encode mutant GLUT-2 transporters or chimeric GLUT-1/GLUT-2 transporters. The polynucleotides may also encode mutant or chimeric transporters that do not transport STZ.

20 The polynucleotides may encode a GLUT-1/GLUT-2 transporter chimera that comprises a contiguous amino acid sequence from SEQ ID NO:2 operatively linked to a contiguous amino acid sequence from SEQ ID NO:4. The polynucleotides may comprises a contiguous nucleic acid sequence from SEQ ID NO:1 operatively linked to a contiguous nucleic acid sequence from SEQ ID NO:3.

25

The polynucleotides may be positioned under the control of a promoter that directs their expression in mammalian cells.

30 The mutant and chimeric glucose transporter proteins and polynucleotides of the present invention are intended for use in administration to a patient to treat diabetes.

- 20 -

Also provided are host cells comprising one of the polynucleotides of the present invention that encodes a glucose transporter that confers physiological glucose sensing capacity to a cell but that does not render the cell subject to diabetic immune destruction, e.g., by small molecule killing. These host cells are intended for use in  
5 administration to a patient to treat diabetes.

Populations of recombinant cells comprising a polynucleotide that encodes a glucose transporter that confers physiological glucose sensing capacity to the cells but that does not render the cells subject to diabetic immune destruction are also provided.  
10

The cells and cell populations may be, or may comprise, cells that comprise a recombinant insulin gene and/or a recombinant hexokinase IV gene.

Medicaments for use in treating diabetes form another aspect of the present invention. The medicaments generally comprise, in a pharmacologically acceptable form, one or more polynucleotides encoding, or cells expressing, a glucose transporter that confers physiological glucose sensing capacity to a cell but that do not render the cell subject to diabetic immune destruction, e.g., by small molecule killing.  
15

Still further aspects of the invention concern methods for conferring glucose sensitivity to a cell, which generally comprise providing to a cell a glucose transporter that confers glucose sensing capacity to a cell but that does not render the cell subject to diabetic immune destruction.  
20

In certain aspects, these methods will preferably include the steps of:  
25

(a) obtaining a polynucleotide encoding a mutant GLUT-2 transporter or a chimeric GLUT-1/GLUT-2 transporter, the transporter not transporting STZ; and  
30

(b) expressing the polynucleotide in the cell.

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Yet further aspects of the present invention concern protein, polynucleotide and cell compositions and methods for treating diabetes, which generally comprise providing to a diabetic animal or patient, a cell that secretes insulin in response to glucose, the cell expressing an engineered glucose transporter that confers glucose  
5 sensing capacity to the cell but that does not render the cell subject to diabetic immune destruction.

The cell that secretes insulin in response to glucose may be a recombinant cell, as described in U.S. Patent 5,427,940 (incorporated herein by reference), which cell  
10 may comprise a recombinant insulin gene or a recombinant hexokinase IV gene.

Cells for use in the treatment methods of this invention may be adapted to express a mutant GLUT-2 transporter or a GLUT-1/GLUT-2 transporter chimera. The mutant or chimeric transporter may be one that does not transport STZ.

15

The cells for use in the treatment methods may be prepared by a method comprising the steps of:

- (a) obtaining a polynucleotide encoding the engineered glucose transporter;  
20 and
- (b) contacting the polynucleotide with the cell to be used in the treatment under conditions effective to allow uptake of the polynucleotide into the cell.

25

The polynucleotide is preferably comprised in an expression vector.

The treatment methods include those in which the polynucleotide is administered to an animal or patient and contacts the cell *in vivo* and those in which  
30 the polynucleotide is contacted with the cell *in vitro* and the cell is subsequently administered to the animal or patient.

**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. Schematic of GLUT-2 expression plasmids. Specific GLUT-2 expression plasmids were constructed as described in Example I. Several variations of expression plasmids have been constructed and validated in cells. These variations include several promoters to drive expression of the GLUT-2 cDNA, including the CMV promoter, RSV promoter, rat Insulin I promoter, human glyceraldehyde-3-phosphate dehydrogenase promoter and mouse metallothionein promoter. In addition, several dominant drug selection marker genes for selection of stable transfectants, including the Neomycin, hygromycin, puromycin, Zeocin, xanthine-guanine phosphoribosyl transferase, HisD and dihydrofolate reductase genes are available and contemplated for use in this manner. (1), amp<sup>R</sup>, ampicillin resistance; (2), first promoter; (3), preferred use of intron; (4), GLUT-2 cDNA; (5), polyadenylation signal; (6), second promoter; (7), drug selection marker gene; (8) and (9), different mRNA transcripts expressed from the first and second promoter.

FIG. 2A, FIG. 2B and FIG. 2C. Blood glucose and insulin levels in nude rats with insulinoma cell implants. FIG. 2A: Control animals without implanted insulinoma cells. FIG. 2B: Animals implanted with RIN cell line 36-7, engineered for expression of GLUT-2. FIG. 2C: Animals implanted with RIN cells lacking GLUT-2 expression. In FIG. 2A, FIG. 2B and FIG. 2C, levels of blood glucose (open squares) and insulin (filled diamonds) were measured as described in Example III. Average values for two rats are presented in each of FIG. 2A, FIG. 2B and FIG. 2C.

FIG. 3A and FIG. 3B. Effect of STZ injection on blood glucose levels in animals with insulinoma implants. FIG. 3A: Three separate nude rats were implanted with RIN GT1-3 cells (transfected with the CB-7 plasmid lacking a GLUT insert).

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FIG. 3B: Three separate rats were implanted with GLUT-2 expressing RIN 35-10 cells. In both FIG. 3A and FIG. 3B, the number "1" and arrow indicate the time point at which the first STZ injection of 110 mg/kg was administered, followed by a second injection of 55 mg/kg 24 hours later. For animals containing implants lacking  
5 GLUT-2 expression (FIG. 3A), the number "2" and arrow indicate the time point at which tumors were surgically resected. Note that diabetes occurred in the animals implanted with GLUT-2 expressing cells (FIG. 3B) without the need for tumor resection.

10 FIG. 4A and FIG. 4B. STZ uptake in RIN and AtT-20ins cell lines. In FIG. 4A, STZ uptake was measured as described in Example V over a range of time points from 15-120 minutes. The abbreviations in the figure legend to the right of the figure refer to the following: AtT-20, untransfected AtT-20ins cells; GT1-8, AtT-20ins cells engineered for overexpression of GLUT-1; CGT-6, AtT-20ins cells engineered for  
15 overexpression of GLUT-2; RIN 36, untransfected RIN 1046-38 cells; RIN 35-7, RIN 1046-38 cells engineered for overexpression of GLUT-2. In FIG. 4B, STZ uptake measured over a range of time points from 30 seconds to 10 minutes for RIN and AtT-20ins cell lines engineered for GLUT-2 overexpression (lines RIN 32-7 and CGT-6, respectively). In both FIG. 4A and FIG. 4B, data points represent the mean  
20  $\pm$  S.E.M. for 3 independent uptake studies.

FIG. 5. GLUT-2 transfected cells transport STZ but not N-nitrosomethylurea. STZ and N-nitrosomethylurea uptake were measured with the same colorimetric assay, as described in Materials and Methods. The abbreviations in the figure legend refer to  
25 the following: CGT-6, Strep, AtT-20ins cells transfected with GLUT-2 and incubated with 10 mM STZ; CGT-6, NMU, AtT-20ins cells transfected with GLUT-2 and incubated with 10 mM N-nitrosomethylurea; GT1-5, NMU, AtT-20ins cells transfected with GLUT-1 and incubated with 10 mM N-nitrosomethylurea. Data points represent the mean  $\pm$  S.E.M. for 3 independent uptake studies.

30

FIG. 6A and FIG. 6B. STZ-mediated toxicity is dose dependent and proportional to GLUT-2 transporter activity. The GLUT-2 specific transporter activity of four RIN

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cell lines was determined as described in the detailed Examples herein and are as follows; RIN81 1.1-4, V<sub>max</sub> of 1.0 mmol glucose/min/liter; RIN 17, V<sub>max</sub> of 7.0; RIN17/C12, V<sub>max</sub> of 25; and RIN 30/10, V<sub>max</sub> of 35 (Ferber, *et al.*, 1994). FIG. 6A demonstrates the dose dependent STZ-mediated toxicity of a 2.5 hour exposure at the indicated drug concentrations. FIG. 6B shows that the relationship between GLUT-2 expression and STZ-mediated toxicity is linear at 2mM STZ.

FIG. 7A and FIG. 7B. STZ sensitivity is a predictive screen for high level expression of the GLUT-2 transgene. FIG. 7A: Northern analysis of 10 independent clones derived from transfection of a STZ resistant RIN line (Parental, Lane 11) with either pCB7/GLUT-2 (Lanes 1-5) or pCB7intron/GLUT-2 (Lanes 6-10). Hygromycin resistant clones were prescreened for sensitivity to 2 mM STZ and then analyzed for GLUT-2 transgene expression as described in Example VI. The message from pCB7intron/GLUT-2 transfected cells runs at a slower mobility due to contributing no-translated 5' and 3' sequences from the expression plasmid. Lane 12 is RNA from RIN 30/10 (Ferber, *et al.*, 1994), known to express high levels of GLUT-2. FIG. 7B: Analysis of the same 10 independent clones for STZ-mediated toxicity demonstrates sensitivity at micromolar concentrations of STZ.

FIG. 8. Rapid STZ-mediated toxicity in cells with high expression of GLUT-2. The exposure time needed for STZ-mediated toxicity was determined using a cell line expressing high levels of GLUT-2 (49/206, FIG. 7A and FIG. 7B) Cells were exposed to 2.0 mM STZ for the indicated times and then viable cells determined 24 hours later as described in the detailed Examples herein.

FIG. 9. Sensitivity to STZ is proportional to the titer of AdCMV-GLUT-2. Following a one hour exposure to recombinant adenovirus encoding GLUT-2, cells were exposed to 2.0 mM STZ for 2.5 hours and then viable cells determined 24 hours later as described in Example VIII.

FIG. 10. STZ-mediated toxicity is conferred upon a wide variety of cell lines by expression of GLUT-2. Gene transfer of GLUT-2 using AdCMV-GLUT-2 confers

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STZ-mediated toxicity to several cell lines. Low passage RIN cells (RIN11), high passage RIN cells (RIN81), monkey kidney CV-1 cells, mouse 3T6 fibroblasts and African green monkey kidney cells were exposed to recombinant adenovirus encoding GLUT-2 for one hours. Subsequently, cells were exposed to 5.0 mM STZ for 2.5  
5 hours and then viable cells determined 24 hours later as described in Example VIII.

FIG. 11A and FIG. 11B. GLUT-2 transgene expression as driven by the CMV promoter is stable *in vitro* and *in vivo*. FIG. 11A. Northern analysis of GLUT-2 transgene expression of a cell line expressing high levels of GLUT-2 (49/206, FIG. 7A and FIG. 7B) is maintained *in vivo* following a 16 or 34 day passage of the  
10 insulinoma in a nude rat model. FIG. 11B. In contrast, the low level of endogenous GLUT-2 expression seen in the parental RIN cells maintained *in vitro* (Lane 1, FIG. 11A and FIG. 11B) is lost following a 24 day passage of the cells *in vivo*.

FIG. 12A, FIG. 12B, FIG. 12C, FIG. 12D, FIG. 12E, FIG. 12F, FIG. 12G and FIG. 12H. Each of the foregoing elements of this figure are schematic representations of mutant and trimeric glucose transporters. GLUT-2 sequences are represented by the unbroken line and GLUT-1 sequences are represented by the broken or dotted line. FIG. 12A, wild-type GLUT-2; FIG. 12B, loop 21; FIG. 12C, Hpa 2-1; FIG. 12D, C12;  
15 FIG. 12E, M12; FIG. 12F, N62Q (no glycosylation); FIG. 12G, loop 12; FIG. 12H, Hpa 1-2; FIG. 12I, C21; and FIG. 12J, M21.  
20

FIG. 13. 293 cells transfected with GLUT-2 transgene and neomycin-resistance gene were selected in G418 and 12 colonies expanded. The resulting 12 cell lines were  
25 tested for resistance to 2 mM STZ and viability determined. The first bar represents 293 cells, and the next 12 bars represent the 12 colonies designated 112/1 through 112/12.

FIG. 14. GLUT-2 expressing cells (49/206) were exposed to STZ at the indicated  
30 concentrations, using 1 M concentration dissolved in DMSO and stored 7 or 21 months at -80°C. The 7 month results are indicated by the solid line and the 21 month results are indicated by the broken line.

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FIG. 15. Three of the higher GLUT-2 expressing cells lines resulting from transfection of GLUT-2 driven by rat insulin I promoter were tested for sensitivity to SyTZ-induced killing at the indicated STZ concentrations.

5

### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

Experimental diabetes can be induced in animals by injection of streptozotocin (Weiss, 1982). Injection of STZ into animals or humans revealed that the major sites  
10 of accumulation of the drug are liver and kidney. Many patients receiving the drug as an antitumor agent have exhibited symptoms of renal and/or hepatic cytotoxicity (Weiss, 1982).

The mechanism of STZ's cytotoxic effect is not clearly understood. Some  
15 theories propose it to be related to reductions in cellular levels of nucleotides such as NAD (Okamoto *et al.*, 1985). Others have hypothesized that STZ toxicity may be in some general way related to glucose transport (Sener *et al.*, 1986).

Glucose is transported by Na/glucose co-transporters and facilitated, equilibrium  
20 glucose transporters. GLUTs 1 through 5 and GLUT 7 are expressed transporters, with GLUTs 1 through 4 being most connected with plasma membrane glucose transport. GLUT-2 has been shown to be an important component of a normal glucose-stimulated insulin secretion response (Hughes, *et al.*, 1992 and 1993, Ferber, *et al.*, 1994, and Newgard, 1992).

25

The inventors contemplated the expression of the GLUT-2 transporter to be the enabling factor that allows modification of proteins leading to streptozotocin toxicity. As shown herein, this was demonstrated to be true by introducing the GLUT-2 gene into neuroendocrine cells to render them susceptible to toxic effects of STZ.

30

As shown herein, rodent insulinoma cells (RIN 1046-38 cells) either expressing or lacking GLUT-2 were implanted into athymic nude rats and allowed to grow as

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solid tumors. After the tumors had reached a palpable size, animals were injected with a large dose (110 mg/kg) of STZ, which has been widely used as a reagent for causing experimental diabetes in animals by virtue of its capacity to specifically kill insulin producing  $\beta$ -cells of the islets of Langerhans (Weiss, 1982). It was found that the  
5 GLUT-2 expressing insulinoma cells, but not those lacking GLUT-2 were killed by the injection of STZ.

This result is further supported by the demonstration herein that STZ preferentially kills GLUT-2 expressing RIN 1046-38 and AtT-20ins cells *in vitro*, and  
10 that overexpression of the structurally related glucose transporter known as GLUT-1 in AtT-20in cells does not confer STZ cytotoxicity. Also as shown herein, the cytotoxic effect of STZ in GLUT-2 expressing cells is likely related to the recognition of the drug as a substrate, since transfection of cell lines with GLUT-2, but not GLUT-1 greatly enhances STZ transport capacity.

15 The current invention takes advantage of the present discovery of the specific capacity of GLUT-2 for streptozotocin transport in providing various novel areas for using the GLUT-2 glucose transporter. The inventors have further discovered that the sensitivity of cells to STZ is proportional to the level of expression of functional  
20 GLUT-2, see, for example, FIG. 6B. As is shown in FIG. 6B, functional GLUT-2 is proportional to the  $V_{max}$ . However, one of skill in the art will realize that various parameters can effect the expression levels of functional GLUT-2, and hence alter  $V_{max}$ . The position of integration of the GLUT-2 construct can alter GLUT-2 expression, due to positional effects. Also, different promoters can be chosen and the  
25 promoter strength of the same promoter could even vary in different cell types (due to levels and combinations of host cell transcription factors, which can effect the expression of functional GLUT-2). In any event, the dose-responsive killing of cells is a reliable indicator of GLUT-2 protein expression. In fact, providing GLUT-2 to a cell is herein shown to confer STZ-mediated killing at STZ concentrations of about  
30 0.25 mM in cells that are normally resistant to 10 mM STZ. Such STZ sensitivity is associated with about a ten-fold increase in GLUT-2 protein expression, which has been correlated in controlled studies, for example, in which a greater than ten-fold

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increase in GLUT-1 expression did not increase sensitivity to STZ in rat insulinoma cells.

5 It is also known to those of skill in the art that different cell types have different resistance to toxic compounds. For example, liver cells (which express cytochrome P450) and lung cells (which express superoxide dismutase) can be more resistant to certain chemical compounds, due to their ability to differentially process the potentially toxic compounds.

10 As disclosed herein, the newly found correlation of GLUT-2 expression and STZ-mediated cell killing allowed the inventors to develop various *in vitro* negative selection assays. Such cell selection techniques are particularly powerful as exposure to STZ is effective to achieve differential killing within about 30 minutes, and as only low levels of STZ need to be employed. In addition to the more straightforward  
15 assays described herein, a consideration of the data available regarding  $\beta$ -cells has led the inventors to develop differential killing techniques. For example, human  $\beta$ -cells, relative to rodent  $\beta$ -cells, appear to be insensitive to STZ toxicity. The inventors believe this to be due to lower GLUT-2 expression in human  $\beta$ -cells in comparison to rat  $\beta$ -cells (Eizirik *et al.*, 1994; DeVos *et al.*, 1995). An aspect of the present  
20 invention therefore includes the use of GLUT-2 transfection to create STZ-sensitive cells that may be killed at STZ concentrations that have only minimal toxic side effects and other human  $\beta$ -cells and/or other cells endogenously expressing GLUT-2.

As will be made clear in the following detailed description and working  
25 examples, the present inventors' use of GLUT-2 and STZ in *in vitro* and *in vivo* selection techniques has further advantages. In particular, where treatments and administration to humans is concerned, the *in vivo* half-life of STZ is advantageously short, which will limit the non-selective cytotoxic actions of this compound *in vivo*. This will provide for advantageous uses over other currently employed negative  
30 selection treatment techniques, such as the use of herpes simplex virus thymidine kinase in combination with gancyclovir. However, given that the present use of GLUT-2 in such treatment embodiments, such as in the treatment of cancer, is

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particularly advantageous, its further combination with other negative selection techniques is proposed to be particularly beneficial. In this manner, the use of GLUT-2 and STZ may be combined with the use of HSV tk and gancyclovir, or cytosine deaminase in combination with drugs such as 5-fluorocytosine.

5

In terms of *in vitro* uses, GLUT-2 also has certain important advantages. The inventors demonstrate herein that GLUT-2 can be effectively transiently expressed, *e.g.*, using adenovirus, and can also be stably expressed in cell lines other than islet cells. Furthermore, effective expression is demonstrated with viral, *e.g.*, CMV and RSV promoters, as well as with tissue-specific promoters, such as the rat insulin gene promoter. In fact, cell killing is herein shown to be effective with adenovirus-producing 293 cells, thus providing an advantageous approach for the elimination of unwanted recombinations during the production of adenoviral vectors. Another primary use of GLUT-2 is in assays for homologous recombination. In fact, data is presented herein to demonstrate the significant enrichment for homologous recombination that is achieved with GLUT-2, which is even better in  $\beta$ -cell lines than the commonly used thymidine kinase. Cytotoxic sensitivity can also be sensitively monitored in a time-dependent manner, and is effective using only a small number of cells.

10  
20

The use of GLUT-2 and STZ in connection with *in vitro* assay systems is further enhanced by the inventors' surprising discovery that STZ can be effectively stored in DMSO. Dissolving STZ in DMSO in the manner provided by the invention provides laboratory workers with protection from exposure to the STZ powder, which exposure was routinely encountered using the prior art methods that required STZ to be weighed in dry powder form immediately prior to preparing and using STZ. The present inventors have found that STZ may be dissolved in DMSO without adverse effects that hamper its future use. The examples presented herein further demonstrate the STZ is stable under these conditions for at least about 18 months.

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## I. Cell Killing

### 1. GLUT-2 Genes

5 Mammalian GLUT-2 cDNAs or genes are generally preferred for use in the present invention. Those from rat (Thorens, *et al.*, 1988, Genbank Accession Number: J03145 and Johnson, *et al.*, 1990) and human (Bell, *et al.*, 1990, Genbank Accession Number: J03810) are examples. Additional GLUT-2 isoforms and their Genbank accession numbers are mouse (M23382 and J04557), and *G. domesticus* (Z22932).  
10 Exemplary GLUT-2 DNA and amino acid sequences are provided herein in the form of SEQ ID NO:3 and SEQ ID NO:4, respectively.

### 2. Killing

15 The cell killing embodiments of the present invention generally concern killing a cell with a cytotoxic substance that binds to, and is transported into the cell by, GLUT-2. STZ is currently the preferred agent for use in this manner, accompanied by appropriate STZ analogues.

20 Generally, to kill a cell in this manner, one would first provide the cell with a functional GLUT-2 transporter protein or a DNA segment that encodes and expresses such a functional GLUT-2 transporter. The GLUT-2-including cell would then be contacted with a composition comprising STZ or a toxic STZ analogue in an amount, and for a period of time sufficient, to kill the cell.

25

"Providing GLUT-2" in the context of cell killing may be achieved by any method that results in incorporation of at least some GLUT-2 molecules into the membrane of the cell, including, e.g., cell fusion, liposome delivery, infection with viral vectors and the like. Preferred methods are generally those that involve the  
30 provision of a recombinant GLUT-2 polynucleotide, cDNA or gene to the cell in a manner that results in GLUT-2 protein production by the cell.

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Most preferably, the cell will be provided with a recombinant expression vector comprising a promoter operatively linked to a GLUT-2 cDNA or gene, wherein the promoter expresses the GLUT-2 gene or cDNA in the cell. The particular type of expression vector to be used is not critical and it is contemplated that virtually any functional vector may be used. Several vectors are described herein and further vectors will be readily known to those of skill in the art in light of the present disclosure and publications such as Sambrook *et. al.* (1989, incorporated herein by reference).

10 The cell killing aspects of the invention have many *in vitro* and *in vivo* uses, as described below. In any event, an advantage of the invention is that only a relatively short exposure to STZ, for example, of between about 10 or even 5 minutes and about 2.5 to 3 hours, is required for effective cellular killing.

15 The time of exposure for complete killing depends on the expression levels for GLUT-2 protein and STZ dose. With  $\beta$ -cell lines tested, several kidney-derived cell lines, and mouse fibroblasts, maximal *in vitro* killing was achieved after 2 hours of incubation with STZ.

20 The amounts of STZ sufficient to kill a cell will be readily determinable by those of ordinary skill in the art in light of the details set forth in the Examples herein. Although optimization may be desired in certain cell types or systems, the optimal amounts will be identifiable without undue experimentation.

25 Currently, using several kidney cell lines (CV-1, VERO, 293), a mouse fibroblast cell line (3T6), and neurosecretory cells (RIN 1046-38, AtT20), cells expressing predominately GLUT-1 and low or no GLUT-2 are resistant to STZ concentrations of at least 10 mM. Some cell lines, for example 3T6, and CV-1 are resistant to STZ concentrations of 20 mM, while others, such as 293 and VERO cells exhibit toxicity at 20 mM. When cells express GLUT-2, the STZ concentrations effective in killing are shifted to lower concentrations, generally less than or equal to about 2 mM. For example, CV-1 cells and RIN-38 cells without GLUT-2 are resistant

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to STZ > 10 mM, upon GLUT-2 expression CV-1 cells are killed by STZ at 2 mM, while RIN-38 cells are killed by STZ at 0.5mM. Given this differential, it is evident that one of ordinary skill in the art could readily determine an effective amount of STZ for use in any given cell type.

5

In addition to the techniques of cell killing, the present invention also provides for the preparation of cells that are toxin- or STZ-sensitive, as may later be used in cell killing embodiments, such as negative selection protocols. To render a cell toxin- or STZ-sensitive according to this invention, one would generally provide the cell with a functional GLUT-2 transporter, as may be achieved by delivering a GLUT-2 protein or a GLUT-2-encoding DNA segment to the cell.

10

STZ compounds, even those modified to have reduced cytotoxicity, may be delivered to a cell by first providing the cell with a functional GLUT-2 transporter protein or a DNA segment that encodes and expresses a functional GLUT-2 protein. The next step is then contacting the resultant cell with a composition comprising STZ or an STZ-like compound.

15

### 3. GLUT-2 in Selection Protocols

20

A first new use of GLUT-2 is as a selection marker for gene transfer studies. In this application, a GLUT-2 cDNA or gene is integrated into a eucaryotic expression vector. The cDNA or gene that the investigator wishes to transfer into a particular cell, i.e., the selected gene, is placed at a second site within the expression vector.

25

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.

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**a) Homologous Recombination**

For homologous recombination studies, the GLUT-2 gene can be used in at least two different fashions. First, in one embodiment, the GLUT-2 gene is placed at one end of a DNA construct that comprises a selected gene to be transferred, flanked by target gene sequences. The expression vector is then contacted with a cell that lacks a GLUT-2 transporter gene. In another embodiment, the selected transfer gene is flanked by, or inserted into, GLUT-2-encoding sequences. The expression vector is then contacted with a cell that has a functional GLUT-2 transporter gene incorporated into its genome.

In the first embodiment, a target gene within a GLUT-2<sup>-</sup> 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'

30

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.

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The principal behind the use of such a vector is as follows. Many recombination events, following introduction of an expression vector into a host cell, are non-homologous. In certain applications, these non-homologous recombination events are undesirable and, thus, it is beneficial to select against these events. When  
5 using a vector as described above, a non-homologous recombination event likely will result in incorporation of all the sequences, including the GLUT-2 gene. Application of a drug to such cells will permit isolation of recombinants, but further application of 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  
10 susceptible to STZ treatment (the original cell was GLUT-2<sup>-</sup>).

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  
15 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<sup>res</sup>/STZ<sup>res</sup>) should yield recombinants that lack the target gene and express the selected gene. Further screens for these phenotypes, either functional or immunologic,  
20 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,  
25 proper recombinants are screened by drug resistance and STZ resistance (the original cell was GLUT-2<sup>-</sup>).

A second homologous recombination protocol also is envisioned by the inventors. In this procedure, a GLUT-2 expressing cell is selected as the initial host.  
30 The selected gene is inserted into an expression vector that contains GLUT-2 homologous sequences such that the GLUT-2 homologous sequences are interrupted by the selected gene, or as described above, the GLUT-2 homologous sequences

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"flank" the selected gene. A proper homologous recombination event that transfers the selected gene will, by definition, interrupt the resident GLUT-2 gene. Such GLUT-2<sup>-</sup> cells can be identified by their lack of sensitivity to STZ.

5           Examples of processes that use negative selection to enrich for homologous recombinants include the disruption of targeted genes in embryonic stem cells or transformed cell lines (Mortensen, 1993, Willnow and Herz, 1994) and the production of recombinant virus such as adenovirus (Imler, *et al.*, 1995).

10                           **b)      Non-Homologous Recombination**

          In other embodiments, the GLUT-2 gene also can be used as a tool for screening for transfer of expression vectors in a non-homologous fashion. In this case, the expression vector contains the selected gene that is to be delivered, a drug  
15   selectable marker gene and a GLUT-2 cDNA or gene. Preferably, the selected gene is interposed between the GLUT-2 transporter gene and the marker gene.

          The expression vector is incorporated into a cell that lacks a functional GLUT-2 transporter. The principle behind the selection process is as follows. While  
20   some recombinants may only achieve partial integration of the construct, i.e., the drug selectable marker or the GLUT-2 sequences, a cell expressing products of both these vector genes also likely contains the intervening sequences, namely, the selected gene. Thus, following recombination, the cells are screened for drug resistance. Of the remaining cells, these are screened for GLUT-2 expression, i.e., sensitivity to STZ.  
25   If the cell satisfies these double-selection criteria, they should also expression the selected gene.

          The drug screen is a conventional positive selection protocol where the surviving cells are those desired. The STZ treatment, being a negative selection,  
30   generally involves preparing replica or duplicate cultures, where a first and a second cell culture of the drug-resistant cells are prepared. One cell culture is contacted with an amount of STZ sufficient to kill a GLUT-2-expressing cell, thereby identifying

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those cells that are sensitive to STZ and, hence, GLUT-2<sup>+</sup>. Further characterization of these doubly-selected clones should confirm expression of the selected gene.

5 Examples of conventional antibiotic resistance markers are those conferring resistance to neomycin, hygromycin, puromycin or zeocin; and xanthine-guanine phosphoribosyl transferase, HisD and dihydrofolate reductase genes. The operation of drug selection or antibiotic resistance marker genes is well known to those of skill in the art.

### 10 c) Screening and Identifying Promoters

The GLUT-2 cDNA or gene can be used in the context of screening for and identifying promoter sequences. For example, GLUT-2-encoding sequences may be placed next to a promoter/enhancer sequence that an investigator wishes to study. The activity of the promoter/enhancer sequence in a particular cell can then be scored by the degree to which its introduction causes STZ cytotoxicity.

Further, novel promoter elements can be identified following the introduction into a cell of a GLUT-2 cDNA in the context of a promoterless cassette. This technique, in which GLUT-2 would presumably integrate randomly into the genome, would be used as a screen to probe the genome for areas that provide strong transcriptional activity. Cells most sensitive to STZ would indicate that GLUT-2 has integrated into the genome at a site responsible for high transcriptional activity, allowing the potential promoter to be rescued from such cells.

25

In such promoter screening methods, one would provide to a cell, generally to a cell lacking a functional GLUT-2 transporter, a polynucleotide comprising a GLUT-2 cDNA that lacks a transcriptional promoter. One would then contact the cell with STZ in an amount and for a period of time sufficient for STZ to kill a GLUT-2 transporter-expressing cell. STZ treatment again preferably involves preparing replica or duplicate cultures that are differentially treated with STZ, as described above. The

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promoter of interest can then be isolated by recloning the inserted GLUT-2 gene and its flanking regions out of the identified cell.

5 An alternative format would employ a "headless horseman" cassette in which a promoterless GLUT-2 gene or cDNA was placed downstream (3') of a cloning site. A genomic library would then be constructed such that the genomic sequences are inserted into the cloning site, just 5' to the GLUT-2 gene. Following introduction into a GLUT-2<sup>-</sup> host cell, screening of cells for STZ sensitivity would identify constructs in which active promoters are present.

10

## II. Screening Assays

A second broad area for using cells expressing the GLUT-2 transporter is in screening assays, for example, in order to identify additional GLUT-2 substrates, STZ analogues or genes that protect against STZ toxicity.

15

### 1. GLUT-2 Substrates

20 In addition to STZ, other compounds that are specifically transported into the cell by GLUT-2 may be identified. These substrates could have cytotoxic effects or could prove to be beneficial to some aspect of cellular function. In light of the present inventors' surprising discovery of the connection between STZ and GLUT-2, cell lines expressing GLUT-2 can now be used to identify such substances.

25 These assays are generally based upon competition of a test substance and STZ. To identify a GLUT-2 substrate one would determine whether the test substrate competes with STZ in any one of a number of suitable assays, such as cell binding, cell transport or cell cytotoxicity assays using GLUT-2 expressing cells.

30 In cell binding and transport assays, the test substrate or STZ may be labeled, e.g., with a radiolabel. The intracellular or extracellular level of the test substrate or STZ may also be determined by immunological detection. Cell cytotoxicity assays are

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generally preferred in certain embodiments, in which case the presence of a GLUT-2 substrate would result in a reduction in cell killing upon exposure to STZ. Suitable STZ uptake and cell killing assays are described herein in detail, for example, in Example V and Example VII, respectively.

5

## 2. STZ Analogues

The type of differential cell binding, transport or cytotoxicity assays described above may be used to test synthetic or natural STZ analogues. For example, STZ analogues may be designed with a view to increasing potency.

10

## 3. STZ Resistance Genes

Following the present discovery that GLUT-2 is responsible for transporting STZ into cells, GLUT-2 expressing cell lines can also now be used to screen for genes that protect against the cytotoxic effects of STZ. In this context, the procedure would be to introduce pools of exogenous genes into GLUT-2 expressing cells, followed by the identification of clones or cells containing protective genes on the basis of resistance to STZ.

15  
20

## III. Tumor Cell Killing

A third novel use of the GLUT-2 gene is in therapeutic ablation of tumors in animals or humans. Support for these methods is to be found in the data presented herein, including the local destruction of GLUT-2-expressing cells *in vivo*, as shown in Example IV, the tumor cell killing of Example VIII, and the stable expression of GLUT-2 *in vivo* shown in Example IX.

25

Previous uses of STZ in animals and humans revealed that the major sites of accumulation of the drug were the liver and kidney and that many patients receiving the drug as an antitumor agent exhibit symptoms of renal and/or hepatic cytotoxicity (Weiss, 1982). The preferential uptake of STZ by kidney and liver among

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extrapancreatic mammalian tissues is now understandable in light of the inventors' findings that GLUT-2 is responsible for STZ transport - as these tissues are amongst the few that express GLUT-2 as their major glucose transporter isoform (Thorens, *et al.*, 1988).

5

One aspect of the proposed cancer treatment methods therefore involves first identifying a patient with a tumor that expresses GLUT-2 and then treating the patient with STZ as a chemotherapeutic agent.

10

In more preferred treatment embodiments, the GLUT-2 gene is introduced into a tumor through such means as viral infection, transfection, gene gunning, liposome or receptor-mediated uptake, and the like. Limited and specific expression is further contemplated, as is facilitated by the use of tissue or cell-specific promoters, such as the insulin promoter for islet  $\beta$ -cells. After allowing time for the GLUT-2 gene to be actively expressed in tumor cells, the tumor is then locally exposed to STZ, which then kills the GLUT-2 expressing cells.

15

20

Recombinant adenovirus is a vector that allows transfer of the GLUT-2 gene to transformed cells with an efficiency approaching 100% (Ferber, *et al.*, 1994) and is therefore contemplated to be particularly appropriate for targeting of the GLUT-2 gene to a high percentage of cells in a tumor.

25

30

The use of GLUT-2 in this way is somewhat analogous in operation to the use of herpes simplex virus thymidine kinase in combination with ganciclovir (HSVTK/ganc) (Wigler *et al.*, 1977; Patent application WO 92/08796; each incorporated herein by reference) and bacterial cytosine deaminase in combination with 5-fluorocytosine (CD/5FC) (U.S. Patent 5,358,866): two other negative selection systems that have been described for reducing or eliminating tumors. However, the current invention of GLUT-2 in combination with STZ offers two advantages over these systems.

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Firstly, a relatively short exposure to STZ of only between about 10 or even about 5 minutes and about 3 hours is required for effective cellular killing; whereas, HSVTK/ganc and CD/5FC each require that cells be exposed to toxin for much longer periods, typically 2-5 days. Secondly, toxicity to STZ is tightly coupled to level of expression of GLUT-2. Cells lacking GLUT-2 are resistant to STZ of concentrations greater than 10mM.

#### IV. GLUT-2 Mutants and Chimeras for Diabetes Therapy

The fourth novel embodiment of this invention was developed from an understanding of the role of GLUT-2 in STZ transport. As the GLUT-2 gene was discovered to be responsible for STZ uptake, the inventors contemplate that GLUT-2 will be involved in the autoimmune destruction of insulin-containing  $\beta$ -cells in insulin dependent diabetes mellitus (IDDM), also known as Type I diabetes.

GLUT-2 is the glucose transporter that is naturally expressed in islet  $\beta$ -cells (Thorens, *et al.*, 1988 and Johnson, *et al.*, 1990). The inventors propose that it is the GLUT-2 expression that is responsible for the specific transport of cytotoxic substances generated by T-cells or macrophages that comprise the cellular infiltrate in IDDM, resulting in  $\beta$ -cell killing.

As a result, the inventors contemplate that GLUT-2 compositions can be used to create glucose transporters that confer physiological glucose sensing capacity but that do not render the cell subject to diabetic immune destruction. One example of such a molecule is a mutant GLUT-2, adapted for such a purpose. Another example of such a transporter is a GLUT-2 chimera that includes portions of GLUT-2 sequence coupled with other transporter sequences, such as those from GLUT-1. Such transporters will likely confer protection against transport of the cytotoxins while allowing glucose transport to continue, thus maintaining physiological or near-physiological glucose-stimulated insulin secretion (GSIS).

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In support of this model, glucagon producing  $\alpha$ -cells express GLUT-1 instead of GLUT-2 (Heimberg, *et al.*, 1995) and are left undamaged in autoimmune diabetes. Furthermore, transplantation of islet grafts into animals with autoimmune diabetes results in graft destruction, even when such studies are carried out in the context of permselective membranes or devices designed to prevent direct contact between T-cells and the graft. These findings can now be understood in the context of the present invention and linked to other studies, e.g., those showing that multiple low dose injections of STZ induce a syndrome of insulinitis and  $\beta$ -cell destruction resembling IDDM (Like and Rossini, 1976).

Further studies have shown that insulinitis and progression to diabetes can be blocked in non-obese diabetic (NOD) mice by injection of high doses of nicotinamide, an agent that suppresses poly (ADP-ribose) synthetase and restores cellular levels of NAD (Yamada, *et al.*, 1982). In light of these data, it has been suggested (Okamoto, 1985) that autoimmune diabetes and diabetes induced by STZ and other chemically related nitroso compounds such as the rodenticide Vacor (Karam, *et al.*, 1980) and N-nitroso compounds in smoked meats (Helgason, *et al.*, 1982) may induce  $\beta$ -cell destruction by related mechanisms.

The demonstration in this application that GLUT-2 can transport a glucose molecule modified by an N-nitroso group (STZ) suggests that  $\beta$ -cell cytotoxicity in IDDM might be induced or accelerated by the accumulation of similarly modified glucose derivatives or more distantly related compounds under conditions of T-cell infiltration and insulinitis. Indeed, islet-infiltrating monocytes have been reported to produce reactive oxygen and nitrogen free radicals (Nathan, 1987) that may be directly cytotoxic or that could react with other molecules such as glucose to produce a new class of  $\beta$ -cell cytotoxins.

U.S. Patent 5,427,940, incorporated herein by reference, demonstrates that the GLUT-2 gene confers glucose-stimulated insulin secretion in secretory cell lines. GLUT-2-including engineered cells for glucose sensing are contemplated for cell-based insulin replacement therapy of IDDM. However, while evidently of significant use,

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in light of the present findings it appears that cells expressing native GLUT-2 may not be optimal for IDDM treatment owing to the likelihood that they will also ultimately be subject to immune attack.

5           Interestingly, GLUT-1 does not substitute for GLUT-2 in imparting glucose sensing into insulin secreting cell lines (Hughes, *et al.*, 1993). Therefore, although GLUT-1-containing cells are not subject to immune attack, they are not useful in IDDM treatment. As a consequence of this invention, GLUT-1 transporters can now  
10           be used to supply non-STZ-binding sequences for use in combination with glucose-sensing portions of GLUT-2 to create a second generation transporter for use in IDDM treatment.

          GLUT-2 genes that contain mutations in individual codons (site-directed mutants) or chimeric glucose transporters that are comprised of various combinations  
15           of GLUT-2 and GLUT-1 segments have been constructed by the inventors. This approach is taken in order to produce molecules that are able to transport glucose efficiently and that can participate in normal glucose signaling, but that do not render a host cell subject to diabetic immune destruction, as exemplified by cells that are  
20           unable to transport STZ.

          Suitable techniques for constructing both mutant and chimeric transporters will be well known to those of ordinary skill in the art. For example, the techniques of site-specific mutagenesis are known, as exemplified by those described in U.S. Patent 4,888,286, incorporated herein by reference, which describes methods for altering  
25           nucleotide sequences. The generation of chimeric molecules through the application of recombinant DNA technology is also standard practice. Chimeric glucose transporters themselves have already been produced for other purposes, and the techniques described in Oka, *et al.*, 1990; Katagiri, *et al.*, 1992; and Cassidy and Newgard, 1994; each incorporated herein by reference, are contemplated for use in  
30           connection with the present invention.

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The techniques for identifying transporters that have the desired properties from the candidate transporters created are all routine techniques, known to those of skill in the art and further described herein. For example, glucose-transport assays are described in Example VI; and STZ transport assays are described in Example V.

5

To interpret the results from the glucose transport assays in terms of normal glucose signaling, one would screen for a mutant or chimeric transporter that exhibited glucose transport properties equivalent to, or in the range of, the normal GLUT-2 molecule.

10

The presence of a physiological GSIS would be determined by adapting the assay to ensure that insulin secretion resulted. In order to obtain a physiological GSIS, the cell must also contain a functional insulin gene and a functional hexokinase IV gene. The cell will preferably have lower levels of a low Km hexokinase, such as hexokinase I. Physiological GSIS is preferably determined as described in Hughes *et. al.* (1993), and is defined as insulin secretion in response to a range of glucose concentrations between 0 and about 20mM.

It is believed that cells that do not transport STZ or that do not transport STZ effectively will be resistant to diabetic immune destruction. This may be confirmed by *in vitro* and *in vivo* assays of cellular destruction and toxicity. "Resistance to diabetic immune destruction", as defined herein, simply means that the cells will survive (e.g., in an appropriate *in vitro* assay), or will have a life-span (e.g., in an appropriate *in vivo* assay), that is increased to any degree relative to a cell lacking a glucose transporter of the present invention. Cells that survive or exist for a significantly increased period of time will naturally be preferred.

Resistance to diabetic immune destruction *in vitro* is preferably measured by an assay that measures small molecule killing. "Small molecule killing", as defined herein, refers to the cytotoxic actions of oxygen free radicals, including the cytotoxic actions of cytokines that are mediated by oxygen free radicals. Cells engineered

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according to the present invention will exhibit prolonged survival in such assays in comparison to cells containing wild type GLUT-2 transporters.

5       The *in vivo* test to confirm that the engineered cells have increased resistance to diabetic immune destruction requires simply that cells be placed within a permselective device, implanted into an animal and monitored for survival. Cells in accordance with this invention will exhibit increased survival or longevity in comparison to cells containing wild type GLUT-2 transporters.

10       Cell lines expressing such chimeric transporters will exhibit a correct GSIS response but will be protected from destructive immune killing when transplanted into IDDM patients in the context of permselective devices. Protection from destruction by small molecule killing of the immune system, as provided by the present invention, will mean that the cells will be substantially free from immune attack, as the  
15       permselective device will also provide the protection from antibody- and cell-mediated killing.

20       There are currently no viable strategies for long-term maintenance of islet or insulin-producing cell line grafts in IDDM patients other than immunosuppression, which renders the patient susceptible to a variety of infectious diseases. Thus, insulin producing cell lines that are responsive to glucose stimulation but also protected against immune destruction represent a major advance in cell-based insulin replacement therapy of IDDM.

25       The present discoveries may be utilized in conjunction with certain techniques that are well-known in the biological arts and that are further described in the following sections.

#### 30       A.     GLUT-2 Sequences

As used herein, in the context of GLUT-2 sequences and other genes, the terms "DNA segment" and "polynucleotide" refer to DNA molecules that have been isolated

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free of total genomic DNA of a particular species. Therefore, a GLUT-2 DNA segment or polynucleotide refers to a DNA segment that encodes GLUT-2 yet is isolated away from, or purified free from, total genomic DNA. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and  
5 also recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

The GLUT-2 polynucleotides or DNA segments may comprise an isolated or purified GLUT-2 gene including GLUT-2 coding sequences and, in certain aspects,  
10 regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences and cDNA sequences.

15 "Isolated substantially away from other coding sequences" means that the gene GLUT-2 gene forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and  
20 does not exclude genes or coding regions later added to the segment by the hand of man.

In particular embodiments, this invention concerns the use of isolated DNA segments and recombinant vectors incorporating DNA sequences that encode a  
25 GLUT-2 protein that includes within its amino acid sequence an amino acid sequence in accordance with SEQ ID NO:4, i.e., a sequence "essentially" as set forth in SEQ ID NO:4. The term "a sequence essentially as set forth in SEQ ID NO:4" means that the sequence substantially corresponds to a portion of SEQ ID NO:4 and has relatively few amino acids that are not identical to, or a biologically functional equivalent of, the  
30 amino acids of SEQ ID NO:4.

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5       The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein. Accordingly, sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:4 will be sequences that are "essentially as set forth in SEQ ID NO:4", with the important provision that to be "equivalent" the encoded protein must function essentially in the same way as GLUT-2.

10       In certain other embodiments, the invention concerns the use of isolated DNA segments and recombinant vectors that include within their sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:3. The term "essentially as set forth in SEQ ID NO:3" is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:3 and has  
15       relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:3. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids (Table 1).

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**Table 1**

| <b>Amino Acids</b> |               |     |   | <b>Codons</b> |     |     |     |     |     |
|--------------------|---------------|-----|---|---------------|-----|-----|-----|-----|-----|
| 5                  | 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 |     |     |     |     |
| 10                 | 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 |
| 15                 | 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 |
| 20                 | 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 |     |     |     |     |

25           Accordingly, DNA segments prepared in accordance with the present invention may also encode biologically functional equivalent proteins or peptides that have variant amino acids sequences. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally

30           equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based

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on considerations of the properties of the amino acids being exchanged, as described herein.

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological GLUT-2 protein activity following expression. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, i.e., introns, which are known to occur within genes.

Excepting intronic or flanking regions, and allowing for the degeneracy of the genetic code, sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of nucleotides that are identical to the nucleotides of SEQ ID NO:3 will be sequences that are "essentially as set forth in SEQ ID NO:3". Sequences that are essentially the same as those set forth in SEQ ID NO:3 may also be functionally defined as sequences that are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NO:3 under relatively stringent conditions. Suitable relatively stringent hybridization conditions are well known to those of skill in the art.

## **B. Biological Functional Equivalents**

Modification and changes may be made in the structure of GLUT-2 and still obtain a molecule having like characteristics for use, e.g., in the various selection protocols described herein. 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, substrates and effectors.

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Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein with like (agonistic) properties. Equally, it is these  
5 same type considerations that are employed to create a protein or polypeptide with counterveiling (or antagonistic) properties, as discussed herein in terms of modifying the GLUT-2 structure to provide second generation molecules that are do not transport STZ and/or that are not subject to immune attack.

10 In terms of maintaining GLUT-2 function essentially as in the wild type, it is thus contemplated by the inventors that various changes may be made in the sequence of GLUT-2 proteins or peptides (or underlying DNA) without appreciable loss of their biological utility or activity.

15 It is also well understood by the skilled artisan that, inherent in the definition of a biologically functional equivalent protein, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent GLUT-2s are thus defined herein as those proteins  
20 in which only certain, not most or all, of the amino acids have been substituted. To be equivalent, the overall function of GLUT-2 cannot be changed. However, a plurality of distinct proteins with different substitutions may easily be made and used in accordance with the invention.

25 It is also well understood that where certain residues are shown to be particularly important to the biological or structural properties of a protein or peptide, e.g., residues in active sites, such residues may not generally be exchanged. This is the case in the present invention where, for equivalents, STZ or glucose-binding residues may not be changed.

30

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity,

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charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents.

To effect more quantitative changes/In making such changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, 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).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  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. It is known that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein.

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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  $\pm$  1); glutamate (+3.0  $\pm$  1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5  $\pm$  1); alanine (-0.5); histidine (-0.5); cysteine (-1.0);  
5 methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those which are  
10 within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

While discussion has focused on functionally equivalent proteins arising from amino acid changes, it will be appreciated that these changes may be effected by  
15 alteration of the encoding DNA; taking into consideration also that the genetic code is degenerate and that two or more codons may code for the same amino acid. Table 1, a table of amino acids and their codons, is presented herein for use in such embodiments, as well as for other uses, such as in the design of probes and primers and the like.

20

### C. Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of modified proteins through specific mutagenesis of the underlying DNA. The technique further  
25 provides a ready ability to prepare and test sequence variants by introducing one or more nucleotide sequence changes into the DNA. The techniques are generally well known, as exemplified by U.S. Patent 4,888,286, incorporated herein by reference.

Site-specific mutagenesis allows the production of mutants through the use of  
30 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

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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.

5           As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage (Messing *et al.*, 1981). These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely  
10 employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

          In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart the two strands of a double  
15 stranded vector which includes within its sequence a DNA sequence which encodes GLUT-2. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Crea *et al.* (1978). This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to  
20 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 which include recombinant vectors bearing the mutated sequence arrangement. Further relevant  
25 publications include Adelman *et al.* (1983).

          The preparation of sequence variants of GLUT-2 using site-directed mutagenesis is provided as one means of producing useful species and is not meant to be limiting as there are other ways in which sequence variants of GLUT-2 may be  
30 obtained. For example, recombinant vectors encoding GLUT-2 may be treated with mutagenic agents to obtain sequence variants (see, *e.g.*, a method described by Eichenlaub, 1979) for the mutagenesis of plasmid DNA using hydroxylamine.

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#### D. Vectors and Promoters

Expression vectors for use in mammalian such cells ordinarily include an origin of replication (as necessary), a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences. The origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (*e.g.*, Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

The promoters may be derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). Further, it is also possible, and may be desirable, to utilize promoter or control sequences normally associated with GLUT-2, provided such control sequences are compatible with the host cell systems.

A number of viral based expression systems may be utilized, for example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication.

Specific initiation signals may also be required for efficient translation of GLUT-2 coding sequences. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may additionally need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be in-frame (or in-phase) with the

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reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription  
5 terminators (Bittner *et al.*, 1987).

In eukaryotic expression, one will also typically desire to incorporate into the transcriptional unit an appropriate polyadenylation site (e.g., 5'-AATAAA-3') if one was not contained within the original cloned segment. Typically, the poly A addition  
10 site is placed about 30 to 2000 nucleotides "downstream" of the termination site of the protein at a position prior to transcription termination.

### 1. $\beta$ -Cell-Specific Promoters

15 It has been documented that the two rat insulin gene promoters, RIP1 (GenBank accession number J00747) and RIP2 (GenBank accession number J00748), as well as the human insulin promoter (HIP; GenBank accession number V00565), direct stringent cell-specific expression of the insulin gene in rodent  $\beta$ -cell insulinoma lines primary islet cells, and in  $\beta$ -cells of transgenic mice.

20

As the sequence and position of the functional promoter elements are well conserved between HIP, RIP1 and RIP2, the transcription factors that interact with these elements are likely to be conserved across species. In fact, HIP can direct cell-specific expression of linked genes in rodent  $\beta$ -cell lines and rat primary islets, albeit,  
25 at a somewhat lower level than that observed for RIP1.

### 2. Modified Promoters

Promoters can be modified in a number of ways in an attempt to increase their  
30 transcriptional activity. Multiple copies of a given promoter can be linked in tandem, mutations which increase activity may be introduced, single or multiple copies of individual promoter elements may be attached, parts of unrelated promoters may be

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fused together, or some combination of all of the above can be employed to generate highly active promoters.

Three nucleotides in the transcriptionally important FLAT E box of the rat insulin I gene promoter (RIP) have been mutated, resulting in a three- to four-fold increase in transcriptional activity of the mutated RIP compared to that of a nonmutated RIP as assayed in transiently transfected HIT cells. Also, the introduction of multiple copies of a promoter element from the *E. coli* tetracycline resistance operon promoter were introduced into the CMV promoter, significantly increasing the activity of this already very potent promoter. Additionally, part of the CMV promoter, which has high but short-lived transcriptional activity in dog myoblasts, was linked to the muscle-specific creatine kinase promoter (MCKp), which has weak but sustained expression in dog myoblasts, resulting in a hybrid promoter that sustained high-level expression for extended periods in dog myoblasts.

### 3. Multimerized Promoters

Several modified rat insulin promoters (modRIP) containing multimerized enhancer elements have been engineered. The currently preferred modRIP contains six multimerized repeats of a 50 base pair region of the *cis* acting enhancer of RIP, placed upstream of an intact copy of RIP.

These novel promoters have been shown to direct expression of transgenes in stably engineered  $\beta$ -cell lines at levels above those attained with unmodified insulin promoters and, in some cases, approaching the levels achieved with the Cytomegalovirus promoter (CMVp). CMVp is one of the strongest activating promoters known, but in a very non-tissue specific manner. Therefore, the present modified rat insulin promoters can be used to direct the tissue specific expression of genes at levels presently achievable only with the non-specific CMVp.

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**E. Drug Selectable Marker Genes**

Although the operation of drug selection or antibiotic resistance techniques is well known in the art, the following additional information is provided regarding the  
5 use of conventional antibiotic resistance markers.

References that describe selection techniques include Sambrook *et al.* (1989), which provides descriptions relevant to various aspects of molecular biology; U.S. Patent 4,752,574, that concerns antibiotic resistance vectors; U.S. Patent 4,430,434 and  
10 Colberre-Garapin *et al.* (1981) that relate to plasmids for conferring streptomycin and neomycin resistance; Santerre *et al.* (1984) and U.S. Patents 4,727,028, 4,960,704 and 4,559,302, that concern hygromycin resistance cloning vectors, modified hygromycin resistance genes and promoters; U.S. Patents 5,238,820, 5,179,017, 4,634,665, 4,399,216 and 5,164,490 and Wigler *et al.* (1980) and O'Hare *et al.* (1981) concern  
15 methods for use with selectable marker genes, including dihydrofolate reductase genes; U.S. Patent 5,021,344, that concerns phleor genes for conferring resistance to phleomycin antibiotics, such as Zeocin; and U.S. Patent 5,128,255 that concerns selection methods employing a gene that encodes a histidinol dehydrogenase. Each of the foregoing references are specifically incorporated herein by reference for the  
20 purposes of providing additional disclosure relating to antibiotic resistance vectors.

The use of phosphoribosyl transferases (Lowy *et al.*, 1980; Szybalska *et al.*, 1962), such as xanthine-guanine phosphoribosyl transferase, is also well established in the art, for example, in the context of monoclonal antibody generation. U.S. Patent  
25 4,196,265 is incorporated herein by reference to provide additional disclosure relating to this subject area.

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## **F. DNA Delivery**

### **1. Transfection**

5           In order to effect expression of a gene construct, the expression construct must be delivered into a cell. As described below, the preferred mechanism for delivery is via viral infection, where the expression construct is encapsidated in an infectious viral particle. However, several non-viral methods for the transfer of expression constructs into cells also are contemplated by the present invention. In one embodiment of the  
10       present invention, the expression construct may consist only of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned which physically or chemically permeabilize the cell membrane.

### **2. Liposome-Mediated Transfection**

15           In a further embodiment of the invention, an 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  
20       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. Also contemplated is an expression construct complexed with Lipofectamine (Gibco BRL).

25           Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful, and the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells has been shown.

30           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

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membrane and promote cell entry of liposome-encapsulated DNA. In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1.

5

Melloul *et al.* (1993) demonstrated transfection of both rat and human islet cells using liposomes made from the cationic lipid DOTAP, and Gainer *et al.* (1996) transfected mouse islets using Lipofectamine-DNA complexes.

10

### **3. Electroporation**

In certain embodiments of the present invention, the expression construct is introduced into the cell via electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge.

15

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes, and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene in this manner.

20

Examples of electroporation of islets include PCT application WO 91/09939.

### **4. Calcium Phosphate Precipitation or DEAE-Dextran Treatment**

25

In other embodiments of the present invention, the expression construct is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5 DNA using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene, and rat hepatocytes were transfected with a variety of marker genes.

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In another embodiment, the expression construct is delivered into the cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells.

5                   **5. Particle Bombardment**

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  
10 pierce cell membranes and enter cells without killing them. 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. The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

15

Gainer *et al.* (1996) have transfected mouse islets with a luciferase gene/human immediate early promoter reporter construct, using biolistic particles accelerated by helium pressure.

20                   **6. Direct Microinjection or Sonication Loading**

Further embodiments of the present invention include the introduction of the expression construct by direct microinjection or sonication loading. Direct microinjection has been used to introduce nucleic acid constructs into *Xenopus* oocytes  
25 and LTK<sup>-</sup> fibroblasts have been transfected with the thymidine kinase gene by sonication loading.

**7. Adenoviral Assisted Transfection**

30                   In certain embodiments of the present invention, the expression construct is introduced into the cell using adenovirus assisted transfection. Increased transfection efficiencies have been reported in cell systems using adenovirus coupled systems, and

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the inventors contemplate using the same technique to increase transfection efficiencies into human islets.

## 8. Receptor Mediated Transfection

5

Still further expression constructs that may be employed to deliver GLUT-2 constructs to target cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis that will be occurring in the target cells. In view of the cell type-specific distribution of various receptors, this delivery method adds another degree of specificity to the present invention.

Certain receptor-mediated gene targeting vehicles comprise a cell receptor-specific ligand and a DNA-binding agent. Others comprise a cell receptor-specific ligand to which the DNA construct to be delivered has been operatively attached. Several ligands have been used for receptor-mediated gene transfer which establishes the operability of the technique.

In other embodiments, the DNA delivery vehicle component of a cell-specific gene targeting vehicle may comprise a specific binding ligand in combination with a liposome. The nucleic acids to be delivered are housed within the liposome and the specific binding ligand is functionally incorporated into the liposome membrane. The liposome will thus specifically bind to the receptors of the target cell and deliver the contents to the cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor-mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

In still further embodiments, the DNA delivery vehicle component of the targeted delivery vehicles may be a liposome itself, which will preferably comprise one or more lipids or glycoproteins that direct cell-specific binding. For example, employing lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes increases uptake of the insulin gene by hepatocytes.

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## 9. Adenoviral Vectors and Infection

One of the preferred methods for delivery of GLUT-2 constructs involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a GLUT-2 that has been cloned therein.

10

Adenovirus vectors, and preferably replication defective vectors, are particularly useful in the context of the present invention. They may be constructed by well-known methods, for example, as achieved through the deletion of the viral early region 1 (E1A) region such that the virus is competent to replicate only in cells, such as human 293 cells, which express adenovirus early region 1 genes from their cellular genome. This is important because the virus will therefore not kill normal cells that do not express early gene products. Techniques for preparing replication defective adenoviruses are well known in the art as exemplified by Berkner *et. al.*, 1983; McGrory *et. al.*, 1988; and Gluzman *et. al.*, 1982). Rosenfeld *et. al.* (1991; 1992) and Stratford-Perricaudet *et. al.* (1990; 1992) also describe uses of adenovirus. Each of the foregoing references are incorporated herein by reference.

Other than the requirement that the adenovirus vector be replication defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of these aspects 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 method of 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.

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The promoter used to express GLUT-2 in an adenovirus is not critical to the present invention. The human cytomegalovirus (CMV) immediate early gene promoter has been used to result in constitutive, high-level expression of the foreign gene. Other promoters are described in Example IX. However, the use of still further viral  
5 or mammalian cellular promoters which are well-known in the art is also suitable to achieve expression of GLUT-2, provided that the levels of expression are sufficient to achieve a physiologic effect.

Promoters that are active specifically in a given cell type can also be used. In  
10 addition to the insulin promoter, other examples include the  $\alpha$ 1-antitrypsin, apolipoprotein AI, liver fatty acid binding protein, LDL receptor, or plasminogen activator inhibitor type 1 (PAI-1) gene promoters for tissue-specific expression in liver cells.

15 Further, the selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of GLUT-2. For example, with the GLUT-2 gene being expressed from the human PAI-1 promoter, expression is inducible by tumor necrosis factor.

20 In that the vectors for use with these aspects of the present invention are replication defective, they will typically not have an adenovirus E1 region. Thus, it will be most convenient to introduce the GLUT-2 coding region at the position from which E1 coding sequences have been removed. However, the position of insertion of the GLUT-2 coding region within the adenovirus sequences is not critical to the  
25 present invention. The GLUT-2 transcription unit may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described previously by Karlsson *et. al.* (1986).

Moreover, where a cDNA insert is employed one will typically desire to  
30 include a polyadenylation signal to effect proper polyadenylation of the GLUT-2 message. 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.

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In further embodiments, the invention relates to pharmaceutical compositions wherein the adenovirus/GLUT-2 gene construct is dispersed in a pharmacologically acceptable solution or buffer. Preferred solutions include neutral saline solutions buffered with phosphate, lactate, Tris, and the like. Of course, one will desire to purify the vector sufficiently to render it essentially free of undesirable contaminant, such as defective interfering adenovirus particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

#### 10. AAV Infection

Adeno-associated virus (AAV) is an attractive vector system for use in the human cell transformation of the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue culture. AAV has a broad host range for infectivity, which means it is applicable for use with human cells. Details concerning the generation and use of rAAV vectors are described in U.S. Patent No. 5,139,941 and U.S. Patent No. 4,797,368, each incorporated herein by reference. Recently, an AAV vector has been approved for phase I human trials for the treatment of cystic fibrosis.

AAV is a dependent parvovirus in that it requires coinfection with another virus (either adenovirus or a member of the herpes virus family) to undergo a productive infection in cultured cells. In the absence of coinfection with helper virus, the wild type AAV genome integrates through its ends into human chromosome 19 where it resides in a latent state as a provirus. rAAV, however, is not restricted to chromosome 19 for integration unless the AAV Rep protein is also expressed. When a cell carrying an AAV provirus is superinfected with a helper virus, the AAV genome is "rescued" from the chromosome or from a recombinant plasmid, and a normal productive infection is established.

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Typically, recombinant AAV (rAAV) virus is made by cotransfecting a plasmid containing the gene of interest flanked by the two AAV terminal repeats and an expression plasmid containing the wild type AAV coding sequences without the terminal repeats, for example pIM45. The cells are also infected or transfected with adenovirus or plasmids carrying the adenovirus genes required for AAV helper function. rAAV virus stocks made in such fashion are contaminated with adenovirus which must be physically separated from the rAAV particles (for example, by cesium chloride density centrifugation). Alternatively, adenovirus vectors containing the AAV coding regions or cell lines containing the AAV coding regions and some or all of the adenovirus helper genes could be used. Cell lines carrying the rAAV DNA as an integrated provirus can also be used.

## 11. Retroviral Infection

15

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. 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.

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. When a recombinant plasmid containing a cDNA, together

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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. The media containing the recombinant  
5 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.

Concern with the use of defective retrovirus vectors is the potential appearance  
10 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.

15

## **12. Other Viral Vectors**

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus and herpesviruses may  
20 be employed. They offer several attractive features for various mammalian cells.

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  
25 and reverse transcription despite the deletion of up to 80% of its genome.

In still further embodiments of the present invention, the nucleic acids to be delivered are housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate  
30 receptors of the target cell and deliver the contents to the cell. 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

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residues to the viral envelope. This modification can permit the specific infection of hepatocytes via sialoglycoprotein receptors.

Another 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. 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*.

10

#### **G. Functional Separation**

The use of temperature sensitive constructs allows for turning gene expression on and off. Promoters capable of driving expression of GLUT-2 in response to an exogenously added compound would allow for conditional expression of GLUT-2. Expression of GLUT-2 is turned off, unless the activating factor is provided. Examples of such systems include the lac repressor system and tetracycline regulatory system (U.S. Patent 5,464,758; incorporated herein by reference).

20 The present invention contemplates the use of the *Cre/Lox* site-specific recombination system (available through Gibco/BRL, Inc., Gaithersburg, Md.) to rescue specific genes out of a genome. Briefly, the system involves the use of a bacterial nucleotide sequence known as a *LoxP* 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 *LoxP* site or excise sequences that lie between two *LoxP* sites. Thus, if a construct for insertion also has flanking *LoxP* sites, introduction of the *Cre* protein, or a polynucleotide encoding the *Cre* protein, into the cell will catalyze the removal of the construct DNA. This technology is enabled in U.S. Patent No. 4,959,317, which is hereby incorporated by reference in its entirety.

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The present invention also contemplates the use of recombination activating genes (RAG) 1 and 2 to rescue specific genes from the genome of transformed cell lines. RAG-1 (GenBank accession number M29475) and RAG-2 (GenBank accession numbers M64796 and M33828) recognize specific recombination signal sequences (RSSs) and catalyze V(D)J recombination required for the assembly of immunoglobulin and T cell receptor genes. Transgenic expression of RAG-1 and RAG-2 proteins in non-lymphoid cells supports V(D)J recombination of reporter substrates. For use in the present invention, is engineered to contain flanking RSSs. Following transformation, the GLUT-2 construct that is internal to the RSSs can be deleted from the genome by the transient expression of RAG-1 and RAG-2 in the cell.

#### H. Cell Implantation

It is proposed that engineered cells of the present invention that respond to glucose by secreting insulin may be introduced into animals with insulin dependent diabetes. Although ideally cells are engineered to achieve glucose dose responsiveness closely resembling that of islets, 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.

Cells that express modified or chimeric GLUT-2 in accordance herewith may be implanted using the alginate-polylysine encapsulation technique of O'Shea and Sun (1986), with modifications as recently 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  $\text{CaCl}_2$ . 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

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rewashed with Krebs balanced salt buffer. Each capsule should contain several hundred cells and have a diameter of approximately 1 mm.

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, *et al.*, 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.

20

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  $\beta$ -cells, although there is no clear evidence that such granules are collected and stored inside macrophages as they are in  $\beta$ -cells (Stossel, 1987).

30

Nevertheless, it may ultimately be possible to use the recombinant DNA for second generation glucose transporters derived from GLUT-2 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.

10

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 that follow represent techniques discovered by the inventors 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 that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

20

## EXAMPLE I

### Cells, Plasmids, Genes and Sequences

**Cell Lines.** Various cell lines derived from the rat insulinoma RIN 1046-38 line (Clark, *et al.*, 1990) are grown in Medium 199 with Earle's salts, containing 11 mM glucose and 5% FBS. The anterior pituitary cell line AtT-20ins (Moore, *et al.*, 1983) and derivatives are grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, New York) containing 25 mM glucose, and supplemented with 10% fetal calf serum (Mediatech, Washington, D. C.), 100 mU/ml penicillin and 100 µg/ml of streptomycin. Modified AtT-20ins cell lines evaluated include CGT-6 and GT1-8 or GT1-15, derived by stable transfection of AtT-20ins cells with the pCB-7 plasmid containing the rat GLUT-2 or human GLUT-1 cDNAs, respectively (Hughes, *et al.*,

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1992 and 1993). 293, 3T6, Vero and CV-1 cell lines were obtained from the ATCC and cultured as recommended.

SEQ ID NO:1 is the sequence of the human GLUT-1 cDNA used in  
5 pCB-7/GLUT-1. The sequence is 1815 base pairs corresponding to sequences -165 to +1650 of the published sequence (Mueckler, *et al.*, 1985). The deduced amino acid sequence encoded by the GLUT-1 cDNA is SEQ ID NO:2. SEQ ID NO:3 is the sequence of the rat GLUT-2 cDNA fragment used in pCB-7/GLUT-2. The sequence is 1943 base pairs corresponding to sequences -108 to + 1835 of the published  
10 sequence (Thorens, *et al.*, 1988). The deduced amino acid sequence encoded by the GLUT-2 cDNA is SEQ ID NO:4.

GLUT-2 expressing rat insulinoma lines were derived from RIN 1046-38 cells by stable transfection with the pCB-7 plasmid containing the rat GLUT-2 cDNA, and  
15 were initially designated RIN 30-5, RIN 30-7, and RIN 30-10 (Ferber, *et al.*, 1994). Note that in referring to these lines, the first number (30) refers to passage number and will vary slightly through the course of the text while the second number (5,7,10) refers to the specific clone.

20 Stable transfectants of pCB7/GLUT-2 were selected in hygromycin using 300 µg/ml hygromycin (Boehringer Mannheim) for 14 days without media changes. Alternatively, the GLUT 2 cDNA of SEQ ID NO:3 (bases -108 to 1835) and the SV40 early polyadenylation signal were cloned in to pCB7intron, generating pCB7intron/GLUT-2 (FIG. 1). pCB7intron is identical to CB7 except for the addition  
25 of a 5' leader sequence of the adenovirus tri-partite leader (+14 to +154 of the 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 (Kaufman and Sharp, 1982). RIN GT1-3 cells were derived from RIN 1046-38 cells by stable transfection with the pCB-7 plasmid without a GLUT-2 insert (Ferber,  
30 *et al.*, 1994).

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**Plasmids.** Several other GLUT-2 expression plasmids utilizing other drug selection markers or alternative promoters have also been designed and are derivatives of the plasmid depicted in FIG. 1. Examples of promoters include the Rous Sarcoma Virus (RSV) Long terminal repeat, the rat Insulin I gene promoter/ enhancer, the human  
5 glyceraldehyde-3-phosphate dehydrogenase promoter, the mouse metallothionein promoter, and the SV40 promoter.

Examples of other drug selection markers include the neomycin resistance gene (NEO) conferring resistance to G418, the puromycin resistance gene (PURO), the  
10 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).

15 By way of example, the following GLUT-2 expression plasmids have been constructed and tested in RIN cells. pCB6/intron was constructed from pCB6 (Brewer, *et al.*, 1994) by addition of a 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  
20 region immunoglobulin gene (Kaufman and Sharp, 1982).

The neomycin resistance gene in pCB6/intron was removed by restriction endonuclease digestion with NarI and BclI, followed by treatment with Klenow fragment. The puromycin resistance gene was isolated from plasmid pPUR  
25 (Clontech, Inc.) as a 723 PstI/ NcoI fragment, treated with Klenow and ligated into the pCB6/intron backbone. The resulting plasmid, pCB10, now contains the puromycin resistance gene driven by the SV40 promoter for selection of stable transfectants as well as the CMV promoter for driving expression of genes of interest.

30 The GLUT-2 cDNA (corresponding to bases -108 to 1642 of the published sequence, Thorens, *et al.*, 1988; SEQ ID NO:3) was cloned into pCB10 generating pCB10/GLUT-2. Subsequently, the CMV promoter was removed by digestion with

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SpeI and EcoRI (removing bases -585 to +1 of the CMV promoter relative to start site of transcription) followed by treating with Klenow fragment. The RSV promoter was isolated as a 637 base SalI/ PvuII restriction fragment from pREP4 (Invitrogen, Inc.), treating with Klenow fragment and ligating into the pCB10/GLUT-2 backbone, generating pRSV8/GLUT-2(SV/PURO).

Alternatively, the rat insulin I promoter was isolated as a 425 base KpnI/HindIII fragment from pacRIP (obtained from Larry Moss), corresponding to bases -416 to +4 relative to the start site of transcription, treated with Klenow and ligated into the pCB10/GLUT-2 backbone, generating pRIP8/GLUT-2(SV/PURO).

Stable transfectants of pCB10/GLUT-2, pRSV8/GLUT-2(SV/PURO), and pRIP8/GLUT-2(SV/PURO) 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.

15

## EXAMPLE II

### Transfer of GLUT-2 into Cell Lines via Recombinant Adenovirus

20 A recombinant adenovirus containing the GLUT-2 cDNA was prepared as previously described (Ferber, *et al.*, 1994 and Becker, *et al.*, 1994; incorporated herein by reference). Briefly, the rat GLUT-2 cDNA consisting of the DNA spanning nucleotide -108 (relative to the translation start) through +1835 (SEQ ID NO:1) (the designation "+" or "-" refers to the AUG codon marking the start site of translation and numbering based on the published sequence of the rat GLUT-2 cDNA (Thorens, 25 *et al.*, 1988)) was cloned in vector pACCMV.pLpA immediately adjacent to its cytomegalovirus (CMV) promoter/enhancer.

The pACCMV.pLpA plasmid containing GLUT-2 was recovered, amplified, 30 and purified by conventional methods, and then combined with the adenovirus plasmid pJM17 to cotransfect the AdE1A-transformed human embryonic kidney cell line 293.

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Following lysis of the cells some two weeks after co-transfection, the lysate was recovered and stored at 4°C.

Since the pJM17 plasmid contains the entire adenovirus 5 genome interrupted  
5 with the pBRX sequence inserted at 3.7 map units, it exceeds the packaging limit. The pACCM.pLpA plasmid contains some 17 map units of the left half of the adenovirus 5 genome, but with the CMV promoter/GLUT-2 sequence substituted for the AdE1A region. Thus, live virus can only be produced in the 293 background after recombination of the two vectors (note, E1A is obtained from the 293 cellular  
10 genome). The resulting virus is replication defective in cells other than 293 cells, but fully infectious, thus serving as an efficient gene transfer vehicle.

### EXAMPLE III

#### Implantation of Insulinoma Cells into Non-Diabetic Rats

15

**Animals and Cell Implantation.** Athymic Fisher nude rats (strain F344/NCr-rnu) were purchased from the NIH National Cancer Institute, Frederick, Maryland and housed in a sterile isolation animal facility with free access to sterile standard laboratory chow and water. Animals received cell implants at 8-12 weeks of age  
20 (130-210 grams).

RIN 36-10 or GT1-3 cells were injected subcutaneously ( $50-100 \times 10^6$  cells per injection) between the shoulder blades of normal (non-diabetic) recipient animals under sodium pentobarbital anesthesia (approximately 35 mg/kg body weight). *In vivo*  
25 studies with the GLUT-2 overexpressing beta-cell line 49/206 was similarly injected subcutaneously, but with  $3-6 \times 10^6$  cells/rat. Blood was collected from ad-lib fed animals at 1-3 day intervals from a tail vein for 2-5 weeks postimplantation to allow measurement of serum insulin and glucose levels.

30 **Blood Analysis.** Blood glucose levels were determined with a glucose oxidase kit (Boehringer Mannheim, Germany). Plasma insulin levels were measured by radioimmunoassay using a porcine insulin standard.

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**Results.** Athymic nude rats received subcutaneous implants of the GLUT-2 expressing RIN cell line 36-10 and the control RIN cell line lacking GLUT-2 expression, RIN GT1-3 (Example I). Nondiabetic animals that received either cell line became hypoglycemic within 2 weeks of cell implantation, apparently in response to an approximate 3-fold increase in the concentration of insulin in the circulation, as shown in FIG. 2A, FIG. 2B and FIG. 2C. Studies were terminated by 12-14 days after cell implantation as the tumors reached a mass of 1-1.5 grams and the animals became severely hypoglycemic (blood glucose concentrations of  $\leq 30$  mg/dl).

The tumors produced insulin in quantities sufficient to impact  $\beta$ -cell function and morphology. Islets from insulinoma-containing rats exhibited marked reductions in the intensity of insulin and GLUT-2 immunofluorescence compared to control rats that were not implanted. The volume fraction of the endocrine pancreas was also reduced from 0.18% in control rats to 0.11% in insulinoma-bearing rats. These data show that the RIN cell lines under study thrive when implanted into nondiabetic nude rats and produce quantities of insulin sufficient to cause hypoglycemia and reduced  $\beta$ -cell mass, even in the face of an intact counterregulatory response.

#### EXAMPLE IV

##### Effect of STZ on Cell Lines *In Vivo*

**Treatment Methods.** Diabetes was induced in nude rats by intraperitoneal injection of STZ (Sigma Chemical Co.), freshly dissolved in 50 mM sodium citrate in 0.45% NaCl, pH 4.5. An initial dose of 110 mg/kg, followed by an injection of 55 mg/kg 24 hours later, was delivered to animals implanted with RIN lines 36-10 or GT1-3. Two of the three animals implanted with line 36-10 also received additional doses of 110 mg/kg and 55 mg/kg at 48 and 72 hours after the initial injection, respectively. The injections were administered approximately 2-3 weeks after cell implantation, a time at which the animals had a clear tumor mass and were becoming hypoglycemic (approximately 50 mg/dl blood glucose). The degree of  $\beta$ -cell destruction in STZ-injected animals was evaluated by surgical resection of the solid tumor and subsequent monitoring of blood glucose levels.

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**Tissue Analysis.** Insulin and GLUT-2 levels were evaluated in islets from animals implanted with RIN cells both before and after STZ injection by indirect immunofluorescence of 5 micron sections of pancreas fixed in Bouin's solution, using methods and antisera that have been described previously (Ogawa, *et al.*, 1992). The volume fraction of insulin positive cells in animals without implanted cell lines and with implanted RIN cells was compared by the stereologic method of Weibel, 1979, on 25 independent frames from each pancreas section. The effect of STZ on the insulinoma tumors expressing or lacking GLUT-2 was evaluated by embedding tissue in paraffin, and staining of 5  $\mu$ m sections with hematoxylin followed by eosin.

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**Results.** RIN 36-10 and GT1-3 cells were injected as described above in Example III, and as the animals began to become hypoglycemic (blood glucose levels of approximately 50 mg/dl), STZ was injected as two large doses (110 mg/kg followed by 55 mg/kg 24 hours later).

15

As seen in FIG. 3A, all three animals implanted with untransfected RIN cells progressed rapidly to severe hypoglycemia, with decreases from 50 mg/dl glucose prior to the first STZ injection to 20 mg/dl 24 hours after the second STZ injection. Upon surgical resection of the tumor, all three animals in the study rapidly developed hyperglycemia, indicating that the STZ had in fact destroyed the islet  $\beta$ -cells but not the insulinoma cells, which had easily compensated for the absence of islet insulin production.

20

Injection of STZ into animals implanted with GLUT-2 expressing RIN cells resulted in rapid onset of diabetes in one animal, and transient increases in blood glucose in the other two animals, as shown in FIG. 3B. In the two animals with transient increases in glucose, further STZ injections resulted in rapid onset of hyperglycemia without requirement for tumor resection. Animals containing RIN cells lacking GLUT-2 could not be injected with additional doses of STZ because of the severity of their hypoglycemia.

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Destruction of islet  $\beta$ -cells in both groups of STZ-injected animals was confirmed by the complete absence of insulin immunofluorescence in Bouin's-fixed pancreas sections. STZ had a marked cytotoxic effect on RIN cells *in vivo* that was specific to GLUT-2 expressing cells. Histologic sections of GLUT-2 expressing cells were characterized by necrotic cells and a heterogenous cellular composition. In contrast, sections from tumors lacking GLUT-2 expression comprised of a largely homogenous cell mass with no signs of necrosis.

### EXAMPLE V

#### STZ Transport *In Vitro*

**Measurement of STZ Uptake.** The various RIN and AtT-20ins cell lines were grown to semi-confluence in 6-well dishes. The culture medium was replaced with RPMI 1640 media containing 1 mM glucose and STZ or N-nitrosomethyl urea (Sigma Chemical Co.) was added to a final concentration of 10 mM, (from a fresh 0.5 M stock solution of these agents in 50 mM sodium citrate, 0.45% NaCl, pH 4.5). Control cells received the sodium citrate/NaCl vehicle alone.

STZ uptake was measured over a range of time points from 30 seconds to 120 minutes, while N-nitrosomethylurea transport was evaluated over the first 10 minutes. Uptake measurements were terminated by addition of 200  $\mu$ l 0.6 M perchloric acid, immediate collection of the cells into a 0.5 ml Eppendorf tube by scraping with a rubber policeman, and centrifugation in a microcentrifuge at approximately 15,000 rpm for 10 minutes.

STZ or N-nitrosomethylurea were detected by mixing 200  $\mu$ l of the cell supernatant with 0.5 ml color reagent (0.5% sulfonilic acid, 0.2% (N-1-Naphthyl) ethyldiamine dihydrochloride in 30% acetic acid) and 0.1 ml 6 N HCl (Forist, 1964). The mixture was incubated at 60°C for 45 minutes and the absorbance at 550 nm was determined with a Beckman DU-64 spectrophotometer and converted to  $\mu$ g of STZ or N-nitrosomethylurea by fitting to the appropriate standard curve prepared with solutions of these reagents in acetate buffer (0.1 M acetic acid, 0.02 M sodium acetate,

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pH 4.0) ranging in concentration from 2-100  $\mu\text{g/ml}$ . Data were normalized against total protein in the cell pellet, measured with the technique of Bradford (Bradford, 1976), with reagents and a protocol supplied by BioRad, Inc. (Hercules, CA).

5     **Results.** In keeping with the cytotoxicity studies (as described in Example VIII), expression of GLUT-2 exhibited a profound effect on cellular uptake of STZ (10mM; FIG. 4A). Untransfected RIN or AtT-20ins cells or AtT-20ins cells engineered for GLUT-1 overexpression (line GT1-8) all exhibited very low rates of STZ transport, with a slow linear increase in accumulation noted over the 120 minute experimental  
10     period. In stark contrast, RIN or AtT-20ins cells transfected with GLUT-2 (lines 36-10 and CGT-6, respectively) transported STZ with high efficiency. The accumulation of STZ in the two GLUT-2 expressing lines are virtually identical. The maximum intracellular STZ content reached 0.32  $\mu\text{g/ml}/\mu\text{g}$  cellular protein, roughly 10 times more than observed for the untransfected or GLUT-1 transfected cells at the  
15     120 minute time point.

In the rapid first phase of STZ transport into GLUT-2 transfected lines half-maximal uptake of STZ was achieved at approximately 1.5-2 minutes, and maximal uptake occurred at 4-6 minutes (FIG. 4B). 3-O-methyl glucose transport into  
20     rat islet cells (Johnson, *et al.*, 1990) or GLUT-2 transfected RIN or AtT-20ins cells (Hughes, *et al.*, 1992 and 1993, Ferber *et al.*, 1994) occurs more rapidly, with maximal uptake (equilibration) achieved within 1 minute, even when the assay is performed at 16°C (the STZ uptake studies described here were performed at room temperature). The slightly slower rate of STZ transport relative to 3-O-methyl glucose  
25     may be related to the fact that STZ is metabolized in cells, with a half life of approximately 10 minutes, while 3-O-methyl glucose is not metabolized.

The inventors' results thus indicate that STZ is specifically transported by GLUT-2 and that the preferential uptake and toxicity of the drug is not due to a  
30     non-specific attribute of the transporter.

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First, the inventors have examined the ability of STZ to inhibit 3-O-methyl glucose uptake into rat islets and find that addition of STZ in a 1:1 molar ratio with 3-O-methyl glucose inhibits uptake of the latter by 50% relative to transport in the absence of the drug. Lower concentrations of STZ have little effect, suggesting that  
5 STZ is a transported substrate that is behaving as a competitive inhibitor in these studies.

Second, NMU is not effectively transported by GLUT-2 expressing cells (FIG. 5) indicating that efficient transport of STZ into GLUT-2 expressing cells  
10 requires the glucose moiety. GLUT-2 transfected CGT-6 cells transport STZ efficiently over 10 minutes, but neither these cells nor GLUT-1 transfected cells take up free N-nitrosomethylurea.

#### EXAMPLE VI

##### 15 GLUT-2 Expression Correlates with STZ Toxicity

**Western Blot Analysis of GLUT-2 Expression.** Cell lysates were prepared from  $\geq$  500,000 cells. The cells were washed with PBS, centrifuged and the pellet was retained and placed on ice. Cell lysates were then prepared by resuspending the cell  
20 pellet in 50-75 $\mu$ l cold homogenization buffer (20 mM KHPO<sub>4</sub>, 100mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml AEBSF) and sonicating for three short bursts (setting 2, Sonic Dismembrator 50, Fisher Sci.). Insoluble material was pelleted with a 10 min. spin at 12,000 rpm in a refrigerated tabletop centrifuge.

25 Protein concentration was determined by the method of Bradford (Bradford, 1976), and 10 $\mu$ g of sample protein in a total volume of 10-15 $\mu$ l was mixed with an equal volume of 2X sample buffer (0.1 M Tris. pH 6.8 with 4% sodium dodecyl sulfate (SDS), 0.2% bromphenol blue, 20% glycerol), and loaded without heating.  
30 Samples were loaded onto 4-20% Tris-glycine gels (Novex, Inc. or Biorad, Inc.). The gel was run at 130V (Novex, Inc.) or 200V (Biorad, Inc.) in 1X running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS).

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The proteins were then transferred to a PVDF membrane (Biorad, Inc.) in 1X transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 for 1hr at 30V (Novex, Inc.) or 100V (Biorad, Inc.). The blot with transferred protein was incubated on a platform shaker in 20-30ml of TBST (150 mM NaCl, 0.05% Tween 20, 10 mM Tris, pH 8.0) with 1% milk for 1hr at RT to block unspecific binding sites. The blot was then incubated with anti-Glut-2 antibody (EastAcres Biologicals, Inc.) at a dilution of 1:5,000 in 10-15ml TBST/milk with 1% BSA (Sigma Chemical Co.) for 4hr on a shaking platform at RT. This incubation was followed with a 20 minute wash of the blot in 50-100ml TBST at RT on a shaking platform. The blot was then incubated with 10ml fresh TBST/1% milk and 1: 8,000 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma Chemical Co.) for 1-2hr on a shaking platform at RT. This was followed by a wash as described above. The immunoreactive proteins were then visualized by incubation in 10ml substrate buffer (66 $\mu$ l NBT and 66 $\mu$ l BCIP in 0.05 M Tris, pH 9) for 1-10min until bands were of desired intensity. The blot was washed with water and dried.

**Northern blotting.** Northern blot analysis was used to detect steady-state levels of mRNAs. Media was aspirated from plates and washed with PBS. RNAzol (Tel-Test, Inc.) was added to the plate (0.9ml per p100 plate), swirled and allowed to sit at room temperature for 2-3 minutes. The lysed cells were removed with a cell scraper and the mixture pipetted several times to mix and disperse. The mixture was transferred to an 1.5ml eppendorf and placed on ice for 5-10 minutes. Chloroform (350ul) was added, and the tube vortexed vigorously, then transferred to ice for 10-15 minutes. The sample was then centrifuged for 15 minutes at maximum speed (11000 to 15000 rpm), and the top, aqueous phase transferred to a clean tube.

The RNA was precipitated by adding an equal volume of isopropanol, placed on ice for 20-30 minutes and centrifuged as above for 30 minutes. The isopropanol was decanted and the pellet washed by adding 300-500  $\mu$ l of 70% ethanol, centrifugation for 10 minutes, and decanting the 70% ethanol. The pellet was dried and subsequently suspended in 1mM Tris, pH7.5/0.5mM EDTA/0.01% SDS. The

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yield of RNA was calculated based on the optical density at 260 nm and the samples stored at -70°C.

RNA was fractionated by electrophoresis in methyl mercury gels (Bailey and Davidson, 1976). The methyl mercury gel was prepared by dissolving 0.75 gm of agarose in 50 ml of gel/ reservoir buffer (0.05M boric acid, 5mM Na<sub>2</sub>B<sub>2</sub>O<sub>7</sub>-10H<sub>2</sub>O, 10mM Na<sub>2</sub>SO<sub>4</sub>, 1mM Na<sub>4</sub>EDTA). The gel was cooled to 55-65°C and 0.25ml 1M methyl mercury hydroxide added. A small amount of this solution was used to plug the bottom edge of a vertical gel apparatus (13x15x0.15cm). The agarose solution was poured into prewarmed plates and a prewarmed comb inserted. The solution was solidified then the RNA sample dissolved in 10 mM methyl mercury hydroxide, 0.5x gel/reservoir buffer, 2.5 % ficoll 400, 0.05% bromophenol blue.

Wells were loaded with 5-10 µg of total RNA or 1-4 µg of polyadenylated RNA per lane. The samples were electrophoresed at 45-50 milliamps with recirculating buffer. The gels were stained with ethidium bromide in 0.5M NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub> for 10 minutes, then destained and washed with several changes of gel/reservoir buffer.

RNA was transferred to nylon and detected following hybridization to a GLUT-2 probe. The RNA was transferred from the gel to a nylon membrane by electroblotting at 4°C in 25mM NaHPO<sub>4</sub>, pH 6 for 4-6 hours at 0.5 amps. The membrane was rinsed in 0.5 X TBE, and the RNA crosslinked to the membrane in a Stratalinker. Hybridization was with a full-length digoxigenin-labeled GLUT-2 cDNA probe and detection with labeled RNA (Genius Non-Radioactive Detection Kit, Boehringer Mannheim, Inc.).

Protocols were followed as recommended by the manufacturer. The membrane was equilibrated in filtered Genius Buffer 1 for 1 minute. Lumigen PPD was warmed to room temperature. The membrane was blocked by gentle agitation in Genius Buffer 2 for 30 to 60 minutes. Genius Buffer 2 was discarded and the membrane incubated in antibody solution (anti-DIG Alkaline Phosphatase:1: 5000 -- 1:10,000 in Genius

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Buffer 2) for 30 minutes. This was followed by 2 washes (15 minutes each) in Genius Buffer 1 using clean dish. The membrane was changed to Genius Buffer 3 for 2 minutes. Diluted Lumigen PPD (1:100 in Genius Buffer 3; 5  $\mu$ l in 500  $\mu$ l) was added (500  $\mu$ l) drop by drop to the membrane (in appropriate bag) using a sterile pipette tip.

5 The membrane was exposed to X-ray film for time needed at room temperature.

**Glucose transport assays.** Glucose uptake was determined with radiolabeled 3-O-methyl  $\beta$ -D-glucose (3-MG), as previously described by Johnson, *et al.*, 1990, incorporated herein by reference.

10

50  $\mu$ l of 1M glucose, 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS), pH 8.0, was placed in the bottom of 400  $\mu$ l microcentrifuge tubes. This solution was overlaid with 150  $\mu$ l of dibutyl phthalate:dinonyl phthalate (4:1) mixture, and centrifuged for 30 s. This was overlaid with 50  $\mu$ l of [ $^{14}$ C] urea (2 mM, 0.5  $\mu$ Ci/ $\mu$ mol) and 20 mM [ $^3$ H]3-MG (5  $\mu$ Ci/ $\mu$ mol) in phosphate-buffered saline (PBS).  
15 The tubes were then preincubated at 15°C for 20 min.

Uptake was initiated by adding 50  $\mu$ l of cell suspension ( $\approx 10^6$  cells) to the top layer that contains 3-MG. Uptake was terminated by centrifuging cells through the dibutyl phthalate:dinonyl phthalate into 1M glucose, 10 mM EDTA and 0.1% SDS.  
20 A sample of the supernatant from the tubes was then used for specific activity calculations and the 1M glucose, 10 mM EDTA and 0.1% SDS phase was used for uptake determination.

25 Samples were counted in a Beckman LS 5801 liquid scintillation counter. Uptake was measured in duplicate for 2, 5, 10 and 20 s. The data was reduced to express uptake as mmol/liter cell space and Eadie-Hofstee plots prepared to determine  $K_m$  and  $V_{max}$  of uptake for each cell line analyzed.

30 **Results.** FIG. 6A presents the viability of  $\beta$ -cell lines treated with various concentrations of STZ relative to controls that were not exposed. The high passage parental cell line (RIN 81 I•I4) does not express endogenous GLUT-2. 17/C12 and

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RIN 30/10 (Ferber, *et al.*, 1994) are cell lines derived from RIN-38 cells that have been stably transfected with pCB7/GLUT-2. As shown, introduction of the GLUT-2 transgene produces an increased sensitivity to STZ (FIG. 6A).

5           FIG. 6B shows that sensitivity to STZ is proportional to the maximal rate of glucose uptake. The maximal velocity of glucose uptake is indicative of the level of expression of functional transporter.

10           The measurement of GLUT-2 activity in these cell lines correlates well with Western blot analyses of GLUT-2 protein in cell extracts. Following resolution of cell extracts by SDS/PAGE and Western blot analysis with an antibody specific to the C-terminal hexadecapeptide of GLUT-2 (East Acres Biologics, Inc.). Density of immunoreactive bands was quantified by acquiring a digital image using a flatbed scanner and the average pixel intensity of each band was determined using NIH Image  
15           software (version 1.56).

          The results were normalized to the highest GLUT-2 expressing line such that Rin10 = 1, 17/C12 = 0.85, and 17/+1 = 0.24. The data in FIG. 6A and FIG. 6B indicate that introduction of a GLUT-2 transgene into cells produces an increased  
20           cytotoxicity of STZ, and the increase in cytotoxicity of STZ is proportional to levels of functional GLUT-2 transporter in the cell.

          Additional evidence for the correlation between levels of GLUT-2 and sensitivity to STZ toxicity has been derived from several approaches. When a  
25           STZ-resistant RIN cell line is transfected with pCB7/GLUT-2 or pCB7intron/GLUT-2 and resulting hygromycin resistant clones are assayed for sensitivity to 2mM STZ, 100 percent of the STZ-sensitive clones express high levels of the GLUT-2 transgene. This result also speaks to the utility of GLUT-2 as a negative selection marker.

30           FIG. 7A is a representative Northern blot using RNA from the parental STZ-resistant or derivative STZ-sensitive clones. All transgenic clones that are sensitive to STZ (lanes 1-5, pCB7/GLUT-2 transfectants and lanes 6-10,

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pCB7intron/GLUT-2 transfectants) express high levels of GLUT-2 mRNA relative to the parental line (lane 11). Lane 12 is RNA from a cell line known to express high levels of GLUT-2 from pCB7 (RIN 30/10 (Ferber, *et al.*, 1994).

5           FIG. 7B shows the dose-dependence of STZ-mediated toxicity in these clones and is in agreement with the Northern data.

## EXAMPLE VII

### STZ Rapidly Kills Cells with High Levels of GLUT-2

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**Cytotoxicity Assays.** STZ toxicity was evaluated *in vitro* with two independent rapid viability assays. In the first of these, rapid metabolic effects on mitochondrial metabolism are revealed using the dye MTT (C,N-diphenyl-N'-4,5-dimethyl thiazol 2-yl tetrazolium bromide), as described for beta cells (Janjic and Wollheim, 1992, 15 incorporated herein by reference).

Cells were grown to near confluence in 24-well plates, then culture media was replaced with RPMI 1640 supplemented with 1 mM glucose and containing 2-20 mM STZ and cells incubated for 6 hours at 37°C. Thereafter, the RPMI media containing 20 STZ was removed and replaced with RPMI 1640 supplemented with 1 mM glucose and 0.5 mg/ml MTT for 1 hour at 37°C. After MTT incubation, the media was replaced with 2.5 ml isopropanol and the conversion of the MTT tetrazolium salt to formazan was quantified by measuring the optical density of the resultant solution at 450 nm in a spectrophotometer. The reduction in optical density induced by STZ 25 incubation was used as an index of viability by comparison to control cells incubated without STZ, which were taken as 100% viable.

The second assay utilizes the uptake by cells of the vital dye- neutral red (Kull and Cuatrecasas, 1981, incorporated herein by reference) to monitor the number of 30 cells that survive for 16-20 hours after exposure to STZ.

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Cells were plated at approximately 70% confluence 18-36 hours prior to exposure to STZ. Incubation of cells with the drug was achieved by removal of culture medium and replacement with RPMI with 1% FBS and a range of STZ concentrations from 0-20 mM for 2.5 hours, routinely (although with select cell lines time courses of 0.5 to 4 h were performed). At the end of the incubation the STZ-containing medium was removed and replaced with normal culture medium.

18-24 hours after exposure to STZ, 25  $\mu$ g of neutral red (0.1% neutral red in acetic acid) was added per ml of culture medium and incubated at room temperature for 20 minutes. The dye solution was removed at the end of the incubation and the cells rinsed with an equal volume of phosphate-buffered saline. Neutral red was extracted from the cell monolayer with a solution of 0.1 M  $\text{NaH}_2\text{PO}_4$  in 50% ethanol. The extracted neutral red taken up by cells was quantified by measuring optical density in a spectrophotometer at 540 nm. The neutral red uptake was determined to be linearly related to viable cell number, as seen in other cell systems (Kull and Cuatrecasas, 1981).

**Results.** The enhanced toxic effect of STZ on GLUT-2 expressing cells *in vivo* was confirmed by *in vitro* analysis. In both AtT20 and RIN-38 derived cell lines GLUT-2 over-expression conferred sensitivity to cytotoxic effects of STZ. In contrast, GLUT-1 over-expression did not confer sensitivity to STZ cytotoxicity.

This line of study was extended as follows: 49/206 represents a cell line with high levels of expression of GLUT-2 as measured by Northern analysis (FIG. 7A, Lane 7) and sensitivity to STZ (FIG. 7B). To determine the STZ exposure time needed for efficient killing, the cells were exposed to 2.0 mM STZ for various times, returned to media lacking the drug for 24 hours and then cell viability determined.

The results of this study are shown in FIG. 8. A 30 minute exposure to the drug resulted in almost complete killing of this cell line, with only a slight improvement seen in longer exposures. This represents a very rapid STZ-mediated toxicity. With high level expression of GLUT-2, lower doses of the drug can be used

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to avoid non-specific killing. Already demonstrated is the complete killing of the 49/206 cell line (and related clones) following a 2.5 hour exposure using 0.5 mM STZ (FIG. 7B). The ability to kill GLUT-2 expressing cells with low doses of the drug for short periods of time has profound benefits for the use of this invention for *in vivo* therapies.

### EXAMPLE VIII

#### Tumor Cell Killing

Recombinant adenovirus represents a high efficiency vehicle for transfer of genes to mammalian cells and cell lines (Berkner, 1988, Graham and Prevec, 1991, Becker, *et al.*, 1994). The inventors have constructed a recombinant adenovirus that contains the cDNA encoding GLUT-2 and have termed it AdCMV-GLUT-2 (Ferber, *et al.*, 1994, incorporated herein by reference; Example II).

The efficacy of this virus for GLUT-2 gene transfer was demonstrated in RIN 38 cells of intermediate passage number in which GLUT-2 is undetectable by immunofluorescent staining (Ferber, *et al.*, 1994). Treatment of these cells with AdCMV-GLUT-2 for 1 hour and analysis of the cells by immunofluorescence 2 days later revealed abundant GLUT-2 protein in essentially 100% of the cells (Ferber, *et al.*, 1994). That the transferred gene encoded a functional glucose transporter was confirmed by assay of 3-O-methyl glucose uptake, which demonstrated rates of uptake similar to those reported for the RIN-10 line that is stably transfected with GLUT-2 (Ferber, *et al.*, 1994).

The inventors now show that when RIN 23-21, a cell line that is resistant to STZ, is treated with AdCMV-GLUT-2 the cells become sensitive to STZ and that the degree of toxicity is proportional to the titer of AdCMV-GLUT-2 virus that is applied (FIG. 9).

In addition to RIN cell lines, transformed cells of various origin can also be rendered sensitive to STZ *via* adenovirus-mediated expression of GLUT-2. FIG. 10

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illustrates the conferral of STZ toxicity following treatment of a variety of cell types with AdCMV-GLUT-2. Included in this analysis are low passage Rin cells (RIN 11), high passage Rin cells (RIN 81), monkey kidney CV1 cells, mouse fibroblast 3T6 cells and monkey kidney VERO cells. Prior to treatment with AdCMV-GLUT-2, these cells are resistant to STZ, but are rendered sensitive to the drug following treatment with the virus. In light of findings disclosed above in which it was shown that stable transfection of AtT-20ins cells with GLUT-2 but not GLUT-1 confers STZ cytotoxicity, it can be concluded that GLUT-2 can serve to transfer this property in a manner that is independent of cell type.

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### EXAMPLE IX

#### Stable Expression of GLUT-2 *In Vivo*

The inventors have demonstrated that GLUT-2 expression in insulinoma cells that are stably transfected with the GLUT-2 gene driven by the CMV or insulin promoter/enhancer sequences can maintained.

Stability of GLUT-2 gene expression is maintained *in vitro* for at least one year in continuous culture when using the CMV promoter/enhancers. Implantation of RIN cell lines transfected with pCB7intron/GLUT-2 into nude rats demonstrates that expression of the GLUT-2 transgene is maintained for the duration of studies performed so far (FIG. 11A). In FIG. 11A, RNA samples in lanes 1 and 2 were isolated from control and GLUT-2 expressing cell lines, respectively. Lanes 3 and 4 correspond to RNA samples isolated from the GLUT-2 transfected cell line following its implantation in nude rats for 16 and 34 days, respectively.

The maintenance of GLUT-2 transgene expression through the course of *in vivo* passage is in contrast to expression of the endogenous GLUT-2 gene which is extinguished in studies conducted by the inventors (FIG. 11B) and in islet transplantation studies that have been conducted by others (Ogawa, *et al.*, 1995). Thus, these studies demonstrate that strong constitutive promoter/enhancers, of which CMV, RSV, and SV40 are examples, or certain cellular promoter/enhancers, of which

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insulin, metallothionein and GAPDH are examples, are able to maintain expression of the GLUT-2 gene for prolonged periods of time in heterologous cells that are transplanted into an *in vivo* setting.

5           In sum, the foregoing data teach that one effective method for introduction of the GLUT-2 gene into tumor cells for the purpose of initiating tumor ablation by STZ treatment is to expose the tumor to AdCMV-GLUT-2 virus or other recombinant viruses that contain the GLUT-2 cDNA and that use the RSV, insulin, or GAPDH promoter/enhancers to direct expression.

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          The *in vitro* and *in vivo* studies performed to date show that several promoters will support stable expression of GLUT-2 *in vivo*. While recombinant adenovirus is not integrated into the genome and thus will not be carried stably in growing tumorigenic cells, the large majority of insulinoma cells treated with AdCMV-GLUT-2  
15 or other recombinant adenoviruses retain expression of the transgene 2 days after treatment (Ferber, *et al.*, 1994), providing support for the efficacy of the method. Based on the findings of the inventors to date, expression of the GLUT-2 gene will likely confer STZ toxicity not only in insulinoma cells but in other types of tumor cells as well.

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#### EXAMPLE X

##### Glucose Transporters Resistant to Immune Destruction

          The mechanisms involved in destruction of xenografts or of insulin producing  
25 cells transplanted into animals or humans with IDDM are not completely understood. These cytotoxic processes may involve chemicals produced by T-cells, macrophages, or other elements of the immune system that are drawn to the site of the cellular transplant. If, as proposed herein, substances are produced in the vicinity of the graft that resemble STZ or that are different from STZ but are still recognized as a substrate  
30 by GLUT-2, the transport of such substances could participate in cytotoxicity to the graft.

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As shown earlier, stable transfection of AtT-20ins cells with the GLUT-1 glucose transporter does not confer STZ killing, while expression of GLUT-2 clearly does. Furthermore, transfection of AtT-20ins cells with GLUT-2 but not GLUT-1 confers glucose sensing (Hughes, *et al.*, 1993). The inventors thus propose that  
5 transfection of cells with mutated GLUT-2 glucose transporters or chimeric transporters consisting of a mixture of GLUT-2 and, for example, GLUT-1 sequences could protect against killing by STZ, as well as other, as yet unidentified toxins.

The inventors have produced a series of mutant and chimeric glucose  
10 transporter molecules, as shown in FIG. 12A, FIG. 12B, FIG. 12C, FIG. 12D, FIG. 12E, FIG. 12F, FIG. 12G, FIG. 12H, FIG. 12I and FIG. 12J. The mutant transporters produced to date include: 1) A chimeric transporter (Hpa 2-1) consisting of GLUT-2 sequence from the N-terminus through the second transmembrane region, and GLUT-1 from that point to the C-terminus; and 2) the inverse chimeric (Hpa 1-2), in which  
15 GLUT-1 sequence from the N-terminus through the second transmembrane region is fused to the GLUT-2 sequence from that point to the C-terminus. These first two chimeric molecules were produced by fusing the GLUT-2 and GLUT-1 sequences *via* a common Hpa-1 restriction endonuclease site found in the two sequences.

20 A third transporter produced, 3) is a GLUT-2 transporter in which the extracellular domain between the first and second transmembrane domains has been replaced with GLUT-1 sequence (Loop 1-2); a fourth, 4) is a chimeric transporter consisting of GLUT-2 with a GLUT-1 C-terminal intracellular domain (2-1); and 5) is a GLUT-2 transporter with a mutated N-linked glycosylation site (N62Q). Further  
25 cDNAs encoding chimeric transporters are summarized in FIG. 12A, FIG. 12B, FIG. 12C, FIG. 12D, FIG. 12E, FIG. 12F, FIG. 12G, FIG. 12H, FIG. 12I and FIG. 12J.

The inventors have expressed mutant and chimeric transporters in mammalian cells, including AtT-20ins and CV-1 cell lines and have found that the Hpa 2-1  
30 chimera is inactive as a glucose transporter, while the Hpa 1-2 chimera transports glucose with kinetics similar to normal GLUT-2. Furthermore, the 2-1 transporter experiences a precipitous drop in both maximal velocity and estimated Km for glucose

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relative to the intact GLUT-2 molecule but is a minimally functional glucose transporter. Finally, the N62Q point mutation of GLUT-2 produces a protein that transports glucose effectively but that has altered affinity for the substrate relative to native GLUT-2.

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Methods for analyzing the properties of the GLUT-2 variants and chimeras are available and are each routine laboratory methods. This allows for the ready identification of chimeric and/or mutated glucose transporters that fail to transport STZ but that do transport glucose effectively. Following such identification, the molecules will be expressed in insulin producing cell lines such as RIN 38 or AtT-20ins. These cells will then be transplanted into normal rats or mice, or into animal models of IDDM such as the NOD mouse in the context of permselective membranes, materials or devices that are designed to prevent contact between cellular elements of the immune system and the graft. It is contemplated that cells expressing chimeric transporters that do not recognize STZ will be protected while cells that express the native GLUT-2 molecule will be destroyed.

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## EXAMPLE XI

### GLUT-2 for Negative Selection to Ensure Homologous

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#### Recombinations for Adenovirus Construction

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The human kidney cell line, 293, is used for production of adenovirus vectors. Demonstration that GLUT-2 conferred sensitivity to STZ is a prerequisite for this system to be useful as a "kill system" for inappropriate recombinations during recombinant virus production. Stable cell lines from 293 cells were engineered that expresses high levels of GLUT-2 RNA and protein, and sensitivity to killing was determined.

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The 293 cells were electroporated with GLUT-2 cDNA, and selected in hygromycin as described in Example I. Twelve colonies were picked and screened for GLUT-2 expression. All twelve clones were tested for sensitivity to 1 mM STZ. FIG. 13 shows the survival of parental 293 cells and the cell lines derived from 293 cells 24

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hours after exposure to STZ. The clones in which  $\geq 90\%$  of the cells did not survive STZ exposure, (2, 5, 6, 8, and 12) were found to express high levels of GLUT-2 by Western and Northern blotting (details of analytic techniques in Example VII). Expression was equivalent to  $\beta$ G 49/107 line shown in FIG. 7A. Thus, GLUT-2  
5 expressing 293 cells are efficiently killed by STZ, illustrating that GLUT-2/STZ selection can be used with this adenovirus producing cell line.

## EXAMPLE XII

### Selective Killing of GLUT-2 Expressing Cells in a Population

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Wild-type 293 cells were mixed with GLUT-2 expressing,  $\beta$ G 112/2 cells of Example XI and subsequently exposed (once) for 2 h to STZ. The surviving cells were then grown up in 10 cm dishes, RNA isolated and analyzed for GLUT-2 expression by Northern analysis (as in Example VII). GLUT-2 mRNA expression was  
15 still evident in cells not exposed to STZ, while GLUT-2 expression of mixed 293 and 112/2 cultures exposed to STZ were distinguishable from background signals of RNA from wild-type 293 cells.

Three engineered  $\beta$ -cell lines were exposed to STZ. Two of the lines consisted  
20 of a mixed population of glucokinase-expressing and GLUT-2 expressing cells (A and B). The third line (C) expressed glucokinase only. Two sets of cultures were plated for each of the three lines, one served as the control culture, the second was exposed to 2 mM STZ for 2 hours. All cultures were then expanded and RNA and protein samples were analyzed as described. The Western blots were scanned and the protein  
25 bands digitized using the program NIH Image.

The two mixed population cultures (A and B) exposed to STZ exhibited decreased GLUT-2 expression, to  $\leq 1\%$  of the protein expression of the parallel cultures not exposed to STZ. This demonstrates at least a 99% removal of GLUT-  
30 expressing cells in the population by STZ treatment. The cells of line C had no detectable GLUT-2 under either treatment. Glucokinase of line C was not altered by treatment with STZ. Lines A and B exhibited 37-fold and 2-fold increases in

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glucokinase expression after the GLUT-2 expressing cells were removed by STZ treatment.

Thus, with sensitive immunologic assays for protein expression, greater than  
5 99% removal of GLUT-2-expressing cells from mixed cell cultures, is demonstrated.

### EXAMPLE XIII

#### GLUT-2 Versus Thymidine Kinase as a Negative Selectable

##### Marker in RIN 1046-38 Cell Lines

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GLUT-2 was compared to herpes simplex virus thymidine kinase (HSV-tk) as a negative selectable marker in RIN 1046-38 cells. Plasmid DNA vectors were created that contained neomycin phosphotransferase (Neo) as a positive selectable marker and either the HSV-tk cDNA (plasmid AX223) or the GLUT-2 cDNA (plasmids BL335  
15 and BN214). AX223 is a derivative of Pol2sneobpA and contains two tandem copies of HSV-tk as previously described (Ishibashi *et al.*, 1993 and Mansour *et al.*, 1988). In BL335 the GLUT-2 cDNA is transcribed from the CMV promoter and the 3' untranslated region. BN214 contains the same Neo and GLUT-2 transcriptional units as BL335, and also contains sequences that are homologous to the rat insulin 1 gene.

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Following electroporation of AX223, BL335, or BN214 into RIN cells, positive selection was first applied for the presence of Neo by the addition of G418 to the media at a final concentration of 500 µg/ml. After 2-3 weeks of growth in G418, negative selection assays were performed. Efficiency of HSV-tk as a negative  
25 selectable marker was assayed by the sensitivity of AX223-derivative clones to gancyclovir. 90 clones were isolated and plated as single colonies into a 96-well tissue culture plate. Following growth of the clones in medium containing G418, clones were exposed to medium supplemented with 6 µM gancyclovir for 3 days, and then maintained in medium containing G418 for 4 days. Only 20% of the clones that  
30 had been electroporated with AX223 and selected for resistance to G418 were susceptible to killing by gancyclovir.

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To assess the efficiency of GLUT-2 as a negative selectable marker, cells that had been electroporated with BL335 and selected for resistance to G418 were treated with trypsin, dispersed, and pooled to create polyclonal populations. The BL335-derivative polyclones were replica-plated at equal densities, grown in medium for 3 days, exposed to 1.5 mM STZ for 2 hours, and then maintained in medium containing G418 for 24 hours. Following washes of the cultures with PBS to remove dead cells, viable cells were dispersed by treatment with trypsin and counted by hemocytometry. In the polyclones that had been exposed to STZ there were 60% fewer viable cells than in the control cultures that were not exposed to STZ.

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In a separate study, individual clones resulting from electroporation with BN214 were analyzed with regard to their STZ sensitivity. 19 clones that had been previously selected with G418 were dispersed with trypsin and replica-plated into 96 well tissue culture dishes. One set of cultures was exposed to 1.5 mM STZ as described for BL335. Significant cell death occurred in 13 of the 19 cultures that were cultured in STZ indicating that about 68% of the BN214-derivative clones expressed functional GLUT-2 transporters.

From these data, it can thus be concluded that GLUT-2 expression in combination with STZ is a superior system of negative selection in RIN cells than HSV-tk in combination with gancyclovir.

#### EXAMPLE XIV

##### Stable GLUT-2 Expression with a Non-Viral, Cell-Specific Promoter.

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The plasmid with rat insulin I promoter driving GLUT-2 expression (as described in Example I) was transfected into RIN-38 cells, (passage 26). The cells were then selected with puromycin for 2 weeks and resistant colonies grown and picked. Forty-eight colonies were picked and screened for sensitivity to killing by exposure to 2 mM STZ. Five of 48 cell lines were found to be killed by 2 mM STZ. FIG. 14 shows the dose-responsive effects of STZ in three of these lines in comparison to a line in which CMV-promoter drives expression of GLUT-2. The

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expression of GLUT-2 in these 5 cell lines was confirmed by Northern analysis of the RNA.

#### EXAMPLE XV

##### 5        Long-Term Stability of Streptozotocin Solutions Dissolved in DMSO

In an effort to minimize handling hazards of streptozotocin, the inventors explored the use of DMSO as a solvent for streptozotocin. Solutions of this compound would avoid the usual practice of weighing streptozotocin immediately  
10 before use. The cell line  $\beta$ G 49/206 was used to test the killing efficacy of the STZ solutions. It was initially found that STZ dissolved in DMSO (1 M stock solution) was equipotent for killing 49/206 cells with STZ dissolved in citrate buffer. The long term stability of STZ dissolved in DMSO was tested with two stocks that had been stored below -70°C for 21 months or 7 months. FIG. 15 demonstrates that the  
15 cytotoxicity of STZ remains after long term storage below -70°C. The killing shown is not different from that obtained with freshly made STZ. Another solvent, propanediol was also tested, however, STZ in this solvent has not proved to be as stable as with DMSO.

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\* \* \*

All of the compositions and 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  
25 preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, 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 that are both chemically and physiologically related may be substituted for the agents  
30 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

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within the spirit, scope and concept of the invention as defined by the appended claims.

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30 32:678-684, 1989.

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10

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

(A) NAME: BOARD OF REGENTS, THE UNIVERSITY OF  
TEXAS SYSTEM

(B) STREET: 201 West 7th Street

10

(C) CITY: Austin

(D) STATE: Texas

(E) COUNTRY: USA

(F) POSTAL CODE (ZIP): 78701

15

(A) NAME: Betagene, Inc.

(B) STREET: 2600 Stemmons Freeway, Suite 125

(C) CITY: Dallas

(D) STATE: Texas

(E) COUNTRY: USA

20

(F) POSTAL CODE (ZIP): 75207-2107

(ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS  
COMPRISING GLUT-2 AND  
GLUT-2 CHIMERAS

25

(iii) NUMBER OF SEQUENCES: 4

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

30

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version  
#1.30 (EPO)

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## (vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/546,934

(B) FILING DATE: 23-OCT-1995

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## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1815 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

10

GTCGACCGCG ACCGTGACGC TTATCGATT CGAACCCGGG TCGGAGTCAG AGTCGCAGTG 60

GGAGTCCCCG GACCGGAGCA CGAGCCTGAG CGGGAGAGCG CCGCTCGCAC GCCCGTCGCC 120

15 ACCCGCGTAC CCGGCGCAGC CAGAGCCACC AGCGCAGCGC TGCCATGGAG CCCAGCAGCA 180

AGAAGCTGAC GGGTCGCCCTC ATGCTGGCTG TGGGAGGAGC AGTGCTTGGC TCCCTGCAGT 240

TTGGCTACAA CACTGGAGTC ATCAATGCCC CCCAGAAGGT GATCGAGGAG TTCTACAACC 300

20

AGACATGGGT CCACCGCTAT GGGGAGAGCA TCCTGCCAC CACGCTCACC ACGCTCTGGT 360

CCCTCTCAGT GGCCATCTTT TCTGTTGGGG GCATGATTGG CTCCTTCTCT GTGGGCCTTT 420

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TCGTTAACCG CTTTGGCCGG CGGAATTCAA TGCTGATGAT GAACCTGCTG GCCTTCGTGT 480

CCGCCGTGCT CATGGGCTTC TCGAAACTGG GCAAGTCCTT TGAGATGCTG ATCCTGGGCC 540

5 GCTTCATCAT CGGTGTGTAC TGCGGCCTGA CCACAGGCTT CGTGCCCATG TATGTGGGTG 600

AAGTGTACCC CACAGCCTTT CGTGGGGCCC TGGGCACCCCT GCACCAGCTG GGCATCGTCG 660

TCGGCATCCT CATGCCCCAG GTGTTGGGCC TGGACTCCAT CATGGGCAAC AAGGACCTGT 720

GGCCCCTGCT GCTGAGCATC ATCTTCATCC CGGCCCTGCT GCAGTGCATC GTGCTGCCCT 780

TCTGCCCCGA GAGTCCCCGC TTCCTGTCTCA TCAACCGCAA CGAGGAGAAC CGGGCCAAGA 840

15 GTGTGCTAAA GAAGCTGCGC GGGACAGCTG ACGTGACCCA TGACCTGCAG GAGATGAAGG 900

AAGAGAGTCG GCAGATGATG CGGGAGAAAG AGGTCACCAT CCTGGAGCTG TTCCGCTCCC 960

CCGCCCTACCG CCAGCCCCATC CTCATCGCTG TGGTGCTGCA GCTGTCCCAG CAGCTGTCTG 1020

GCATCAACGC TGTCTTCTAT TACTCCACGA GCATCTTCCA GAAGGCGGGG GTGCAGCAGC 1080

CTGTGTATGC CACCATTTGGC TCCGGTATCG TCAACACGGC CTTCACTGTC GTGTCGCTGT 1140

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TTGTGGTGGA GCGAGCAGGC CGGCGGACCC TGCACCTCAT AGCCTCGCT GGCATGGCGG 1200  
GTTGTGCCAT ACTCATGACC ATCGCGCTAG CACTGCTGGA GCAGCTACCC TGGATGTCCT 1260  
5 ATCTGAGCAT CGTGGCCATC TTTGGCTTTG TGGCCTTCTT TGAAGTGGGT CCTGGCCCCA 1320  
TCCCATGGTT CATCGTGGCT GAACTCTTCA GCCAGGGTCC AGTCCAGCT GCCATTGCCG 1380  
TTGCAGGCTT CTCCAACCTGG ACCTCAAATT TCATTGTGG CATGTGCTTC CAGTATGTGG 1440  
10 AGCAACTGTG TGGTCCCTAC GTCTTCATCA TCTTCACTGT GCTCCTGGTT CTGTTCTTCA 1500  
TCTTCACCTA CTTCAAAGTT CCTGAGACTA AAGCCCGGAC CTTGATGAG ATCGCTTCCG 1560  
15 GCTTCCGGCA GGGGGGAGCC AGCCAAAGTG ATAAGACACC CGAGGAGCTG TTCCATCCCC 1620  
TGGGGGCTGA TTCCCAAGTG TGAGTCGCCC CAGATCACCA GCCCGGCCTG CTCCCAGCAG 1680  
CCCTAAGGAT CTCTCAGGAG CACAGGCAGC TGGATGAGAC TTCCAAACCT GACAGATGTC 1740  
20 AGCCGAGCCG GGCCTGGGC TCCTTTCTCC AGCCAGCAA TGATGTCCAG GTACCGAAT 1800  
TCCTCGACGG TCGAC 1815

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 492 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |    |    |    |    |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|----|----|----|
| Met | Glu | Pro | Ser | Ser | Lys | Lys | Leu | Thr | Gly | Arg | Leu | Met | Leu | Ala | Val | 1  | 5  | 10 | 15 |
| Gly | Gly | Ala | Val | Leu | Gly | Ser | Leu | Gln | Phe | Gly | Tyr | Asn | Thr | Gly | Val | 20 | 25 | 30 |    |
| Ile | Asn | Ala | Pro | Gln | Lys | Val | Ile | Glu | Glu | Phe | Tyr | Asn | Gln | Thr | Trp | 35 | 40 | 45 |    |
| Val | His | Arg | Tyr | Gly | Glu | Ser | Ile | Leu | Pro | Thr | Thr | Leu | Thr | Thr | Leu | 50 | 55 | 60 |    |

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Trp Ser Leu Ser Val Ala Ile Phe Ser Val Gly Gly Met Ile Gly Ser  
65 70 75 80

Phe Ser Val Gly Leu Phe Val Asn Arg Phe Gly Arg Arg Asn Ser Met  
85 90 95

Leu Met Met Asn Leu Leu Ala Phe Val Ser Ala Val Leu Met Gly Phe  
100 105 110

10 Ser Lys Leu Gly Lys Ser Phe Glu Met Leu Ile Leu Gly Arg Phe Ile  
115 120 125

Ile Gly Val Tyr Cys Gly Leu Thr Thr Gly Phe Val Pro Met Tyr Val  
130 135 140

15 Gly Glu Val Ser Pro Thr Ala Phe Arg Gly Ala Leu Gly Thr Leu His  
145 150 155 160

Gln Leu Gly Ile Val Val Gly Ile Leu Ile Ala Gln Val Phe Gly Leu  
165 170 175

20 Asp Ser Ile Met Gly Asn Lys Asp Leu Trp Pro Leu Leu Ser Ile  
180 185 190

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Ile Phe Ile Pro Ala Leu Leu Gln Cys Ile Val Leu Pro Phe Cys Pro  
 195 200 205  
  
 Glu Ser Pro Arg Phe Leu Leu Ile Asn Arg Asn Glu Glu Asn Arg Ala  
 210 215 220  
  
 Lys Ser Val Leu Lys Lys Leu Arg Gly Thr Ala Asp Val Thr His Asp  
 225 230 235 240  
  
 Leu Gln Glu Met Lys Glu Glu Ser Arg Gln Met Met Arg Glu Lys Lys  
 245 250 255  
  
 Val Thr Ile Leu Glu Leu Phe Arg Ser Pro Ala Tyr Arg Gln Pro Ile  
 260 265 270  
  
 Leu Ile Ala Val Val Leu Gln Leu Ser Gln Gln Leu Ser Gly Ile Asn  
 275 280 285  
  
 Ala Val Phe Tyr Tyr Ser Thr Ser Ile Phe Glu Lys Ala Gly Val Gln  
 290 295 300  
  
 Gln Pro Val Tyr Ala Thr Ile Gly Ser Gly Ile Val Asn Thr Ala Phe  
 305 310 315 320

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Thr Val Val Ser Leu Phe Val Val Glu Arg Ala Gly Arg Arg Thr Leu  
325 330 335

His Leu Ile Gly Leu Ala Gly Met Ala Gly Cys Ala Ile Leu Met Thr  
340 345 350

Ile Ala Leu Ala Leu Leu Glu Gln Leu Pro Trp Met Ser Tyr Leu Ser  
355 360 365

10 Ile Val Ala Ile Phe Gly Phe Val Ala Phe Phe Glu Val Gly Pro Gly  
370 375 380

Pro Ile Pro Trp Phe Ile Val Ala Glu Leu Phe Ser Gln Gly Pro Arg  
385 390 395 400

15 Pro Ala Ala Ile Ala Val Ala Gly Phe Ser Asn Trp Thr Ser Asn Phe  
405 410 415

Ile Val Gly Met Cys Phe Gln Tyr Val Glu Gln Leu Cys Gly Pro Tyr  
420 425 430

Val Phe Ile Ile Phe Thr Val Leu Leu Val Leu Phe Phe Ile Phe Thr  
435 440 445

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Tyr Phe Lys Val Pro Glu Thr Lys Gly Arg Thr Phe Asp Glu Ile Ala  
450 455 460

Ser Gly Phe Arg Gln Gly Gly Ala Ser Gln Ser Asp Lys Thr Pro Glu  
465 470 475 480

Glu Leu Phe His Pro Leu Gly Ala Asp Ser Gln Val  
485 490

10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1943 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

20

ACTCCGCTTG CTCCTCCTCC TACAATGTAT AACCAGGCAG AGCTGAGGAC AGCTACTCAT 60

CCACATTGG AACAGGACCT GGATTGAGAC AACAACTCCG CACACAACAT GTCAGAAGAC 120

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180 AAGATCACCG GAACCTTGGC TTTCACCTGTC TTCACTGCAG TGCTGGGTTC CTTCAGTTC  
240 GGATATGACA TCGGTGTGAT CAATGCACCT CAAGAGGTAA TAATATCCCA TTATCGACAT  
300 GTTTTGGGTG TTCCTCTGGA TGACCGAAGA GCTACCATTA ACTATGACAT CAATGGCACA  
360 GACACCCAC TCATAGTCAC ACCAGCACAT ACGACACCAG ACGCCTGGGA AGAAGAGACT  
420 GAAGGATCTG CTCACATAGT CACTATGCTC TGGTCTCTGT CTGTGTCCAG CTTTGCAGTA  
480 GCGGGAATGG TCGCCTCGTT CTTTGGTGGG TGGCTTGGGG ACAAACTCGG AAGGATCAAA  
540 GCCATGTTGG CTGCAAAACAG CCTCTCGTTG ACTGGAGCCC TCTTGATGGG GTGTTCCAAA  
600 TTTGGACCGG CACATGCTCT CATCATTTGCT GGAAGAAGCG TATCAGGACT GTACTGTGGG  
660 CTAATTTCAG GACTGGTTCC AATGTACATT GCGGAGATTG CTCCAACCAC ACTCAGGGGT  
720 GCCCTGGGCA CTCCTCACC AACTGGCTCTT GTCACAGGCA TTCTTATTAG TCAGATTGCT  
780 GGCCTCAGCT TCATTCTGGG CAATCAGGAT TACTGGCACA TCCTACTTGG CCTATCTGCT  
840 GTACCAGCTC TCTTGCAGTG TCTGCTACTG CTCTTCTGTC CAGAAAGCCC CAGATACCTT

5  
10  
15  
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-113-

TACCTAAATT TGGAAGAGGA AGTCAGGGCA AAGAAAAGCT TGAAAAGACT AAGGGGCACT 900

GAGGACATCA CCAAAGATAT TAATGAGATG AGGAAAGAAA AGGAAGAGGC GTCGACTGAG 960

5 CAGAAGGTCT CCGTGATCCA GCTCTTCAAG GATCCGAAAT ATCGGCAGCC CATGTGTGGT 1020

GCACTGATGC TACACTTGGC TCAGCAGTTC TCTGGAATCA ATGGGATATT TTA CTATTCA 1080

ACCAGCATTT TTCAGACAGC TGGCATCAGC CAGCCTGTGT ATGCAACCAT CGGCCGTTGGT 1140

GCCATCAACA TGATCTTCAC AGCTGTCTCT GTGCTGCTTG TGGAGAAGGC GGGCGGCGG 1200

ACCCTGTTCC TGGCCGGGAT GATTGGCATG TTTTCTCTGT CCGTCTTCAT GTCGCTGGGA 1260

15 CTGGTGTTC TGGATAAGTT CACCTGGATG AGTTATGTGA GCATGACGGC CATCTTCCTC 1320

TTCGTCAGTT TCTTTGAGAT TGGGCCAGGT CCAATCCCTT GGTTCATGGT TGCTGAATTT 1380

TTCAGCCAAG GACCCCGTCC CACGGCTCTG GCACTGGCTG CCTTAGCAA CTGGGTCTGC 1440

20 AATTTCATCA TCGCCCTCTG CTTCCAGTAC ATTGCGGACT TCCTCGGGCC TTACGTGTTC 1500

TTCCCTTTTTC CTGGGGTGGT CCTGGTCTTC ACCCTGTTC CA TTTT TTA AGTTCAGAA 1560

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ACCAAAGGAA AGTCTTTTGA CGAAATTGCT GCAGAATTCC GGAAGAAGAG TGGTTCGGCC 1620  
CCACCACGCA AAGCCACTGT ACAAAATGGAA TTCCTGGGGT CATCAGAGAC TGTGTGAGGA 1680  
5 TGAGCTGCCT AAAATCCAGG AACAGACCAG AAGGGAACCA GGTGCTCCCT CTTAACTCGA 1740  
ATCCTTCATG AGGTAGGAC TACACCCAGG AGGATCATTT ATTCTTTTGG AAGAAATTTGT 1800  
ACAGACTCCG ATTAGAAACG TCAACATATA TCATTACCAA AGAAAGTATT TTTTAACTTT 1860  
10 AGAGAACTCA TTTTGGATGG TAAAACTCTT CAGTAAACCA GAGGCTGCTC TGCTTCTCTA 1920  
CAGGGAAGC ACGTTATAAG CTT 1943

15 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 522 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

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-115-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Ser | Glu | Asp | Lys | Ile | Thr | Gly | Thr | Leu | Ala | Phe | Thr | Val | Phe | Thr |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     | 15  |     |     |

5

Ala Val Leu Gly Ser Phe Phe Gln Phe Gly Tyr Asp Ile Gly Val Ile Asn

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ala | Pro | Gln | Glu | Val | Ile | Ile | Ser | His | Tyr | Arg | His | Val | Leu | Gly | Val |
|     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |     |     |     |

10

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Pro | Leu | Asp | Asp | Arg | Arg | Ala | Thr | Ile | Asn | Tyr | Asp | Ile | Asn | Gly | Thr |
|     | 50  |     |     |     |     | 55  |     |     |     | 60  |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asp | Thr | Pro | Leu | Ile | Val | Thr | Pro | Ala | His | Thr | Thr | Pro | Asp | Ala | Trp |
| 65  |     |     |     |     | 70  |     |     |     | 75  |     |     |     |     |     | 80  |

Glu Glu Glu Thr Glu Gly Ser Ala His Ile Val Thr Met Leu Trp Ser

85 90 95

20

Leu Ser Val Ser Ser Phe Ala Val Gly Gly Met Val Ala Ser Phe Phe

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Gly Gly Trp Leu Gly Asp Lys Leu Gly Arg Ile Lys Ala Met Leu Ala  
115 120 125

Ala Asn Ser Leu Ser Leu Thr Gly Ala Leu Leu Met Gly Cys Ser Lys  
130 135 140

Phe Gly Pro Ala His Ala Leu Ile Ile Ala Gly Arg Ser Val Ser Gly  
145 150 155 160

10 Leu Tyr Cys Gly Leu Ile Ser Gly Leu Val Pro Met Tyr Ile Gly Glu  
165 170 175

Ile Ala Pro Thr Thr Leu Arg Gly Ala Leu Gly Thr Leu His Gln Leu  
180 185 190

15 Ala Leu Val Thr Gly Ile Leu Ile Ser Gln Ile Ala Gly Leu Ser Phe  
195 200 205

Ile Leu Gly Asn Gln Asp Tyr Trp His Ile Leu Leu Gly Leu Ser Ala  
210 215 220

20 Val Pro Ala Leu Leu Gln Cys Leu Leu Leu Phe Cys Pro Glu Ser  
225 230 235 240

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Pro Arg Tyr Leu Tyr Leu Asn Leu Glu Glu Val Arg Ala Lys Lys  
245 250 255

Ser Leu Lys Arg Leu Arg Gly Thr Glu Asp Ile Thr Lys Asp Ile Asn  
260 265 270

Glu Met Arg Lys Glu Lys Glu Glu Ala Ser Thr Glu Gln Lys Val Ser  
275 280 285

Val Ile Gln Leu Phe Thr Asp Pro Asn Tyr Arg Gln Pro Ile Val Val  
290 295 300

Ala Leu Met Leu His Leu Ala Gln Gln Phe Ser Gly Ile Asn Gly Ile  
305 310 315 320

Phe Tyr Tyr Ser Thr Ser Ile Phe Gln Thr Ala Gly Ile Ser Gln Pro  
325 330 335

Val Tyr Ala Thr Ile Gly Val Gly Ala Ile Asn Met Ile Phe Thr Ala  
340 345 350

Val Ser Val Leu Leu Val Glu Lys Ala Gly Arg Arg Thr Leu Phe Leu  
355 360 365

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Ala Gly Met Ile Gly Met Phe Phe Cys Ala Val Phe Met Ser Leu Gly  
 370 375 380  
 Leu Val Leu Leu Asp Lys Phe Thr Trp Met Ser Tyr Val Ser Met Thr  
 385 390 395 400  
 Ala Ile Phe Leu Phe Val Ser Phe Phe Glu Ile Gly Pro Gly Pro Ile  
 405 410 415  
 Pro Trp Phe Met Val Ala Glu Phe Phe Ser Gln Gly Pro Arg Pro Thr  
 420 425 430  
 Ala Leu Ala Leu Ala Ala Phe Ser Asn Trp Val Cys Asn Phe Ile Ile  
 435 440 445  
 Ala Leu Cys Phe Gln Tyr Ile Ala Asp Phe Leu Gly Pro Tyr Val Phe  
 450 455 460  
 Phe Leu Phe Ala Gly Val Val Leu Val Phe Thr Leu Phe Thr Phe Phe  
 465 470 475 480  
 Lys Val Pro Glu Thr Lys Gly Lys Ser Phe Asp Glu Ile Ala Ala Glu  
 485 490 495

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Phe Arg Lys Lys Ser Gly Ser Ala Pro Pro Arg Lys Ala Thr Val Gln  
500 505 510

Met Glu Phe Leu Gly Ser Ser Glu Thr Val  
515 520

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**CLAIMS:**

1. A method for killing cells in a cell population, comprising contacting a cell population that includes GLUT-2-expressing cells with a composition comprising  
5 STZ in an amount effective and for a period of time sufficient to kill at least about 50% of the GLUT-2-expressing cells in said cell population.
2. The method of claim 1, wherein said method kills at least about 55% of the  
10 GLUT-2-expressing cells in said cell population.
3. The method of claim 2, wherein said method kills at least about 60% of the  
15 GLUT-2-expressing cells in said cell population.
4. The method of claim 3, wherein said method kills at least about 65% of the  
20 GLUT-2-expressing cells in said cell population.
5. The method of claim 4, wherein said method kills at least about 70% of the  
GLUT-2-expressing cells in said cell population.
- 25 6. The method of claim 5, wherein said method kills at least about 75% of the  
GLUT-2-expressing cells in said cell population.
7. The method of claim 6, wherein said method kills at least about 80% of the  
30 GLUT-2-expressing cells in said cell population.

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8. The method of claim 7, wherein said method kills at least about 85% of the GLUT-2-expressing cells in said cell population.

5 9. The method of claim 8, wherein said method kills at least about 90% of the GLUT-2-expressing cells in said cell population.

10 10. The method of claim 9, wherein said method kills at least about 95% of the GLUT-2-expressing cells in said cell population.

15 11. The method of claim 10, wherein said method kills at least about 96% of the GLUT-2-expressing cells in said cell population.

20 12. The method of claim 11, wherein said method kills at least about 97% of the GLUT-2-expressing cells in said cell population.

13. The method of claim 12, wherein said method kills at least about 98% of the GLUT-2-expressing cells in said cell population.

25 14. The method of claim 13, wherein said method kills at least about 99% of the GLUT-2-expressing cells in said cell population.

30 15. The method of any preceding claim, wherein said GLUT-2-expressing cells are prepared by providing to cells within the cell population a functional GLUT-2 glucose transporter protein.

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16. The method of claim 15, wherein said GLUT-2-expressing cells are prepared by providing to cells within the cell population a polynucleotide that expresses GLUT-2 in said cells.

5

17. The method of claim 16, wherein said GLUT-2-expressing cells are prepared by providing to cells within the cell population a recombinant vector comprising a promoter operatively linked to a GLUT-2 gene, the promoter expressing said GLUT-2 gene in said cell.

10

18. The method of claim 16 or 17, wherein cells within said cell population are provided with said polynucleotide by transfection.

15

19. The method of claim 17, wherein cells within said cell population are provided with said recombinant vector by infection with a recombinant virus that comprises said recombinant vector.

20

20. The method of claim 19, wherein said recombinant vector is provided to cells within the cell population by infection with a recombinant retrovirus, adeno-associated virus (AAV) or adenovirus that comprises said recombinant vector.

25

21. The method of claim 20, wherein said recombinant vector is an adenoviral vector comprised within a recombinant adenovirus.

30

22. The method of claim 21, wherein said recombinant vector is an adenoviral vector comprised within a replication deficient recombinant adenovirus.

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23. The method of any one of claims 17 through 22, wherein said recombinant vector comprises a constitutive promoter that expresses said GLUT-2 gene.

5 24. The method of claim 23, wherein said recombinant vector comprises a GAPDH, SV40 IE, CMV or an RSV LTR promoter.

25. The method of claim 24, wherein said recombinant vector comprises a  
10 CMV or an RSV LTR promoter.

26. The method of any one of claims 17 through 22, wherein said recombinant vector comprises a tissue-specific promoter that expresses said GLUT-2 gene in  
15 specific cells of said cell population.

27. The method of claim 26, wherein said recombinant vector comprises a pancreatic  $\beta$  cell-specific promoter that specifically expresses said GLUT-2 gene in  
20 pancreatic  $\beta$  cells within a cell population.

28. The method of claim 27, wherein said recombinant vector comprises an insulin gene promoter.  
25

29. The method of claim 28, wherein said recombinant vector comprises a rat insulin gene promoter (RIP).

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30. The method of claim 29, wherein said recombinant vector comprises a RIP1 promoter.

5 31. The method of claim 29, wherein said recombinant vector comprises a RIP2 promoter.

32. The method of claim 28, wherein said recombinant vector comprises a  
10 human insulin gene promoter (HIP).

33. The method of any one of claims 17 through 32, wherein said recombinant  
vector comprises a modified promoter having increased transcriptional activity.

15

34. The method of claim 33, wherein said recombinant vector comprises a  
modified promoter that comprises multimerized promoter elements.

20

35. The method of claim 34, wherein said recombinant vector comprises a  
modified promoter that comprises multimerized insulin gene promoter elements.

25 36. The method of any preceding claim, wherein said GLUT-2-expressing cells  
further comprise at least one exogenous gene.

37. The method of claim 36, wherein said exogenous gene is a negatively  
30 selectable marker gene.

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38. The method of claim 37, wherein said exogenous gene is a cytosine deaminase gene.

5 39. The method of claim 36, wherein said exogenous gene is a drug selectable marker gene.

40. The method of claim 39, wherein said exogenous gene is a drug selectable  
10 marker gene that confers resistance to neomycin, hygromycin, puromycin, zeocin, mycophenolic acid, histidinol or methotrexate.

41. The method of any preceding claim, wherein said GLUT-2-expressing cells  
15 are tumor cells.

42. The method of claim 41, wherein said GLUT-2-expressing cells are  
insulinoma cells.

20

43. The method of any one of claims 1 through claim 40, wherein said  
GLUT-2-expressing cells are primary cells.

25

44. The method of any preceding claim, wherein said GLUT-2-expressing cells  
are secretory cells.

30 45. The method of any one of claims 1 through 43, wherein said GLUT-2-expressing cells are non-secretory cell.

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46. The method of any preceding claim, wherein said cell population is contacted with said composition comprising STZ for a period of time of between about 10 minutes and about 3 hours.

5

47. The method of claim 46, wherein said cell population is contacted with said STZ-containing composition for a period of time of about 30 minutes.

10

48. The method of any preceding claim, wherein said cell population is contacted with a composition that comprises STZ at a concentration of between about 0.1 mM and about 10 mM.

15

49. The method of claim 48, wherein said cell population is contacted with a composition that comprises STZ at a concentration of between about 0.1 mM and about 2 mM.

20

50. The method of claim 49, wherein said cell population is contacted with a composition that comprises STZ at a concentration of between about 0.1 mM and about 1 mM.

25

51. The method of claim 50, wherein said cell population is contacted with a composition that comprises STZ at a concentration of between about 0.1 mM and about 0.5 mM.

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52. The method of claim 51, wherein said cell population is contacted with a composition that comprises STZ at a concentration of between about 0.1 mM and about 0.25 mM.

5

53. The method of claim 52, wherein said cell population is contacted with a composition that comprises STZ at a concentration of about 0.1 mM.

10

54. The method of any preceding claim, wherein said cell population is contacted with a composition that comprises STZ dissolved in DMSO.

15

55. The method of any preceding claim, further defined as a method for selecting a cell having a selected gene integrated at a site-specific point, the method comprising the steps of:

20

(a) obtaining a cell population, the cells of which comprise a GLUT-2 transporter gene incorporated into their genome;

(b) providing to the cells of said cell population a polynucleotide comprising said selected gene, wherein said selected gene is flanked by GLUT-2 sequences; and

25

(c) contacting said cell population with STZ in an amount effective and for a period of time sufficient for said STZ to kill at least about 60% of the GLUT-2 transporter-expressing cells in said cell population.

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56. A method for selecting a cell having a selected gene integrated at a site-specific point, comprising the steps of:

- 5 (a) obtaining a cell with a GLUT-2 transporter gene incorporated into its genome;
- (b) providing to said cell a polynucleotide comprising said selected gene, wherein said selected gene is flanked by GLUT-2 sequences; and
- 10 (c) contacting said cell with STZ in an amount effective and for a period of time sufficient for said STZ to kill a GLUT-2 transporter-expressing cell.

15 57. The method of claim 56, comprising the steps of:

- (a) obtaining a cell population, the cells of which comprise a GLUT-2 transporter gene incorporated into their genome;
- 20 (b) providing to the cells of said cell population a polynucleotide comprising said selected gene, wherein said selected gene is flanked by GLUT-2 sequences; and
- (c) contacting said cell population with STZ in an amount effective and  
25 for a period of time sufficient for said STZ to kill at least about 60% of the GLUT-2 transporter-expressing cells in said cell population.

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58. A method for selecting a cell, comprising the steps of:

- 5
- (a) obtaining a starting cell that substantially lacks a functional GLUT-2 transporter gene;
- (b) providing said cell with a polynucleotide comprising (i) a drug selectable marker gene, wherein said drug selectable marker gene is flanked by target gene sequences and (ii) a GLUT-2 transporter gene adjacent thereto;
- 10
- (c) contacting said cell with a drug in an amount effective and for a period of time sufficient to kill a cell not expressing said drug selectable marker; and
- 15
- (d) contacting said cell with STZ in an amount effective and for a period of time sufficient to kill a cell expressing a functional GLUT-2 transporter, in comparison to a cell substantially lacking a functional GLUT-2 transporter gene.

20

59. The method of claim 58, comprising the steps of:

- (a) obtaining a cell population, the cells of which substantially lack a functional GLUT-2 transporter gene;
- 25
- (b) providing to the cells of said cell population a polynucleotide comprising (i) a drug selectable marker gene, wherein said drug selectable marker gene is flanked by target gene sequences and (ii) a GLUT-2 transporter gene adjacent thereto;

30

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- 5 (c) contacting said cell population with a drug in an amount effective and for a period of time sufficient to kill cells of said cell population that do not express said drug selectable marker, thereby preparing a purged cell population; and
- 10 (d) contacting said purged cell population with STZ in an amount effective and for a period of time sufficient to kill at least about 60% of the cells in the purged cell population that express a functional GLUT-2 transporter, and to not kill significant amounts of cells that substantially lack a functional GLUT-2 transporter gene.
- 15 60. The method of claim 58 or 59, wherein said polynucleotide further comprises a selected gene flanked by said target gene sequences.
- 20 61. The method of any one of claims 58 through 60, wherein said drug selectable marker gene confers resistance to neomycin, hygromycin, puromycin, zeocin, mycophenolic acid, histidinol or methotrexate.
- 25 62. A method for selecting a cell with an integrated selected gene, comprising the steps of:
- (a) providing to said cell a polynucleotide comprising (i) a GLUT-2 transporter gene, (ii) a drug selectable marker gene and (iii) a selected gene, wherein said selected gene is interposed between said transporter gene and said marker gene;

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- (b) contacting said cell with a drug in amount effective and for a period of time sufficient for said drug to kill a cell not expressing said drug selectable marker gene; and
- 5 (c) contacting said cell with STZ in an amount effective and for a period of time sufficient for said STZ to kill a GLUT-2 transporter-expressing cell.
- 10 63. The method of claim 62, wherein step (c) further comprises the steps of:
- (i) preparing a first and a second cell culture of said cell;
- (ii) contacting said first cell culture with STZ in an amount effective and  
15 for a period of time sufficient to kill a GLUT-2 transporter-expressing cell; and
- (iii) identifying a cell from said first cell culture that is sensitive to STZ.
- 20 64. The method of claim 62, comprising the steps of:
- (a) providing to cells of a cell population a polynucleotide comprising  
25 (i) a GLUT-2 transporter gene, (ii) a drug selectable marker gene and (iii) a selected gene, wherein said selected gene is interposed between said transporter gene and said marker gene;
- (b) contacting said cell population with a drug in amount effective and  
30 for a period of time sufficient for said drug to kill cells of said cell population that do not express said drug selectable marker gene, thereby preparing a purged cell population; and

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- (c) contacting said purged cell population with STZ in an amount effective and for a period of time sufficient for said STZ to kill at least about 60% of the GLUT-2 transporter-expressing cells of the purged cell population.

5

65. The method of claim 64, wherein step (c) further comprises the steps of:

- (i) preparing a first and a second cell culture of said purged cell population;
- (ii) contacting said first cell culture with STZ in an amount effective and for a period of time sufficient to kill at least about 60% of the GLUT-2 transporter-expressing cells of the purged cell population; and
- (iii) identifying a cell from said first cell culture that is sensitive to STZ.

10

15

20 66. The method of claim 63 or 65, further comprising isolating a cell with an integrated selected gene from the corresponding cell of said second cell culture.

25 67. The method of any one of claims 62 through 66, wherein said drug selectable marker gene confers resistance to neomycin, hygromycin, puromycin, zeocin, mycophenolic acid, histidinol or methotrexate.

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68. A method for identifying a promoter element, comprising the steps of:

- 5
- (a) providing to a cell a polynucleotide comprising a GLUT-2 cDNA that lacks a transcriptional promoter;
  - (b) preparing a first and a second cell culture of said cell;
  - (c) contacting said first cell culture with an amount of STZ effective to kill a GLUT-2 transporter-expressing cell; and
  - 10 (d) identifying a cell from said first cell culture that is sensitive to STZ.

69. The method of claim 68, comprising the steps of:

- 15
- (a) providing to cells of a cell population a polynucleotide comprising a GLUT-2 cDNA that lacks a transcriptional promoter;
  - (b) preparing a first and a second cell culture of said cell population;
  - 20 (c) contacting said first cell culture with STZ in an amount effective and for a period of time sufficient to kill at least about 60% of the GLUT-2 transporter-expressing cells in said first cell culture; and
  - 25 (d) identifying a cell from said first cell culture that is sensitive to STZ.

70. The method of claim 68 or 69, further comprising the step of obtaining the promoter element from the corresponding cell of said second cell culture.

30

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71. A method for identifying a polynucleotide conferring STZ resistance, comprising:

- 5 (a) providing to a GLUT-2-expressing cell a polynucleotide encoding a putative STZ resistance gene;
- (b) contacting said cell with STZ in an amount effective and for a period of time sufficient to kill a GLUT-2-expressing cell; and
- 10 (c) identifying an STZ resistant cell.

72. The method of claim 71, comprising the steps of:

- 15 (a) providing to a cell within a population of GLUT-2-expressing cells a polynucleotide encoding a putative STZ resistance gene;
- (b) contacting said population of cells with STZ in an amount effective and for a period of time sufficient to kill at least about 60% of the  
20 GLUT-2-expressing cells within said population of cells; and
- (c) identifying an STZ resistant cell from said population of cells.

25 73. The method of claim 71 or 72, further comprising the step of obtaining the STZ resistance gene from said STZ resistant cell.

74. A method for identifying a polynucleotide that encodes an STZ transporter  
30 protein, comprising:

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- 5 (a) obtaining a cell that does not substantially express a functional GLUT-2 transporter;
- (b) providing to said cell a polynucleotide encoding a putative STZ transporter protein;
- (c) contacting said cell with STZ in an amount effective and for a period of time sufficient to kill an STZ-transporting cell; and
- 10 (d) identifying an STZ sensitive cell.
75. The method of claim 74, comprising the steps of:
- 15 (a) obtaining a population of cells that do not substantially express functional GLUT-2 transporters;
- (b) providing to a cell of said population of cells a polynucleotide encoding a putative STZ transporter protein;
- 20 (c) contacting said population of cells with STZ in an amount effective and for a period of time sufficient to kill at least about 60% of the STZ-transporting cells within said STZ-contacted population of cells; and
- 25 (d) identifying an STZ sensitive cell from said STZ-contacted population of cells.

- 30 76. The method of claim 75, wherein step (d) further comprises the steps of:

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- (i) preparing a first and a second cell culture of said STZ-contacted population of cells;
- (ii) contacting said first cell culture with STZ in an amount effective and for a period of time sufficient to kill at least about 60% of the STZ-transporting cells within said STZ-contacted population of cells; and
- (iii) identifying a cell from said first cell culture that is sensitive to STZ.

10

77. The method of any one of claims 74 through 76, further comprising the step of obtaining the polynucleotide that encodes the STZ transporter protein from said STZ sensitive cell.

15

78. The method of claim 76, further comprising isolating a cell containing a polynucleotide that encodes an STZ transporter protein from the cell of said second cell culture that corresponds to the STZ sensitive cell of said first cell culture.

20

79. A method for identifying a GLUT-2 substrate, comprising determining whether a test substrate competes with STZ in a cell binding, cell transport or cell cytotoxicity assay using a GLUT-2 expressing cell.

25

80. The method of claim 79, wherein the method comprises a cell binding assay.

30

81. The method of claim 79, wherein the method comprises a cell transport assay.

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82. The method of any one of claims 79 through 81, wherein said test substrate is labeled.

5

83. The method of any one of claims 79 through 81, wherein said STZ is labeled.

10 84. The method of any one of claims 79 through 81, wherein said test substrate and said STZ each comprises a different label.

15 85. The method of any one of claims 79 through 84, wherein said test substrate or said STZ comprises a radiolabel.

86. The method of any one of claims 79 through 85, wherein the intracellular level of said test substrate or said STZ is determined.

20

87. The method of any one of claims 79 through 86, wherein the extracellular level of said test substrate or said STZ is determined.

25

88. The method of any one of claims 79 through 87, wherein the amount of said test substrate or said STZ is measured by immunological detection.

30 89. The method of claim 79, comprising a cell cytotoxicity assay.

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90. The method of claim 89, comprising determining whether said test substrate competes with STZ in a cell cytotoxicity assay using a cell population of GLUT-2-expressing cells, wherein the addition of STZ to said cell population results in the killing of at least about 50% of the GLUT-2-expressing cells in said cell  
5 population.

91. A kit comprising, in suitable container means, a GLUT-2 glucose transporter gene composition and a negatively selectable marker gene composition.  
10

92. The kit of claim 91, wherein said GLUT-2 glucose transporter gene and said negatively selectable marker gene are comprised within distinct expression vectors.

93. The kit of claim 91, wherein said GLUT-2 glucose transporter gene and said negatively selectable marker gene are comprised within a single expression vector.  
15

94. The kit of any one of claims 91 through 93, further comprising a pharmaceutically acceptable STZ composition.  
20

95. The kit of any one of claims 91 through 93, further comprising an STZ composition dissolved in DMSO.  
25

96. The kit of any one of claims 91 through 94, wherein said negatively selectable marker gene composition comprises a cytosine deaminase gene.  
30

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97. The kit of claim 96, further comprising a pharmaceutically acceptable fluorocytosine composition.

5 98. A GLUT-2 glucose transporter composition for use in a negative selection protocol or treatment regimen.

99. The GLUT-2 composition of claim 98, wherein said negative selection  
10 protocol comprises providing GLUT-2 to cells within a cell population *in vitro* and contacting said cell population with a composition comprising an effective amount of STZ.

100. The GLUT-2 composition of claim 99, wherein said negative selection  
15 protocol comprises providing GLUT-2 to cells within a cell population *in vitro* and contacting said cell population with a composition comprising STZ in an amount effective and for a period of time sufficient to kill at least about 50% of the GLUT-2-expressing cells in said cell population.

20 101. The GLUT-2 composition of claim 98, wherein said treatment regimen comprises providing GLUT-2 to cells of a tumor *in vivo* and contacting said tumor with a composition comprising a biologically effective amount of STZ.

25 102. The GLUT-2 composition of claim 101, wherein said treatment regimen comprises providing GLUT-2 to a population of tumor cells within an animal and contacting said population of tumor cells with a composition comprising STZ in an amount effective and for a period of time sufficient to kill at least about 50% of  
30 the GLUT-2-expressing cells in said tumor cell population.

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103. The GLUT-2 composition of any one of claims 98 through 102, wherein said composition comprises a first polynucleotide comprising a first coding region that encodes GLUT-2.

5

104. The GLUT-2 composition of claim 103, wherein said composition comprises a recombinant vector that comprises a promoter operatively linked to said first coding region, the promoter expressing GLUT-2 in cells of said cell population.

10

105. The GLUT-2 composition of claim 104, wherein said composition comprises a recombinant virus that comprises said recombinant vector.

15

106. The GLUT-2 composition of claim 104 or 105, wherein said recombinant vector comprises a constitutive promoter that expresses GLUT-2 in a cell.

20

107. The GLUT-2 composition of claim 104 or 105, wherein said recombinant vector comprises a tissue-specific promoter that expresses GLUT-2 in selected cells of a cell population.

25

108. The GLUT-2 composition of claim 107, wherein said recombinant vector comprises a pancreatic  $\beta$  cell-specific promoter.

30

109. The GLUT-2 composition of any one of claims 103 through 108, wherein said composition comprises a second polynucleotide coding region comprising at least one exogenous gene.

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110. The GLUT-2 composition of claim 109, wherein said second coding region is comprised within a first recombinant vector that expresses GLUT-2 and said exogenous gene.

5

111. The GLUT-2 composition of claim 109, wherein said second coding region is comprised within a second, distinct recombinant vector that expresses said exogenous gene.

10

112. The GLUT-2 composition of any one of claims 109 through 111, wherein said exogenous gene is a negatively selectable marker gene.

15

113. The GLUT-2 composition of claim 112, wherein said exogenous gene is a cytosine deaminase gene.

20

114. Use of a GLUT-2 composition in a negative selection protocol or treatment regimen.

25

115. A GLUT-2 composition for use in a method of treating cancer, wherein said method comprises providing a GLUT-2 glucose transporter to cells of a tumor within an animal and contacting said tumor with a composition comprising STZ in a therapeutically effective amount sufficient to kill said cells.

30

116. The GLUT-2 composition of claim 115, wherein said method comprises providing a GLUT-2 glucose transporter to a population of tumor cells within an animal and administering to said animal a composition comprising STZ in an

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amount effective and for a period of time sufficient to kill at least about 50% of the GLUT-2-expressing cells in said tumor cell population.

- 5     117.    The GLUT-2 composition of claim 115 or 116, wherein said method further comprises providing a second negatively selectable marker gene to said tumor cells and administering to said animal a composition comprising a therapeutically effective amount of a compound that kills cells expressing said second negatively selectable marker gene.

10

118.    The GLUT-2 composition of claim 117, wherein said second negatively selectable marker gene is a cytosine deaminase gene and wherein a therapeutically effective amount of fluorocytosine is administered to said animal.

15

119.    Use of a GLUT-2 composition in the preparation of a medicament for use in treating cancer.

- 20     120.    An STZ composition for use in a GLUT-2-mediated negative selection protocol or treatment regimen.

25     121.    The STZ composition of claim 120, wherein said STZ composition is dissolved in DMSO.

122.    A glucose transporter that confers physiological glucose sensing capacity to a cell but that does not render said cell subject to diabetic immune destruction.

30

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123. The glucose transporter of claim 122, further defined as a GLUT-2 transporter that includes an amino acid mutation.

5 124. The glucose transporter of claim 122 or 123, further defined as a GLUT-1/GLUT-2 transporter chimera.

125. The glucose transporter of any one of claims 122 through 124, further  
10 defined as a GLUT-1/GLUT-2 transporter chimera that does not transport STZ.

126. The glucose transporter of claim 124 or 125, wherein said GLUT-1/GLUT-2 transporter chimera has an extracellular domain that comprises at least a portion of  
15 an extracellular domain of a GLUT-1 transporter.

127. The glucose transporter of any one of claims 124 through 126, wherein said GLUT-1/GLUT-2 transporter chimera has an intracellular domain that comprises at least a portion of an intracellular domain of a GLUT-1 transporter.

20

128. The glucose transporter of any one of claims 124 through 127, wherein said GLUT-1/GLUT-2 transporter chimera has a transmembrane domain that comprises at least a portion of a transmembrane domain of a GLUT-2 transporter.

25

129. The glucose transporter of any one of claims 122 through 128, preparable by a process that comprises the steps of:

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- (a) providing a putative glucose transporter to a secretory cell that includes an insulin gene and a hexokinase IV gene but that substantially lacks an effective GLUT-2 gene; and
- 5 (b) identifying a cell that has a physiological glucose sensing capacity and that is resistant to diabetic immune destruction.
130. The glucose transporter of claim 129, wherein said providing comprises the steps of:
- 10 (a) obtaining a polynucleotide encoding said putative glucose transporter; and
- 15 (b) expressing said polynucleotide in said cell.
131. The glucose transporter of claim 125, preparable by a process that comprises the steps of:
- 20 (a) providing a putative GLUT-1/GLUT-2 glucose transporter to a secretory cell that includes an insulin gene and a hexokinase IV gene but that substantially lacks an effective a GLUT-2 gene;
- 25 (b) identifying a cell that has a physiological glucose sensing capacity and that does not transport STZ.

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132. The glucose transporter of claim 131, wherein said providing comprises the steps of:

5 (a) obtaining a polynucleotide encoding said putative GLUT-1/GLUT-2 glucose transporter; and

(b) expressing said polynucleotide in said cell.

10 133. The glucose transporter of any one of claims 122 through 132, for use in administration to a patient to treat diabetes.

15 134. A polynucleotide encoding a glucose transporter that confers physiological glucose sensing capacity to a cell but that does not render said cell subject to diabetic immune destruction.

20 135. The polynucleotide of claim 134, wherein said polynucleotide encodes a mutant GLUT-2 transporter that does not transport STZ.

25 136. The polynucleotide of claim 134 or 135, wherein said polynucleotide encodes a GLUT-1/GLUT-2 transporter chimera that does not transport STZ.

30 137. The polynucleotide of claim 136, wherein said polynucleotide encodes a GLUT-1/GLUT-2 transporter chimera that comprises a contiguous amino acid sequence from SEQ ID NO:2 operatively linked to a contiguous amino acid sequence from SEQ ID NO:4.

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138. The polynucleotide of claim 137, wherein said polynucleotide comprises a contiguous nucleic acid sequence from SEQ ID NO:1 operatively linked to a contiguous nucleic acid sequence from SEQ ID NO:3.

5

139. The polynucleotide of any one of claims 134 through 138, wherein the polynucleotide is positioned under the control of a promoter that directs its expression in a mammalian cell.

10

140. The polynucleotide of any one of claims 134 through 139, for use in administration to a patient to treat diabetes.

15

141. The polynucleotide of claim 140, wherein said polynucleotide is expressed in a cell to prepare a recombinant cell that secretes insulin in response to glucose and wherein said cell is administered to a patient to treat diabetes.

20

142. A host cell comprising a polynucleotide that encodes a glucose transporter that confers physiological glucose sensing capacity to a cell but that does not render said cell subject to diabetic immune destruction.

25

143. The host cell of claim 142, wherein said cell comprises a recombinant insulin gene or a recombinant hexokinase IV gene.

30

144. The host cell of claim 142 or 143, for use in administration to a patient to treat diabetes.

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145. A population of recombinant cells comprising a polynucleotide that encodes a glucose transporter that confers physiological glucose sensing capacity to the cells but that does not render said cells subject to diabetic immune destruction.

5

146. The population of cells of claim 145, wherein the cells of said population comprises a recombinant insulin gene or a recombinant hexokinase IV gene.

10 147. The population of cells of claim 145 or 146, for use in administration to a patient to treat diabetes.

15 148. The population of cells of any one of claims 145 through 147, wherein said cells are encapsulated within a semi-permeable coating or are housed within an implantable device.

20 149. A method for conferring glucose sensitivity to a cell, comprising providing to the cell a glucose transporter that confers glucose sensing capacity to a cell but that does not render said cell subject to diabetic immune destruction.

25 150. The method of claim 149, wherein said providing comprises the steps of:

(a) obtaining a polynucleotide encoding a mutant GLUT-2 transporter or a chimeric GLUT-1/GLUT-2 transporter, the transporter not transporting STZ; and

30 (b) expressing said polynucleotide in said cell.

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151. Use of a polynucleotide that encodes a mutant or chimeric glucose transporter that confers physiological glucose sensing capacity to a cell but that does not render said cell subject to diabetic immune destruction in the preparation of a medicament for use in treating diabetes.

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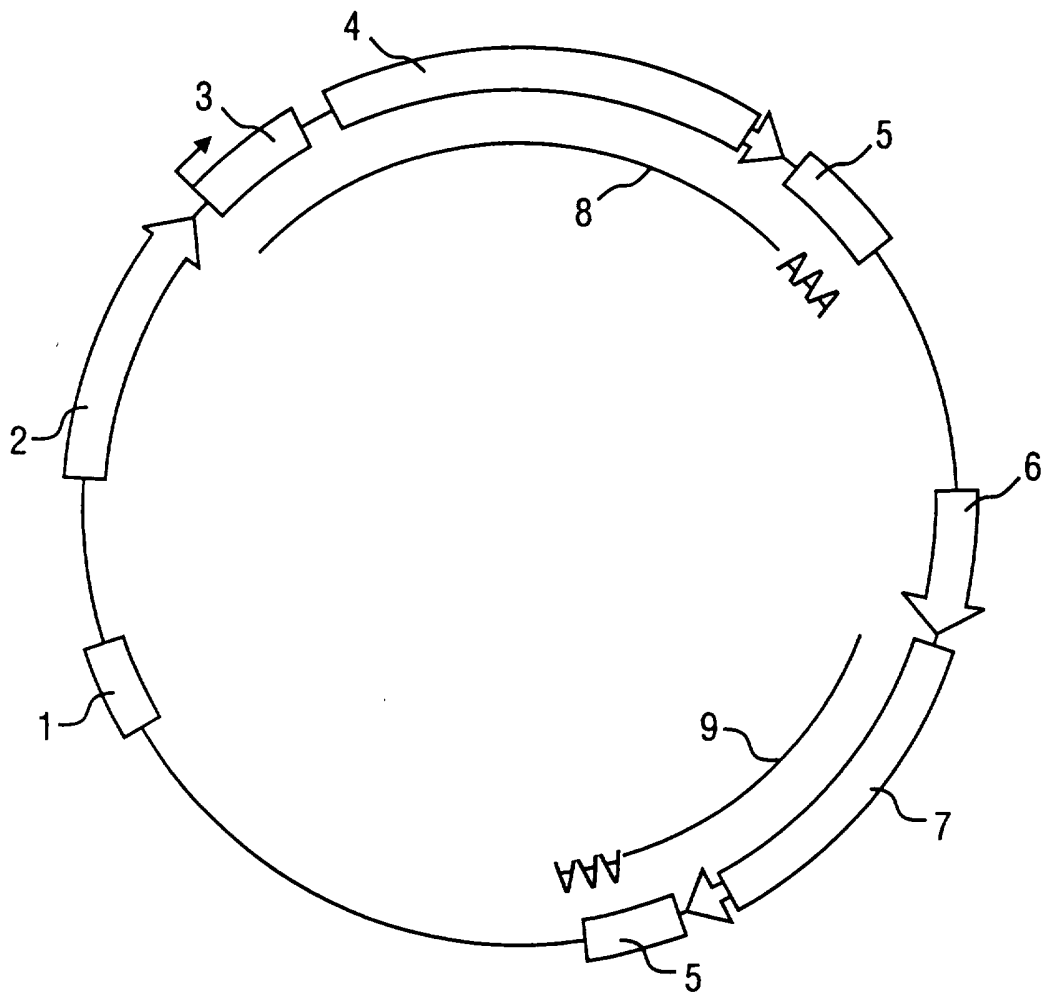


FIG. 1

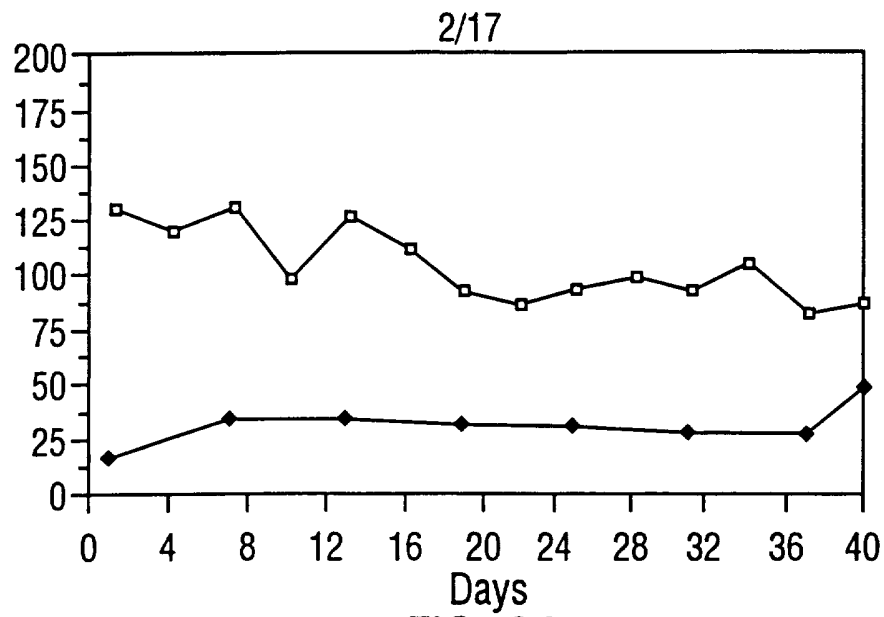


FIG. 2A

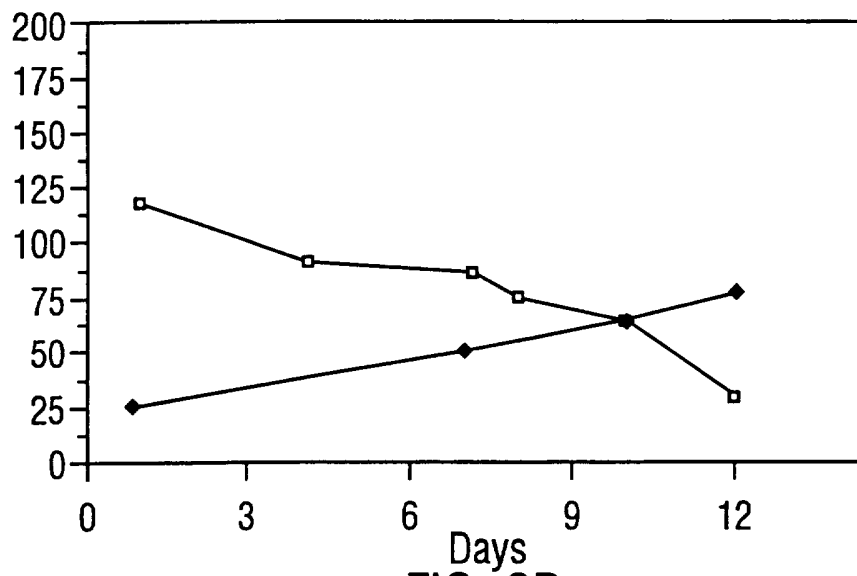


FIG. 2B

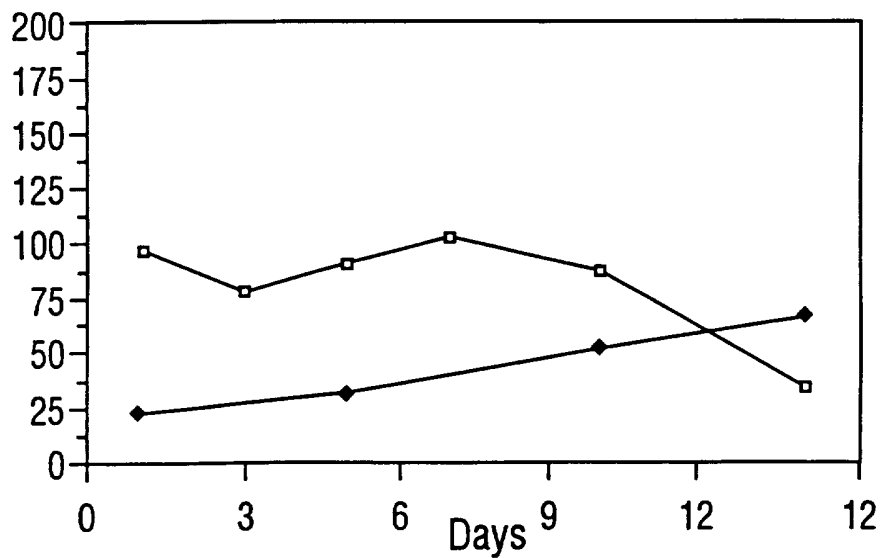


FIG. 2C

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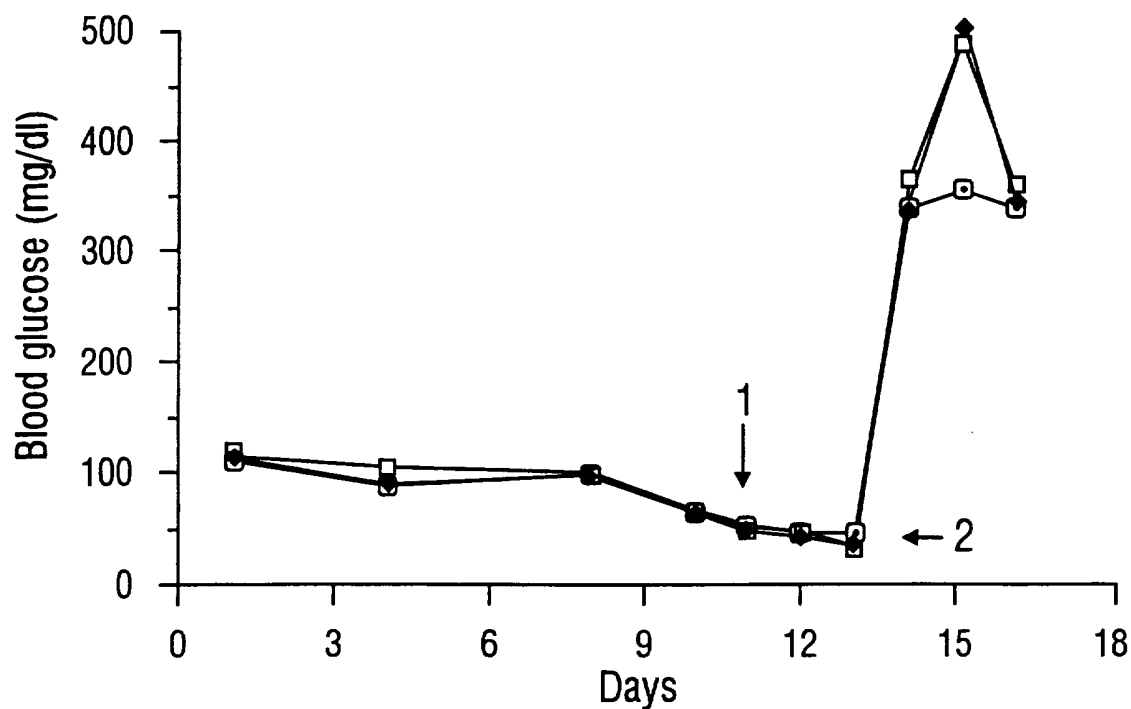


FIG. 3A

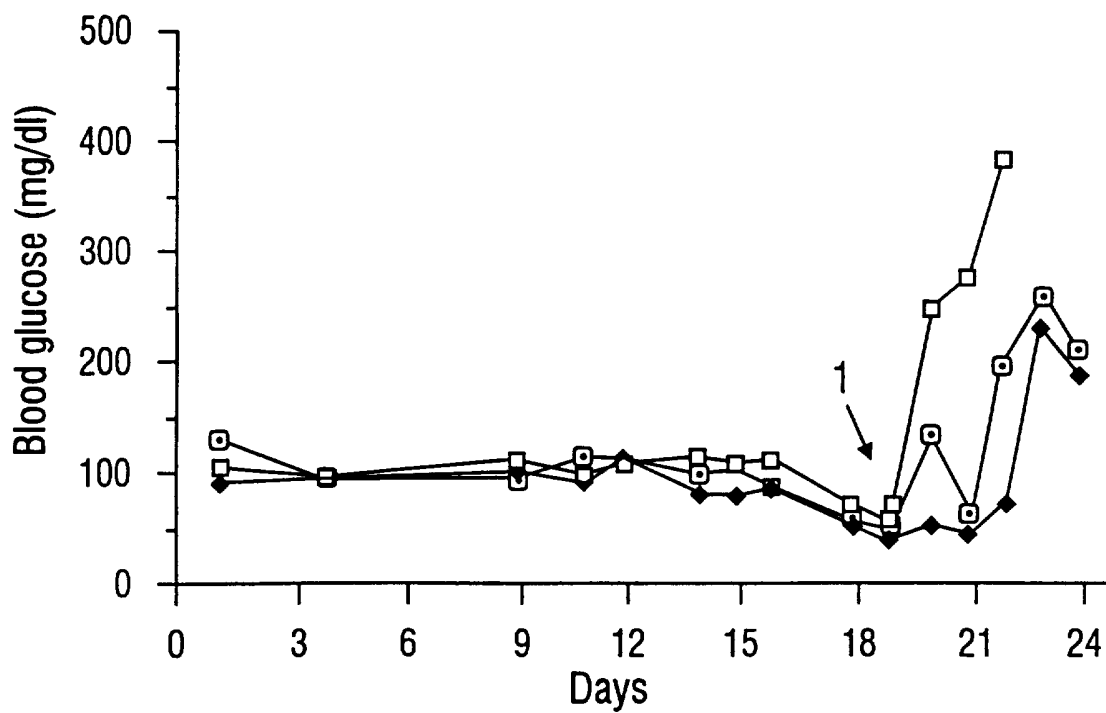


FIG.3B

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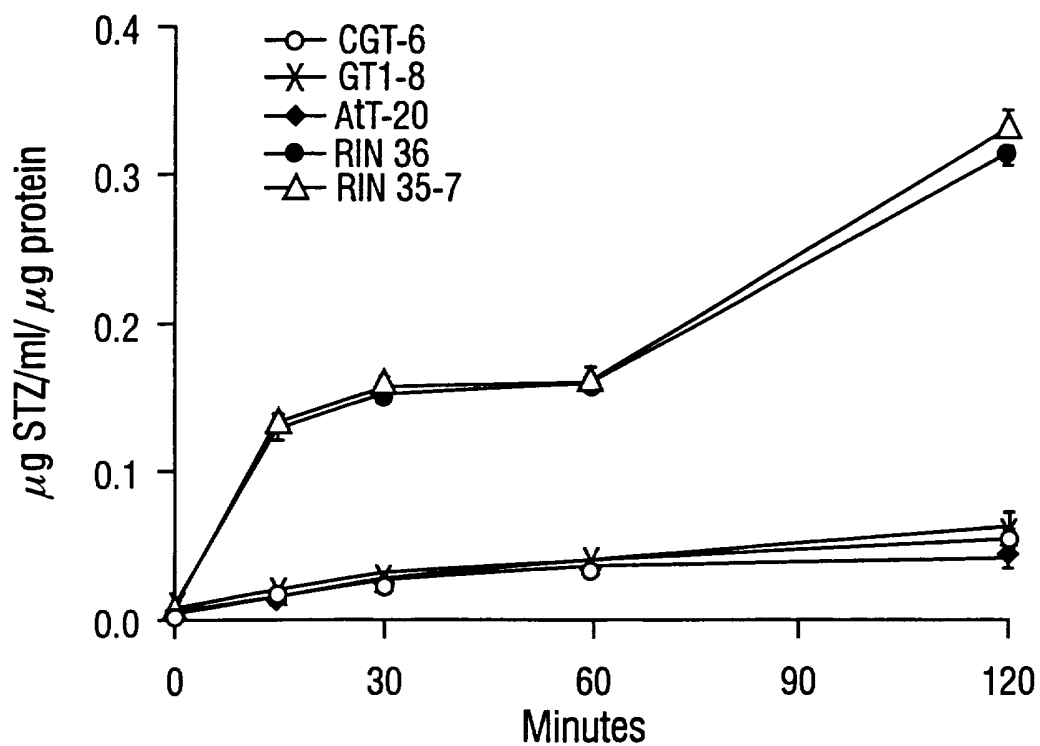


FIG. 4A

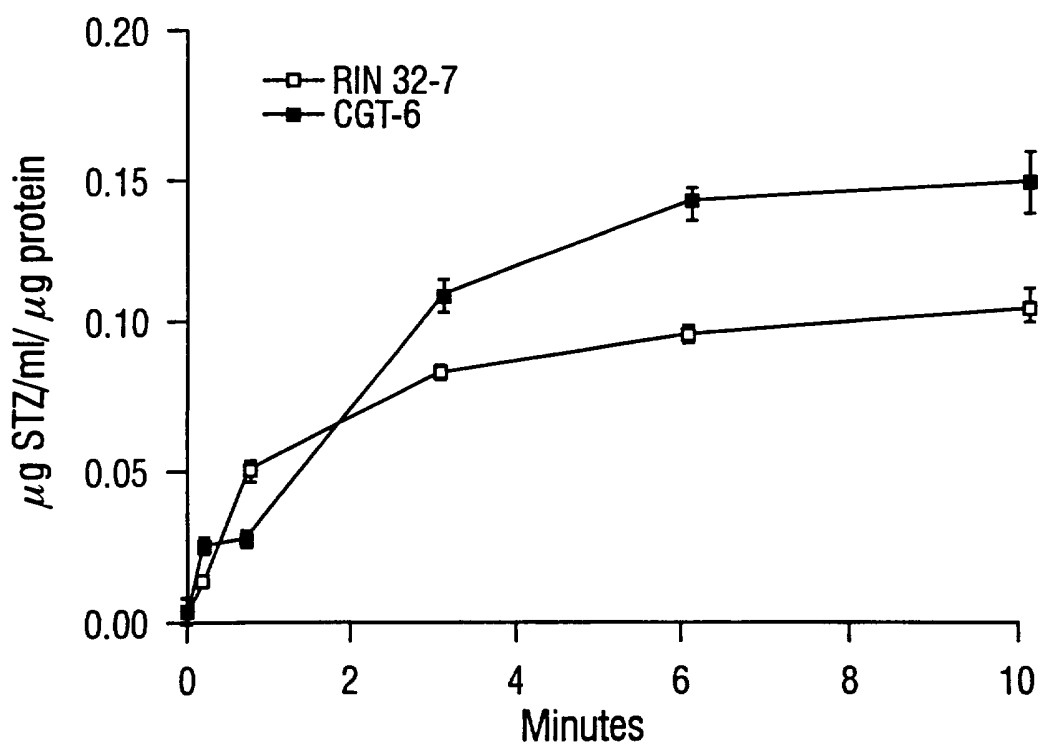
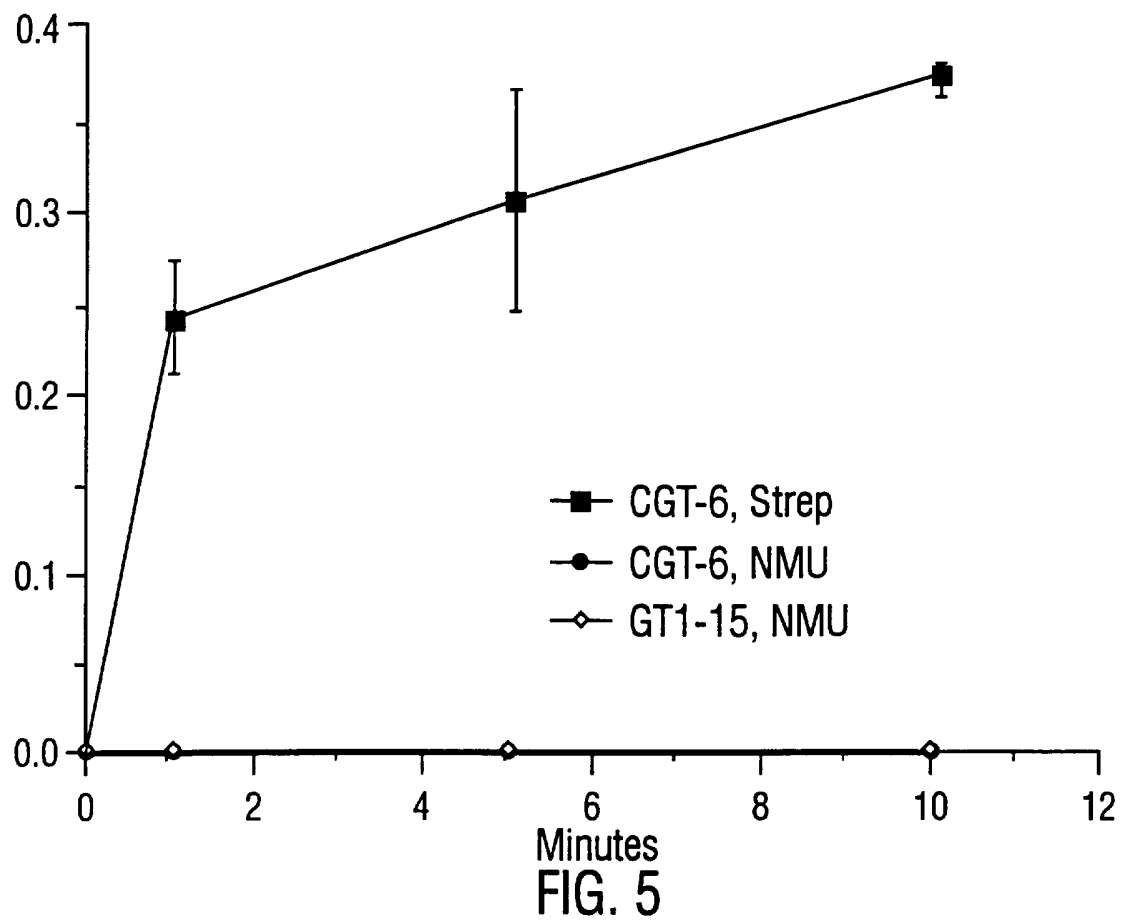


FIG. 4B

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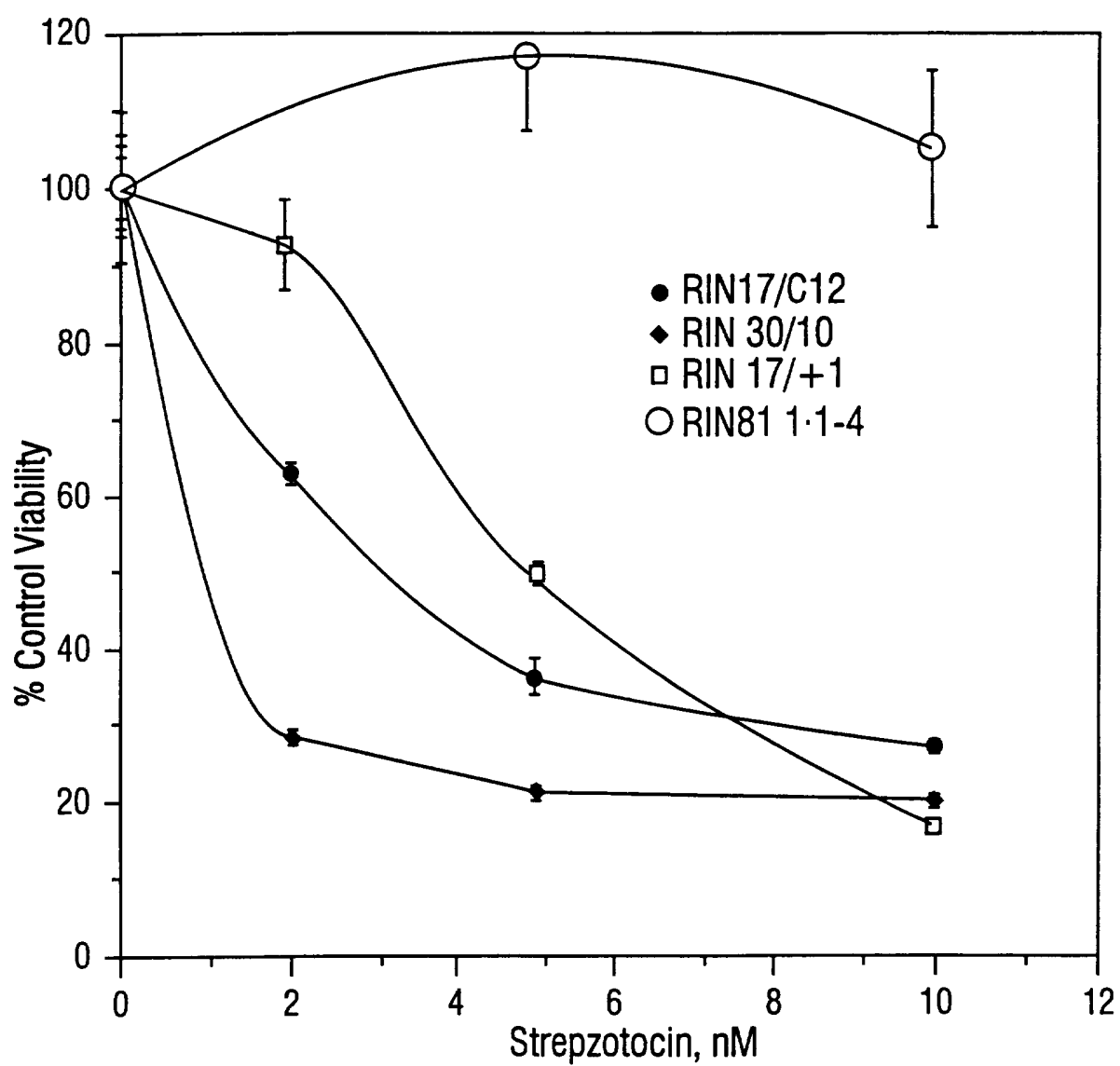


FIG. 6A

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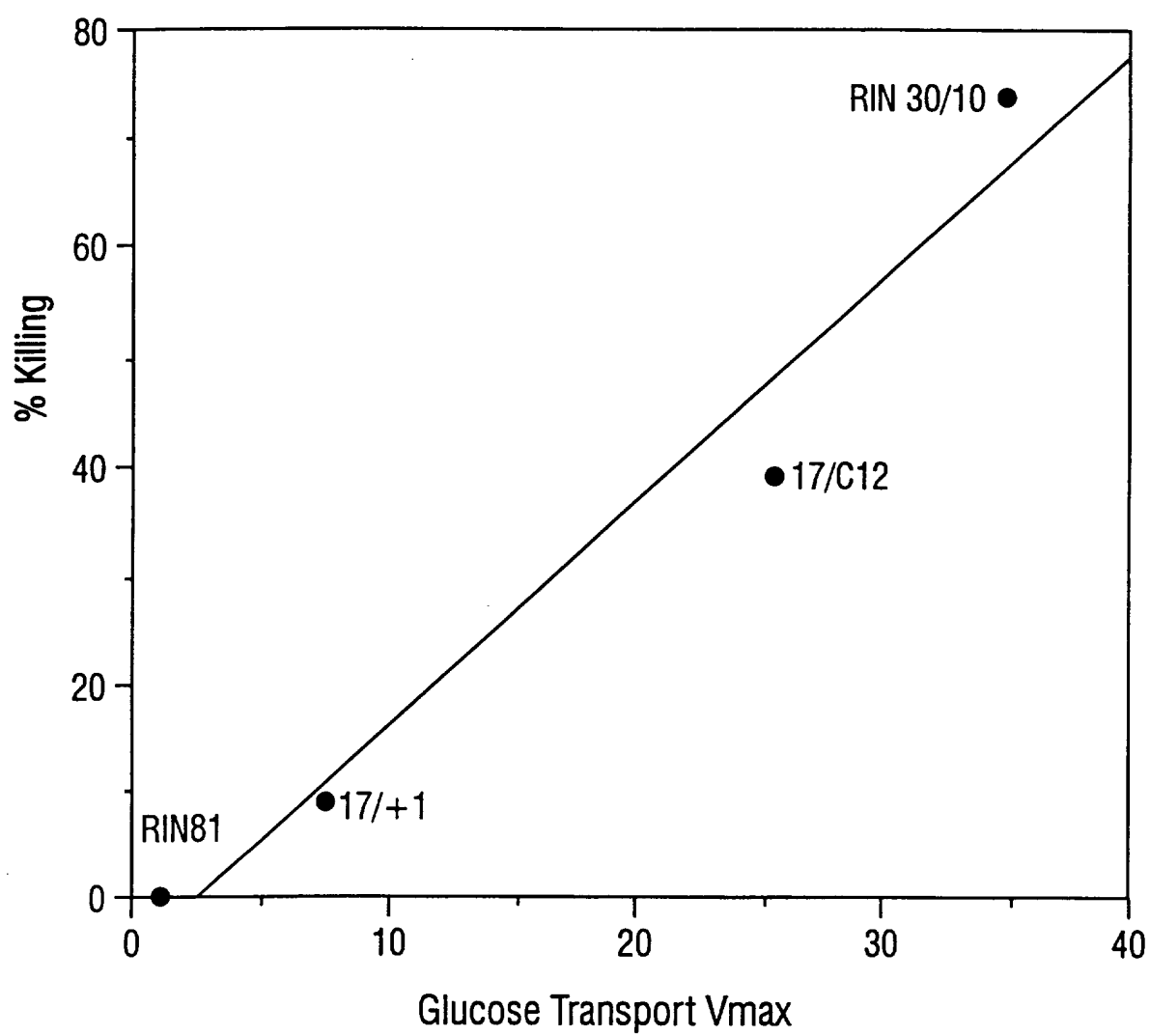
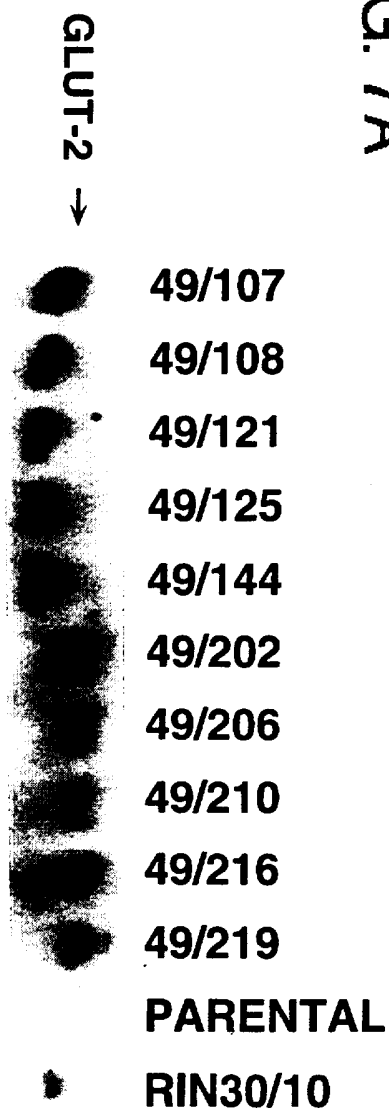


FIG. 6B

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FIG. 7A



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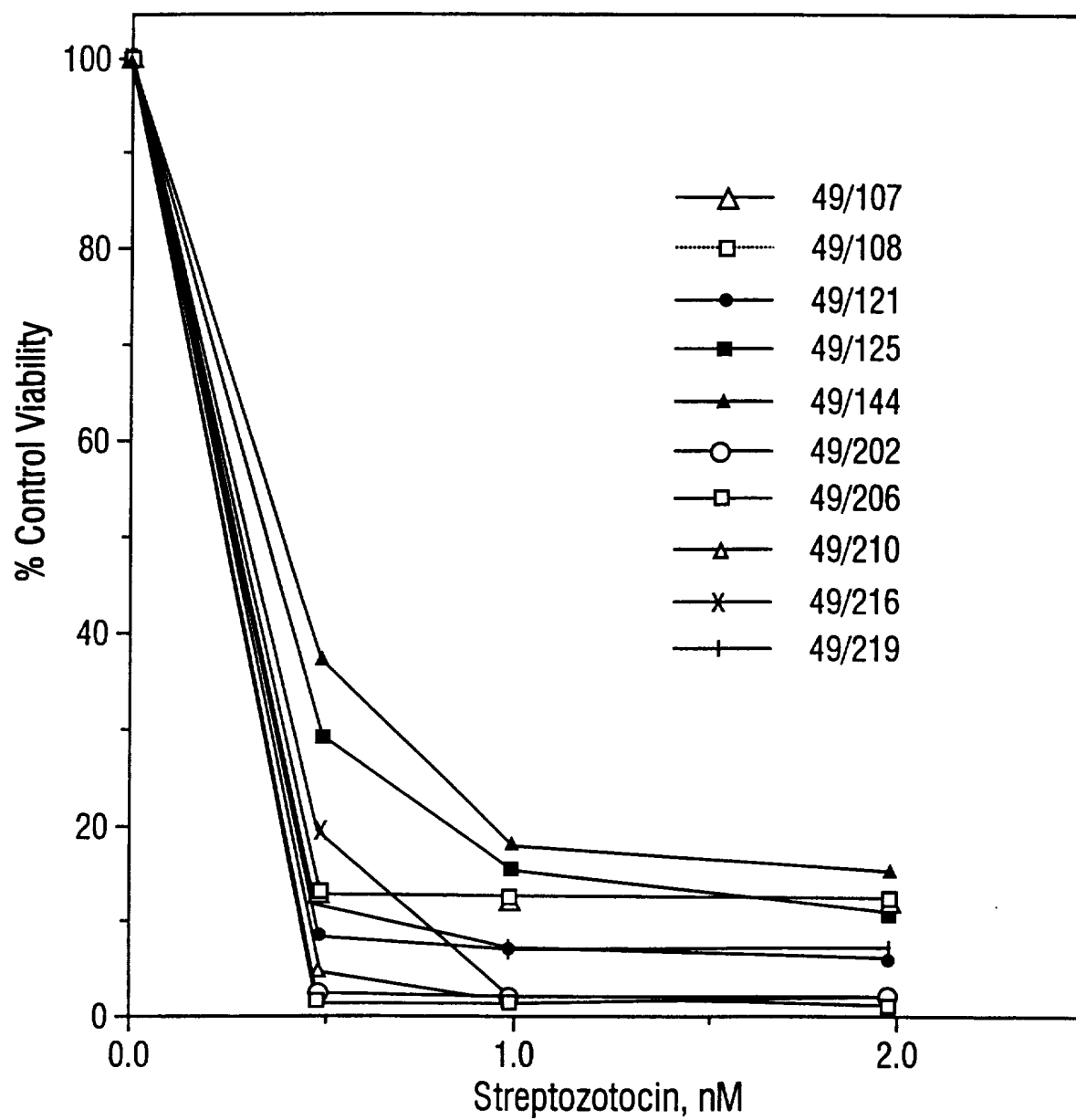


FIG. 7B

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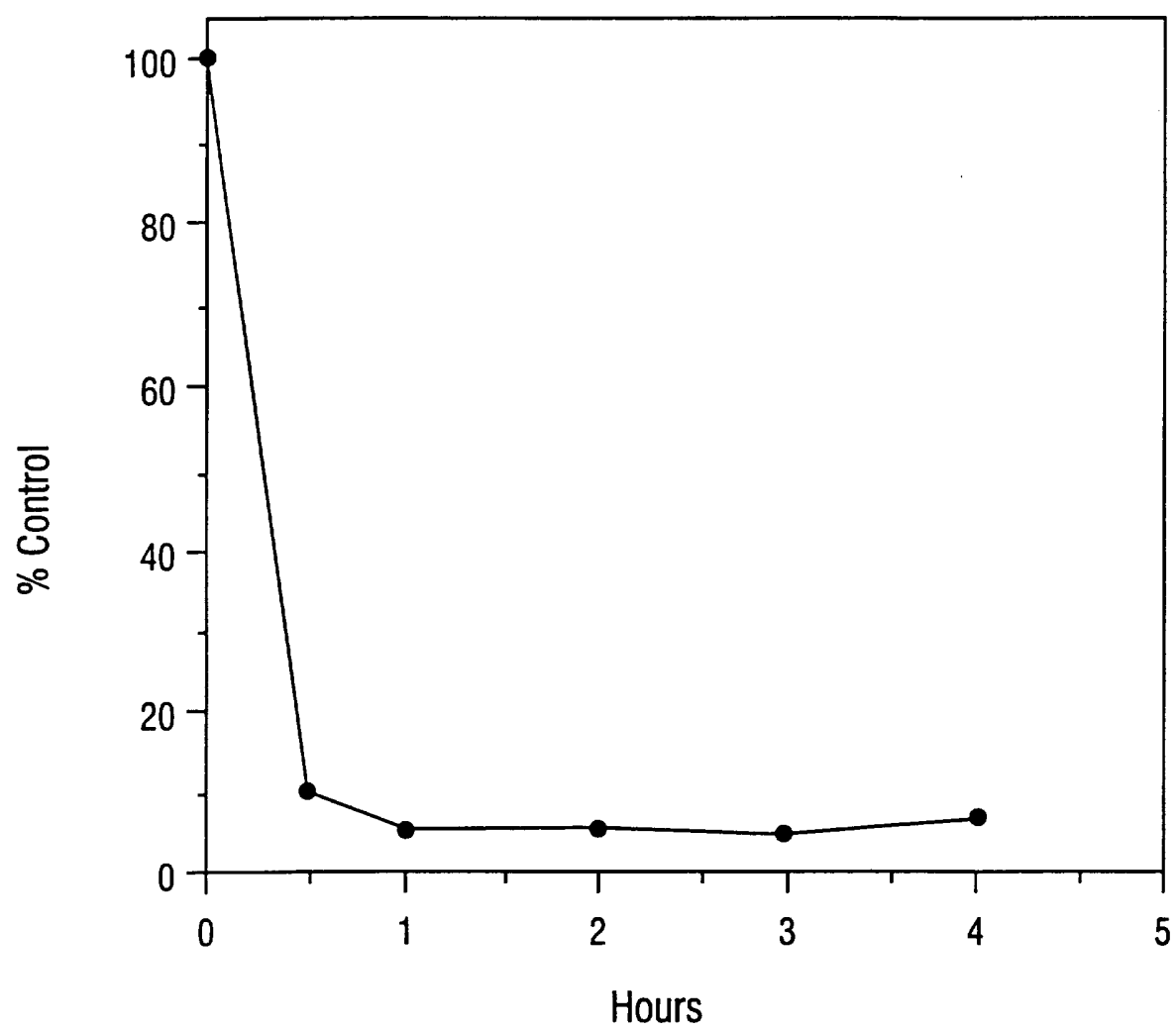


FIG. 8

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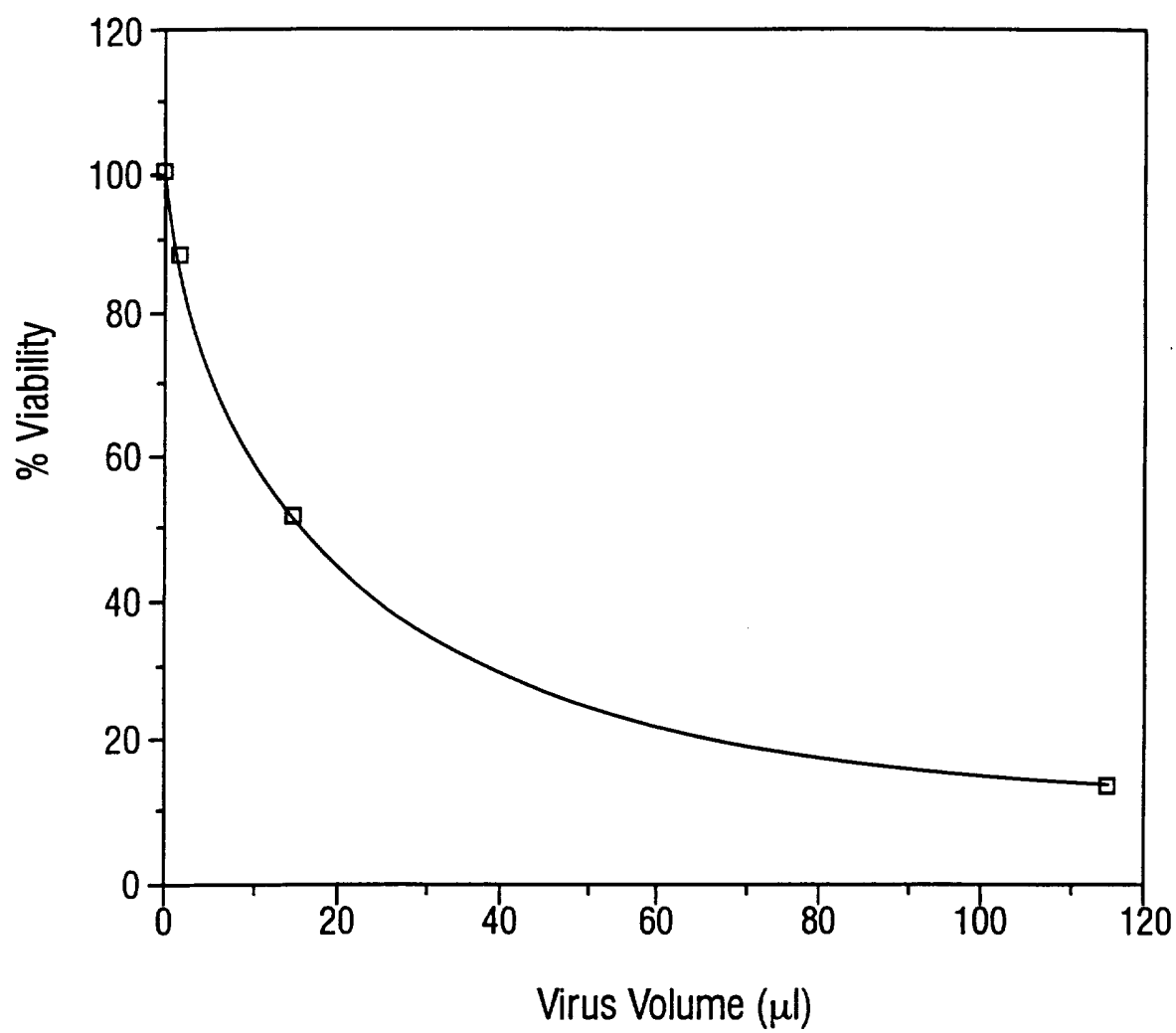


FIG. 9

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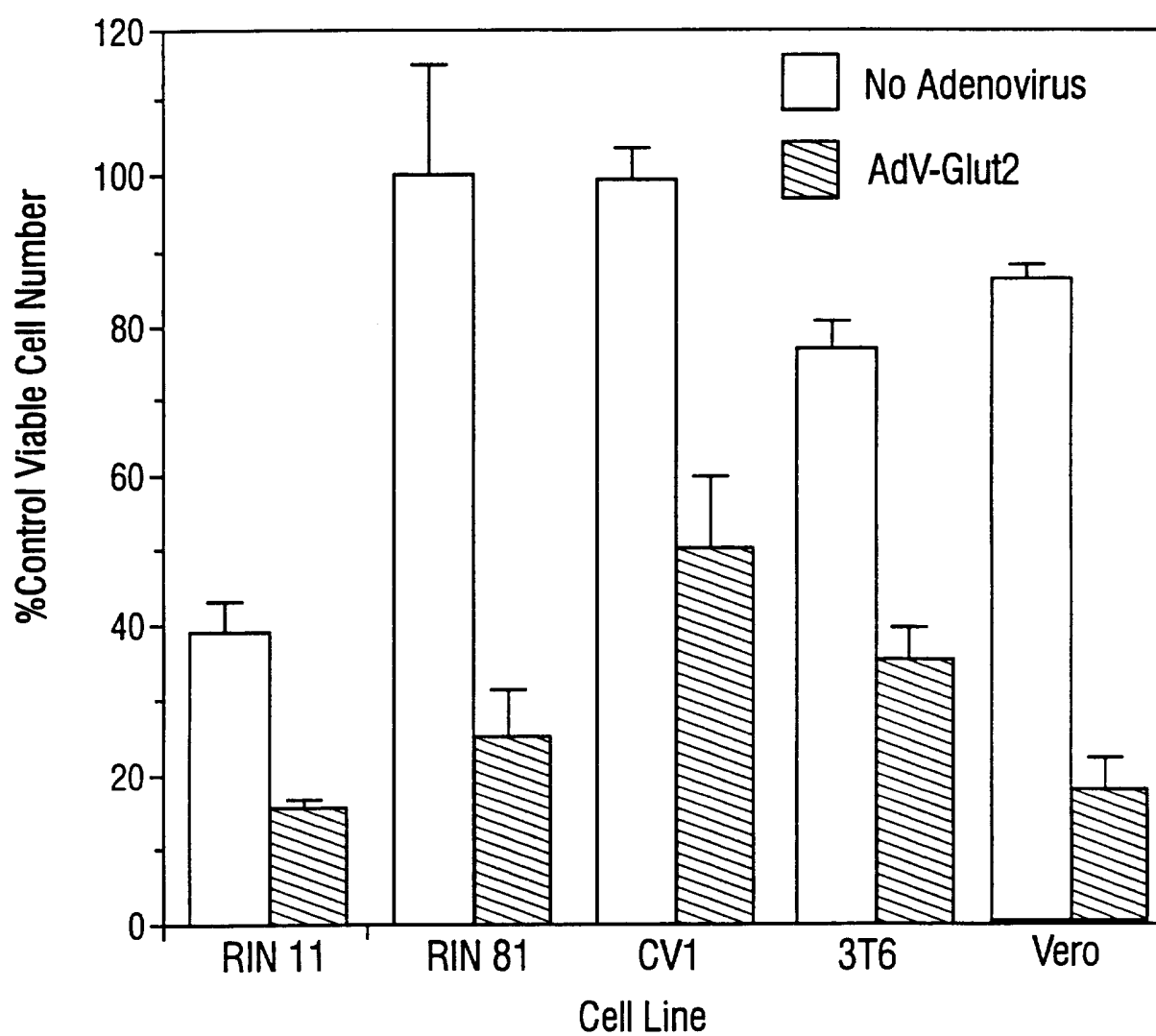


FIG. 10

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FIG. 11A

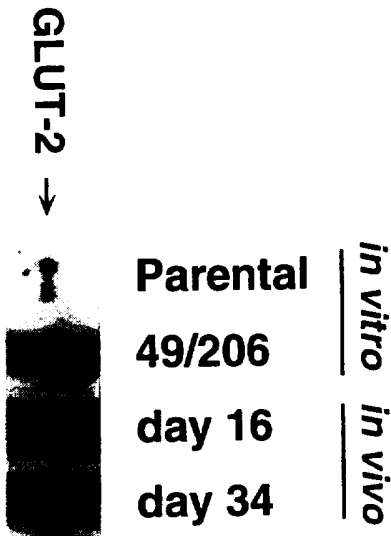
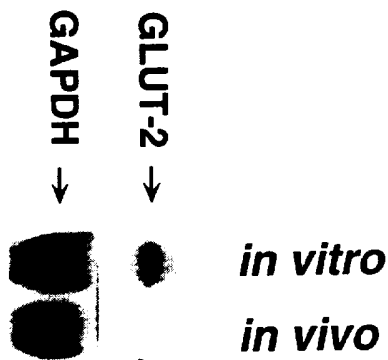


FIG. 11B



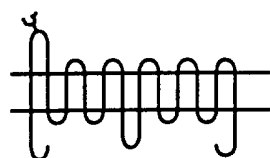


FIG. 12A

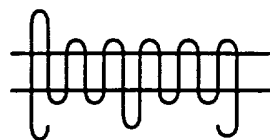


FIG. 12F

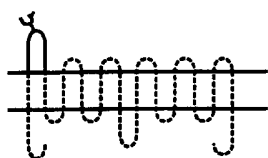


FIG. 12B

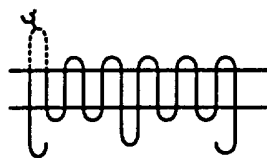


FIG. 12G

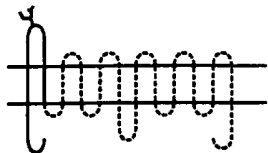


FIG. 12C

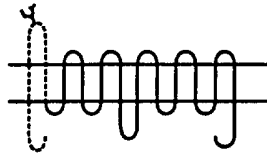


FIG. 12H

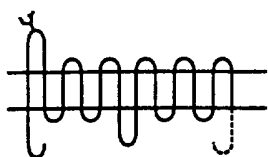


FIG. 12D

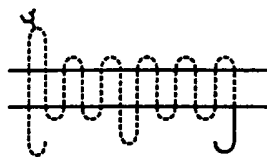


FIG. 12I

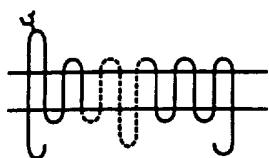


FIG. 12E

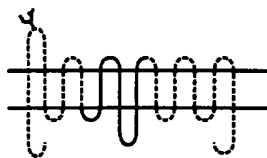


FIG. 12J

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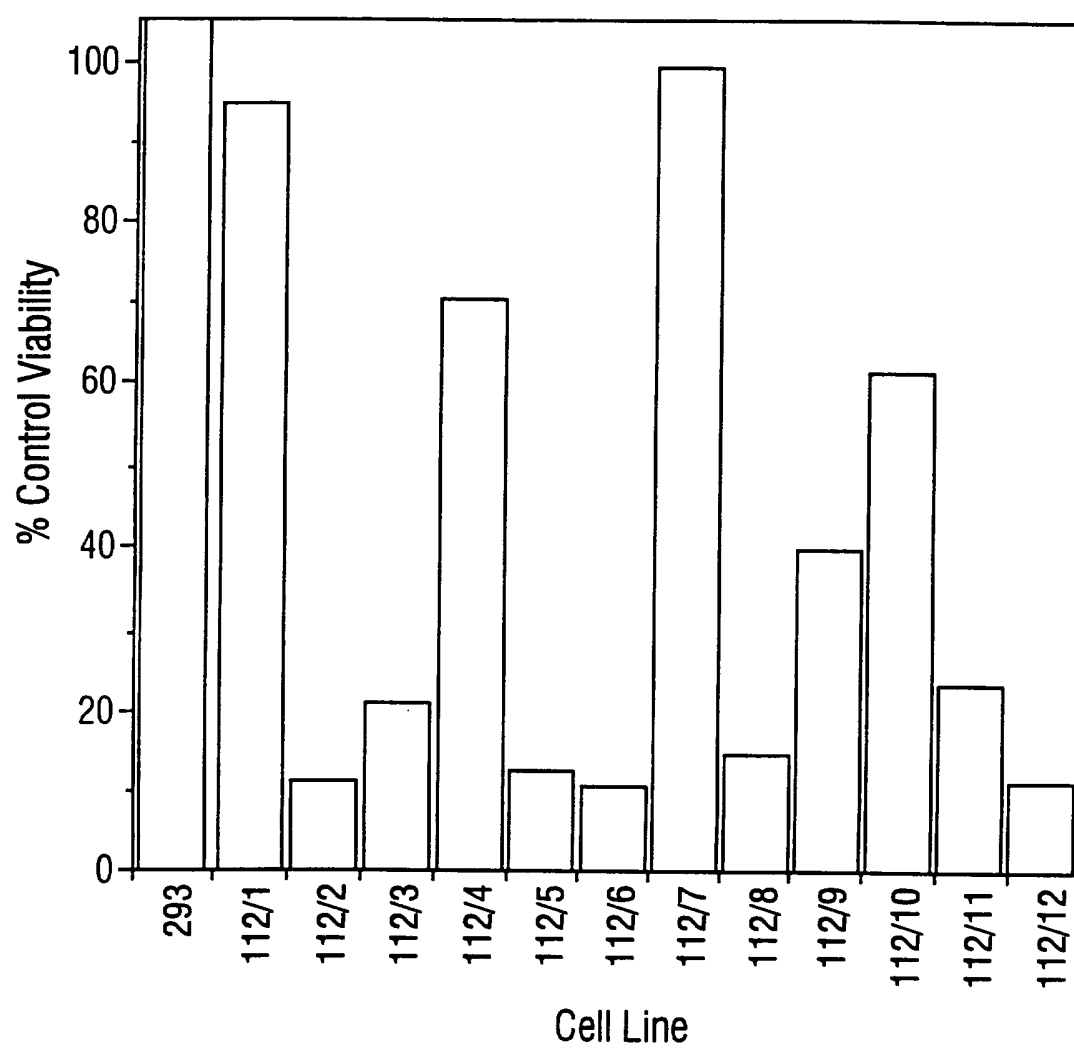


FIG. 13

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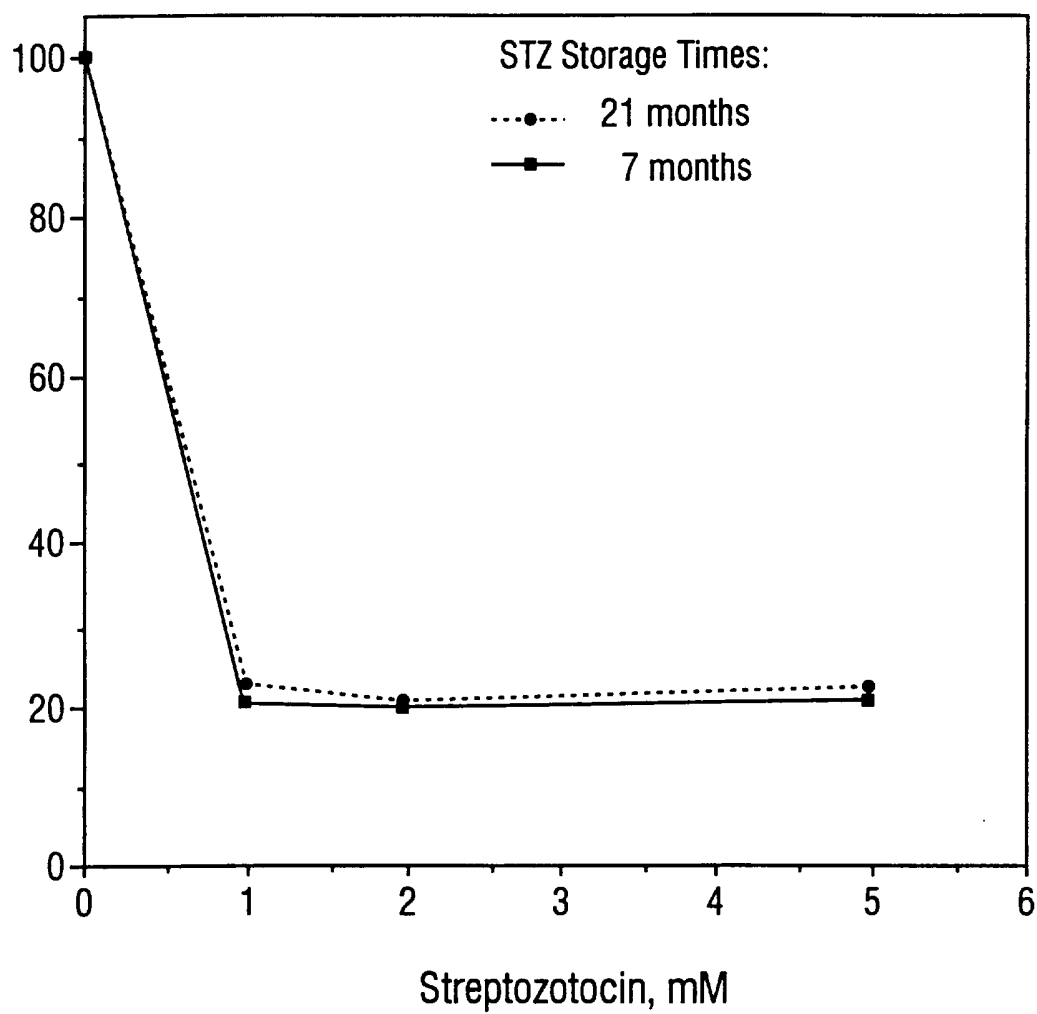


FIG. 14

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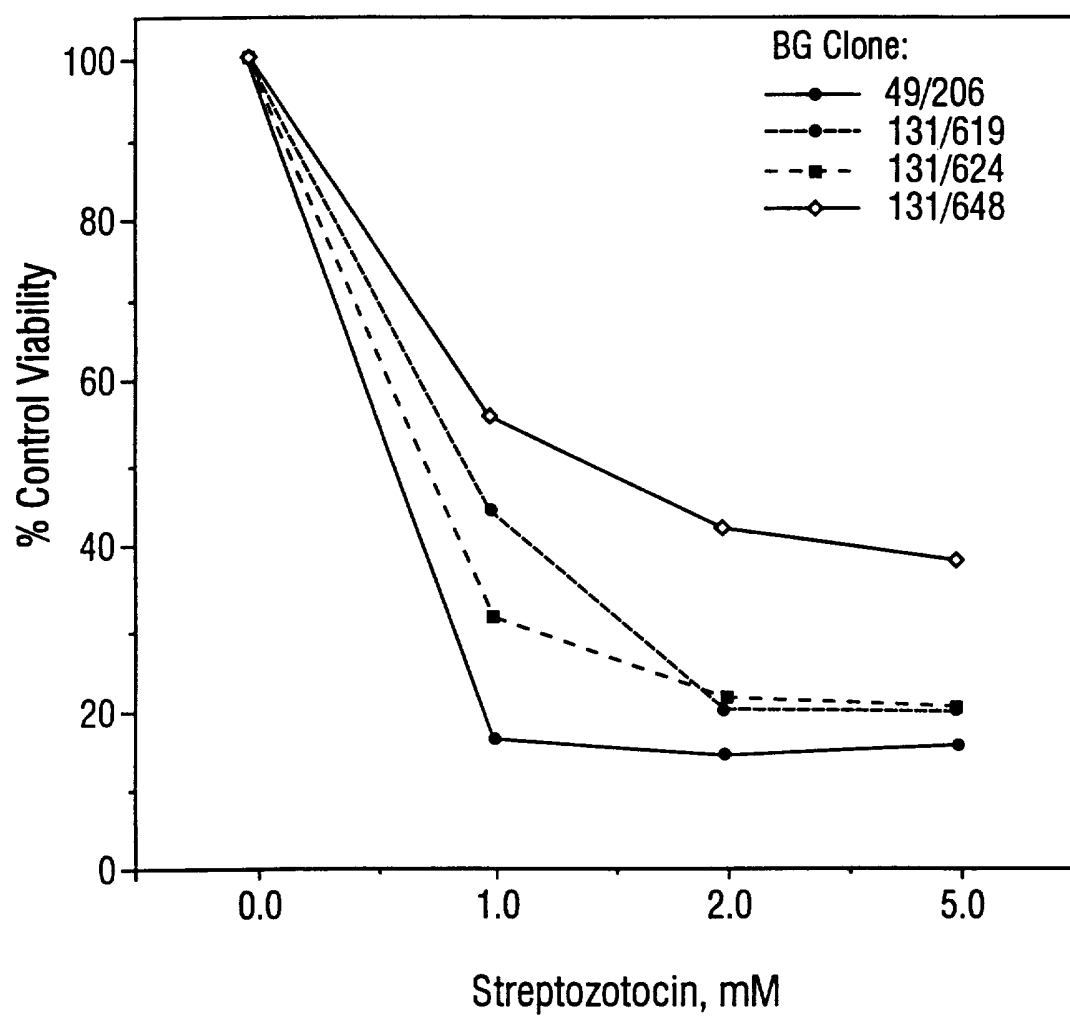


FIG. 15