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(54) Title: CLONED DNA ENCODING A UDP-GALNAc-POLYPEPTIDE,N-ACETYLGALACTOS AMINYLTRANSFERASE		
(57) Abstract The present invention relates to a method for the isolation and expression of a glycosyltransferase enzyme for use in the synthesis of oligosaccharide or polysaccharide structures on glycoproteins, glycolipids, or as free molecules. The gene coding for the enzyme N-acetylgalactosaminyltransferase and the polypeptide sequence of the acceptor peptide for the enzyme N-acetylgalactosaminyltransferase have been isolated and used for the control of glycosylation of a protein.		

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A CLONED DNA ENCODING A UDP-GALNAc: POLYPEPTIDE,
N-ACETYL GALACTOSAMINYLTRANSFERASE

BACKGROUND OF THE INVENTION

The present invention relates to glycosyltransferase enzymes and the genes
5 corresponding to such enzymes. In particular, the present invention relates to the
enzyme N-acetylgalactosaminyltransferase. Specifically, the invention relates to the
isolation and sequencing of the enzyme N-acetylgalactosaminyltransferase. The
invention also relates to the construction of proteins capable of expressing the
acceptor peptide for the enzyme N-acetylgalactosaminyltransferase.

10 Carbohydrates are an important class of biological compounds. In cells,
carbohydrates function as structural components where they regulate viscosity, store
energy, or are key components of cell surfaces. Nearly all site specific intercellular
interactions involve cell surface carbohydrates. For example, union of sperm and
egg as well as the implantation of fertilized egg are both mediated by cell surface
15 carbohydrates. Likewise, a number of proteins that function as cell adhesion
molecules, including GMP-140, ELAM-1, and lymphocyte adhesion molecules like
Mel-14, exhibit structural features that mimic lectins, and are thought to bind
specific cell surface carbohydrate structures (Stoolman, Cell (1989) 56:907-910).
Glycosylated proteins as tumor-associated antigens are now being used to identify
20 the presence of numerous carcinomas. Even isolated oligosaccharides have been
found to exhibit biological activity on their own.

Specific galactose glycosaccharides are known to inhibit the agglutination of
uropathogenic coliform bacteria with red blood cells (U.S. Patent No. 4,521,592).
Other oligosaccharides have been shown to possess potent antithrombic activity by
25 increasing the levels of plasminogen activator (U.S. Patent No. 4,801,583). This
same biological activity has been used, by binding oligosaccharides, in conjunction
with an amino glycoprotein, to medical instruments to provide medical surfaces
which have anticoagulation effects (U.S. Patent No. 4,810,784). Still other
oligosaccharides have found utility as gram positive antibiotics and disinfectants
30 (U.S. Patent Nos. 4,851,338 and 4,665,060). Further, oligosaccharides have been
used as bacteria receptor sites in the diagnosis and identification of specific bacteria
(U.S. Patent Nos. 4,657,849 and 4,762,824).

It is also well recognized that oligosaccharides have an influence on the
protein or lipid to which they are conjugated (Rademacher et al., Ann. Rev.,
35 Biochem., (1988), 57:785). Specific oligosaccharides have been shown to influence
proteins, stability, rate of proteolysis, rate of *in vivo* clearance from the bloodstream,

thermal stability and solubility. Changes in the oligosaccharide portion of cell surface carbohydrates have been noted in cells which have become cancerous. Other oligosaccharide changes have been detected during cell differentiation (Toone et al., Tetrahedron Report (1989) 45(17):5365-5422). As such, the significance of oligosaccharides to biological function cannot be understated.

O-glycosidically linked (mucin type) oligosaccharides have been reported on a number of different types of glycoproteins (Sadler, (1984) Biology of Carbohydrates, (Ginsburg and Robbins, eds.) pp. 199-213, Vol. 2, John Wiley and Sons, New York). These structures have been assigned a diverse array of functions, ranging from quite specific such as being involved in cell-cell recognition and host-pathogen interaction, to more general such as providing protection from proteolytic degradation or supplying the appropriate charge and water binding properties to mucous secretions (Sadler (1984) Biology of Carbohydrates (*supra*); Paulson (1989) Trends Biochem. Sci., 14:272-275; and Jentoft (1990) Trends Biochem. Sci., 15:291-294).

The initial reaction in O-linked oligosaccharide biosynthesis is the transfer of an N-acetylgalactosamine residue from the nucleotide sugar UDP-N-acetylgalactosamine to a serine or threonine residue on the protein acceptor. This reaction, which can occur post-translationally, is catalyzed by UDP-GalNAc:polypeptide, N-acetylgalactosaminyltransferase (hereinafter referred to as GalNAc-transferase or GalNAcT) an intracellular membrane bound enzyme believed to be localized in the secretory pathway.

The exact location(s) of GalNAc-transferase is still controversial. It has been reported that the initial addition of N-acetylgalactosamine to the acceptor protein can take place early (even co-translationally) in the rough endoplasmic reticulum (ER). Other authors have suggested that this reaction is a post-translational event occurring in later ER compartments and/or in the cis region of the Golgi complex (e.g. Hanover *et al.* (1982) J. Biol. Chem. 257:10172-10177; Roth (1984) J. Cell Biol. 98:399-406; Elhammer and Kornfeld (1984) J. Cell Biol. 98:327-331; Tooze *et al.* (1988) J. Cell Biol. 106:1475-1487; Deschuyteneer *et al.* (1988) J. Biol. Chem. 263:2452-2459; Ulmer and Palade (1989) Proc. Natl. Acad. Sci. (U.S.A.) 89:663-667; Wertz *et al.* (1989) J. Virol. 63:4767-4776; Piller *et al.* (1989) Eur. J. Biochem. 183:123-135; Piller *et al.* (1990) J. Biol. Chem. 265:9264-9271. Finally, evidence has also been presented for a model in which transfer of N-acetylgalactosamine to Ser/Thr may occur in several compartments in the secretory pathway, including compartments later than the Golgi complex (Schachter and Brockhausen (1992) Glycoconjugates, Allen and Kisailus, eds., pp. 263-332, Marcel Dekker Inc., New

York). Elongation and termination of O-linked oligosaccharides is accomplished by sequential addition of individual monosaccharides by specific transferases (Roseman (1970) Chem. Phys. Lipids 5:270-280); current data suggest that these reactions are localized primarily in the Golgi apparatus (Schachter and Brockhausen, *supra*).

5 The fundamental role of oligosaccharides, particularly, O-glycosidically linked (mucin type) oligosaccharides, to biological function in molecular biology has made them the object of considerable research, in particular, considerable efforts have been made in organic synthesis to synthesize these materials. Although synthetic approaches to making carbohydrates are quite developed, this technique suffers
10 notable difficulties which relate to the selective protection and deprotection steps required in the available synthetic pathways. These difficulties, combined with difficulties associated with isolating and purifying carbohydrates, and determining their structures, has made it essentially impossible for synthetic organic chemistry to economically produce valuable carbohydrates.

15 Enzyme-mediated catalytic synthesis would offer dramatic advantages over the classical synthetic organic pathways, producing very high yields of carbohydrates (e.g., oligosaccharides and/or polysaccharides) economically, under mild conditions in aqueous solutions, and without generating notable amounts of undesired side products. To date, such enzymes, which include glycosyltransferase, are however
20 difficult to isolate, especially from eukaryotic, e.g., mammalian sources, because these proteins are only found in low concentrations, and tend to be membrane-bound. In addition to being difficult to isolate, the acceptor (peptide) specificity of GalNAc-transferase is poorly understood.

 It has been reported that in at least three different proteins the acceptor sites
25 glycosylated by the N-acetylglucosaminyltransferase have a common feature. This feature, which appears to lead to nuclear and cytoplasmic O-GlcNAc structures, is an acidic amino acid followed by serine, proline, and then a run of serines and threonines (Haltiwanger *et al.*, 1990). A more narrowly defined acceptor site has been reported for the proteoglycan xylosyltransferase: the acceptor site for this
30 enzyme consists of acidic amino acids closely followed by the tetrapeptide Ser-Gly-Xaa-Gly, where Xaa may be any amino acid (Bourdon *et al.* 1987). In spite of attempts to define it either by studying the amino acid sequences surrounding glycosylated serine and threonine residues of known location (Hagopian *et al.*, 1971; Hill *et al.*, 1977; Gooley *et al.*, 1991) or by performing *in vitro* studies on synthetic
35 peptides (Young *et al.*, 1979; Briand *et al.*, 1981; Hughes *et al.*, 1988; Wang *et al.*, 1992), these studies have yielded little conclusive information. In light of the above-

noted considerable value of carbohydrates, there is accordingly a strongly felt need for an improved method for isolation of glycosyltransferase enzyme as well as for studies of the acceptor (peptide) specificity of the enzyme to facilitate its use in carbohydrate synthesis.

5

INFORMATION DISCLOSURE

Placement of a reference within the following Information Disclosure does not constitute an admission or acknowledgement that the reference constitutes "prior art" to the present application.

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35

SUMMARY OF THE INVENTION

The present invention is based upon the discoveries of the gene coding for the

enzyme N-acetylgalactosaminyltransferase, the amino acid sequence of the enzyme N-acetylgalactosaminyltransferase, and the polypeptide sequence of the acceptor peptide for the enzyme N-acetylgalactosaminyltransferase. These discoveries allow for the control of glycosylation of a protein.

5 The present invention involves controlling the glycosylation of a protein, either within a cell or in vitro, by introducing into the DNA sequence encoding the protein at least one gene which is capable of expressing the acceptor peptide for the enzyme N-acetylgalactosaminyltransferase, expressing a protein having an acceptor cite for that enzyme, and exposing the expressed protein to that enzyme.

10 The present invention also provides a process for altering the glycosylation of a protein produced by a cell where the process involves introducing into the cell at least one gene which is capable of expressing the enzyme N-acetylgalactosaminyltransferase followed by expressing a sufficient amount of the enzyme in the cell to thereby alter the glycosylation of the protein in the cell.

15 The above discussed and many other features and attendant advantages of the present invention will become better understood by reference to the following detailed description when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Separation of bovine colostrum GalNAc-transferase on SDS-
20 polyacrylamide electrophoresis. Panel A: The purified bovine colostrum enzyme separated by SDS-PAGE on a 10% polyacrylamide gel and visualized by silver staining. Panel B: *In vitro* ¹²⁵I-labeled, purified bovine colostrum enzyme visualized by autoradiography. Left lane, products from digestion with peptide N-glycosidase F; right lane, enzyme incubated as for peptide N-glycosidase F digestion but without
25 the glycosidase. The migration of molecular weight markers is indicated to the right.

Figure 2. N-terminal sequence of bovine colostrum GalNAc-transferase, sequence
of oligonucleotide primers, restriction map for cDNA clones (pCR1000-91B and
pCR1000-52A) containing the GalNAc-transferase and the sequencing strategy. (A)
30 N-terminal amino acid sequence (34 amino acids) [SEQ ID NO:1] obtained from purified bovine colostrum GalNAc-transferase. The oligonucleotide (oligos A-E) [SEQ ID NOS: 2-6, respectively] sequence of the primers and probes used in PCR reactions and Southern blot analysis are shown below the amino acid sequence. The degeneracy of oligonucleotides A, B and C are 512, 64 and 64, respectively. (B)
35 Nucleotide sequence of the region surrounding the EcoRI cloning site of the λ gt10 vector. Oligonucleotides F and G [SEQ ID NOS: 7 and 8, respectively] were

synthesized and used in PCR reactions with the bovine small intestine cDNA library cloned in λ gt10 [SEQ. ID NO:16] (see text). (C) Restriction map of cDNA clones pCR1000-91B and pCR1000-52A. The protein coding region of the GalNAc-transferase protein is represented by the open box, the noncoding regions by the straight solid line and vector sequences by a solid box. The arrows beneath the 91B clone and above the 52A clone indicate the direction and extent of sequencing of the clones.

Figure 3. Amino acid sequence [SEQ ID NO:9] of the cloned GalNAc-transferase inferred from the nucleotide sequence of cDNA clones 91B [SEQ ID NO:10] and 52A.

The proposed transmembrane sequence is indicated by the solid boxed residues. Potential sites for N-linked glycosylation are indicated by the dashed boxed residues and predicted sites for O-linked glycosylation are marked with a dot under the appropriate amino acid. The N-terminus of the soluble bovine GalNAc-transferase (determined by N-terminal sequencing) is indicated by the arrow. The consensus poly A+ sequence (AATAAA) is indicated with a solid box and the sequence of the 93 bp insert of pCR1000-93I and the 621 bp insert of pCR1000-600 are indicated by the dashed underline (93I) or solid underline (600). The numbering of the nucleotide (upper) [SEQ ID NO:10] or amino acid sequence (lower) [SEQ ID NO:9] is indicated to the right of the sequence. The first ATG codon obtained from the 91B clone [SEQ ID NO:10] represents the beginning of the 1680 base pair nucleotide sequence for GalNAc-transferase [SEQ ID NO:11]. Genbank accession number L07780.

Figure 4. Predicted transmembrane domain and O-linked glycosylation sites for the cloned GalNAc-transferase. The amino acid sequence of the cloned molecule was analyzed for putative transmembrane segment(s) as described by Kyte and Doolittle (1982) (Panel A) and for O-linked glycosylation sites as outlined by Elhammer *et al.* (1993) (Panel B).

Figure 5. Northern blot analysis. Two μ g of poly A⁺ mRNA isolated from bovine mammary tissue, MDBK cells and 8 different human tissues were probed with the ³²P-labeled, 600 bp insert isolated from the pCR1000-600 (see Fig. 3). (A) lanes 1 and 2 contain mRNA from MDBK cells and bovine mammary tissue, respectively. (B) lanes 1 through 8 contain mRNA isolated from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, respectively.

Figure 6. Immunoprecipitation of *in vivo* ³⁵S-methionine labeled GalNAc-transferase expressed in baculovirus infected Sf9 cells. The cloned GalNAc-transferase DNA was expressed in Sf9 cells using a baculovirus vector. The infected cells were switched to culture medium containing ³⁵S-methionine 24 hours post-

infection and harvested after another 24 hours. The cells were lysed in a detergent containing buffer and the labeled transferase was immunoprecipitated from the cell lysates and the corresponding culture media. The washed immunoprecipitates were separated by SDS-PAGE on a 10% polyacrylamide gel. Lanes 1, 3 and 5 contain radioactivity precipitated from cell lysates of cells infected with virus containing the constructs GalNAcT 2-1.A, GalNAcT 2-1.B and CMV Pol-1, respectively. Lanes 2, 4 and 6 contains radioactivity immunoprecipitated from the corresponding culture media. The two molecular mass forms of the immunoprecipitated protein is indicated by the arrow heads. The migration of molecular weight markers is indicated to the right.

Figure 7. Probabilities of glycosylation. Probabilities were calculated from the amino acid sequence, using Eq 1 and the specificity parameters from Table 5. A: Human granulocyte-macrophage colony-stimulating factor. B: Human choriogonadotropin β -chain. C: Subtilisin BPN'. D: Bovine cytochrome C.

Figure 8. Probabilities of glycosylation. Probabilities were calculated from the amino acid sequence, using Eq 1 and the specificity parameters from Table 5. A: Bovine rhodanese. B: Chimeric protein constructed from the first two domains of human CD4 and the last three domains of *Pseudomonas* exotoxin. C: Human LDL receptor protein. D: Human Alzheimer amyloid protein precursor.

Figure 9. Lineweaver-Burk plots of GalNAc-transferase reaction velocities. The transfer of ^3H -acetylgalactosamine to the acceptor peptides by bovine colostrum GalNAc-transferase was assayed as outlined in Materials and Methods. A representative experiment for each peptide is shown. The substrates were (A): RTPPP [SEQ ID NO:12]; and (B): PPASTSAPG [SEQ ID NO:14].

Figure 10. Characterization of the reaction products from *in vitro* glycosylation of PPASTSAPG [SEQ ID NO:14] and PPASSSAPG [SEQ ID NO:15]. Products from glycosylation of the acceptor peptides PPASTSAPG [SEQ ID NO:14] (Panels A,B and C) and PPASSSAPG [SEQ ID NO:15] (Panels D, E and F) were isolated by BioGel P-2 chromatography and separated on descending paper chromatography in pyridine-ethyl acetate-glacial acetic acid-water (5:5:1:3, v:v:v:v). Panels A and D: the untreated glycosylated peptides; Panels B and E: products from digestion with *Patella vulgata* α -N-acetylgalactosaminidase; Panels C and F: products from mild alkaline sodium borohydride treatment. The migration distances of standards are indicated by arrows. 1: Gal β 1-3GalNAc-ol; 2: GalNAc-ol.

Figure 11. Determination of acceptor peptide amino acids conjugated with radioactive N-acetylgalactosamine after glycosylation with bovine colostrum

GalNAc-transferase. Glycosylated acceptor peptides were isolated from assay mixtures by BioGelP-2 chromatography and subjected to automated Edman degradation as described in Materials and Methods. The radioactivity eluted in each sequencing cycle was determined by scintillation counting. A and B show fractions
5 collected from sequencing of glycosylated PPASTSAPG [SEQ ID NO:14] and PPASSSAPG [SEQ ID NO:15], respectively.

Figure 12. The domain structure of bovine UDP-GalNAc:polypeptide, N-acetylgalactosaminyltransferase; construction of the secreted, soluble enzyme.

GalNAcT denotes the full-length transferase; the domain structure of the molecule is
10 high-lighted by the symbols described in the key. GalNAcTs denotes the soluble fusion molecule; the melittin signal sequence and 5 amino acids forming the linkage between the signal sequence and the GalNAc-transferase sequence, are represented by the solid bar. The arrow indicates the signal peptidase cleavage site.

Figure 13. The nucleotide sequence of cloned, re-engineered (to a soluble enzyme)

15 UDP-GalNAc:polypeptide, N-acetylgalactosaminyl-transferase. To express a secreted form of the bovine GalNAc-transferase, the sequences coding for the cytoplasmic and membrane spanning domains of the full-length cDNA (141 nucleotides) were replaced with sequences that code for the honeybee melittin signal peptide and five linker amino acids (78 nucleotides) [SEQ ID NO:18]. The honeybee melittin signal
20 sequence was chosen since the intended expression system for the construct was baculovirus/Sf9 cells.

Figure 14. Separation of soluble GalNAc-transferase on SDS-polyacrylamide

electrophoresis. Silver staining detected only one protein band on the 10% polyacrylamide gel. A molecular mass of approximately 61 kDa could be detected by
25 Coomassie Blue staining.

Figure 15. The nucleotide sequence of UDP-GalNAc:polypeptide, N-

acetylgalactosaminyl-transferase. The depicted nucleotide sequence [SEQ ID NO:11] codes for the enzyme N-acetylgalactosaminyltransferase.

Figure 16. The amino acid sequence of UDP-GalNAc:polypeptide, N-

30 acetylgalactosaminyl-transferase. The amino acid sequence of the enzyme N-acetylgalactosaminyltransferase [SEQ ID NO:9] is depicted.

Figure 17. An amino acid sequence of a soluble form UDP-GalNAc:polypeptide, N-acetylgalactosaminyl-transferase. The amino acid sequence of a secreted form of the enzyme N-acetylgalactosaminyltransferase [SEQ ID NO:19] is depicted.

35 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The term "N-acetylgalactosaminyltransferase (GalNAcT)" as used herein

refers to enzymes substantially homologous to, and having substantially the same biological activity as, the enzyme coded for by the nucleotide sequence depicted in Fig. 15 [SEQ ID NO:11] and the amino acid sequence depicted in Fig. 16 [SEQ ID NO:9]. This definition is intended to encompass natural allelic variations in the GalNAcT sequence, and all references to GalNAcT, and nucleotide and amino acid sequences thereof are intended to encompass such allelic variations, both naturally-occurring and man-made. Cloned genes of the present invention may code for the GalNAcT enzyme of any species of origin, but preferably code for enzymes of mammalian, most preferably bovine, origin.

The production of proteins such as the enzyme GalNAcT from cloned genes by genetic engineering is well known. See, e.g., U.S. Patent No. 4,761,371 to Bell et al. at Col. 6, line 3 to Col. 9 line 65. (The disclosure of all U.S. patent references cited herein is to be incorporated herein by reference.) The discussion which follows is accordingly intended as an overview of this field, and is not intended to reflect the full state of the art.

DNA which encodes the enzyme GalNAcT may be obtained, in view of the instant disclosure, by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures. Screening of mRNA or genomic DNA may be carried out with oligonucleotide probes generated from the GalNAcT gene sequence information provided herein. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with known procedures and used in conventional hybridization assays. In the alternative, GalNAcT gene sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the GalNAcT gene sequence provided herein. See U.S. Patent Nos. 4,683,195 to Mullis et al. and 4,683,202 to Mullis.

The GalNAcT enzyme may be synthesized in host cells transformed with vectors containing DNA encoding the GalNAcT enzyme. A vector is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding the GalNAcT enzyme and/or to express DNA which encodes the GalNAcT enzyme. An expression vector is a replicable DNA construct in which a DNA sequence encoding the GalNAcT enzyme is operably linked to suitable control sequences capable of effecting the expression of the GalNAcT enzyme in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a

transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate
5 in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous recombination). An example of a
10 useful vector is a baculovirus expression vector. The vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. Transformed host cells are cells which have been transformed or transfected with the GalNAcT
15 enzyme constructed using recombinant DNA techniques. Transformed host cells ordinarily express the GalNAcT enzyme, but host cells transformed for purposes of cloning or amplifying the GalNAcT enzyme DNA need not express the GalNAcT enzyme. When expressed, the GalNAcT enzyme will typically be located in the host cell membrane.

20 DNA regions are operably linked when they are functionally related to each other. For example, a promoter is operably linked to a coding sequence if it controls the transcription of the sequence. A ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leader sequences, contiguous and in the
25 same translational reading frame.

Cultures of cells derived from multicellular organisms are a desirable host for recombinant GalNAcT enzyme synthesis. In principal, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. However, mammalian and insect cells are preferred. Propagation of such cells in cell culture
30 has become a routine procedure. Examples of useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI138, BHK, COS-7, CV, MDBK and Sf9 cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome binding site, RNA splice site (if intron-containing
35 genomic DNA is used), a polyadenylation site, and a transcriptional termination sequence.

The transcriptional and translation control sequences in expression vectors to be used in transforming vertebrate cells are often provided by viral sources. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and Simian Virus 40 (SV40). The early and late promoters of SV40 are useful because
5 both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Further, the GalNAcT enzyme promoter, control and/or signal sequences, may also be used, provided such control sequences are compatible with the host cell chosen.

An origin of replication may be provided either by construction of the vector
10 to include an exogenous origin, such as may be derived from SV40 or other viral source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter may be sufficient.

GalNAcT enzyme made from cloned genes in accordance with the present invention may be used for designing new compounds containing oligosaccharides for
15 a variety of healthcare and industrial applications. For example, host cells may be transformed with a vector of the present invention, GalNAcT enzyme expressed in that host, the cells lysed, and the enzyme isolated from the lysed cells. The enzyme can then be used in vitro to begin the initial reaction in the O-linked oligosaccharide biosynthesis of the transfer of an N-acetylgalactosamine residue from the nucleotide
20 sugar UDP-N-acetylgalactosamine to a serine or threonine residue on the protein acceptor.

Cloned genes and vectors of the present invention are useful in molecular biology to transform cells which do not ordinarily express the GalNAcT enzyme to thereafter express this enzyme. Such cells are useful as intermediates for producing
25 the enzyme. Such cells are also useful for the in vivo biosynthesis of an O-linked oligosaccharide to a protein acceptor.

Milk (and colostrum) contains a number of glycosyltransferase activities (*e.g.* Prieels *et al.*, 1975; Paulson *et al.*, 1977; Bushway *et al.*, 1979; Parodi *et al.*, 1984). Previous work has shown that bovine colostrum contains what appears to be a
30 soluble form of N-acetylgalactosaminyl transferase (GalNAcT) (Elhammer and Kornfeld, 1986) but did not provide a procedure for the purification of sufficient amounts of GalNAcT for N-terminal sequencing. The following procedure describes the purification of GalNAcT from bovine colostrum. Following collection of sufficient samples, the amino acid sequence of the enzyme is determined by N-terminal
35 sequencing. This information is then used to isolate a cDNA clone encoding a full-length (membrane bound) transferase which upon expression in the insect cell line

Sf9 resulted in the synthesis of a fully active enzyme. The acceptor specificity of the enzyme is then determined using a semiquantitative analysis of the amino acids surrounding known glycosylation sites in 16 different proteins followed by *in vitro* glycosylation studies of synthetic peptides. This work has demonstrated that certain amino acid residues are overrepresented in glycosylated peptide segments and that residues in specific positions surrounding glycosylated serine and threonine residues may have a more marked influence on acceptor efficiency than other amino acid moieties.

The examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

Materials

The following materials are used in the examples. [α - ^{32} P]dATP (300 Ci/mmol), UDP-[1- ^{3} H]N-acetylgalactosamine (8.3 Ci/mmol) and Na[^{125} I] (15.2 mCi/ μ g) are purchased from Amersham. [α - ^{33} P]dATP is from NEN/Dupont and ^{35} S-methionine is from ICN (Trans S-35 label, 1 mCi/ml). Bovine colostrum is obtained from a local farmer. UDP-N-acetylgalactosamine, UDP, PMSF, chymostatin, leupeptin, antipain, pepstatin, aprotinin, bovine submaxillary mucin, Nonidet P-40 (NP-40), Triton X-100, taurodeoxycholate, Sephadex G-100 Superfine, rabbit anti-chicken IgG antibodies, ATP, myelin basic protein, subtilisin, rhodanese and cytochrome C (reduced and carboxymethylated as described by Heinrikson, R.L., 1973) are from Sigma. DEAE-Sephacel, Sepharose 6B and Protein A-Sepharose are from Pharmacia. IODOGEN is from Pierce. Peptide, N-glycosidase F is from Oxford Glycosystems. Geneamp Kit (for PCR) is obtained from Perkin Elmer/Cetus. A bovine small intestine cDNA library cloned in a λ gt10 vector is purchased from Clontech (catalog # BL1010a). The TA cloning vector pCR1000 is from Invitrogen. Sequenase version 2.0 is from U.S. Biochemical Corp. The baculoGold transfection kit is from PharMingen. 1 cc Bond Elut C₁₈ columns were from Varian. Serum-free Grace's insect medium, Insect Express, was from BioWhitaker. The vector pVt-Bac was a gift from Dr. Thierry Vernet at the Biotechnology Research Institute, National Research Council of Canada. *Patella vulgata* α -N-acetylgalactosaminidase is from V-Labs, Inc. Restriction enzymes and all other reagents are from standard sources.

In addition, the following buffers are used. Buffer A: 25 mM Imidazole, pH 7.2, 6 mM MnCl₂, 30 mM NaCl; buffer B: 25 mM imidazole, pH 7.2, 1 M NaCl, 1% Triton X-100, 20 mM EDTA; buffer C: 25 mM Imidazole, pH 7.2, 30 mM MnCl₂, 20 mM NaCl; buffer D: 25 mM Imidazole pH 7.2, 0.5 M NaCl, 20 mM EDTA; buffer E:

25 mM Imidazole, pH 7.2, 10 mM $MnCl_2$, 20% glycerol; buffer F: 25 mM Imidazole, pH 7.2, 30 mM $MnCl_2$, 100mM NaCl; buffer G: 25 mM Imidazole, pH 7.2, 80 mM NaCl, 0.1% taurodeoxycholate, 10% glycerol; buffer H: 25 mM Imidazole, pH 7.2, 100 mM NaCl, 0.1% Triton X-100, 10% glycerol.

5 Example 1 - Isolation of N-acetylgalactosaminyltransferase from Bovine Colostrum

The first four steps in the purification of the transferase are identical to the procedure described by Elhammer and Kornfeld (1986) (which is herein incorporated by reference) except that the samples loaded on the affinity columns are adjusted to 1 mM ATP (in addition to the reported buffer, salt and UDP concentrations) to
10 compensate for an apparently higher pyrophosphatase activity(ies) in the colostrum used. Equilibration, loading, washing and elution buffer volumes are adjusted (scaled up) for the larger columns used. All steps in the purification procedure are performed at +4°C and enzyme activity is assayed with the following standard assay throughout the purification.

15 The standard assay for UDP-GalNAc:polypeptide, N-acetylgalactosaminyltransferase activity during purification contained the following components in a final volume of 80 μ l: 50 mM Imidazole pH 7.2, 10 mM $MnCl_2$, 0.5% Triton X-100, 15 μ M UDP-GalNAc, UDP[1-³H-] GalNAc (27,000 cpm/assay), 0.15 mg/ml apomucin and varying amounts of enzyme (see individual experiments). The reaction
20 mixture is incubated at 37°C for 5-10 minutes (see individual experiments) and the reaction product is TCA precipitated and radioactivity measured as described. Assays for activity in lysates from *Spodoptera frugiperda* cell line 9 (hereinafter referred to as Sf9 cells) are carried out as described by Thomsen *et al.*, (1990).

Step 1: Separation of lipid globules and particles

25 Crude frozen colostrum obtained from a local farmer is thawed and centrifuged at 15,000 g for 30 minutes. The resulting yellowish lipid layer is removed and discarded. The colostrum is then dialyzed against 20 volumes of buffer A for 16 hours with two buffer changes. The dialyzed material is centrifuged at 100,000 g for 60 minutes. The upper lipid layer is removed and discarded and the clear supernatant is
30 carefully collected. The pellet and fluffy layer at the bottom is discarded.

Step 2: DEAE-Sephacel chromatography

The supernatant from the 100,000 g centrifugation is loaded directly on a DEAE-Sephacel column equilibrated in buffer A. For optimum results, the bed volume of this column should be approximately equal to the amount of 100,000 g supernatant loaded
35 (or ~750 ml/L crude colostrum). The run-through fractions are assayed for GalNAcT, and the fractions with activity are collected and pooled. Typically more than 90% of the

applied activity can be recovered after passage through the column.

Step 3: Apomucin affinity chromatography I

The affinity chromatography steps are carried out on apomucin-Sepharose columns with a bed volume of ~60 ml. Apomucin (deglycosylated mucin) is prepared from bovine submaxillary mucin by the method of Hagopian and Eylar with minor modifications. The carbohydrate content of the apomucin preparation is determined by the method of Reinhold. CNBr-activated Sepharose is prepared from Sepharose 6B essentially as described by Cautrecasas. The apomucin is coupled to the activated Sepharose in 0.1 M sodium carbonate buffer pH 9.2 at 4°C overnight. The protein concentration during the reaction is 2.5 mg/ml. All subsequent steps are carried out as in Affinity Chromatography, Principles and Methods (1979), pp. 15-18, Pharmacia Fine Chemicals, Piscataway, NJ. The coupling efficiency is nearly 100% and the final apomucin-Sepharose contained ~5 mg of bound apomucin/ml sedimented gel.

The columns are run by gravity at a pressure of ~30 cm H₂O during loading and ~60 cm H₂O during washing, elution and regeneration. Before loading, the column is washed with 400 ml buffer B (regeneration buffer) followed by 500 ml buffer C and 150 ml buffer C containing 0.25 mM UDP. Prior to loading the column the sample (~200U enzyme activity per 50 ml column in the first affinity step) is supplemented with MnCl₂ and UDP to final concentrations of 30 mM and 1.25 mM, respectively. The column is washed with 4 column volumes of buffer C containing 0.25 mM UDP and six 40 ml fractions are collected. The column is then eluted with buffer D. Due to the specific elution pattern of these columns the eluate is routinely collected as follows: fractions 1 and 2: 25 ml each, normally contains no, or very little activity; fractions 3 and 4: 50 ml each contains the bulk of the activity; fractions 3 and 4: 50 ml each, contains the bulk of the activity; fractions 5 through 7: 25 ml each, contains in some cases smaller amounts of activity. The individual fractions are dialyzed against 4 liters of buffer E (2 changes) immediately after elution, and assayed for enzyme activity. Typically only fractions 3 and 4 are used in the subsequent purification.

Step 4: Apomucin affinity chromatography II

In this step the same type column is used as in the previous one. Before loading, the column is first washed with 400 ml buffer B followed by 500 ml buffer F and 150 ml buffer F, containing 0.25 mM UDP. Prior to running the column, dialyzed fractions 3 and 4 from step 3 are supplemented with 1 M MnCl₂, 4 M NaCl and UDP to achieve final concentrations of 30 mM, 100 mM and 1.25 mM respectively. Approximately 600U enzyme activity per run could be located during this step. After loading the sample, the column is washed with 2 column volumes of buffer F followed by two column volumes

of buffer F containing 0.5 M NaCl and finally with two column volumes of buffer F containing 1 M NaCl. All the wash buffers contained 0.25 mM UDP. The washes are collected in 40 ml fractions. Elution is then carried out in the same manner as in step 3 but with buffer D containing only 100 mM NaCl. The eluted fractions are dialyzed and assayed for transferase activity as described for step 3.

Step 5: Gel filtration chromatography on Sephadex G-100 superfine

The dialyzed fractions from three step 4 runs are pooled, 1/50 volume 5% taurodeoxycholate is added, and the material is concentrated to 2.5 ml on an Amicon YM-10 filter under 40 psi pressure. Half of this material, 1.25 ml, is loaded on a Sephadex G-100 Superfine column (20-50 μ m bead size; 1.5 x 100 cm) equilibrated in buffer G having 300 mM NaCl. The column is run at a pressure of 30 cm H₂O, which resulted in a flow of approx. 2.3 ml/hour and fractions (100 total) are collected at 40 min. intervals and assayed for activity. The fractions comprising the activity peak are pooled and concentrated as described above but without any further addition of detergent. Analytical gel filtration to determine the molecular weight of the transferase is carried out using the same procedure but with a smaller column (0.9 x 100 cm) and collecting 1.06 ml fractions. The recoveries from this step using the conditions described above typically ranged from 80-90%.

The purified GalNAcT preparation contains only one polypeptide, with a molecular mass of approximately 70 kDa, detectable with silver staining (Figure 1A). A portion of the purified preparation is labeled *in vitro* with ¹²⁵I and separated on SDS-PAGE before and after digestion with peptide N-glycosidase F. Figure 1B shows that this treatment results in an approximately 6 kDa shift in the apparent molecular mass of the protein.

Example 2 - N-terminal Sequencing of the Purified Molecule

N-terminal sequencing of the purified bovine colostrum GalNAcT is done by automated Edman degradation in an Applied Biosystems Sequencer (Model 470) fitted with an on-line HPLC analyzer (Model 120-A) for phenylthiohydantoin. Quantitation of the latter is afforded by the Nelson Analytical Turbochrom chromatography data system connected in parallel with the recorder to the output from the HPLC system. The 34 amino acid sequence is shown in Figure 2A [SEQ ID NO:1].

Example 3 - Isolation and Characterization of cDNA Clones Encoding Bovine GalNAc-Transferase

Oligonucleotide primers are synthesized based on the partial N-terminal amino acid sequence of the purified bovine colostrum enzyme with an Applied Biosystems DNA Synthesizer, model 380B. The oligonucleotide (oligos A-E) [SEQ ID NOS: 2-6,

respectively,] sequence of the primers and probes used in the Polymerase Chain Reaction (hereinafter referred to as PCR) and later in a Southern Blot analysis are shown in Fig. 2A below the GalNAcT amino acid sequence. The degeneracy of oligonucleotides A, B and C are 512, 64 and 64, respectively. The PCR is carried out
5 in 0.1 ml of solution containing 50 mM KCl, 10 mM Tris-HCL pH 8.3, 1.5 mM MgCl₂, 0.2 mM each of the four dNTP's, 1 µM of each oligonucleotide, either 5 µl of the bovine intestine cDNA library or 10 ng of plasmid or λ DNA and 2.5 units of Taq polymerase. The reaction is covered with 0.1 ml of mineral oil and subjected to a temperature step cycle. When degenerate oligonucleotides are used the steps are 94°C (1 min), 37°C (2
10 min), 72°C (3 min) for a total of 35 cycles. For nondegenerate oligonucleotides the steps are 94°C (1 min), 55°C (2 min), 72°C (3 min) for a total of 25 cycles. Standard DNA manipulations are performed as described in Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning.

The cDNA encoding the GalNAcT gene is cloned using the following approach.
15 Oligonucleotides A [SEQ ID NO:2] and C [SEQ ID NO:4] are used as opposing primers in a PCR reaction. A bovine small intestine cDNA library cloned into a λgt10 vector is used as the template for the reaction. On the basis of the amino acid sequence, the predicted size of the amplified PCR product is 93 bp. The products of the PCR reaction are analyzed by Southern blot analysis using oligonucleotide B [SEQ ID NO:3] as a
20 probe (Figure 2A). Although the PCR reaction yields a number of ethidium bromide staining bands, only a single band of approximately 90 bp hybridizes to the probe. This fragment is gel purified and cloned into the TA cloning vector pCR1000, to yield plasmid pCR1000-93I. Determination of the DNA sequence (Fig. 3) of the pCR1000-93I insert reveals that the deduced amino sequence perfectly matches amino acids 4-34 in
25 the N-terminal sequence of the purified transferase [SEQ ID NO:1] (compare Figure 2A).

In an attempt to PCR amplify and clone the GalNAcT gene from the bovine λgt10 library, oligonucleotide primers D-G [SEQ ID NOS: 5-8, respectively] (Figure 2A and B) are synthesized. Oligonucleotides D [SEQ ID NO:5] and E [SEQ ID NO:6] are
30 derived from the sequence of the pCR1000-93I insert and F [SEQ ID NO:7] and G [SEQ ID NO:8] are primers that directly flank either side of the EcoRI cloning site of λgt10 (Figure 2B). PCR reactions are run using the bovine cDNA library as template with oligonucleotides D+F or D+G as primers. The resulting PCR products are analyzed by Southern blot analysis using oligonucleotide E [SEQ ID NO:6] as a probe.

35 No hybridizing bands are seen in PCR reactions when the D+F primers are used, but the D+G combination yield a single hybridizing fragment of approximately 600 bp.

This fragment is gel purified and cloned into the TA cloning vector to yield pCR1000-600 and the sequence of this insert is determined (Fig. 3). The 621 bp insert contains a 207 amino acid open reading frame with the first 23 amino acids of that open reading frame being a perfect match to amino acids 12-34 of the purified protein (Figure 2A)

5 [SEQ ID NO:1].

Assuming that the 621 bp fragment contains a portion of the GalNAcT gene, this fragment is labeled with [α - ^{32} P]dATP by nick translation (Goldin *et al.*, 1981) and is used as a probe to screen the bovine cDNA library. The cDNA library (containing 2.5×10^6 independent clones) is screened by plaque hybridization using the above labeled

10 DNA fragment as a probe. Seven positive plaques are obtained from the primary screen and each isolate is plaque purified three times. Five of the seven isolates are found to contain inserts of 600 bp or smaller while the two remaining isolates contain inserts of approximately 1600 and 2300 bp. The two larger inserts are PCR amplified and cloned (using oligonucleotides F and G as primers) into the TA cloning vector to yield

15 pCR1000-52A (1600 bp insert) and pCR1000-91B (2300 bp insert). The size of the λ inserts are analyzed on 1% agarose gels following restriction digest with EcoRI or by PCR using oligonucleotides F and G as primers.

Example 4 - DNA Sequence Analysis of PCR Inserts and Predicted Amino Acid Sequence

20 The inserts in pCR1000-93I, pCR1000-600, pCR1000-91B (2294 bp) and pCR1000-52A (1582 bp) are sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 with [α - ^{33} P]dATP. Double stranded DNA sequencing (Ausubel *et al.* 1987) is done with 20-mer oligonucleotide primers, synthesized according to the sequence of the cDNA insert. The sequencing strategy is

25 shown in Figure 2C. Sequence analysis is performed using the Sequence Analysis software package of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

As can be seen in Fig. 3, the first ATG codon of the sequence obtained from the 91B clone is present at nucleotide 53. The translated sequence from the ATG predicts

30 a polypeptide of 559 amino acids [SEQ ID NO:9] with a predicted $M_r = 64,173$ which is in good agreement with the M_r for the purified bovine GalNAcT protein (Figure 1A). The sequence of the 52A clone demonstrated that it is a truncated version of the 91B clone in that the sequence of this clone starts at nucleotide 162 and ends at nucleotide 1744 of the larger 91B clone. The 52A insert covers nearly all of the open reading

35 frame sequences (missing codons for the first 37 amino acids) found in the 91B clone. The nucleotide sequence of the 52A clone is identical to the 91B clone with the

exception that nucleotide 358 is a G in the 52A clone instead of an A. This base change is in the wobble position (AGA to AGG) of codon 102 so it does not alter the arginine at that position. The 3'-untranslated region of the 91B clone is 562 bp in length, contains a consensus polyadenylation signal (nucleotides 2176-2182) and a track of 25
5 A residues at the end of the clone (Fig. 3), indicating that the 91B clone contains all the 3' terminal sequences of the GalNAcT mRNA [SEQ ID NO:10].

Comparisons of the nucleotide and predicted amino acid sequences of the cloned molecule with the sequences in the GenBank data base and the Swissprot protein sequence data base yielded no significant similarities. Inspection of the predicted
10 amino acid sequence shows that the cloned molecule has the characteristics of a type II membrane protein with the same general domain structure as other cloned glycosyltransferases (Paulson and Colley, 1989). Also similar is the insignificant sequence homology with other glycosyltransferases as well as with other previously reported sequences. A Kyte-Doolittle hydropathicity analysis (Kyte and Doolittle, 1982)
15 of the molecule resulted in a predicted transmembrane domain between residues #9 and 28 (Figure 4A); secondary structure analysis suggests that this domain of the molecule has an α -helical conformation (Garnier *et al.*, 1978). Further, the predicted amino acid sequence contains three sites for N-linked glycosylation, asparagines #95, 141 and 552; as well as four predicted (Elhammer *et al.*, 1993) sites for O-linked glycosylation, serine
20 119 and threonines 117, 118 and 288 (Figure 4B).

Example 5 - RNA Isolation and Northern Hybridization

Expression of the GalNAcT mRNA from Madin-Darby bovine kidney cells (hereinafter referred to as MDBK cells) cells, bovine mammary tissue and various human tissues is analyzed by Northern blot analysis using the 600 bp insert of
25 pCR1000-600 as a hybridization probe. Total RNA and poly A⁺ RNA is prepared from bovine mammary tissue and from MDBK using the Invitrogen Fastrack kit, following the manufacturers procedure. Two μ g of poly A⁺ RNA are denatured by glyoxylation and Northern blot analysis is performed as previously described (Homa *et al.*, 1986). A human multiple tissue Northern blot (Clontech (Cat # 7760-1)) is prehybridized in
30 50% formamide, 5 x SSC, 1 x Denhart's, 1% SDS, 100 μ g per ml denatured salmon testes DNA, at 42°C for 2 h and then hybridized overnight at 42°C with the ³²P-labeled 600 bp insert isolated from the pCR1000-600. Filters are washed three times for 15 min in 0.1 X SSC, 0.1% SDS at 55°C. As shown in Figure 5, at least two different sized GalNAcT mRNA's are detected from all the samples. The size of the bovine messages
35 are approximately 4.1 and 3.2 kb, while all the human tissues express messages of 4.8 and 3.9 kb. In addition, a third mRNA of approximately 1.5 kb is detected in the

skeletal muscle sample.

Example 6 - Expression of the pCR1000-91B Insert in Sf9 Cells

The putative GalNAcT coding region, pCR1000-91B, is digested with SstII and HindIII (both enzymes cut only in pCR1000 sequences that flank the insert; Figure 2C) and these sites are blunted using T4 DNA polymerase so that it can be cloned into a baculovirus expression vector. BamHI linkers are then ligated onto the blunted ends and the resulting sample is ligated into the BamHI site of the baculovirus expression vector pAC373 (Summers and Smith, 1986). The resulting isolates are screened for proper orientation of the GalNAcT open reading frame with respect to the baculovirus polyhedron promoter, to yield pAC373-GalNAcT. Cotransfection of Sf9 cells with pAC373-GalNAcT and linearized baculovirus DNA from PharMingen's baculoGold transfection kit is performed using calcium phosphate precipitation (Summers & Smith, 1986). The baculovirus DNA provided in the PharMingen transfection kit contains a lethal mutation that can be corrected by homologous recombination with sequences contained in the pAC373 vector. Therefore, following transfection, only recombinant viruses will grow on Sf9 cells. Transfections are done in duplicate and the resulting virus samples are referred to as GalNAcT 2-1A and GalNAcT 2-1B. Cells are harvested 48 hours post infection and lysed in a detergent containing buffer. Following sedimentation of undissolved material, the cleared lysates are assayed for GalNAcT activity. Lysates from uninfected cells or from cells infected with either a baculovirus containing an unrelated gene, CMV-POL (human cytomegalovirus DNA polymerase gene), or two separate baculovirus isolates of the GalNAcT gene, GalNAcT 2-1A and GalNAcT 2-1B, are assayed.

Expression of this construct in Sf9 cells results in an almost 100 fold increase (compared to cells expressing an unrelated protein or uninfected cells) in intracellular GalNAcT activity (Table 1). The marked increase in a protein(s) with a molecular mass similar to that predicted from the amino acid sequence further supports this conclusion (Fig. 6). Increased transferase activity (approximately 4 times) is also detected in the culture medium from the infected cells. Without wishing to be bound to any particular theory, we believe it likely that this represents the intact or a proteolytic fragment(s) of the molecule leaking from dead or dying cells; the total activity in the medium is never more than 4% of the total activity in the cells.

Example 7 - Immunoprecipitation and SDS-PAGE ANALYSIS OF GalNAc-Transferase

The baculovirus expressed protein is further examined by immunoprecipitation and SDS-PAGE analysis. Baculovirus infected cells are labeled from 24 to 48 hours postinfection with [³⁵S]methionine. GalNAcT is immunoprecipitated from lysates and

culture media of the labeled cells using a chicken polyclonal antibody raised against the purified bovine colostrum enzyme. A chicken is injected with 100 µg purified enzyme axillary, intramuscularly (with Freund's complete adjuvant). One month later the chicken is boosted with another 50 µg antigen subcutaneously (with Freund's incomplete adjuvant); a second booster, 50 µg enzyme axillary, intra-muscularly, is administered after an additional 21 days. Test bleeds are done two weeks after each booster. After the second test bleed (which upon analysis is found to contain anti-GalNAcT antibodies) eggs are collected each day and used as a source for antibodies. IgG is isolated from egg yolk as described by Jensenius *et al.*, 1981.

10 Immunoprecipitation of the *in vivo* ^{35}S -methionine labeled enzyme, is done from crude cell lysates. Infected cells are labeled between 24-48 hours postinfection with 50 µCi/ml ^{35}S -methionine in medium that contains one tenth the normal methionine concentration. Approximately 1.5×10^6 labeled, infected cells are dissolved in 670 µl PBS containing 0.5% Triton X-100, 0.5% taurodeoxycholate, 0.05% SDS, 0.1 TIU/ml of Aprotinin and 10 µg/ml each of leupeptin, antipain, chymostatin and pepstatin. Following incubation on ice for >2 hours, any undissolved debris is sedimented at 15 10,000 x g for 20 minutes and the supernatant is collected. Immunoprecipitation is carried out by the addition of 4 µl (approximately 20 µg chicken IgG) of chicken anti GalNAcT antibodies; purified IgG isolated from egg yolk is used for all 20 immunoprecipitation experiments. Following incubation at 4°C over night, the antigen-antibody complexes are isolated by over night adsorption to 22 µl (volume of sedimented gel) of protein A-Sepharose coated with rabbit anti-chicken IgG antibodies. The coated protein A-Sepharose is prepared by incubating 330 µl sedimented protein A-Sepharose with 2.3 mg rabbit anti-chicken IgG antibodies (an affinity purified IgG fraction) in 1 25 ml of PBS over night; the coated protein A-Sepharose is washed three times with 1 ml PBS containing 0.5% Triton X-100, 0.5% taurodeoxycholate, 0.05% SDS. Following adsorption of the antigen, the immunosorbent is sedimented by centrifugation and washed extensively essentially as described by Dunphy *et al.* (1985). The washed antigen-antibody-immunosorbent complexes are suspended in 50 µl SDS-PAGE sample 30 buffer (Laemmli, 1970) and heated for five minutes on a boiling water bath to release the bound antigen. Following sedimentation of the protein A-Sepharose the antigen containing supernatants are aspirated and loaded on SDS-PAGE. SDS-PAGE, and fluorography of the dried gels is done as described previously (Davis *et al.*, 1986) (Fig. 6).

35 The results from separation of the precipitated material on SDS-PAGE is shown in Figure 6. Two closely spaced proteins with molecular masses of approximately 67

and 63.5 kDA are detected in both lysates and media from cells infected with viruses containing the GalNAcT gene (Figure 6). This is close to the molecular mass predicted for the cloned protein and is also comparable to the molecular mass of the purified bovine colostrum enzyme (Figure 1). The endogenously expressed enzyme is only barely detectable in this experiment but appears to have a similar molecular mass (Figure 6, lanes 5 and 6); phosphor screen autoradiography of the gel shows that approximately 75 times more radioactivity is incorporated in the cloned enzyme as compared to the endogenous one (Data not shown). The additional lower molecular weight bands seen on the gel probably represents nonspecifically precipitated material since the same bands are found in the control samples (Fig. 6, lanes 5 and 6).

The amino acid sequence [SEQ ID NO:9] predicted for the larger clone isolated provides a plausible explanation for the water solubility of the bovine colostrum GalNAcT. This enzyme apparently lacks the N-terminal 40 amino acids of the membrane bound molecule, a segment which includes both the cytoplasmic and membrane spanning domains. The Kyte-Doolittle hydropathicity plot from the cloned enzyme (Figure 4A) shows only one sequence segment, residues #9 through 28, with a high membrane spanning probability. It is not clear, at present, if the soluble bovine colostrum enzyme is the result of proteolytic cleavage of a membrane bound molecule or if it represents a *bona fide* secretory protein. Soluble, enzymatically active forms of a β 1-4 galactosyltransferase and a α 2-6 sialyltransferase have been reported, both of which appear to be the result of proteolytic cleavage of membrane bound proteins (Paulson and Colley, 1989 and references therein). In addition, the translation products from the different mRNA species related to both these molecules appears in most tissues to be membrane bound molecules (Joziassse, 1992). By analogy it appears likely that the two mRNA's observed in our Northern blotting experiments (Figure 5) both code for membrane bound enzymes and thus that the soluble bovine colostrum enzyme (again in analogy with β 1-4 galactosyltransferase and α 2-6 sialyltransferase) must represent a proteolytic fragment of a membrane bound enzyme. On the other hand, work reported on the different transcripts synthesized for rat kidney α 2-6 sialyltransferase suggest that some of these molecules contain start codons which when translated would yield proteins without cytoplasmic and membrane anchoring domains (Svenson *et al.*, 1990; Wang *et al.*, 1990; Wen *et al.*, 1992). Clearly, further experiments are needed to positively answer this question.

The larger sizes of the two GalNAcT messages (as compared to the cloned DNA) are presumably related to untranslated sequences larger than those recovered in the isolated clones, in the 5' and/or 3' ends of the native molecules. Messenger RNA

molecules from previously characterized cloned glycosyltransferases frequently contain extensive 5' and 3' untranslated sequences (*e.g.* Weinstein *et al.*, 1987; Larsen *et al.*, 1989; Russo *et al.*, 1990; Scocca *et al.*, 1990; Sarkar *et al.*, 1991; Nagata *et al.*, 1992). There is also a precedence for more than one mRNA species for at least 4 glycosyltransferases; the genes coding for these enzymes appear to be under control of more than one promoter (reviewed by Joziassse, 1992). In addition, a GalNAcT has been described which catalyzes the synthesis of a specific oncofetal epitope on fibronectin (Matsuura *et al.*, 1988; Matsuura *et al.*, 1989). This observation is consistent with the existence of more than one form of the enzyme.

Expression of the cloned sequence in Sf9 cells resulted in a large increase in intracellular transferase activity, thus establishing the identity of the cloned sequence with a GalNAc-to-Ser/Thr transferase. The marked increase in a protein(s) with a molecular mass similar to that predicted from the amino acid sequence further supports this conclusion (Fig 6). A closer examination of the protein(s) immunoprecipitated in this experiment reveals two closely spaced polypeptide bands with an approximately 3.5 kDa difference in molecular mass. The exact identity of these two proteins is not known at present; they may represent different glycoforms of the enzyme or, perhaps more likely, the lower molecular mass form may be a proteolytic fragment, similar to the enzyme purified from bovine colostrum. The latter possibility is supported by two observations: 1), the mass difference between the two molecules is roughly equal to that of the sequence (40 amino acids) missing in bovine colostrum enzyme and 2), while the immunoprecipitates from cell lysates contains predominantly the higher molecular mass form of the enzyme, the culture medium appears to be enriched in the lower mass form. High-speed centrifugation of the culture medium failed to sediment more than approximately 30% of the enzymatic activity (Data not shown). The smaller molecular mass of the insect cell produced molecule as compared to the predicted mass of a membrane bound bovine enzyme (the molecular mass of the soluble colostrum enzyme plus approximately 4 kDa for the transmembrane and cytoplasmic domains), may be the result of differences in glycosylation of the two molecules.

Insect cells typically synthesize truncated, non-sialylated N- and O-linked oligosaccharides (*e.g.* Hsieh and Robbins, 1984; Domingo and Throwbridge, 1988; Kuroda *et al.*, 1990; Thomsen *et al.*, 1990; Wathen *et al.*, 1991; Chen and Bahl, 1991); this results in a reduced molecular mass of insect cell produced glycoproteins on SDS-PAGE. The identity of higher molecular mass bands, approximately 120-180 kDa, on the gel is not clear. We have observed these bands previously in immunoprecipitates (by our anti-GalNAc-transferase antibody) from *in vivo* labeled mammalian and insect

cells (Elhammer, unpublished observations). They may represent unspecifically precipitated material, another protein(s) containing an epitope(s) recognized by our polyclonal antibody or aggregated GalNAcT. The latter possibility appears less likely however, since immunoprecipitates of purified GalNAcT contains only one protein band.

- 5 The fact that the intensity and molecular mass of these bands appears to vary (while the GalNAc-transferase band remains constant) between different experiments and cell types suggest that they represent unspecifically adsorbed contaminants (compare lanes 1-4 and 5-6 in this experiment).

Example 8 - Construction and Expression in Sf9 cells of a Soluble GalNAc-transferase
10 (GalNAcTs)

- In order to express a secreted form of the bovine GalNAc-transferase, the sequences coding for the cytoplasmic and membrane spanning domains of the full-length cDNA were replaced with sequences that code for the honeybee melittin signal peptide (Fig. 12). The honeybee melittin signal sequence was chosen since the intended
15 expression system for the construct was baculovirus/Sf9 cells. It has been demonstrated that use of an insect derived signal peptide often results in increased secretion (as compared to a signal peptide of heterologous origin) of the recombinant molecule when expressed in Sf9 cells (Tessier et al., 1991). The fusion site for the signal peptide was chosen based on the N-terminal sequence of the soluble colostrum enzyme.

- 20 The plasmid pAC373-GalNAcT (Homa et al., 1993) which contains the full length GalNAc-transferase gene under the control of the baculovirus polyhedron promoter was digested with XbaI and BglII, which generated a 150 bp fragment, and with BglII and XhoI, which generated a 9700 bp vector fragment. Both fragments were gel purified. The XbaI site used is located 7 amino acids from the N-terminus of the soluble
25 colostrum enzyme, in a portion of the molecule corresponding to what is referred to as the "stem region" in other glycosyltransferases (reviewed by Shaper & Shaper, 1992). Soluble forms of several glycosyltransferases are generated by proteolytic cleavages in this region. Consequently, it is believed to be unimportant for catalytic activity (Joziassse, 1992; Shaper & Shaper, 1992).

- 30 pVT-BAC (Tessier et al, 1991), which contains the coding sequences of the honeybee melittin signal peptide, was digested with SmaI and a 12 bp XbaI linker was ligated onto the SmaI site. The sample was then digested with XbaI and XhoI and the 2100 bp fragment generated by this digest was gel purified. The three gel purified fragments were added to the same tube and ligated. The resulting plasmid contains
35 a GalNAc-transferase gene under the control of the baculovirus polyhedron promoter in which the first 47 amino acids (141 nucleotides) have been replaced with 21 amino

acids (63 nucleotides) of the honeybee melittin signal peptide plus five (5) amino acids (15 nucleotides) that link the two domains together (Fig. 13) [SEQ ID NO:18]. This construct, pAC373-GalNAcTs-Mel, was then used to prepare a recombinant baculovirus that expresses a secreted form of GalNAc-transferase, GalNAcTs, using the BaculoGold
5 transfection kit (Summers & Smith, 1986; Homa et al, 1993)(Fig. 17)[SEQ ID NO:19].

GalNAcTs was routinely expressed in one liter batches. Approximately 1×10^9 cells in one liter Grace's serum-free insect medium (Insect Express) were infected with 5 pfu/cell of GalNAcTs-Mel. The infected cells were cultured in shaker flasks for 65 hours before harvest. Expression of the construct GalNAcTs-Mel in Sf9 cells resulted
10 in 130-fold increase in GalNAc-transferase activity in the culture medium, as compared to uninfected cells (Table 2) or cells infected with an unrelated molecule ($\alpha 6-3$). This is more than 35 times the amount recovered in the medium of cells expressing the full length molecule (Homa & Elhammer, unpublished observations). A significant portion (36%) of the total enzymatic activity resulting from expression of the soluble molecule
15 was, however, retained inside the cells; the reason for this is not clear at present.

Example 9 - Isolation and Characterization of GalNAcTs

Purification of the soluble molecule was accomplished in one step, by chromatography on apomucin-Sepharose. The procedure used for the purification of the recombinant enzyme was a modification of the second affinity chromatography step in
20 the purification of the bovine colostrum enzyme (Elhammer & Kornfeld, 1986). The affinity column used for purification of the recombinant enzyme was prepared as described (Elhammer & Kornfeld, 1986). Total bed volume was approximately 200 ml; ligand density was approximately 3.5 mg/ml gel. Before each separation run the column was washed with 1000 ml Buffer B and equilibrated with 1000ml Buffer F
25 followed by 420 ml Buffer F containing 0.25 mM UDP. Crude conditioned cell culture medium was fractionated on this column in 1 liter batches as follows.

The medium was first dialyzed against 25 mM Imidazole, pH 7.2, 100 mM NaCl, 30 mM MnCl_2 with three buffer changes. Following centrifugation at 12,000Xg for 20 minutes to remove precipitated material (no enzyme activity was lost in this step), the
30 dialyzed medium was supplemented with UDP to 1.25 mM and loaded directly on the affinity column; the column run-through was collected in five 200 ml fractions. The column was then washed sequentially with 500 ml buffer F, 250 ml Buffer F containing 0.2% Triton X-100, 250 ml Buffer F and 250 ml Buffer F containing 1.0 M NaCl. All wash buffers contained 0.25 mM UDP and washes were collected in 250 ml fractions.
35 The bound enzyme was eluted from the column with 720 ml Buffer D and collected in seven 80 ml fractions. Run-through, wash, and eluted fractions were all dialyzed

against Buffer E containing 300 mM NaCl (three changes) prior to assay for GalNAc-transferase activity. The recovery of enzyme activity on the column was invariably over 90%. The following concentration of the eluted enzyme, however, led to significant losses in activity. In fact this step accounted for the largest losses in the preparation
5 procedure. Dialyzing the enzyme into a buffer containing 300 mM NaCl prior to concentration was an absolute necessity to avoid even higher losses in this step. The enzyme isolated from bovine colostrum shows a similar behavior in this regard (Elhammer & Kornfeld, 1986). The purified preparation was concentrated by ultrafiltration on a YM-10 membrane at 45 psi pressure.

10 The purified, concentrated preparation, together with an aliquot of the crude conditioned cell culture medium, was analyzed on SDS-PAGE essentially as described by Laemmli (1970). The crude medium sample was precipitated as described by Wessel and Flugge (1984) prior to electrophoresis; precipitate corresponding to approximately 250 μ l medium was loaded. NH_2 -terminal sequencing of the purified molecule was
15 done as described in Example 2. Interestingly, this purification procedure yielded a homogenous preparation only if expression of the molecule was carried out in serum-free medium. Preparations from serum containing medium appeared homogenous on SDS-PAGE but N-terminal sequencing and separation on capillary electrophoresis revealed that they, in addition to GalNAc-transferase, also contained considerable
20 amounts of bovine fetuin (Data not shown). Consequently the purified enzyme characterized herein was isolated from material produced in serum-free medium.

The amino acid composition of the purified GalNAcTs was determined by automated ion-exchange chromatography on a Beckman amino acid analyzer (Beckman 6300) and was found to correspond with the composition predicted from the nucleic acid
25 sequence shown in Fig. 3 [SEQ ID NO:9]. Samples were hydrolyzed for 24 hours *in vacuo* at 110°C in 6N HCl. Dried hydrolysates were dissolved in buffer at pH 2.2 (NaS; Beckman) prior to application to the analyzer. Quantitation of the latter was afforded by the Nelson Analytical Turbochrom chromatography data system connected in parallel with the recorder to the output of the dual-channel spectrophotometer. NH_2 -
30 terminal sequencing of the purified, concentrated enzyme demonstrated that the melittin leader sequence was cleaved from the molecule at the predicted site (Fig. 12). Furthermore, only one sequence was detected in the preparation, consistent with an essentially pure enzyme preparation. The purity of the enzyme was also investigated on SDS-PAGE. As can be seen in Fig. 14, only one band, with a molecular mass of
35 approximately 61 kDa, could be detected by Coomassie Blue staining. Silver staining also failed to detect more than one protein band on the gel and separation of the

purified enzyme on capillary electrophoresis resulted in only one UV absorbing peak eluting at a position consistent with the molecular mass of approximately 61 kDa (Data not shown).

Example 10 - Statistical Analysis of the Peptide Acceptor For GalNAc-Transferase

5 The efficient production of the cloned molecule using the baculovirus expression system facilitated preparation of GalNAc-transferase in amounts sufficient for detailed biochemical and enzymatic studies to determine the acceptor substrate specificity of GalNAc-transferase from a database of *in vivo* substrates and from the *in vitro* glycosylation of proteins and peptides. These studies have been facilitated by the
10 availability of information regarding the presence of glycosylated serine and threonine residues in proteins obtained during protein sequencing. This information is registered in the NBRF protein sequence repository. cursory inspection of the amino acid sequences surrounding these reactive Ser and Thr residues revealed that the enzyme must have a broad specificity and that there is no unique, readily identifiable amino
15 acid sequence associated with enzymatic specificity. Indeed, the enzyme is even capable of glycosylating N-terminal and C-terminal Ser or Thr residues, which indicates that the specificity cannot be limited to one or two specific subsites flanking the reactive hydroxyamino acid.

 Recently, a statistical method for analyzing the broad specificity of enzymes
20 which act on protein segments through extended multisite attachment has been developed and applied with some success to determine the specificity of the HIV-1 protease (Poorman *et al.* 1991). The number of reported sequences surrounding glycosylated Ser and Thr residues was large enough to warrant their analysis by this statistical method. This approach was used to outline the crucial features of the
25 peptide acceptor for GalNAc-transferase. The validity of this information was then determined in a series of experiments involving *in vitro* glycosylation of both synthetic and naturally occurring substrates. The results indicate that both serine and threonine residues can be glycosylated by the same enzyme and that the broad specificity of GalNAc-transferase is the consequence of an extended binding site.

30 A search of the NBRF protein database yields several hundred definite or probable Thr and Ser O-glycosylation sites. From these, only those with reasonably unambiguous assignments are chosen and all proteoglycans are excluded since they contain primarily glycosaminoglycan chains where the anchoring sugar is xylose and not GalNAc. Also included into the reference set are the O-glycosylation sites identified
35 in some recently analyzed glycoproteins. In the case of proteins listed for several different species, homologous proteins are included only when the glycosylation sites

-30-

themselves show no homology. The complete reference set consists of the 196 glycosylated peptide segments (shown in Table 1 in Elhammer *et al.*, 1993). The glycosylated peptides are listed as enneapeptide (*ennea* Greek, nine) segments, with the reactive Ser or Thr in the central position, designated as P0. Accordingly, the amino acid side chains toward the N-terminus are designated as the subsites P1 to P4 and those toward the C-terminus as subsites P1' to P4'. A length of nine residues is chosen as a starting point, with the option that, depending on the results on the selectivity of the subsites, the portion of the peptides subject to analysis may be extended or truncated. The sequences show that besides the obvious need for Ser or Thr in P0, no other subsite has an absolute requirement for any given amino acid. This then suggests that specificity of the enzyme may be the result of the cooperation of several subsites, none of them essential, but all of them contributing to catalytic efficiency.

In order to explore whether the cooperative-subsite model is a valid description of GalNAc-transferase, the standard set of peptides is analyzed according to the method applied with some success to the specificity of the HIV-1 protease (Poorman *et al.* 1991). In brief, the frequencies of individual amino acids at each subsite are compared (Table 3) to those expected on the basis of their abundance in a reference set of globular proteins (Nakashima *et al.* 1986). Table 4 shows that Ser, Thr, and Pro are significantly surabundant in almost all positions. Specificity parameters, s_{ij} — defined as the observed abundances divided by the abundances expected for globular proteins — are calculated from these data; they are listed in Table 5. It has been shown (Poorman *et al.* 1991) that if the enzyme has independent subsites then the probability, h , that any Ser or Thr-containing enneapeptide will react with the enzyme can be calculated from the equation:

$$h = \frac{RP}{RP + \frac{1}{PS}} \quad \text{Eq. 1}$$

where PS is the abundance of glycosylatable peptides in all proteins and RP is the cumulative probability calculated as the product of all relevant s_{ij} values:

$$RP = \prod_{i=1}^9 s_{ij} \quad \text{Eq. 2}$$

Using this algorithm, a Ser or Thr-containing peptide may be predicted to be a

substrate for the enzyme if the probability, h , is higher than a certain cutoff value, h_c . In order to achieve the best compromise between overpredictions and underpredictions, PS and h_c are iteratively optimized using the proteins in the Kabsch-Sander database (Kabsch and Sander 1983), as described (Poorman *et al.* 1991). It is found that the best
 5 compromise is reached with PS = 1/56 h_c = 0.19, resulting in a 21.4% underprediction in the reference data set. Thus, a given peptide is predicted to be a glycosyl acceptor, if

$$10 \quad h = \frac{\prod_{i=1}^9 s_{ij}}{56 + \prod_{i=1}^9 s_{ij}} \geq 0.19 = h_c$$

Overprediction is expected to be variable and in some cases may be rather high since glycosylation of a fully folded protein presumably requires not only the proper
 15 local primary structure but also accessibility from the aqueous environment. For example, applying the predictive algorithm to human granulocyte-macrophage colony-stimulating factor (fqhugm.aa, Figure 7A), it is found that the four experimentally observed O-glycosylation sites are correctly predicted at sequence positions 22, 24, 26, and 27 (Kaushansky *et al.* 1992) but four additional sites are also
 20 predicted: Ser₁₅ ($h=0.29$), Thr₁₀₈ ($h=0.24$), Thr₁₁₁ ($h=0.66$), and Ser₁₁₂ ($h=0.66$). Similarly, human choriogonadotropin β chain (kthub.aa, Figure 7B) yields one underprediction, Ser₁₃₈ ($h=0.01$) and one overprediction at Thr₁₆₀ ($h=0.28$). The probability pattern of these two proteins is shown in Figure 7. It is interesting to note that the calculated probabilities for these two proteins are not distributed uniformly
 25 between the two extremes. Rather, a small number of residues are associated with very high probabilities whereas the rest of the sequence indicates uniformly low probabilities. Furthermore, the residues with high probabilities are clustered into one or two distinct segments where the clustering of Ser and Thr residues may perhaps be a necessary but certainly not a sufficient criterion for creating a highly glycosylated
 30 protein segment.

Taken together, these observations suggest that glycosylation is determined as much by the specificity of the enzyme toward a given sequence of amino acids surrounding the reactive residue as by the exposure of this peptide chain to the aqueous environment. This inference is further strengthened by the distribution of potential
 35 glycosylation sites in the Kabsch/Sander proteins, none of which are glycosylated *in vivo*. For example, subtilisin BPN' (subsn.aa, Figure 7C) which is produced by a

microbial system incapable of O-linked oligosaccharide biosynthesis, contains a number of randomly distributed potential glycosylation sites, while very few nonglycosylated mammalian proteins contain any potential glycosylation sites. It is perhaps more typical to find no potential glycosylation sites at all, as in the case of horse hemoglobin (hbho.aa) or that of bovine cytochrome C (ccpg.aa, Figure 7D).

It appears as if the sequence of mammalian proteins which are not destined to be glycosylated are in fact selected against containing potential glycosylation sites. On the other hand, according to current understanding of the intracellular topology of the GalNAc-to-serine/threonine transferase, the amino acid sequence of a cytoplasmic protein would not be selected against having potential glycosylation sites for the anchoring of mucin type oligosaccharides. For example, it is found that bovine rhodanese (robo.aa, Figure 8A) does contain three strong glycosylation sites at Thr₁₄₁ (h=0.95), Ser₁₄₂ (h=0.49), Ser₁₄₅ (h=0.61), see Figure 8. Since this region of the protein consists of a fully exposed segment linking the two homologous domains of the enzyme, exposure of native or mildly denatured rhodanese to GalNAc-transferase should result in glycosylation of the molecule. Similarly, the chimeric protein, CD4PE40, constructed from two domains of the human CD4 protein and three domains of the *Pseudomonas* exotoxin, shows two prominent potential glycosylation sites, both at regions linking individual domains, see Figure 8B. In the same vein, one would predict that subtilisin could also be extensively glycosylated, if not in the native form then at least after mild denaturation.

The potential of the predictive method is perhaps best illustrated by its application to the LDL receptor (ldlrec.aa, Figure 8C) and the Alzheimer precursor protein (alz.aa, Figure 8D), which have both been shown to be extensively O-glycosylated, each in a known, narrow segment of the polypeptide. As shown in Figure 8, the present method not only correctly identifies these regions of glycosylation but also specifically predicts which Ser and Thr residues may be modified.

The above analysis allows one to hypothesize about the salient features of the enzyme active site responsible for the specificity of glycosylation. Table 4 indicates that high selectivity is expressed at all subsites, but only toward Ser, Thr, and Pro. The selectivity of a given subsite depends on how many times more frequent are at that site the surabundant residues than all the other amino acids. Also, selectivity is higher when the surabundant residue is one which occurs with low frequency in globular proteins. To quantitate the selectivity, one defines a specificity parameter for the subsite i, S_i , as the number of surabundant residues found at that site, divided by the number of these same residues expected at that site from random distribution. This

ratio is then multiplied by the fraction of surabundant residues at that site. The values of S_j , reported in Table 3, suggest that the binding site extends at least from P3 to P4' and perhaps even P4 is included in the substrate-enzyme interactions.

Besides the three specific amino acids, no other amino acid is found in
5 surabundance at any site, although Gly, Ala, Val, and Met are permitted to occur randomly, as indicated by their $s_{ij} \approx 1$, see Table 5. Perhaps more important is the fact that Asp, Asn, Arg, Tyr, Leu, Phe, Lys, Cys, and Trp occur only at very low frequencies and their presence at any site strongly decreases the probability of glycosylation. In other words, neither strongly hydrophobic nor strongly hydrophilic residues (see
10 consensus hydrophobicity scale, Eisenberg 1984) are recognized by the glycosyltransferase. If a single intrinsic property of the amino acid side chains — such as hydrophobicity, size, surface, electric charge, etc — is responsible for the specific interaction with the active site, then s_{ij} should depend critically on that property. Attempts to correlate the s_{ij} distribution with a variety of parameters possibly
15 characterizing the individual amino acids met with little success. Most importantly, no correlation has been found with a parameter predicting β -turns, although such a correlation has been inferred for xylosyltransferase (M.A. Bourdon *et al.*, 1987).

The question of whether any positive or negative cooperativity could exist between specific amino acid side chains at any two subsites has also been explored. For
20 this purpose, in a series of trials the amino acids in the reference peptide set are randomized within each subsite in turn. Any positive or negative cooperativity between subsites should disappear in the randomized set and one would observe a significant change in the distribution of h values. Only random fluctuations are in fact observed, thereby indicating the absence of any strong cooperativity. In other words, the subsites
25 behave as if they are independent of the presence of specific amino acids at any other subsite.

The possibility still remained that the enzyme would recognize a specific secondary structure of the target enneapeptide and that this secondary structure would be induced not by the cooperative action of several constituent amino acids but by their
30 intrinsic tendency to form a given secondary structure. In order to test this possibility, the potential glycosylation sites predicted for proteins in the Kabsch-Sander (Kabsch and Sander, 1983) database is used since no secondary structural information is available at the present time for any O-glycosylated proteins. For this analysis, the 50 potential sites with the highest h values are selected. As shown in Table 6, these
35 potential glycosylation sites are very strongly selected against α -helical conformation. The preferred conformations appear to be a random coil, a sharp bend, or a β -strand

from P4 to P0 followed by a turn. Thus, there seems to be no unique secondary structure recognized by the enzyme but, rather, extended conformations seem to be preferred. Stated in other terms, the enzyme does not require a preformed secondary structure but imposes one upon binding of the substrate. The hydration index of the amino acids in the potential glycosylation sites, also shown in Table 6, indicates that most peptides are reasonably exposed to the aqueous environment.

Example 11 - Transfer of N-acetylgalactosamine to Native Protein Acceptors by GalNAc-Transferase

The above conclusions and the predictions of the specificity algorithm are validated experimentally by studying the ability of bovine colostrum GalNAc-transferase to transfer N-acetylgalactosamine to a variety of acceptors, both native proteins and synthetic acceptor peptides. The transfer of N-acetylgalactosamine to protein acceptors is assayed as described by Elhammer and Kornfeld, 1986, with minor modifications. The concentration of UDP-GalNAc is saturating in all assays; a K_m of 8 μ M is reported for bovine colostrum GalNAc-transferase (Elhammer and Kornfeld, 1986).

The reaction products are characterized using alkaline sodium borohydride treatment essentially as described by Carlson (1968). Digestion with *Patella vulgata* α -N-acetylgalactosaminidase (approximately 1 unit/ml) is done in 25 mM citrate buffer pH 4.0 in a final volume of 30 μ l for 24 hours. Released radioactive sugars are separated on descending paper chromatography in pyridine-ethyl acetate-glacial acetic acid-water (5:5:1:3; v:v:v:v).

Table 7 shows that, as predicted, both bovine rhodanese and, to a lesser extent, the bacterial protein subtilisin do indeed function as acceptors for the enzyme, although neither of them reacts unless reduced and carboxymethylated prior to exposure to the enzyme. Conversely, bovine cytochrome C which contains one Ser and eight Thr residues but no predicted potential sites, is not an acceptor for the enzyme, whether in the native, or in the reduced and carboxymethylated state. Myelin basic protein, a molecule which previously has been shown to be an efficient acceptor for GalNAc-transferase (Hagopian *et al.*, 1971) is included as a positive control in this experiment.

The experiments with native protein acceptors demonstrate that proteins which do not contain O-linked oligosaccharides *in situ* but do contain exposed sequences with the predicted features of an acceptor, can function as acceptors for GalNAc-transferase *in vitro*. It should be noted, nevertheless, that neither subtilisin nor rhodanese showed any acceptor activity without prior reduction and carboxymethylation. Although the

most probable of the predicted sites in rhodanese, Thr₁₄₁, is located in an exposed segment on the molecule, inspection of the three-dimensional structure of rhodanese revealed that the hydroxyl group of this amino acid is oriented not toward the surrounding solvent but toward the hydrophobic core. This then probably accounts for the need for mild denaturation for acceptor activity. Rhodanese contains two additional predicted acceptor sites, Ser₁₄₂ and Ser₁₄₅, (Fig. 8). However, due to the low rates of transfer to serine residues under our standard assay conditions, transfer to these sites should not contribute significantly to the total transfer in the assay. The lower rate of transfer to reduced and carboxymethylated rhodanese, compared to that of myelin basic protein, may be related to incomplete exposure of the acceptor sites even by the reduction and carboxymethylation procedure, and/or differences in rate constants between the acceptor sequences on the two molecules. Myelin basic protein contains one site predicted with high probability and three additional low probability sites. The molecule can reportedly be glycosylated with 1.2 to 1.5 N-acetylgalactosamines per molecule (Cruz and Moscarello, 1983).

The bacterial protein subtilisin contains four predicted serine sites with probabilities higher than 0.6 (Fig. 8). Three of the serines have a high exposure index in the native protein (Kabsch and Sander, 1983), but the three-dimensional structure of the protein indicates that the hydroxyls are located in a restrained environment. Again, this could account for the need for reduction and carboxymethylation for acceptor activity. The 35 times slower transfer rate to denatured subtilisin, as compared to myelin basic protein, indicates again a slower transfer to serines than to threonines, under the conditions used. Factors such as those discussed for rhodanese may also contribute to the low levels of transfer. Finally, cytochrome C, which does not contain any predicted acceptor site, is completely inactive as an acceptor, whether in the native or in the reduced and carboxymethylated form.

Example 12 - Transfer of N-acetylgalactosamine to Synthetic Acceptor Peptides by GalNAc-Transferase

The ability of GalNAc-transferase to glycosylate a series of synthetic acceptor peptides is shown in Figure 9 and Table 8. The synthetic acceptor peptides Arg-Thr-Pro-Pro-Pro (RTPPP) [SEQ ID NO:12], Arg-Ser-Pro-Pro-Pro (RSPPP) [SEQ ID NO:13], Pro-Pro-Ala-Ser-Thr-Ser-Ala-Pro-Gly (PPASTSAPG) [SEQ ID NO:14], Pro-Pro-Ala-Ser-Ser-Ser-Ala-Pro-Gly (PPASSSAPG) [SEQ ID NO:15] and Pro-Pro-Ala-D-Ser-Thr-D-Ser-Ala-Pro-Gly (PPAdSTdSAPG) are synthesized by solid phase methodology on an Applied Biosystems Inc 430A peptide synthesizer (ABI, Foster City, CA) using double couple cycles with standard t-Boc chemistry on a 0.5 mmol scale.

The t-Boc-amino acids and the PAM resin solid supports are supplied by ABI. The completed peptides are removed from the supporting resin, concurrently with the side chain-protecting groups, by a standard HF cleavage procedure using anisole as a cation scavenger (10% v/v). The crude peptides are purified by preparative reverse phase chromatography on a C18 Vydac column (2.5 x 30 cm) using a water/acetonitrile gradient, each phase containing 0.1% TFA. Each purified peptide is characterized by FAB/MS and shows a single symmetrical peak on analytical HPLC.

Sequence analysis for the identification of the glycosylated amino acid(s) in the acceptor peptides PPASTSAPG [SEQ ID NO:14] and PPASSSAPG [SEQ ID NO:15] is performed using an Applied Biosystems (ABI) 470A sequencer equipped with an on-line ABI 120A PTH analyzer. PTH derivatives are dissolved in 120 µl 20% acetonitrile in water; 50 µl of this solution is injected onto the HPLC and the remainder diverted to a fraction collector. Cartridge filters are prepared with 1.5 mg polybrene using the ABI program 03RPRE. Samples are sequenced using the ABI program 03RPTH. Peptides are dissolved in 60 µl of 50% acetic acid and loaded onto the filter in two 30 µl aliquots.

The experiments shown in Figure 10 demonstrate that the incorporated radioactivity in the acceptor peptides PPASTSAPG [SEQ ID NO:14] and PPASSSAPG [SEQ ID NO:15] is in the form of N-acetylgalactosamine. Digestion of the two glycosylated peptides with *Patella vulgata* α-N-acetylgalactosaminidase releases only GalNAc and alkaline sodium borohydride treatment results in the release GalNAc-ol both from PPASTSAPG [SEQ ID NO:14] and PPASSSAPG [SEQ ID NO:15].

The glycosylated amino acids in the peptides PPASTSAPG [SEQ ID NO:14] and PPASSSAPG [SEQ ID NO:15] are identified by sequencing of the reaction products from the corresponding assay. Fig. 11 shows that for both glycosylated peptides the majority of the sugar-linked radioactivity is associated with residue #5, the central amino acid, be it threonine, as in PPASTSAPG [SEQ ID NO:14], or serine, as in PPASSSAPG [SEQ ID NO:15]. The measurable amounts of radioactivity associated with the residues following residue 5 are presumably due to the large load of peptide in the sequencer necessitated by the low specific radioactivity of the sample. Nevertheless, since the radioactivities associated with residues 7 and 8 extrapolate smoothly to that of residue 6, it is most likely that, within our experimental error, residue 6 is not labelled.

The transfer of N-acetylgalactosamine to peptide acceptors is assayed by two different assays. The concentration of UDP-GalNAc is saturating in all assays; a K_m of 8µM is reported for bovine colostrum GalNAc-transferase (Elhammer and Kornfeld, 1986). For the determination of kinetic parameters the reaction mixture contains 50 mM Imidazole, pH 7.2, 10 mM $MnCl_2$, 0.5% Triton X-100, 150 µM UDP-GalNAc,

approximately 120,000 cpm of UDP-[1-³H]-GalNAc, varying concentrations of acceptor (see individual experiments) and approximately 5 mU of enzyme in a final volume of 40 µl; standard incubation time is 20 minutes. The reaction is terminated by placing the samples in a boiling water bath for 1.5 minutes. The reaction product (glycosylated peptide) is separated from unreacted UDP-GalNAc by chromatography on Dowex-2 columns (0.5 ml bed volume) equilibrated in water; the run-through fraction (2.5 ml) containing the glycosylated peptide is collected, supplemented with scintillation fluid and counted for radioactivity. For the determination of the low levels of transfer to serine, the assay conditions are as follows: 50 mM Imidazole, pH 7.2, 10 mM MnCl₂, 0.5% Triton X-100, 150 µM UDP-GalNAc, approximately 260,000 cpm UDP-[³H]-GalNAc and 3.2 mM acceptor peptide (the concentration of RSPPP is 3.7 mM). The assays are incubated for 20 minutes (PPASTSAPG) [SEQ ID NO:14] or 8 hours (PPASSSAPG [SEQ ID NO:15], PPAAdSSdSAPG and RSPPP [SEQ ID NO:13]). Following incubation, the enzyme is inactivated by placing the samples on a boiling water bath for 1.5 minutes. The samples are then allowed to cool and the reaction products are separated from unreacted UDP-GalNAc and free GalNAc by chromatography on a Biogel P-2 column (1 X 50 cm) equilibrated in 7% isopropanol; thirty 1.3 ml fractions are collected.

The peptide PPASTSAPG [SEQ ID NO:14] is designed to contain a single Thr, at P0. The proline residues at P4, P3 and P3' provide maximum probabilities at those positions; serine residues at P1 and P1' result in good probabilities without much steric constraint. Finally, the alanine residues at P2 and P2' and the glycine at P4 are indifferent as to the probability of glycosylation but allow for flexibility of the peptide backbone. The aggregate probability of the peptide is still optimal: $h = 0.985$ and the peptide is predicted by the algorithm to be an ideal acceptor. Tables 8 and 9 show that this peptide is the most efficient of the acceptors tested and comparative assays show that its reactivity is very close to that of bovine apomucin (data not shown). Furthermore, the kinetic parameters for the two peptides, determined under our conditions, are quite comparable to those of the purified porcine submaxillary GalNAc-transferase-catalyzed glycosylation of peptides whose structure is derived from sites identified in porcine submaxillary mucin (Wang *et al.*, 1992).

The peptide RTPPP [SEQ ID NO:12], derived from the major acceptor sequence in myelin basic protein (Hagopian *et al.*, 1971), has a K_m lower than that of PPASTSAPG [SEQ ID NO:14] but also a much lower V_{max} and, hence, its catalytic efficiency is only half of that of PPASTSAPG [SEQ ID NO:14]. The activities of the two corresponding peptides containing serine instead of threonines are measurable but too

low for determining the kinetic parameters under the conditions used. Clearly, however, bovine colostrum GalNAc-transferase is capable of transferring GalNAc to the serine of these peptides, albeit — under our *in vitro* conditions — approximately 35 times slower than to threonine (Table 9).

5 Substitution of the two amino acids flanking the acceptor threonine in PPASTSAPG [SEQ ID NO:14] with D-amino acids results in a 250-fold drop in the rate of transfer, clearly demonstrating that the active site of the enzyme recognizes a peptide segment which extends beyond the acceptor amino acid itself. Indeed, the finite residual activity of the D-Ser peptide indicates that none of the recognition subsites is
10 essential and that the P3, P4, and P3' residues are able to compensate partly for the loss of the specific P1 and P1' residues. Interestingly, the peptide Arg-Ser-Pro-Pro-Pro (RSPPP) [SEQ ID NO:13], the myelin basic protein acceptor sequence where the acceptor Thr is replaced by Ser, is also glycosylated, albeit three times slower than PPAAdSSdSAPG. Again, at least *in vitro*, the enzyme seems to prefer
15 threonine-containing acceptors.

In contrast to the enzyme recently purified from porcine submaxillary gland (Wang *et al.*, 1992), however, the bovine colostrum GalNAc-transferase is definitely capable of glycosylating both threonine and serine residues. In this context it should be noted that in experiments reported by O'Connel *et al.* (1992), bovine colostrum
20 GalNAc-transferase failed to glycosylate serine in a peptide derived from human erythropoietin. This phenomenon may be related to the specific acceptor peptide used, even though a serine in this position is glycosylated *in vivo*. Alternatively, given the considerably slower rate of transfer to serine residues by bovine colostrum GalNAc-transferase *in vitro*, the short incubation times used may have been insufficient
25 to produce measurable amounts of product. Under our *in vitro* conditions, the transfer to the serine of PPASSSAPG [SEQ ID NO:15] is approximately 35-fold slower than to the threonine of PPASTSAPG [SEQ ID NO:14]. This observation immediately raises the question whether serine is an acceptor at all for *in situ* glycosylations.

It is likely that Ser is indeed an *in vivo* acceptor, for the following reasons: 1)
30 numerous reports identify unambiguously O-glycosylated serine residues in glycoproteins; 2) preliminary experiments indicate that the Ser- and Thr-glycosylating activities have similar pH optima and that both are independent of calcium concentrations in the assays; 3) the affinity matrix for isolation of the enzyme is bovine apomucin immobilized on Sepharose. This ligand contains comparable numbers of
35 serine and threonine residues and should consequently not distinguish between the two activities (compare below); 4) a mere 35-fold difference in rate is quite usual and is, in

fact, rather small as a range of specificity for multisubstrate enzymes. Slight changes in experimental conditions may well obviate, or even reverse the reactivities observed under our conditions. It is unlikely that the serine transferase activity of the enzyme preparation would be caused by a contaminating serine transferase since: a) the enzyme
5 is pure as judged by SDS-PAGE/silver staining as well as by N-terminal sequencing; b) an antibody raised against the purified transferase precipitates both Ser- and Thr-glycosylating activities; c) assay of samples of crude, partially purified, and pure enzyme yielded the same ratio of Ser- and Thr-transferase activities.

The specificity of GalNAc-transferase is adequately described by a cumulative
10 specificity model, where the independent contribution of several subsites can produce a range of reactivities toward peptide segments. Additional important features of the acceptor peptide segments are exposure on the surface of the protein substrate and an extended conformation. The acceptor amino acid is surrounded preferentially by Ser, Thr, Pro, Ala and Gly residues, but in no specific order. Both serine and threonine are
15 acceptor amino acids, at least for the bovine colostrum enzyme.

Example 13 - Transfer of N-acetylgalactosamine to Synthetic Acceptor Peptides by Soluble GalNAc-Transferase

The ability of GalNAcTs to glycosylate a series of synthetic acceptor peptides was also studied. Assays for the determination of kinetic parameters for peptide acceptors
20 were carried out as described by Elhammer et al. (1993) but using a modification of the method described by O'Connell and Tabak (1993) for isolation of the acceptor peptides: One ml Bond Elut columns containing 100 mg packing material were used. Before loading the assay samples, the columns were washed with 2 ml methanol followed by 2 ml 0.1% TFA (in water). The assay samples (40 µl) were diluted to 1 ml with 0.1%
25 TFA and loaded on the columns. Unbound radioactivity was then washed out with 4 ml 0.1% TFA, after which the glycosylated acceptor peptides were eluted with 1.5 ml 35% acetonitrile, 0.1% TFA (in water), directly into scintillation vials. Calculation of kinetic parameters was done from double reciprocal plots (1/v versus 1/S) using standard procedures. Determination of kinetic parameters for the cloned soluble
30 GalNAc-transferase resulted in the following numbers: The K_m for UDP-GalNAc is approximately 1.7 µM and the K_m s for the threonine containing acceptor peptide PPASTSAPG [SEQ ID NO:14] and the serine containing acceptor peptide PPDAASAAPLR [SEQ ID NO:17] are approximately 6.5 and 3.6 mM, respectively. Transfer by GalNAcTs to another serine containing acceptor peptide PPASSSAPG [SEQ
35 ID NO:15] is approximately 70 times slower than to PPASTSAPG [SEQ ID NO:14] (Data not shown). The specific activity of the purified enzyme preparation, using bovine

apomucin as acceptor, is approximately 2,160 U/mg protein (Table 4).

The enzymatic properties of the purified GalNacTs appear to be similar to the those determined for the enzymes purified from bovine colostrum and porcine submaxillary gland (Elhammer and Kornfeld, 1986; Elhammer et al., 1993; Wang et al., 1992; Wang et al., 1993). The K_m for the acceptor peptide PPASTSAPG [SEQ ID NO:14] is almost identical for the colostrum enzyme and the baculo expressed molecule, 6.0 vs. 6.5 mM. The serine containing acceptor peptide PPDAASAAPLR [SEQ ID NO:17] (O'Connel et al. 1992; Wang et al., 1993), also has a K_m in the low mM range, 3.5 mM, in assays with GalNacTs. This is similar to the K_m determined for this acceptor using the GalNac-transferase purified from porcine submaxillary glands, 4.5 mM (Wang et al., 1993). A K_m for PPDAASAAPLR [SEQ ID NO:17] using the bovine colostrum enzyme, has not been determined. Instead, it has been reported that this peptide is not an acceptor for the colostrum enzyme (O'Connel et al., 1993). Both GalNacTs and the bovine colostrum enzyme glycosylate the peptide PPASSSAGP [SEQ ID NO:15] are at least 35 times slower than PPASTSAPG [SEQ ID NO:14] (Elhammer et al., 1993). The K_m for UDP-GalNac in assays using GalNacTs is lower than those determined for the bovine colostrum and the porcine submaxillary gland enzymes, 1.7 μ M vs. 8 μ M and 6 μ M, respectively (Table V; Elhammer & Kornfeld, 1986; Wang et al., 1992). The reason for this is not clear at present. The amino acid sequence of the bovine colostrum and the cloned molecules should be identical except for five amino acids in the NH_2 -terminal end of the molecule; the colostrum enzyme sequence also contains an additional two amino acids at the NH_2 -terminus. A possible explanation is that differences in post-translational processing, in particular glycosylation, of the Sf9 produced vs. the bovine molecule may, to some extent, influence the kinetic characteristics of the two molecules.

As discussed above, the oligosaccharide structures on the insect produced molecule are most likely of high mannose and/or truncated high mannose type, while results from endoglycosidase digestion experiments suggest that the colostrum enzyme contains complex type oligosaccharides (Elhammer & Kornfeld, 1986). It is likely that the N-linked oligosaccharide structures on the porcine enzyme also would be of the types normally synthesized by mammalian cells; peptide: N-glycosidase F digestion experiments suggest that this molecule contains 9 kDa of N-linked oligosaccharides (Wang et al., 1992). Further experiments will however be needed to clarify this question. Taken together the experimental data suggest that the recombinant, soluble enzyme has *in vitro* enzymatic characteristics quite similar to those determined for the native (colostrum) enzyme. The availability of this molecule should facilitate further

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studies on the enzymatic properties of GalNAc-transferase as well as experiments on *in vitro* synthesis of O-linked glycoconjugates.

Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary
5 only and that various other alternatives, adaptations and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments illustrated herein, but is only limited by the following claims.

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Table 1

Expression of baculovirus isolates of the GalNAc-transferase

Construct	Specific activity, Units/mg protein	Relative rate
Uninfected cells	0.16	1
CMW Pol-1	0.13	0.8
GalNAcT 2-1.A	13.9	87
GalNAcT 2-1.B	11.9	74

5

Table 2

Expression of baculovirus isolates of soluble GalNAc-transferase

Construct	Total activity (U ^a)	
	Cell lysate	Culture medium
Uninfected cells	0.15	0.44
α6-3	0.12	0.40
GalNAcT 2-1A	16.22	1.61
GalNAcT-Mel	32.15	57.80

5 The GalNAc-transferase constructs GalNAcT 2-1A and GalNAcTs-Mel together with a
 10 construct coding for an unrelated molecule, α6-3 (a γ-aminobutyric acid receptor
 subunit), were expressed in Sf9 cells. Cells (1×10^6) were infected with recombinant
 virus containing GalNAcTs-Mel (5 pfu/cell). The cells were harvested 65 hours post
 infection, lysed in a detergent containing buffer and the GalNAc-transferase activity was
 determined in the cell lysates and the corresponding culture media; lysate and culture
 15 medium from uninfected cells were assayed as control. The numbers have been adjusted
 for differences in protein content in the cell lysates; the volume of the culture media
 was 5 ml.

20 ^a1 unit equals one mole N-acetylgalactosamine transferred to apomucin per minute,
 under assay conditions.

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TABLE 3

Glycosylation sites. Abundance of amino acids at subsites

5	AMINO	% S.D.	# EXPECTED	# FOUND IN 196	RESIDUES								
	ACID	AVERAGE	IN 196	A.A.	P4	P3	P2	P1	P0	P1'	P2'	P3'	P4'
10	D	5.7+/-2.2	11.17+/-	4.31	6	2	3	2	0	2	6	3	6
	N	4.4+/-2.0	8.62+/-	3.92	5	4	6	3	0	4	7	3	4
	E	6.4+/-2.9	12.54+/-	5.68	10	10	8	5	0	15	13	7	11
	Q	3.9+/-1.7	7.64+/-	3.33	4	7	6	5	0	5	8	6	7
	S	6.6+/-2.7	12.94+/-	5.29	21	29	25	28	90	35	33	21	24
15	G	7.8+/-3.0	15.29+/-	5.88	15	12	11	13	0	16	14	9	15
	H	2.2+/-1.3	4.31+/-	2.55	2	6	4	2	0	2	3	3	5
	R	4.8+/-2.5	9.41+/-	4.90	3	2	3	7	0	3	7	3	4
	T	5.8+/-2.3	11.37+/-	4.51	20	21	26	27	106	31	21	28	27
	A	8.7+/-3.7	17.05+/-	7.25	20	16	20	24	0	22	20	17	16
20	P	4.5+/-2.0	8.82+/-	3.92	24	30	25	30	0	24	25	49	25
	Y	3.3+/-1.9	6.47+/-	3.72	2	3	5	2	0	1	2	3	2
	V	7.0+/-2.5	13.72+/-	4.90	12	12	12	15	0	10	11	12	13
	M	2.1+/-1.3	4.12+/-	2.55	6	4	6	4	0	3	2	5	3
	I	5.2+/-2.3	10.19+/-	4.51	6	5	9	9	0	8	6	6	5
25	L	8.2+/-3.2	16.07+/-	6.27	11	10	11	11	0	6	8	7	9
	F	3.9+/-1.9	7.64+/-	3.72	2	4	3	4	0	1	0	3	3
	K	6.8+/-3.3	13.33+/-	6.47	5	4	4	1	0	4	5	2	5
	C	1.6+/-1.5	3.14+/-	2.94	1	0	0	1	0	2	1	1	1
	W	1.2+/-1.0	2.35+/-	1.96	1	1	1	2	0	0	1	1	1
30	NUMBER OF SIGNIFICANTLY ABUNDANT RESIDUES				24	80	76	85	196	90	79	77	76
	SELECTIVITY OF SUBSITES, S _i				0.4	1.1	0.9	1.1	8.1	1.3	1.0	1.6	0.9

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TABLE 4

Glycosylation. Surabundant amino acids surrounding the reactive Ser or Thr
 Surabundance at a given subsite for a given amino acid is expressed as the number
 of that amino acid found at the site in excess to that expected from random
 5 distribution, divided by the S.D. of the expected distribution. The excess of
 surabundant residues is equal to or higher than twice the S.D. of the expected
 residue

10	AMINO									
	ACID	P4	P3	P2	P1	P0	P1'	P2'	P3'	P4'
	S	(1.5)	3.0	2.3	2.8	14.6	4.2	3.8	(1.5)	2.1
	T	(1.9)	2.1	3.2	3.5	21.0	4.4	2.1	3.7	3.5
15	P	3.9	5.4	4.1	5.4		3.9	4.1	10.3	4.1

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TABLE 5

Glycosylation specificity parameters, s_{ij}

AMINO										
	ACID	P4	P3	P2	P1	P0	P1'	P2'	P3'	P4'
5	-----									
	D	0.54	0.18	0.27	0.18	0.00	0.18	0.54	0.27	0.54
	N	0.58	0.46	0.70	0.35	0.00	0.46	0.81	0.35	0.46
	E	0.80	0.80	0.64	0.40	0.00	1.20	1.04	0.56	0.88
	Q	0.52	0.92	0.78	0.65	0.00	0.65	1.05	0.78	0.92
10	S	1.62	2.24	1.93	2.16	6.96	2.71	2.55	1.62	1.86
	G	0.98	0.78	0.72	0.85	0.00	1.05	0.92	0.59	0.98
	H	0.46	1.39	0.93	0.46	0.00	0.46	0.70	0.70	1.16
	R	0.32	0.21	0.32	0.74	0.00	0.32	0.74	0.32	0.43
	T	1.76	1.85	2.29	2.38	9.32	2.73	1.85	2.46	2.38
15	A	1.17	0.94	1.17	1.41	0.00	1.29	1.17	1.00	0.94
	P	2.72	3.40	2.83	3.40	0.00	2.72	2.83	5.56	2.83
	Y	0.31	0.46	0.77	0.31	0.00	0.15	0.31	0.46	0.31
	V	0.87	0.87	0.87	1.09	0.00	0.73	0.80	0.87	0.95
	M	1.46	0.97	1.46	0.97	0.00	0.73	0.49	1.21	0.73
20	I	0.59	0.49	0.88	0.88	0.00	0.78	0.59	0.59	0.49
	L	0.68	0.62	0.68	0.68	0.00	0.37	0.50	0.44	0.56
	F	0.26	0.52	0.39	0.52	0.00	0.13	0.01	0.39	0.39
	K	0.38	0.30	0.30	0.08	0.00	0.30	0.38	0.15	0.38
	C	0.32	0.03	0.03	0.32	0.00	0.64	0.32	0.32	0.32
25	W	0.43	0.43	0.43	0.85	0.00	0.04	0.43	0.43	0.43

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TABLE 6.

Distribution of mean hydration and secondary structure index in the fifty peptides of the Kabsch-Sander database with the highest probability of O-glycosylation

5

NUMBER OF RESIDUES IN DATABASE = 10747

NUMBER OF ENNEAPEPTIDES IN DATABASE = 10235

	SS	INDEX	TOTAL	P4	P3	P2	P1	P0	P1'	P2'	P3'	P4'	EXPECTED
10	B	β -bridge	204	3	0	0	1	1	1	1	1	3	1.0
	E	β -strand	2091	18	14	18	15	13	11	7	6	4	10.2
	G	3_{10} -helix	308	0	0	0	0	0	0	0	0	0	1.5
	H	α -helix	2743	2	2	2	2	2	2	2	2	3	13.4
	I	π -helix	0	0	0	0	0	0	0	0	0	0	
15	0												
	S	bend	1377	7	14	13	12	13	13	15	15	14	6.7
	T	turn	1349	4	3	3	6	5	3	6	10	9	6.6
	n	none	2675	16	17	14	14	16	20	19	16	17	13.1
20	HYD.	INDEX	TOTAL	P4	P3	P2	P1	P0	P1'	P2'	P3'	P4'	
	0		2068	11	7	4	7	5	7	4	3	8	
	1		1208	5	6	4	4	4	5	1	7	6	
	2		944	3	9	7	4	3	6	6	5	5	
	3		797	7	4	7	7	5	7	8	4	6	
	4		725	5	2	5	4	1	5	3	4	1	
	5		752	6	3	1	2	4	3	1	4	7	
	6		685	4	3	7	7	4	1	4	4	2	
	7		594	2	2	5	1	6	4	4	3	1	
	8		534	2	4	3	4	5	2	4	3	4	
	9		511	3	4	0	0	5	4	5	2	3	
	10		1929	2	6	7	10	8	6	10	11	7	
MEAN HYD.			4.5	3.7	4.4	4.7	4.8	5.6	4.4	5.7	5.2	4.4	

TABLE 7

Transfer of N-acetylgalactosamine to protein acceptors

- 5 The transfer of N-acetylgalactosamine to protein acceptors was assayed under standard conditions (see Material and Methods). The acceptor concentration was 65 μ M, the enzyme concentration approximately 65 mU/ml and assay time was 60 minutes. The transfer to both native and reduced-carboxymethylated acceptors was assayed.

10

Acceptor	Product formed nmol/min/mg	
	Native	Reduced and carboxymethylated
Myelin basic protein	22.2	21.3 ^a
Bovine rhodanese	<0.1	3.3
Subtilisin	<0.1	0.6
15 Bovine cytochrome C	<0.1	<0.1

^a Myelin basic protein was not reduced and carboxymethylated in this experiment.

TABLE 8

Determination of kinetic parameters for acceptor peptides

- 5 Assays were carried out as described in Materials and Methods. Assay time was 20 minutes. The values are given as means $\pm$ S.E. (n=3)

10	Acceptor	K_m mM	$10^3 \times V_{max}^a$ s^{-1}	V_{max}/K_m $M^{-1} s^{-1}$
	RTPPP [SEQ ID NO:12]	3.3 ± 0.6	604 ± 83	183
	PPASTSAPG [SEQ ID NO:14]	6.0 ± 0.3	$1,808 \pm 357$	301
15	PPASSSAPG [SEQ ID NO:15]	n.a.	n.a.	$\approx 8.5^b$
	RSPPP [SEQ ID NO:13]	n.a.	n.a.	$\approx 0.4^b$
20	PPAdSTdSAPG	n.a.	n.a.	$\approx 1.2^b$

n.a., Not applicable

^a Calculations based on a 70 kDa molecular mass of the enzyme.

^b Calculated assuming that $S_0 \ll K_m$

TABLE 9

Comparison of different synthetic peptides as acceptors for
bovine colostrum GalNAc-transferase

5

Assays were done as described in Materials and Methods. The products were separated by Biogel P-2 chromatography. Assay times were 20 minutes for PPASTSAPG [SEQ ID NO:14] and 8 hours for PPASSSAPG [SEQ ID NO:15], PPAdSTdSAPG and RSPPP [SEQ ID NO:13].

10

Acceptor	Product formed, nmol/min/mg	Relative rate
PPASTSAPG	503.3	1
PPASSSAPG	14.3	1/35
PPAdSTdSAPG	2.0	1/252
RSPPP	0.67	1/751

15

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SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: Elhammer, Ake P.
Homa, Fred L.
- (ii) TITLE OF INVENTION: A Cloned DNA Encoding a UDP-GalNAc:
Polypeptide, N-Acetylgalactosaminyltransferase
- (iii) NUMBER OF SEQUENCES: 19
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: The Upjohn Company, Corp. Intellectual
Property Law
(B) STREET: 301 Henrietta Street
(C) CITY: Kalamazoo
(D) STATE: Michigan
(E) COUNTRY: USA
(F) ZIP: 49001
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Darnley Jr., James D.
(B) REGISTRATION NUMBER: 33,673
(C) REFERENCE/DOCKET NUMBER: 4755.P CP
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 616-385-5210
(B) TELEFAX: 616-385-6897
(C) TELEX: 224401
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 34 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Leu | Pro | Ala | Gly | Asp | Val | Leu | Glu | Pro | Val | Gln | Lys | Pro | His | Glu |
| 1 | | | | 5 | | | | 10 | | | | | 15 | | |
| Gly | Pro | Gly | Glu | Met | Gly | Lys | Pro | Val | Val | Ile | Pro | Lys | Glu | Asp | Gln |
| | | | 20 | | | | 25 | | | | | | 30 | | |
| Glu | Lys | | | | | | | | | | | | | | |

(2) INFORMATION FOR SEQ ID NO:2:

-52-

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
5 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
GCNCGNGAYG TNCTWGARCC 20
- 15 (2) INFORMATION FOR SEQ ID NO:3:
- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
20 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
CARAARCCNC AYGARGG 17
- 30 (2) INFORMATION FOR SEQ ID NO:4:
- (i) SEQUENCE CHARACTERISTICS:
35 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
45 TTYCTYCTRG TYCTYTTY 18
- (2) INFORMATION FOR SEQ ID NO:5:
- (i) SEQUENCE CHARACTERISTICS:
50 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 55 (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
60 CAAAGCCTC ATGAAGGTCC 20
- (2) INFORMATION FOR SEQ ID NO:6:
- 65 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCATTCCTAA AGAGGACC

18

10 (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 22 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAAGTTCAGC CTGTTAAGT CC

22

25 (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 23 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGAAAAATGG GACCTTCTTT ATG

40

23

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 559 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: protein

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

55 Met Arg Lys Phe Ala Tyr Cys Lys Val Val Leu Ala Thr Ser Leu Ile
 1 5 10 15
 Trp Val Leu Leu Asp Met Phe Leu Leu Leu Tyr Phe Ser Glu Cys Asn
 20 25 30
 Lys Cys Asp Glu Lys Lys Glu Arg Gly Leu Pro Ala Gly Asp Val Leu
 35 40 45
 Glu Pro Val Gln Lys Pro His Glu Gly Pro Gly Glu Met Gly Lys Pro
 50 55 60
 Val Val Ile Pro Lys Glu Asp Gln Glu Lys Met Lys Glu Met Phe Lys
 65 70 75 80

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	Ile	Asn	Gln	Phe	Asn	Leu	Met	Ala	Ser	Glu	Met	Ile	Ala	Leu	Asn	Arg	
					85					90					95		
5	Ser	Leu	Pro	Asp	Val	Arg	Leu	Glu	Gly	Cys	Lys	Thr	Lys	Val	Tyr	Pro	
				100					105					110			
	Asp	Asn	Leu	Pro	Thr	Thr	Ser	Val	Val	Ile	Val	Phe	His	Asn	Glu	Ala	
			115					120					125				
10	Trp	Ser	Thr	Leu	Leu	Arg	Thr	Val	His	Ser	Val	Ile	Asn	Arg	Ser	Pro	
		130					135					140					
	Arg	His	Met	Leu	Glu	Glu	Ile	Val	Leu	Val	Asp	Asp	Ala	Ser	Glu	Arg	
15		145				150					155					160	
	Asp	Phe	Leu	Lys	Arg	Pro	Leu	Glu	Ser	Tyr	Val	Lys	Lys	Leu	Lys	Val	
					165					170					175		
20	Pro	Val	His	Val	Ile	Arg	Met	Glu	Gln	Arg	Ser	Gly	Leu	Ile	Arg	Ala	
				180					185					190			
	Arg	Leu	Lys	Gly	Ala	Ala	Val	Ser	Lys	Gly	Gln	Val	Ile	Thr	Phe	Leu	
			195					200					205				
25	Asp	Ala	His	Cys	Glu	Cys	Thr	Val	Gly	Trp	Leu	Glu	Pro	Leu	Leu	Ala	
		210					215					220					
	Arg	Ile	Lys	His	Asp	Arg	Lys	Thr	Val	Val	Cys	Pro	Ile	Ile	Asp	Val	
30		225				230					235					240	
	Ile	Ser	Asp	Asp	Thr	Phe	Glu	Tyr	Met	Ala	Gly	Ser	Asp	Met	Thr	Tyr	
					245					250					255		
35	Gly	Gly	Phe	Asn	Trp	Lys	Leu	Asn	Phe	Arg	Trp	Tyr	Pro	Val	Pro	Gln	
				260					265					270			
	Arg	Glu	Met	Asp	Arg	Arg	Lys	Gly	Asp	Arg	Thr	Leu	Pro	Val	Arg	Thr	
			275					280					285				
40	Pro	Thr	Met	Ala	Gly	Gly	Leu	Phe	Ser	Ile	Asp	Arg	Asp	Tyr	Phe	Gln	
			290				295					300					
	Glu	Ile	Gly	Thr	Tyr	Asp	Ala	Gly	Met	Asp	Ile	Trp	Gly	Gly	Glu	Asn	
45		305				310					315					320	
	Leu	Glu	Ile	Ser	Phe	Arg	Ile	Trp	Gln	Cys	Gly	Gly	Thr	Leu	Glu	Ile	
					325					330					335		
50	Val	Thr	Cys	Ser	His	Val	Gly	His	Val	Phe	Arg	Lys	Ala	Thr	Pro	Tyr	
				340					345					350			
	Thr	Phe	Pro	Gly	Gly	Thr	Gly	Gln	Ile	Ile	Asn	Lys	Asn	Asn	Arg	Arg	
			355					360					365				
55	Leu	Ala	Glu	Val	Trp	Met	Asp	Glu	Phe	Lys	Asn	Phe	Phe	Tyr	Ile	Ile	
			370				375					380					
	Ser	Pro	Gly	Val	Thr	Lys	Val	Asp	Tyr	Gly	Asp	Ile	Ser	Ser	Arg	Leu	
60			385			390					395					400	
	Gly	Leu	Arg	His	Lys	Leu	Gln	Cys	Arg	Pro	Phe	Ser	Trp	Tyr	Leu	Glu	
					405					410					415		
65	Asn	Ile	Tyr	Pro	Asp	Ser	Gln	Ile	Pro	Arg	His	Tyr	Phe	Ser	Leu	Gly	
				420					425					430			
	Glu	Ile	Arg	Asn	Val	Glu	Thr	Asn	Gln	Cys	Leu	Asp	Asn	Met	Ala	Arg	
			435					440					445				

-55-

Lys Glu Asn Glu Lys Val Gly Ile Phe Asn Cys His Gly Met Gly Gly
 450 455 460
 5 Asn Gln Val Phe Ser Tyr Thr Ala Asn Lys Glu Ile Arg Thr Asp Asp
 465 470 475 480
 Leu Cys Leu Asp Val Ser Lys Leu Asn Gly Pro Val Thr Met Leu Lys
 485 490 495
 10 Cys His His Leu Lys Gly Asn Gln Leu Trp Glu Tyr Asp Pro Val Lys
 500 505 510
 Leu Thr Leu Gln His Val Asn Ser Asn Gln Cys Leu Asp Lys Ala Thr
 515 520 525
 15 Asp Glu Asp Ser Gln Val Pro Ser Ile Arg Asp Cys Ser Gly Ser Arg
 530 535 540
 20 Ser Gln Gln Trp Leu Leu Arg Asn Val Thr Leu Pro Glu Ile Phe
 545 550 555

(2) INFORMATION FOR SEQ ID NO:10:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2294 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGAAGTAACC CTGAAGTTAG AATTGGATTA CTTTCATTTG ACTTAAAGTG CCATGAGAAA 60
 ATTTGCATAC TGCAAGGTGG TCCTAGCCAC CTCCTTGATT TGGGTACTCT TGGATATGTT 120
 40 CCTGCTGCTT TACTTCAGTG AATGCAACAA ATGTGATGAA AAAAAAGAGA GAGGACTTCC 180
 TGCTGGGGAT GTTCTAGAGC CAGTACAAAA GCCTCATGAA GGTCTGGAG AAATGGGGAA 240
 45 ACCAGTCGTC ATTCCTAAAG AGGATCAAGA AAAGATGAAA GAGATGTTTA AAATCAATCA 300
 GTTCAATTTA ATGGCAAGTG AGATGATTGC ACTCAACAGA TCTCTACCAG ATGTTAGATT 360
 AGAAGGGTGT AAAACAAAGG TGTATCCAGA TAACCTTCCT ACAACCAGTG TGGTGATTGT 420
 50 TTTCCACAAT GAGGCTTGGA GCACACTTCT GCGAACTGTC CATAGCGTCA TTAATCGCTC 480
 ACCAAGGCAC ATGCTAGAAG AAATTGTTCT AGTAGATGAT GCCAGTGAAA GAGACTTTTT 540
 55 AAAAAGACCT CTAGAGAGTT ACGTGAAAAA ATTAAAAGTA CCCGTTACG TCATTCTGAAT 600
 GGAGCAGCGT TCTGGATTGA TCAGAGCTAG GTTAAAAGGT GCTGCTGTGT CTAAAGGCCA 660
 AGTGATCACC TTTTGTAGACG CGCACTGTGA GTGCACAGTG GGGTGGCTGG AGCCTCTCTT 720
 60 AGCCAGGATC AAACATGACA GGAAGACAGT GGTCTGTCCC ATCATAGATG TGATCAGTGA 780
 TGACACTTTC GAGTACATGG CAGGTTCTGA CATGACCTAT GGCGGGTTCA ACTGGAAGCT 840
 CAACTTTCGC TGGTATCCTG TTCCCCAAAG AGAAATGGAC AGAAGGAAAG GTGATCGGAC 900
 65 TCTTCCTGTG AGAACACCTA CAATGGCAGG AGGCCTTTTT TCAATAGACA GAGATTACTT 960
 TCAGGAAATT GGAACATATG ATGCTGGAAT GGATATTTGG GGAGGAGAAA ACCTAGAAAT 1020

-56-

TTCCTTTAGG ATTTGGCAGT GTGGAGGAAC TTTGGAGATT GTTACTTGCT CACATGTTGG 1080
 ACATGTGTTT CGGAAAGCTA CACCCTACAC GTTTCAGGA GGCACGGGGC AGATTATCAA 1140
 5 TAAAAATAAC AGACGACTTG CAGAAGTATG GATGGATGAA TTCAAGAATT TCTTCTATAT 1200
 AATTTCTCCA GGTGTTACAA AGGTAGATTA TGGAGATATA TCATCAAGAC TTGGTCTAAG 1260
 10 GCACAACTC CAATGCAGAC CATTCTCTTG GTACCTAGAG AATATTTATC CTGATTCTCA 1320
 GATTCCTCGT CACTATTTCT CTTTGGGAGA GATACGAAAT GTGGAAACAA ATCAGTGTCT 1380
 AGATAACATG GCTAGAAAAG AGAATGAAAA AGTTGGAATT TTTAACTGTC ATGGTATGGG 1440
 15 AGGTAATCAG GTTTTCTCTT AACTGCCAA CAAAGAAATT AGAACAGATG ACCTTTGCTT 1500
 GGATGTCTCC AAACCTAATG GCCCAGTCAC AATGCTCAA TGCCACCACC TAAAAGGCAA 1560
 20 CCAGCTTTGG GAGTATGACC CGGTGAAGTT GACCCTGCAG CATGTGAACA GTAACCAGTG 1620
 CCTGGACAAA GCCACAGACG AGGACAGCCA GGTGCCAGC ATCAGAGACT GCAGCGGAAG 1680
 CCGATCCCAG CAGTGGCTTC TTCGGAACGT CACCCTTCCA GAAATATTCT GAGACCAAAT 1740
 25 TTACAAAAAA AGGAAAACGT AAGGACTGAC TGGGCTACCT CAGCATAAT TTCTGCCACA 1800
 TTCTTAAGTA GCAAAAAAAG GAAAAGTGCT TTCCTTCTGC AGGATGTAAG GTTTATCAGC 1860
 30 CATTAAGTAA TTATAGACTG CCCTCGCTTC CACTAGCTGT GAACCAGCCT TCCTGTCCCA 1920
 GGACGTGCAA CTACGTAGTA GCGAGACTGT GCACACTGAT GTTTACAAGA TTGAAAGAGT 1980
 CGGTCATCAA GAATCCTCGT AAAGAATACT CAGACTGAAA CAGTCTGCGA ACTGTGCTTT 2040
 35 CCAGAGAGCT GCGCCTTTTA TGGTTTGCCT GCACAGCAGT GAGTTCCGCT GACTGTGCTG 2100
 TCATAATGAA GAGACGTCTA AGATTTTTTT TCTGATTAGA ACTGGTAGCC AGTATATTAA 2160
 40 ATACTGATAT AAAAATAAAT GAACTGGAAC CAGATTCAGA ATCATGAAAA CATTTTTTACA 2220
 ATTTAAAAAA AACAAACTA TATTAAACAG GGTTTAAAGG AATTAAAAACA AAAAAAAAAA 2280
 AAAAAAAAAA AAAA 2294

45 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1680 base pairs
 (B) TYPE: nucleic acid
 50 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

60 ATGAGAAAAT TTGCATACTG CAAGGTGGTC CTAGCCACCT CCTTGATTG GGTACTCTTG 60
 GATATGTTCC TGCTGCTTTA CTTCAGTGAA TGCAACAAAT GTGATGAAAA AAAAGAGAGA 120
 GGACTTCCTG CTGGGGATGT TCTAGAGCCA GTACAAAAGC CTCATGAAGG TCCTGGAGAA 180
 65 ATGGGGAAAC CAGTCGTCAT TCCTAAAGAG GATCAAGAAA AGATGAAAGA GATGTTTAAA 240
 ATCAATCAGT TCAATTTAAT GGCAAGTGAG ATGATTGCAC TCAACAGATC TCTACCAGAT 300
 GTTAGATTAG AAGGGTGTA AACAAAGGTG TATCCAGATA ACCTTCCTAC AACCAGTGTG 360

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GTGATTGTTT TCCACAATGA GGCTTGGAGC ACACTTCTGC GAACTGTCCA TAGCGTCATT 420
 AATCGCTCAC CAAGGCACAT GCTAGAAGAA ATTGTTCTAG TAGATGATGC CAGTGAAAGA 480
 5 GACTTTTAA AAAGACCTCT AGAGAGTTAC GTGAAAAAAT TAAAAGTACC CGTTCACGTC 540
 ATTCGAATGG AGCAGCGTTC TGGATTGATC AGAGCTAGGT TAAAAGGTGC TGCTGTGTCT 600
 10 AAAGGCCAAG TGATCACCTT TTTAGACGCG CACTGTGAGT GCACAGTGGG GTGGCTGGAG 660
 CCTCTCTTAG CCAGGATCAA ACATGACAGG AAGACAGTGG TCTGTCCCAT CATAGATGTG 720
 ATCAGTGATG ACACTTTCGA GTACATGGCA GGTTCGTGACA TGACCTATGG CGGGTTCAAC 780
 15 TGGAAGCTCA ACTTTCGCTG GTATCCTGTT CCCCAAAGAG AAATGGACAG AAGGAAAGGT 840
 GATCGGACTC TTCCTGTGAG AACACCTACA ATGGCAGGAG GCCTTTTTTC AATAGACAGA 900
 20 GATTACTTTC AGGAAATTGG AACATATGAT GCTGGAATGG ATATTTGGGG AGGAGAAAAC 960
 CTAGAAATTT CCTTTAGGAT TTGGCAGTGT GGAGGAACTT TGGAGATTGT TACTTGCTCA 1020
 CATGTTGGAC ATGTGTTTCG GAAAGCTACA CCCTACACGT TTCCAGGAGG CACGGGGCAG 1080
 25 ATTATCAATA AAAATAACAG ACGACTTGCA GAAGTATGGA TGGATGAATT CAAGAATTTT 1140
 TTCTATATAA TTTCTCCAGG TGTTACAAAG GTAGATTATG GAGATATATC ATCAAGACTT 1200
 GGTCTAAGGC ACAAACCTCA ATGCAGACCA TTCTCTTGGT ACCTAGAGAA TATTTATCCT 1260
 30 GATTCTCAGA TTCCTCGTCA CTATTTCTCT TTGGGAGAGA TACGAAATGT GGAAACAAAT 1320
 CAGTGTCTAG ATAACATGGC TAGAAAAGAG AATGAAAAAG TTGGAATTTT TAACTGTCAT 1380
 35 GGTATGGGAG GTAATCAGGT TTTCTCTTAC ACTGCCAACA AAGAAATTAG AACAGATGAC 1440
 CTTTGCTTGG ATGTCTCCAA ACTTAATGGC CCAGTCACAA TGCTCAAATG CCACCACCTA 1500
 40 AAAGGCAACC AGCTTTGGGA GTATGACCCG GTGAAGTTGA CCCTGCAGCA TGTGAACAGT 1560
 AACCAGTGCC TGGACAAAGC CACAGACGAG GACAGCCAGG TGCCCAGCAT CAGAGACTGC 1620
 AGCGGAAGCC GATCCCAGCA GTGGCTTCTT CGGAACGTCA CCCTTCCAGA AATATTCTGA 1680

45

(2) INFORMATION FOR SEQ ID NO:12:

50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

60 Arg Thr Pro Pro Pro
 1 5

(2) INFORMATION FOR SEQ ID NO:13:

65 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

-58-

(ii) MOLECULE TYPE: peptide

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Arg Ser Pro Pro Pro
1 5

10 (2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
15 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

25 Pro Pro Ala Ser Thr Ser Ala Pro Gly
1 5

(2) INFORMATION FOR SEQ ID NO:15:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

40 Pro Pro Ala Ser Ser Ser Ala Pro Gly
1 5

45 (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAAGTTCAGC CTGGTTAAGT CCAAGCTGAA TTCTTTTGCT TTTTACCCTG GAAGAAATAC

60

60

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
65 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

-59-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

5 Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg
 1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1617 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

	ATGAAATTCT TAGTCAACGT TGCCCTTGTT TTTATGGTCG TGTACATTTT TTACATCTAT	60
	GCGGATCCAA GCCCTGCTCT AGAGCCAGTA CAAAAGCCTC ATGAAGGTCC TGGAGAAATG	120
25	GGGAAACCAG TCGTCATTCC TAAAGAGGAT CAAGAAAAGA TGAAAGAGAT GTTTAAATC	180
	AATCAGTTCA ATTTAATGGC AAGTGAGATG ATTGCACTCA ACAGATCTCT ACCAGATGTT	240
30	AGATTAGAAG GGTGTAAAAC AAAGGTGTAT CCAGATAACC TTCCTACAAC CAGTGTGGTG	300
	ATTGTTTTCC ACAATGAGGC TTGGAGCACA CTTCTGCGAA CTGTCCATAG CGTCATTAAT	360
	CGCTCACCAA GGCACATGCT AGAAGAAATT GTTCTAGTAG ATGATGCCAG TGAAAGAGAC	420
35	TTTTTAAAAA GACCTCTAGA GAGTTACGTG AAAAAATTAA AAGTACCCGT TCACGTCATT	480
	CGAATGGAGC AGCGTTCTGG ATTGATCAGA GCTAGGTTAA AAGGTGCTGC TGTGTCTAAA	540
40	GGCCAAGTGA TCACCTTTTT AGACGCGCAC TGTGAGTGCA CAGTGGGGTG GCTGGAGCCT	600
	CTCTTAGCCA GGATCAAACA TGACAGGAAG ACAGTGGTCT GTCCCATCAT AGATGTGATC	660
	AGTGATGACA CTTTCGAGTA CATGGCAGGT TCTGACATGA CCTATGGCGG GTTCAACTGG	720
45	AAGCTCAACT TTCGCTGGTA TCCTGTTCCC CAAAGAGAAA TGGACAGAAG GAAAGGTGAT	780
	CGGACTCTTC CTGTGAGAAC ACCTACAATG GCAGGAGGCC TTTTTCATAT AGACAGAGAT	840
50	TACTTTCAGG AAATTGGAAC ATATGATGCT GGAATGGATA TTTGGGGAGG AGAAAACCTA	900
	GAAATTTCCCT TTAGGATTTG GCAGTGTGGA GGAACCTTGG AGATTGTTAC TTGCTCACAT	960
	GTTGGACATG TGTTTCGGAA AGCTACACCC TACACGTTTC CAGGAGGCAC GGGGCAGATT	1020
55	ATCAATAAAA ATAACAGACG ACTTGCAGAA GTATGGATGG ATGAATTCAA GAATTTCTTC	1080
	TATATAATTT CTCCAGGTGT TACAAAGGTA GATTATGGAG ATATATCATC AAGACTTGGT	1140
60	CTAAGGCACA AACTCCAATG CAGACCATTG TCTTGGTACC TAGAGAATAT TTATCCTGAT	1200
	TCTCAGATTG CTCGTCACCT TTTCTCTTTG GGAGAGATAC GAAATGTGGA AACAAATCAG	1260
	TGTCTAGATA ACATGGCTAG AAAAGAGAAT GAAAAAGTTG GAATTTTAA CTGTCATGGT	1320
65	ATGGGAGGTA ATCAGGTTTT CTCTTACACT GCCAACAAAG AAATTAGAAC AGATGACCTT	1380
	TGCTTGGATG TCTCCAAACT TAATGGCCCA GTCACAATGC TCAAATGCCA CCACCTAAAA	1440

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GGCAACCAGC TTTGGGAGTA TGACCCGGTG AAGTTGACCC TGCAGCATGT GAACAGTAAC 1500
 CAGTGCCTGG ACAAAGCCAC AGACGAGGAC AGCCAGGTGC CCAGCATCAG AGACTGCAGC 1560
 5 GGAAGCCGAT CCCAGCAGTG GCTTCTTCGG AACGTCACCC TTCCAGAAAT ATTCTGA 1617

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 517 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

10	Asp	Pro	Ser	Pro	Ala	Leu	Glu	Pro	Val	Gln	Lys	Pro	His	Glu	Gly	Pro
	1				5					10					15	
25	Gly	Glu	Met	Gly	Lys	Pro	Val	Val	Ile	Pro	Lys	Glu	Asp	Gln	Glu	Lys
				20					25					30		
	Met	Lys	Glu	Met	Phe	Lys	Ile	Asn	Gln	Phe	Asn	Leu	Met	Ala	Ser	Glu
			35					40					45			
30	Met	Ile	Ala	Leu	Asn	Arg	Ser	Leu	Pro	Asp	Val	Arg	Leu	Glu	Gly	Cys
		50				55						60				
35	Lys	Thr	Lys	Val	Tyr	Pro	Asp	Asn	Leu	Pro	Thr	Thr	Ser	Val	Val	Ile
	65				70					75					80	
	Val	Phe	His	Asn	Glu	Ala	Trp	Ser	Thr	Leu	Leu	Arg	Thr	Val	His	Ser
				85						90					95	
40	Val	Ile	Asn	Arg	Ser	Pro	Arg	His	Met	Leu	Glu	Glu	Ile	Val	Leu	Val
			100						105					110		
	Asp	Asp	Ala	Ser	Glu	Arg	Asp	Phe	Leu	Lys	Arg	Pro	Leu	Glu	Ser	Tyr
			115					120					125			
45	Val	Lys	Lys	Leu	Lys	Val	Pro	Val	His	Val	Ile	Arg	Met	Glu	Gln	Arg
		130				135						140				
50	Ser	Gly	Leu	Ile	Arg	Ala	Arg	Leu	Lys	Gly	Ala	Ala	Val	Ser	Lys	Gly
	145				150					155						160
	Gln	Val	Ile	Thr	Phe	Leu	Asp	Ala	His	Cys	Glu	Cys	Thr	Val	Gly	Trp
					165					170					175	
55	Leu	Glu	Pro	Leu	Leu	Ala	Arg	Ile	Lys	His	Asp	Arg	Lys	Thr	Val	Val
			180						185					190		
	Cys	Pro	Ile	Ile	Asp	Val	Ile	Ser	Asp	Asp	Thr	Phe	Glu	Tyr	Met	Ala
			195					200					205			
60	Gly	Ser	Asp	Met	Thr	Tyr	Gly	Gly	Phe	Asn	Trp	Lys	Leu	Asn	Phe	Arg
		210				215						220				
65	Trp	Tyr	Pro	Val	Pro	Gln	Arg	Glu	Met	Asp	Arg	Arg	Lys	Gly	Asp	Arg
	225					230				235					240	
	Thr	Leu	Pro	Val	Arg	Thr	Pro	Thr	Met	Ala	Gly	Gly	Leu	Phe	Ser	Ile
					245					250					255	

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[illegible]

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CLAIMS

1. A process for altering the glycosylation of a protein produced by a cell, said process comprising the steps of:
 - introducing into said cell at least one gene which is capable of
 - 5 expressing the enzyme N-acetylgalactosaminyltransferase; and
 - expressing a sufficient amount of said enzyme in said cell to thereby alter the glycosylation of said protein in said cell.
2. A process according to claim 1 wherein said cell is a eukaryotic cell.
- 10 3. A process according to claim 2 wherein said cell is selected from the group consisting of Chinese hamster ovary cells, mouse L cells, mouse A9 cells, baby hamster kidney cells, C127 cells, PC8 cells, Sf9 cells, Drosophila cells, and other insect and mammalian cells.
- 15 4. A process according to claim 2 wherein said gene is introduced into said cell by transfection with a vector comprising cDNA which codes for said enzyme.
- 20 5. A process according to claim 4 wherein said cDNA has the nucleotide sequence as set forth in Fig. 15 [SEQ ID NO:11] of this specification.
6. A process according to claim 4 wherein said vector is selected from the group consisting of pAC373, pECE, pMSG, pAV009/A+, pMT 101/A-, actin 5 α , metallothionine.
- 25 7. A process according to claim 6 wherein said vector is pAC373.
8. A process according to claim 7 wherein said cDNA has the nucleotide sequence as set forth in Fig. 15 [SEQ ID NO:11] of this specification.
- 30 9. A process according to claim 2 including the further step of recovering the protein for which glycosylation was altered.
10. A process according to claim 1 wherein said cell is a prokaryotic cell.
- 35 11. A process according to claim 10 wherein said cell is selected from the group

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consisting of *E. coli* and *Bacillus subtilis*.

12. A process according to claim 10 wherein said gene is introduced into said cell by transfection with a vector comprising cDNA which codes for said enzyme.

5

13. A process according to claim 12 wherein said cDNA has the nucleotide sequence as set forth in Fig. 15 [SEQ ID NO:11] of this specification.

14. A process according to claim 12 wherein said vector is selected from the group consisting of Trp, T7, Tac, Lac, and Pl.

10

15. A process according to claim 14 wherein said vector is Trp.

16. A process according to claim 15 wherein said cDNA has the nucleotide sequence as set forth in Fig. 15 [SEQ ID NO:11] of this specification.

15

17. A process according to claim 10 including the further step of recovering the protein for which glycosylation was altered.

18. A cDNA sequence encoding the enzyme N-acetylgalactosaminyltransferase.

20

19. A recombinant DNA molecule comprising a DNA sequence encoding the enzyme N-acetylgalactosaminyltransferase.

20. The recombinant DNA molecule of claim 19 wherein the N-acetylgalactosaminyltransferase comprises the nucleotide sequence as set forth in Fig. 15 [SEQ ID NO:11] of this specification.

25

21. A protein encoded by the DNA sequence depicted in claim 20.

30

22. The protein of claim 21 having the amino acid sequence as set forth in Fig. 16 [SEQ ID NO:9] of this specification.

23. The recombinant DNA molecule of claim 20 wherein the sequences coding for the cytoplasmic and membrane spanning domains of the full-length cDNA have been substantially deleted.

35

24. A protein encoded by the DNA sequence depicted in claim 23.

25. A process for altering the glycosylation of a protein produced by a cell, said process comprising the steps of:

5 introducing into said cell at least one gene which is capable of expressing the acceptor peptide for the enzyme N-acetylgalactosaminyltransferase;

expressing a protein having an acceptor cite for the enzyme N-acetylgalactosaminyltransferase; and

exposing said protein to N-acetylgalactosaminyltransferase.

10

26. A process for altering the gylcosylation of a protein, said process comprising the steps of:

introducing into the DNA sequence encoding a protein at least one gene which is capable of expressing the acceptor peptide for the enzyme N-

15 acetylgalactosaminyltransferase;

expressing said DNA sequence including said gene to produce a protein having an acceptor cite for the enzyme N-acetylgalactosaminyltransferase; and exposing said protein to N-acetylgalactosaminyltransferase.

20 27. A polypeptide having the sequence of the acceptor peptide for the enzyme N-acetylgalactosaminyltransferase as follows:

PPASTSAPG

[SEQ ID NO:14] wherein Proline is P, Alanine is A, Serine is S, Threonine is T, and Glycine is G.

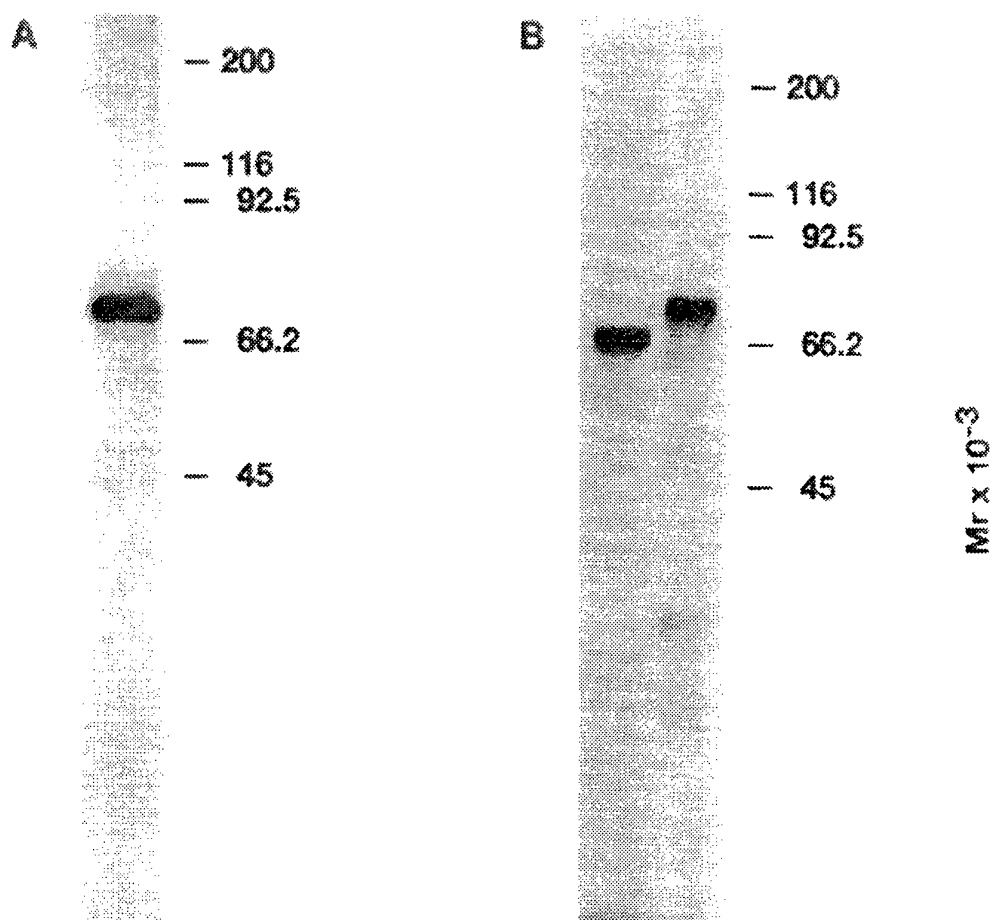
25

28. A polypeptide having the sequence of the acceptor peptide for the enzyme N-acetylgalactosaminyltransferase as follows:

PPASSSAPG

[SEQ ID NO:15] wherein Proline is P, Alanine is A, Serine is S, and Glycine is G.

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Fig. 1

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FIGURE 2

A.

1	5	10	15
GLY LEU PRO ALA GLY ASP VAL LEU GLU PRO VAL GLN LYS PRO HIS			
	20	25	30
GLU GLY PRO GLY GLU MET GLY LYS PRO VAL VAL ILE PRO LYS GLU ASP GLN GLU LYS			
			34
			[SEQ ID NO:1:]

5'-GCX GGX GAN GTX CTZ GAR CC-3'	OLIGO A	[SEQ ID NO:2]
5'-CAR AAR CCX CAN GAR GG-3'	OLIGO B	[SEQ ID NO:3]
3'-TTN CTN CTR GTN CTN TTN-5'	OLIGO C	[SEQ ID NO:4]
5'-CAA AAG CCT CAT GAA GGT CC-3'	OLIGO D	[SEQ ID NO:5]
5'-TC ATT CCT AAA GAG GAC C-3'	OLIGO E	[SEQ ID NO:6]

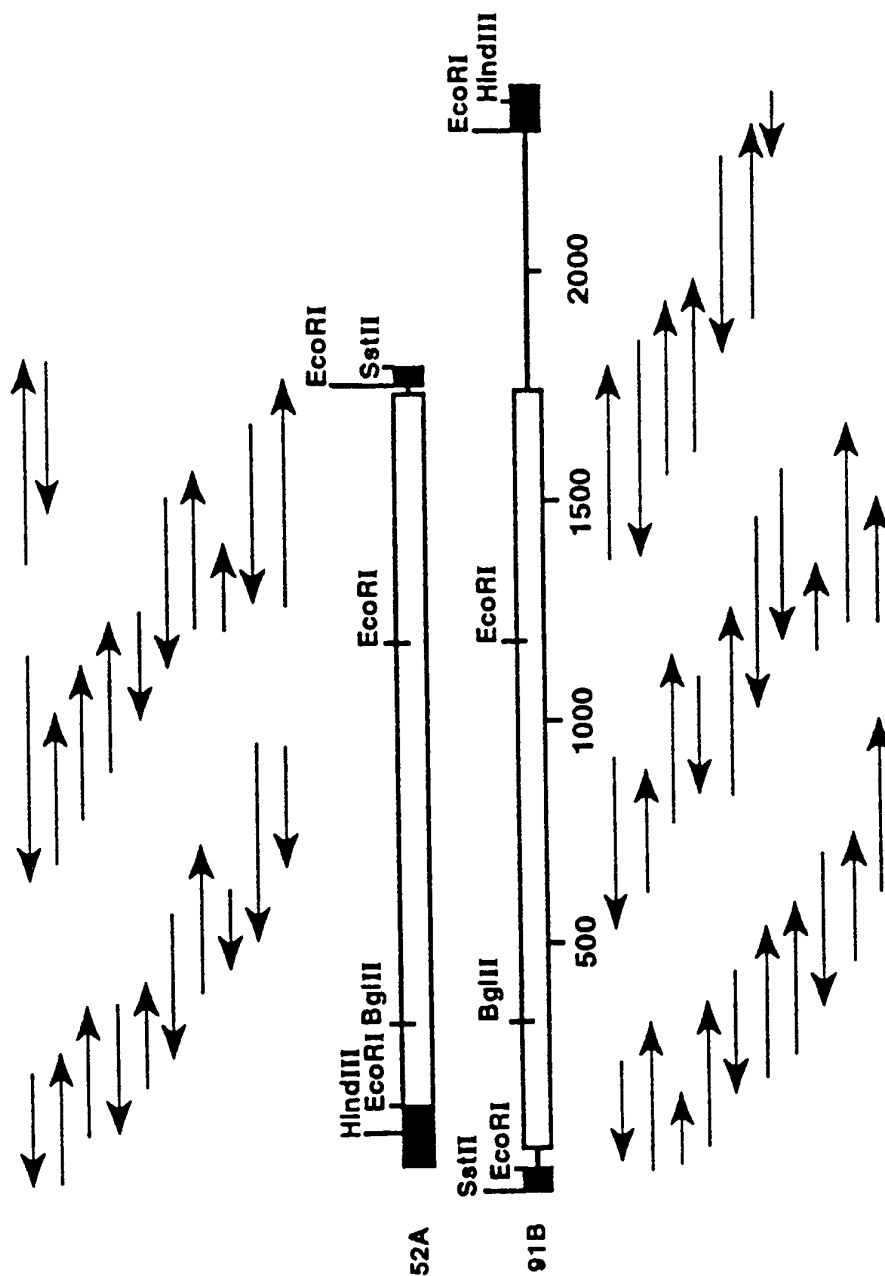
X=G+A+T+C
 N=T+C
 R=A+G
 Y=C+G
 Z=A+T

B.

CAAGTTCAGCCTGGTTAAGTCCAAAGCTGAATTTCTTTTGCTTTTACCCCTGGAAGAATAC	Ygt10	[SEQ ID NO:16]
5'-CAAGTTCAGCCTGGTTAAGTCC-3'	OLIGO F	[SEQ ID NO:7]
3'-CGAAAAATGGGACCTTCTTTATG-5'	OLIGO G	[SEQ ID NO:8]

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FIGURE 2 (CONT'D.)



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FIGURE 3A

SEQUENCE ID No. 10

GGA	ACT	AAC	CCT	GAA	GTT	AGA	ATT	GGA	TACT	TTTC	ATT	GACT	TAA	AGT	GCC	ATG	AGA	AAA	TTT	64
																Met	Arg	Lys	Phe	4
SEQUENCE ID No. 9 →																				
ATG	TTC	CTG	CTG	CTT	TAC	TTC	AGT	GAA	TGC	AAC	AAA	TGT	GAT	GAA	AAA	AAA				166
Met	Phe	Leu	Leu	Leu	Tyr	Phe	Ser	Glu	Cys	Asn	Lys	Cys	Asp	Glu	Lys	Lys				38
GAA	GGT	CCT	GGA	GAA	ATG	GGG	AAA	CCA	GTC	GTC	ATT	CCT	AAA	GAG	GAT	CAA				268
Glu	Gly	Pro	Gly	Glu	Met	Gly	Lys	Pro	Val	Val	Ile	Pro	Lys	Glu	Asp	Gln				72

GAG	ATG	ATT	GCA	CTC	AAC	AGA	TCT	CTA	CCA	GAT	GTT	AGA	TTA	GAA	GGG	TGT				370
Glu	Met	Ile	Ala	Leu	Asn	Arg	Ser	Leu	Pro	Asp	Val	Arg	Leu	Glu	Gly	Cys				106
TTC	CAC	AAT	GAG	GCT	TGG	AGC	ACA	CTT	CTG	CGA	ACT	GTC	CAT	AGC	GTC	ATT				472
Phe	His	Asn	Glu	Ala	Trp	Ser	Thr	Leu	Leu	Arg	Thr	Val	His	Ser	Val	Ile				140
AGT	GAA	AGA	GAC	TTT	TTA	AAA	AGA	CCT	CTA	GAG	AGT	TAC	GTG	AAA	AAA	TTA				574
Ser	Glu	Arg	Asp	Phe	Leu	Lys	Arg	Pro	Leu	Glu	Ser	Tyr	Val	Lys	Lys	Leu				174
GCT	AGG	TTA	AAA	GGT	GCT	GCT	GTG	TCT	AAA	GGC	CAA	GTG	ATC	ACC	TTT	TTA				676
Ala	Arg	Leu	Lys	Gly	Ala	Ala	Val	Ser	Lys	Gly	Gln	Val	Ile	Thr	Phe	Leu				208
ATC	AAA	CAT	GAC	AGG	AAG	ACA	GTG	GTC	TGT	CCC	ATC	ATA	GAT	GTG	ATC	AGT				778
Ile	Lys	His	Asp	Arg	Lys	Thr	Val	Val	Cys	Pro	Ile	Ile	Asp	Val	Ile	Ser				242
AAC	TGG	AAG	CTC	AAC	TTT	CGC	TGG	TAT	CCT	GTT	CCC	CAA	AGA	GAA	ATG	GAC				880
Asn	Trp	Lys	Leu	Asn	Phe	Arg	Trp	Tyr	Pro	Val	Pro	Gln	Arg	Glu	Met	Asp				276
GGC	CTT	TTT	TCA	ATA	GAC	AGA	GAT	TAC	TTT	CAG	GAA	ATT	GGA	ACA	TAT	GAT				982
Gly	Leu	Phe	Ser	Ile	Asp	Arg	Asp	Tyr	Phe	Gln	Glu	Ile	Gly	Thr	Tyr	Asn				310

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FIGURE 3B

GCA TAC TGC AAG GTG GTC CTA GCC ACC TCC TTG ATT TGG GTA CTC TTG GAT	115
Ala Tyr Cys Lys Val Val Leu Ala Thr Ser Leu Ile Trp Val Leu Leu Asp	21
GAG AGA GGA CTT CCT GCT GGG GAT GTT CTA GAG CCA GTA CAA AAG CCT CAT	217
Glu Arg Gly Leu Pro Ala Gly Asp Val Leu Glu Pro Val Gln Lys Pro His	55
GAA AAG ATG AAA GAG ATG TTT AAA ATC AAT CAG TTC AAT TTA ATG GCA AGT	319
Glu Lys Met Lys Glu Met Phe Lys Ile Asn Gln Phe Asn Leu Met Ala Ser	89
AAA ACA AAG GTG TAT CCA GAT AAC CTT CCT ACA ACC AGT GTG GTG ATT GTT	421
Lys Thr Lys Val Tyr Pro Asp Asn Leu Pro Thr Thr Ser Val Val Ile Val	123
AAT CGC TCA CCA AGG CAC ATG CTA GAA GAA ATT GTT CTA GTA GAT GAT GCC	523
Asn Arg Ser Pro Arg His Met Leu Glu Glu Ile Val Leu Val Asp Asp Ala	157
AAA GTA CCC GTT CAC GTC ATT CGA ATG GAG CAG CGT TCT GGA TTG ATC AGA	625
Lys Val Pro Val His Val Ile Arg Met Glu Gln Arg Ser Gly Leu Ile Arg	191
GAC GCG CAC TGT GAG TGC ACA GTG GGG TGG CTG GAG CCT CTC TTA GCC AGG	727
Asp Ala His Cys Glu Cys Thr Val Gly Trp Leu Glu Pro Leu Leu Ala Arg	225
GAT GAC ACT TTC GAG TAC ATG GCA GGT TCT GAC ATG ACC TAT GGC GGG TTC	829
Asp Asp Thr Phe Glu Tyr Met Ala Gly Ser Asp Met Thr Tyr Gly Gly Phe	259
AGA AGG AAA GGT GAT CGG ACT CTT CCT GTG AGA ACA CCT ACA ATG GCA GGA	931
Arg Arg Lys Gly Asp Arg Thr Leu Pro Val Arg Thr Pro Thr Met Ala Gly	293
GCT GGA ATG GAT ATT TGG GGA GGA GAA AAC CTA GAA ATT TCC TTT AGG ATT	1033
Ala Gly Met Asp Ile Trp Gly Gly Glu Asn Leu Glu Ile Ser Phe Arg Ile	327

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FIGURE 3C

TGG CAG TGT GGA GGA ACT TTG GAG ATT GTT ACT TGC TCA CAT GTT GGA CAT	1084
Trp Gln Cys Gly Gly Thr Leu Glu Ile Val Thr Cys Ser His Val Gly His	344
ATC AAT AAA AAT AAC AGA CGA CTT GCA GAA GTA TGG ATG GAT GAA TTC AAG	1186
Ile Asn Lys Asn Asn Arg Arg Leu Ala Glu Val Trp Met Asp Glu Phe Lys	378
ATA TCA TCA AGA CTT GGT CTA AGG CAC AAA CTC CAA TGC AGA CCA TTC TCT	1288
Ile Ser Ser Arg Leu Gly Leu Arg His Lys Leu Gln Cys Arg Pro Phe Ser	412
TCT TTG GGA GAG ATA CGA AAT GTG GAA ACA AAT CAG TGT CTA GAT AAC ATG	1390
Ser Leu Gly Glu Ile Arg Asn Val Glu Thr Asn Gln Cys Leu Asp Asn Met	446
GGT AAT CAG GTT TTC TCT TAC ACT GCC AAC AAA GAA ATT AGA ACA GAT GAC	1492
Gly Asn Gln Val Phe Ser Tyr Thr Ala Asn Lys Glu Ile Arg Thr Asp Asp	480
CAC CAC CTA AAA GGC AAC CAG CTT TGG GAG TAT GAC CCG GTG AAG TTG ACC	1594
His His Leu Lys Gly Asn Gln Leu Trp Glu Tyr Asp Pro Val Lys Leu Thr	514
AGC CAG GTG CCC AGC ATC AGA GAC TGC AGC GGA AGC CGA TCC CAG CAG TGG	1696
Ser Gln Val Pro Ser Ile Arg Asp Cys Ser Gly Ser Arg Ser Gln Gln Trp	548
GGAAAACGTAAGGACTGACTGGGCTACCTCAGCATACATTTCTGCCACATTCTTAAGTAGCAAAAAA	1819
CTTCCACTAGCTGTGAACCAGCCTTCCTGTCCCAGGACGTGCAACTACGTAGTAGCGAGACTGTGCAC	1954
AGTCTGCCAACTGTGCTTTCCAGAGAGCTGCGCCTTTTATGGTTTGCGTGACAGCAGTGAGTTCCGC	2089
TTAAATACTGATATAAAATATAAATGAACTGGAACCAGATTCAGAATCATGAAAACATTTTTACAATTT	2224

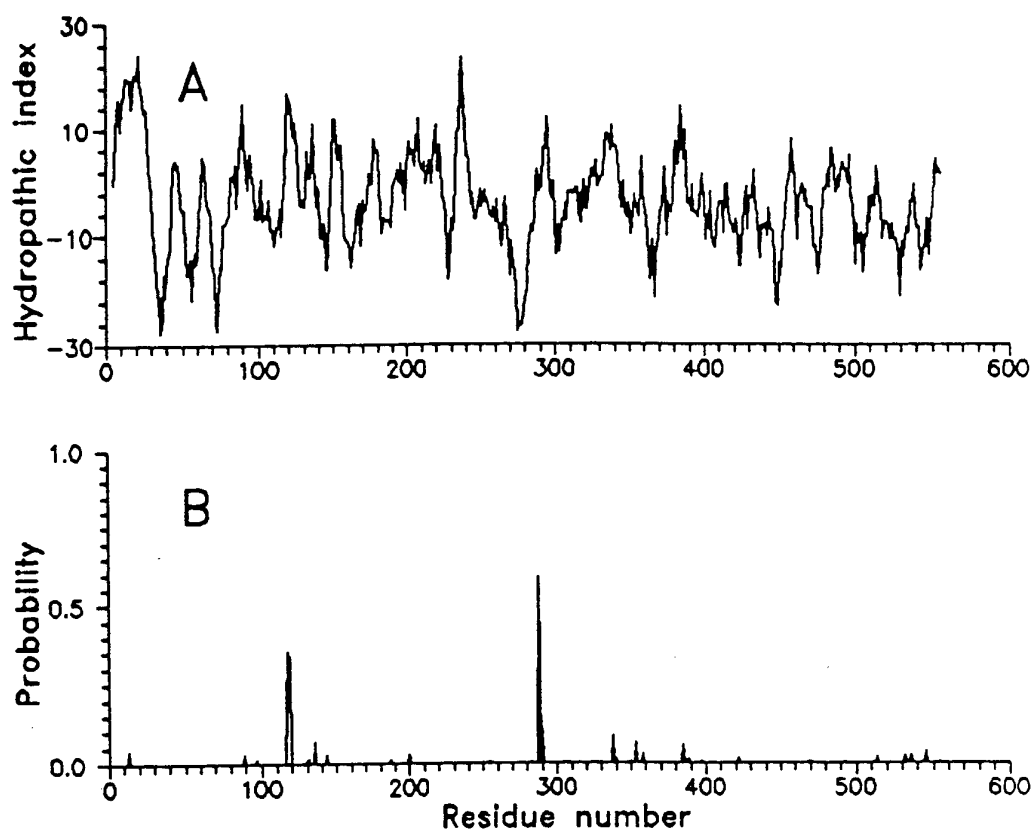
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FIGURE 3D

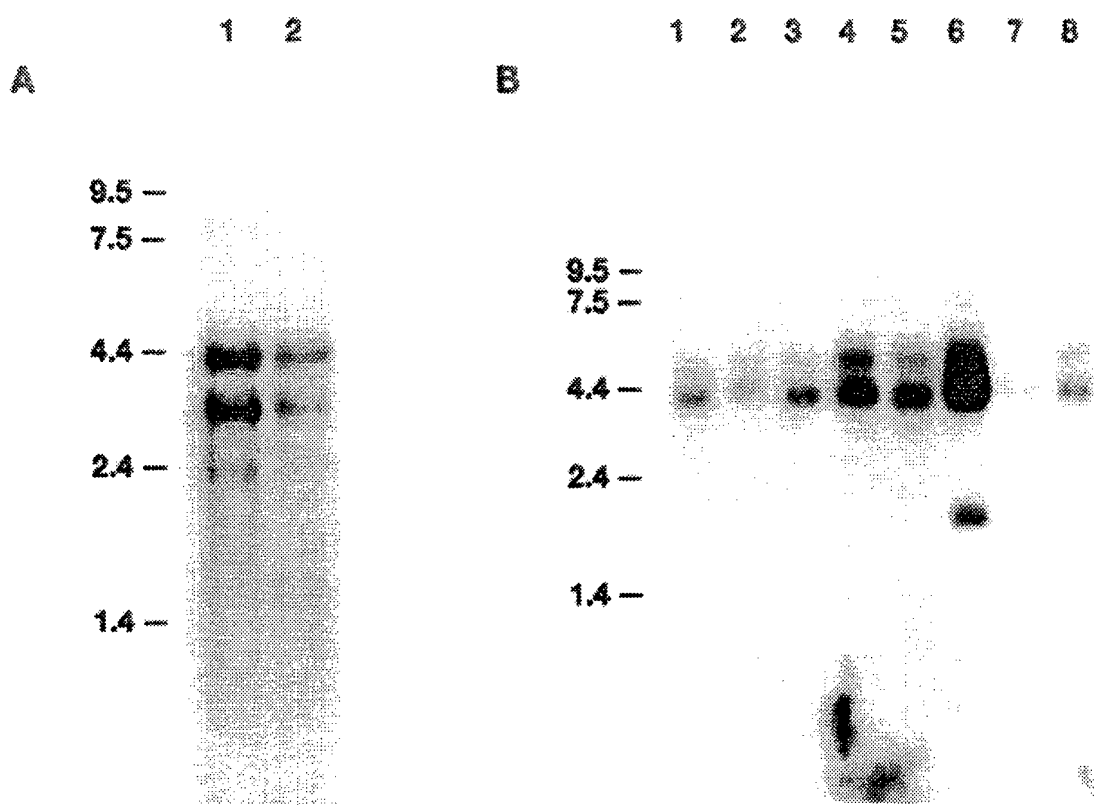
GTG TTT CGG AAA GCT ACA CCC TAC ACG TTT CCA GGA GGC ACG GGG CAG ATT	1135
Val Phe Arg Lys Ala Thr Pro Tyr Thr Phe Pro Gly Gly Thr Gly Gln Ile	361
AAT TTC TTC TAT ATA ATT TCT CCA GGT GTT ACA AAG GTA GAT TAT GGA GAT	1237
Asn Phe Phe Tyr Ile Ile Ser Pro Gly Val Thr Lys Val Asp Tyr Gly Asp	395
TGG TAC CTA GAG AAT ATT TAT CCT GAT TCT CAG ATT CCT CGT CAC TAT TTC	1339
Trp Tyr Leu Glu Asn Ile Tyr Pro Asp Ser Gln Ile Pro Arg His Tyr Phe	429
GCT AGA AAA GAG AAT GAA AAA GTT GGA ATT TTT AAC TGT CAT GGT ATG GGA	1441
Ala Arg Lys Glu Asn Glu Lys Val Gly Ile Phe Asn Cys His Gly Met Gly	463
CTT TGC TTG GAT GTC TCC AAA CTT AAT GGC CCA GTC ACA ATG CTC AAA TGC	1543
Leu Cys Leu Asp Val Ser Lys Leu Asn Gly Pro Val Thr Met Leu Lys Cys	497
CTG CAG CAT GTG AAC AGT AAC CAG TGC CTG GAC AAA GCC ACA GAC GAG GAC	1645
Leu Gln His Val Asn Ser Asn Gln Cys Leu Asp Lys Ala Thr Asp Glu Asp	531
CTT CTT CGG <u>AAC</u> <u>GTC</u> <u>ACC</u> CTT CCA GAA ATA TTC TGA GACCAAATTTACAAAAAAA	1751
Leu Leu Arg <u>Asn</u> <u>Val</u> <u>Thr</u> Leu Pro Glu Ile Phe End	559
GGAAAAGTGCTTTCCTTCTGCAGGATGTAAGGTTTATCAGCCATTAAAACTTTATAGACTGCCCTCG	1886
ACTGATGTTTACAAGATTGAAAGAGTCGGTCATCAAGAATCCTCGTAAAGAATACTCAGACTGAAAC	2021
TGACTGTGCTGTCATAATGAAGAGACGTCTAAGATTTTTTTTCTGATTAGAACTGGTAGCCAGTATA	2156
AAAAAAAAACAAAACCTATATTAAACAGGGTTTAAAGGAATTAAACAAAAAAAAAAAAAAAAAAAAA	2294

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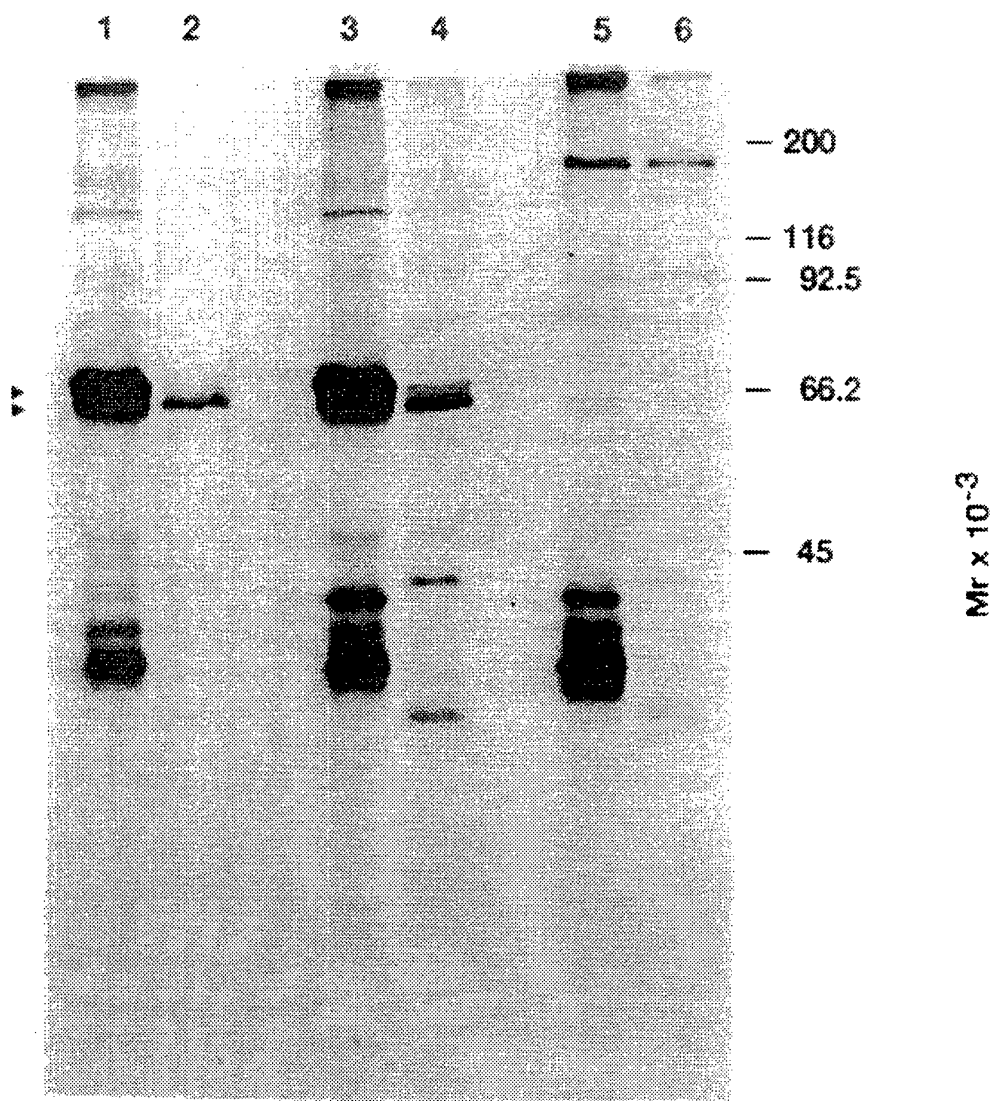
FIGURE 4



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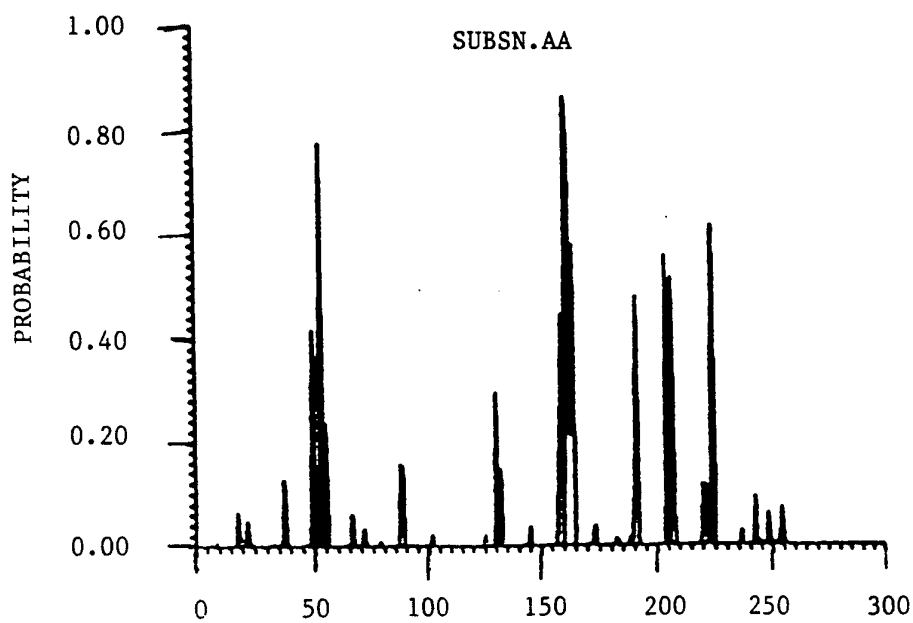
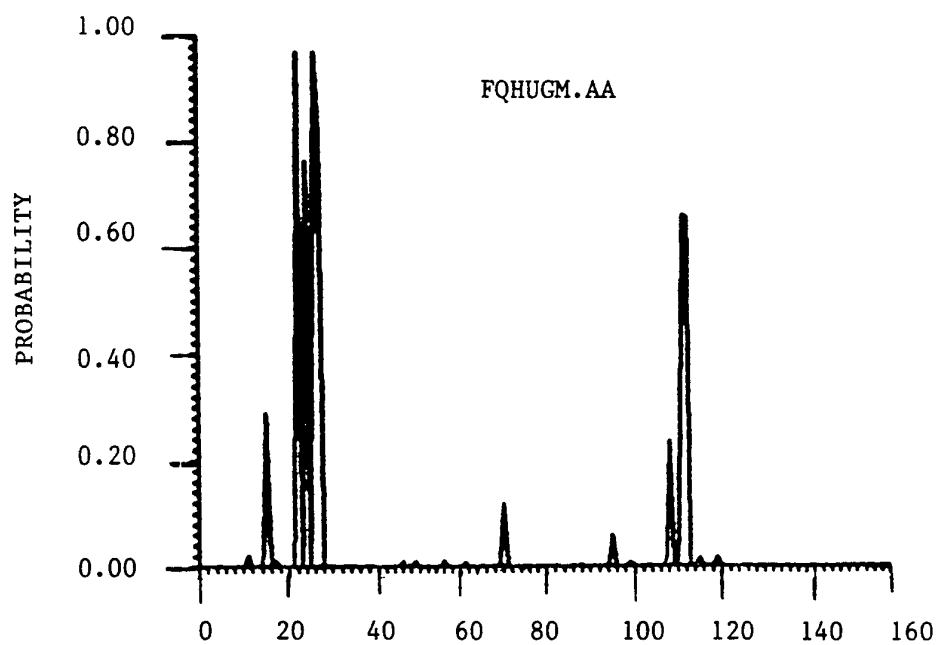
Fig. 5

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Fig. 6

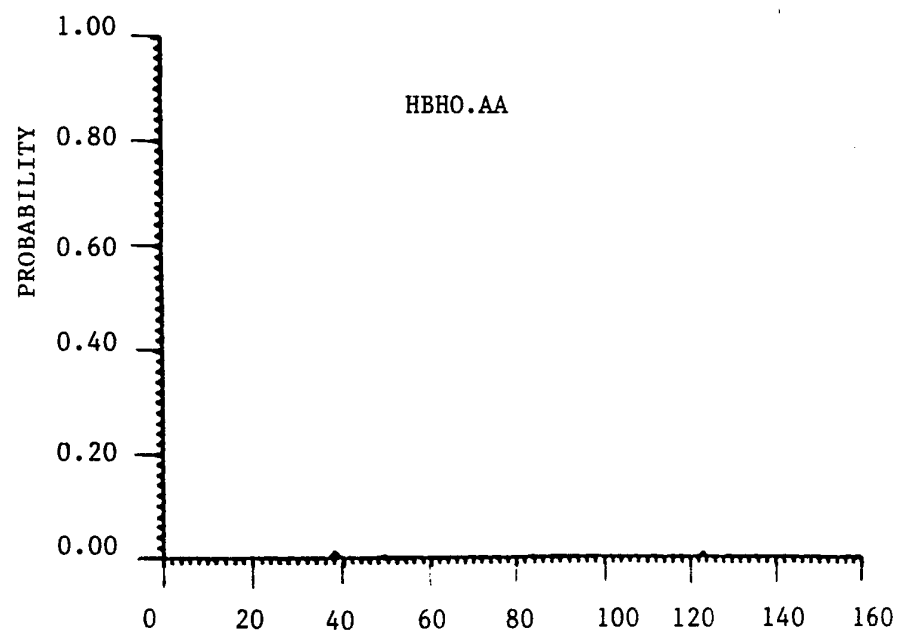
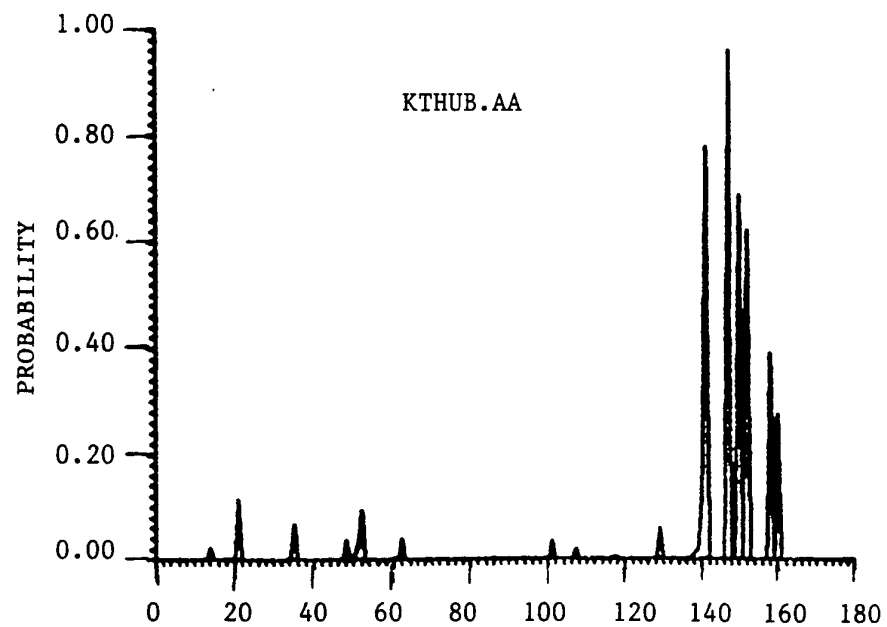
11/25

FIGURE 7A



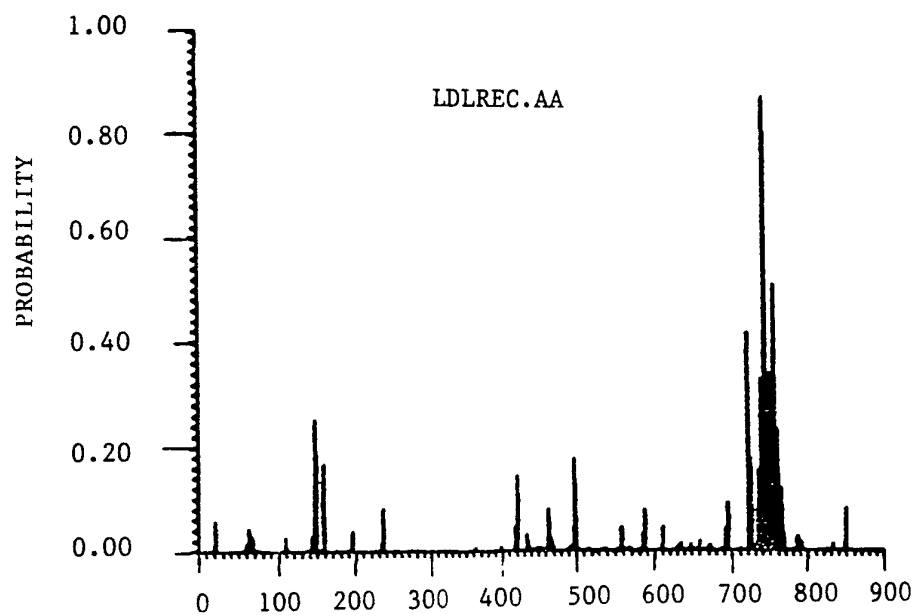
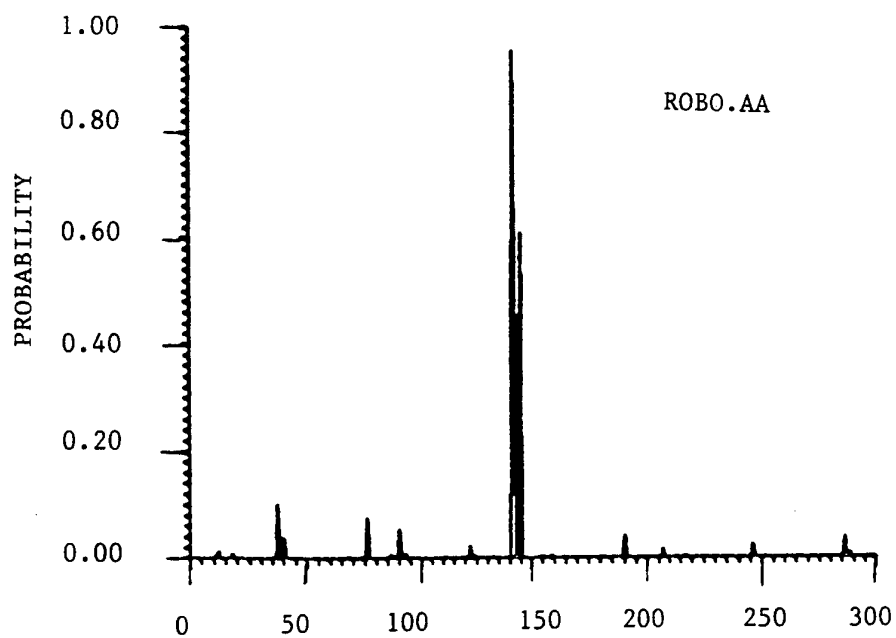
SUBSTITUTE SHEET (RULE 26)

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FIGURE 7B



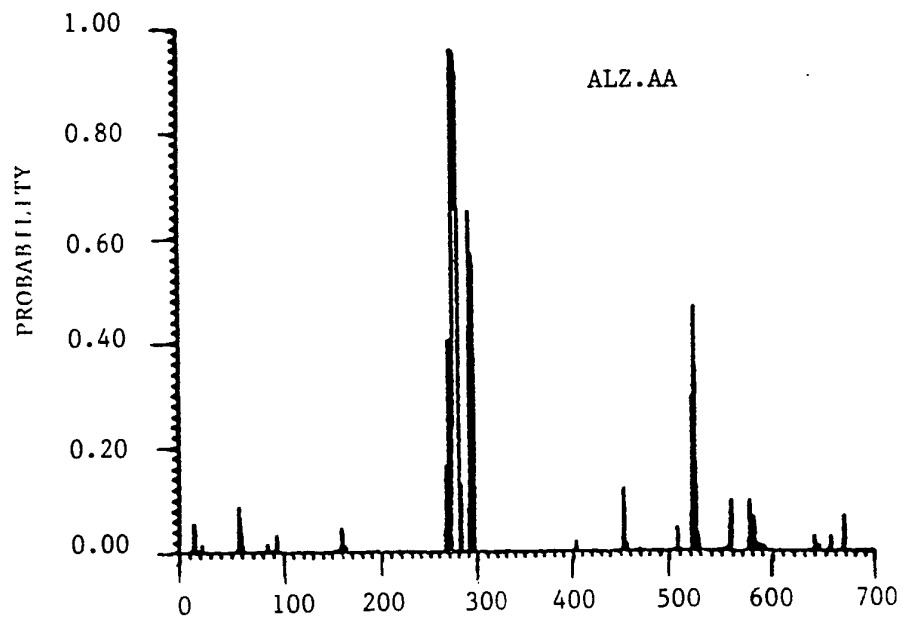
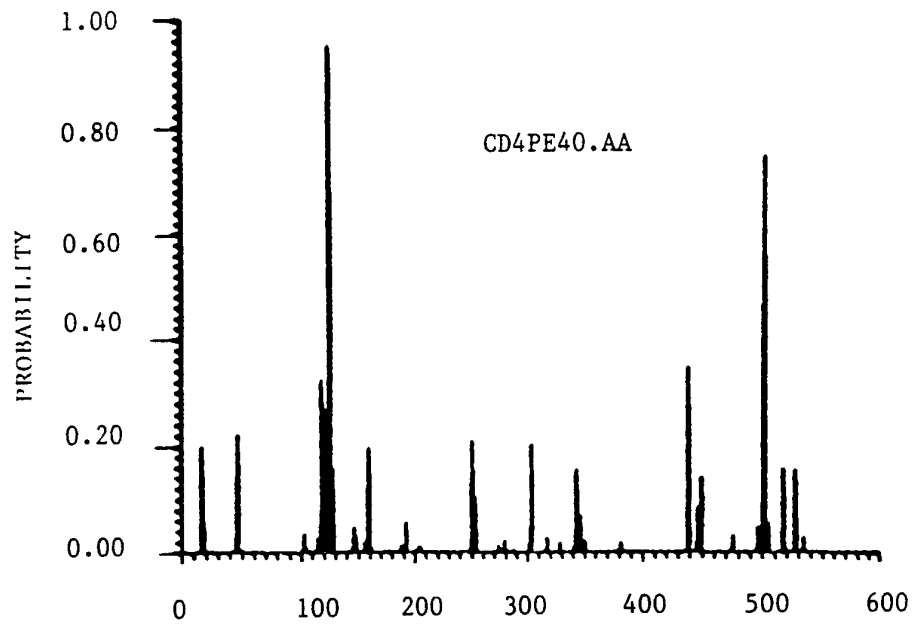
13/25

FIGURE 8A



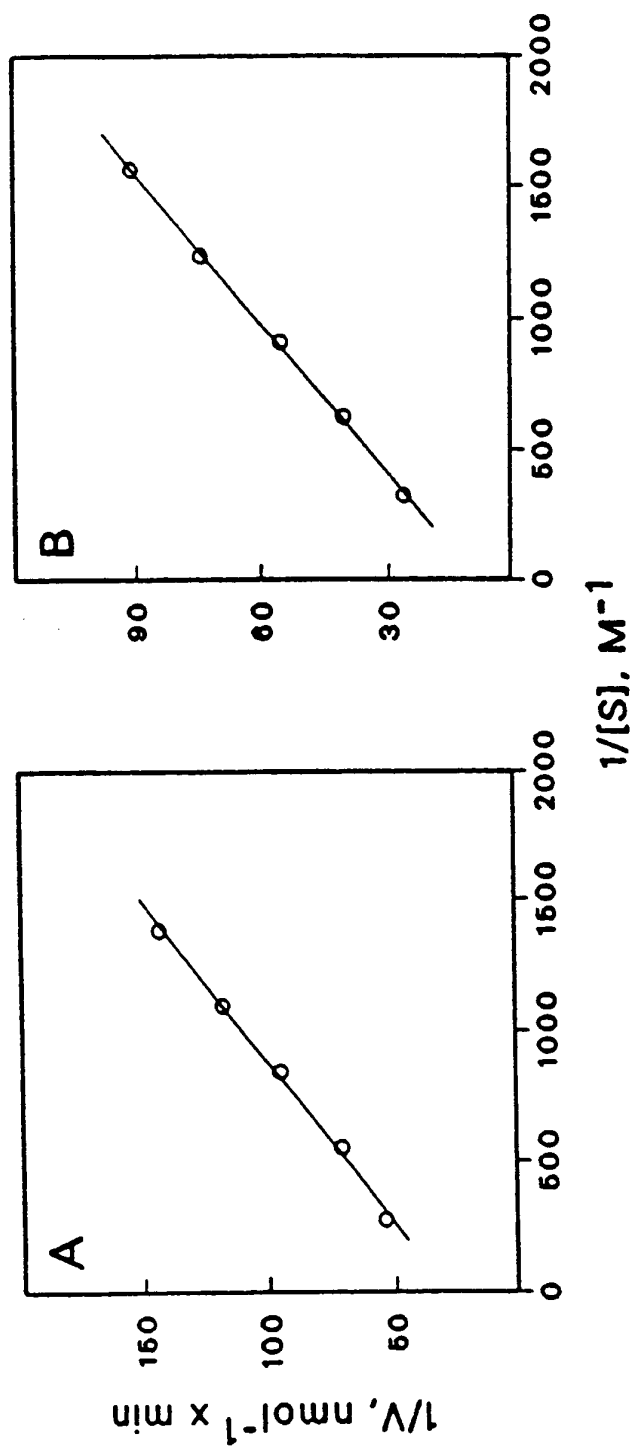
14/25

FIGURE 8B



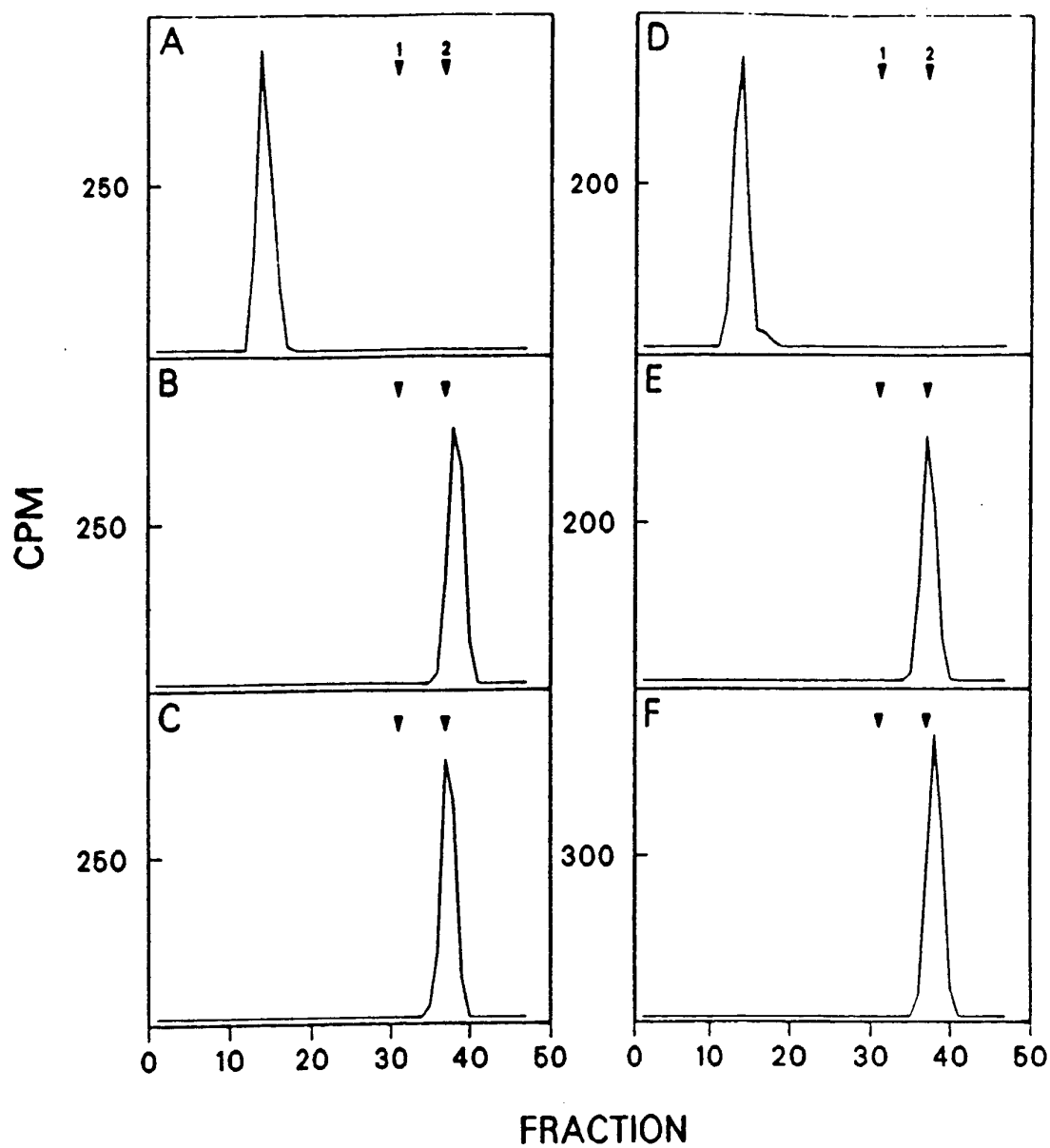
15/25

FIGURE 9

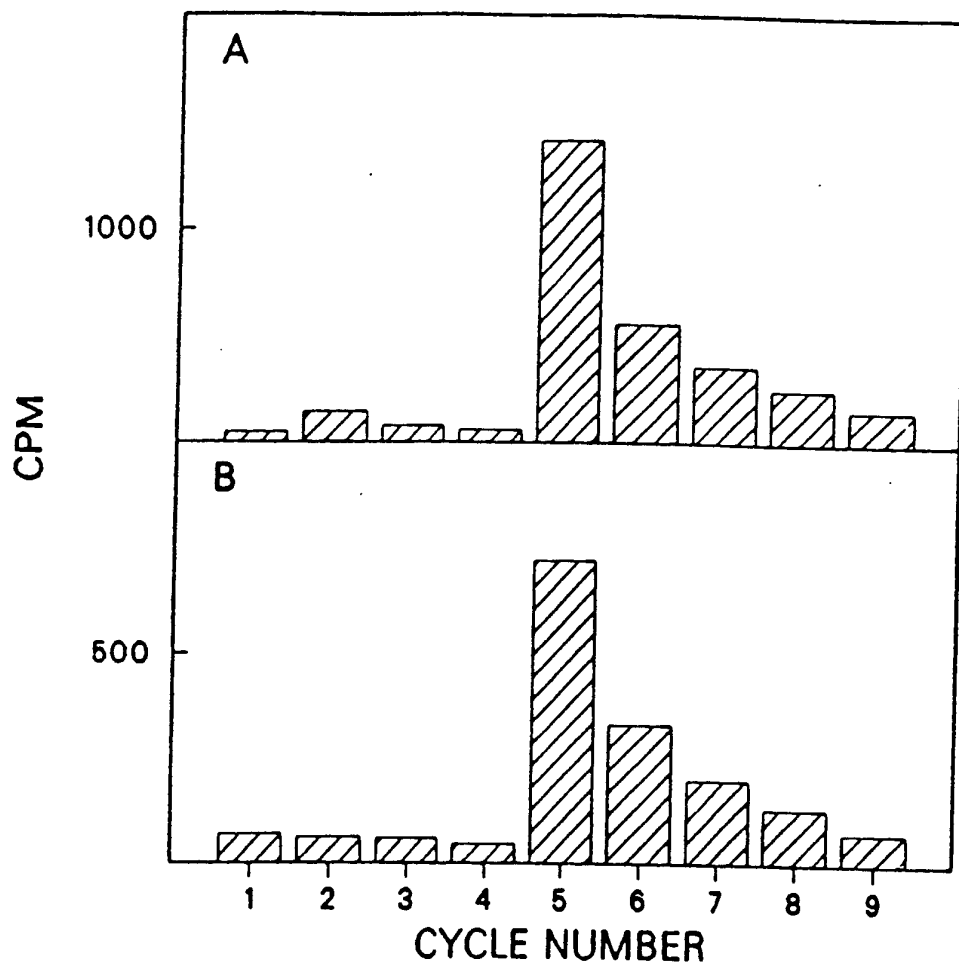


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FIGURE 10

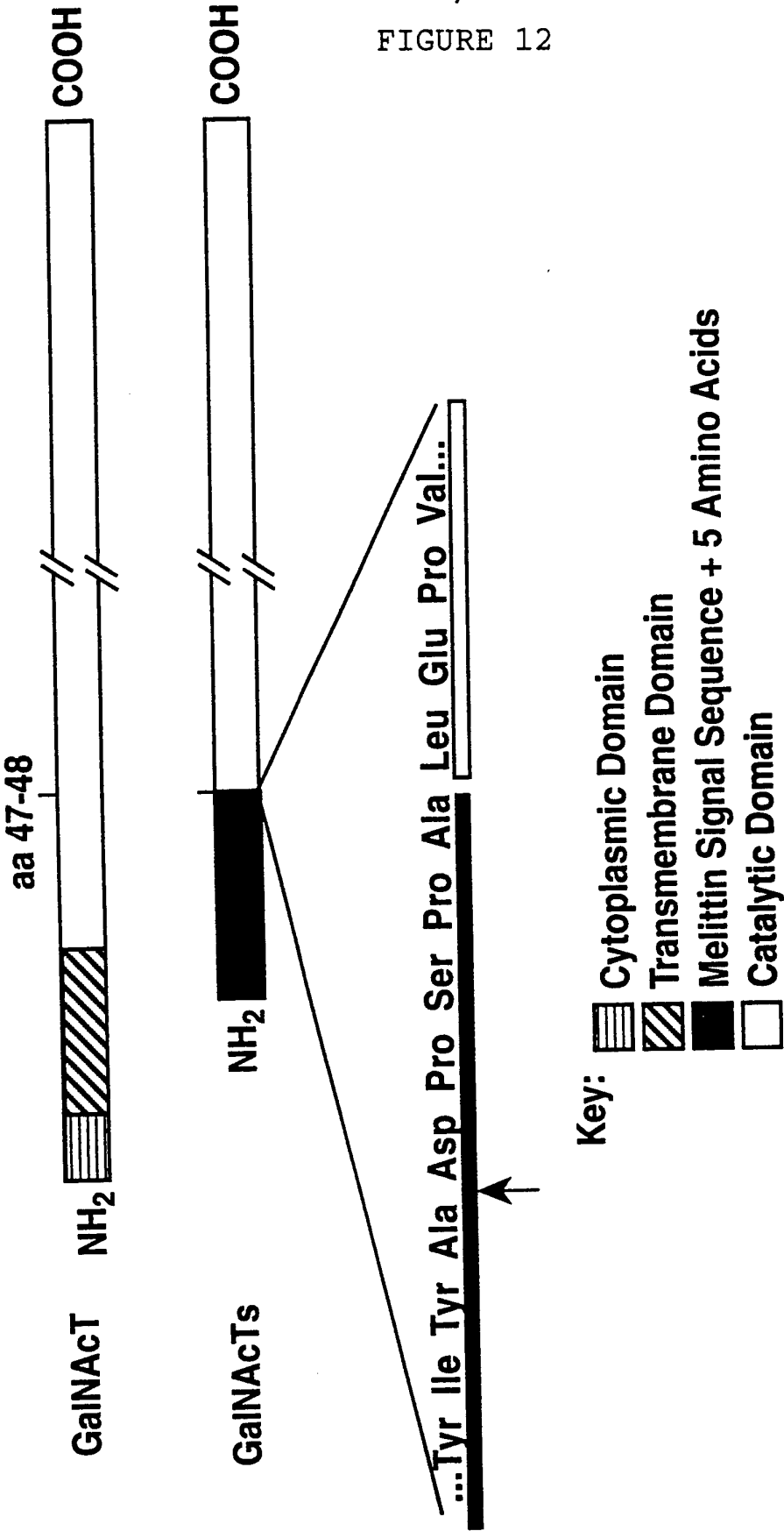


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FIGURE 11



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FIGURE 12



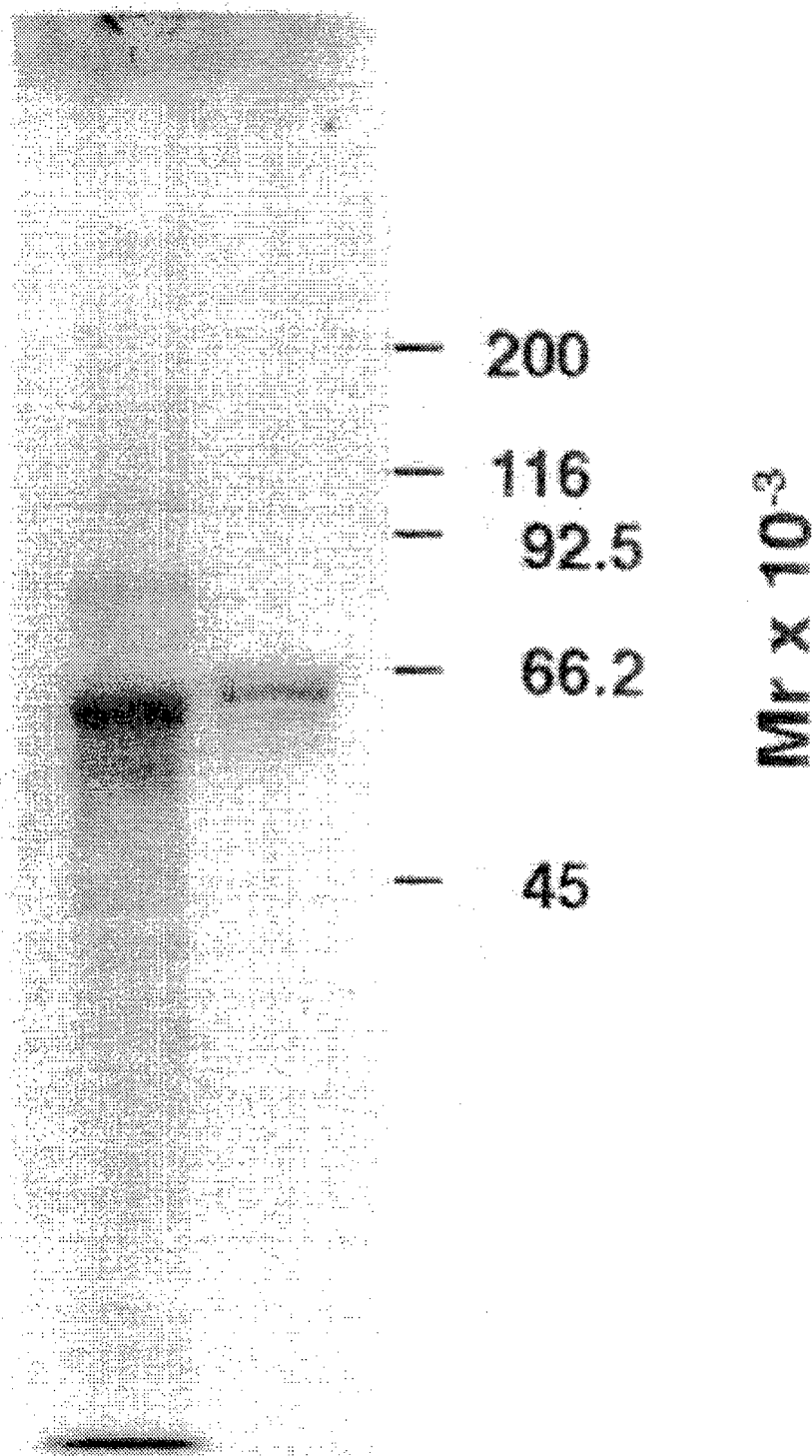
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FIGURE 13

(SEQUENCE ID NO:18:)

ATGAAATTCT	TAGTCAACGT	TGCCCTTGTT	TTTATGGTCG	TGTACATTTC	TTACATCTAT	60
GCGGATCCAA	GCCCTGCTCT	AGAGCCAGTA	CAAAAGCCTC	ATGAAGGTCC	TGGAGAAATG	120
GGGAAACCAG	TCGTCAATTCC	TAAAGAGGAT	CAAGAAAAGA	TGAAAGAGAT	GTTTAAAATC	180
AATCAGTTCA	ATTTAATGGC	AAGTGAGATG	ATTGCACTCA	ACAGATCTCT	ACCAGATGTT	240
AGATTAGAAG	GGTGTA AAC	AAAGGTGTAT	CCAGATAACC	TTCCTACAAC	CAGTGTGGTG	300
ATTGTTTTCC	ACAAATGAGC	TTGGAGCACA	CTTCTGCGAA	CTGTCCATAG	CGTCATTAAT	360
CGCTCACCAA	GGCACATGCT	AGAAGAAATT	GTTCTAGTAG	ATGATGCCAG	TGAAAGAGAC	420
TTTTTAAAAA	GACCTCTAGA	GAGTTACGTG	AAAAAATTAA	AAGTACCCGT	TCACGTCATT	480
CGAATGGAGC	AGCGTCTGG	ATTGATCAGA	GCTAGGTTAA	AAGGTGCTGC	TGTGTCTAAA	540
GGCCAAGTGA	TCACCTTTTT	AGACGCGCAC	TGTGAGTGCA	CAGTGGGGTG	GCTGGAGCCT	600
CTCTTAGCCA	GGATCAAACA	TGACAGGAAG	ACAGTGGTCT	GTCCCATCAT	AGATGTGATC	660
AGTGATGACA	CTTTCGAGTA	CATGGCAGGT	TCTGACATGA	CCTATGGCGG	GTTCAACTGG	720
AAGCTCAACT	TTCTGCTGTA	TCCTGTTCCC	CAAAGAGAAA	TGGACAGAAG	GAAAGGTGAT	780
CGGACTCTTC	CTGTGAGAAC	ACCTACAATG	GCAGGAGGCC	TTTTTTCAAT	AGACAGAGAT	840
TACTTTTCAGG	AAATTGGAAC	ATATGATGCT	GGAATGGATA	TTTGGGGAGG	AGAAAACCTA	900
GAAATTTTCT	TTAGGATTTG	GCAGTGTGGA	GGAACTTTGG	AGATTGTTAC	TTGCTCACAT	960
GTTGGACATG	TGTTTCGGAA	AGCTACACCC	TACACGTTTC	CAGGAGGCAC	GGGGCAGATT	1020
ATCAATAAAA	ATAACAGACG	ACTTGCAGAA	GTATGGATGG	ATGAATTCAA	GAATTTCTTC	1080
TATATAATTT	CTCCAGGTGT	TACAAAGGTA	GATTATGGAG	ATATATCATC	AAGACTTGGT	1140
CTAAGGCACA	AACTCCAATG	CAGACCATTG	TCTTGGTACC	TAGAGAATAT	TTATCCTGAT	1200
TCTCAGATTG	CTCGTCACTA	TTTCTCTTTG	GGAGAGATAC	GAAATGTGGA	AACAAATCAG	1260
TGTCTAGATA	ACATGGCTAG	AAAAGAGAAT	GAAAAAGTTG	GAATTTTAA	CTGTCATGGT	1320
ATGGGAGGTA	ATCAGGTTTT	CTCTTACACT	GCCAACAAAG	AAATTAGAAC	AGATGACCTT	1380
TGCTTGGATG	TCTCCAAACT	TAATGGCCCA	GTCACAATGC	TCAAATGCCA	CCACCTAAAA	1440
GGCAACCAGC	TTTGGGAGTA	TGACCCGGTG	AAGTTGACCC	TGCAGCATGT	GAACAGTAAC	1500
CAGTGCCTGG	ACAAAGCCAC	AGACGAGGAC	AGCCAGGTGC	CCAGCATCAG	AGACTGCAGC	1560
GGAAGCCGAT	CCCAGCAGTG	GCTTCTTCGG	AACGTCACCC	TTCCAGAAAT	ATTCTGA	1617

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Fig. 14

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FIGURE 15

(SEQ ID NO:11)

ATGAGAAAAT TTGCATACTG CAAGGTGGTC CTAGCCACCT CCTTGATTG GGTACTCTTG	60
GATATGTTCC TGCTGCTTAA CTTCAAGTGA TGCAACAAAT GTGATGAAAA AAAAGAGAGA	120
GGACTTCCTG CTGGGGATGT TCTAGAGCCA GTACAAAAGC CTCATGAAGG TCCTGGAGAA	180
ATGGGGAAAC CAGTCGTCAT TCCTAAAGAG GATCAAGAAA AGATGAAAGA GATGTTTAAA	240
ATCAATCAGT TCAATTTAAT GGCAAGTGAG ATGATTGCAC TCAACAGATC TCTACCAGAT	300
GTTAGATTAG AAGGGTGTA AACAAAGGTG TATCCAGATA ACCTTCCTAC AACCAGTGTG	360
GTGATTGTTT TCCACAATGA GGCTTGAGC ACACTTCTGC GAACTGTCCA TAGCGTCATT	420
AATCGCTCAC CAAGGCACAT GCTAGAAGAA ATTGTTCTAG TAGATGATGC CAGTGAAAGA	480
GACTTTTAA AAAGACCTCT AGAGAGTTAC GTGAAAAAAT TAAAAGTACC CGTTCACGTC	540
ATTCGAATGG AGCAGCGTTC TGGATTGATC AGAGCTAGGT TAAAAGGTGC TGCTGTGTCT	600
AAAGGCCAAG TGATCACCTT TTTAGACGCG CACTGTGAGT GCACAGTGGG GTGGCTGGAG	660
CCTCTCTTAG CCAGGATCAA ACATGACAGG AAGACAGTGG TCTGTCCCAT CATAGATGTG	720
ATCAGTGATG ACACTTTCGA GTACATGGCA GGTCTTGACA TGACCTATGG CGGGTTCAAC	780
TGGAAGCTCA ACTTTCGCTG GTATCCTGTT CCCCAGAG AAATGGACAG AAGGAAAGGT	840
GATCGGACTC TTCCTGTGAG AACACCTACA ATGGCAGGAG GCCTTTTTTC AATAGACAGA	900
GATTACTTTC AGGAAATTGG AACATATGAT GCTGGAATGG ATATTTGGGG AGGAGAAAAC	960
CTAGAAATTT CCTTTAGGAT TTGGCAGTGT GGAGGAAC TTGGAGATTGT TACTTGCTCA	1020
CATGTTGGAC ATGTGTTTCG GAAAGCTACA CCCTACACGT TTCCAGGAGG CACGGGGCAG	1080
ATTATCAATA AAAATAACAG ACGACTTGCA GAAGTATGGA TGGATGAATT CAAGAATTTT	1140
TTCTATATAA TTTCTCCAGG TGTTACAAAG GTAGATTATG GAGATATATC ATCAAGACTT	1200
GGTCTAAGGC ACAAACCTCCA ATGCAGACCA TTCTCTTGGT ACCTAGAGAA TATTTATCCT	1260
GATTCTCAGA TTCCTCGTCA CTATTTCTCT TTGGGAGAGA TACGAAATGT GGAAACAAAT	1320
CAGTGTCTAG ATAACATGGC TAGAAAAGAG AATGAAAAAG TTGGAATTTT TAACTGTCAT	1380
GGTATGGGAG GTAATCAGGT TTTCTCTTAC ACTGCCAACA AAGAAATTAG AACAGATGAC	1440
CTTTGCTTGG ATGTCTCCAA ACTTAATGGC CCAGTCACAA TGCTCAAATG CCACCACCTA	1500
AAAGGCAACC AGCTTTGGGA GTATGACCCG GTGAAGTTGA CCCTGCAGCA TGTGAACAGT	1560
AACCAGTGCC TGGACAAAGC CACAGACGAG GACAGCCAGG TGCCCAGCAT CAGAGACTGC	1620
AGCGGAAGCC GATCCCAGCA GTGGCTTCTT CGGAACGTCA CCCTTCCAGA AATATTCTGA	1680

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FIGURE 16

(SEQ ID NO:9)

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Met Arg Lys Phe Ala Tyr Cys Lys Val Val Leu Ala Thr Ser Leu Ile
1          5          10          15

Trp Val Leu Leu Asp Met Phe Leu Leu Leu Tyr Phe Ser Glu Cys Asn
          20          25          30

Lys Cys Asp Glu Lys Lys Glu Arg Gly Leu Pro Ala Gly Asp Val Leu
          35          40          45

Glu Pro Val Gln Lys Pro His Glu Gly Pro Gly Glu Met Gly Lys Pro
          50          55          60

Val Val Ile Pro Lys Glu Asp Gln Glu Lys Met Lys Glu Met Phe Lys
65          70          75          80

Ile Asn Gln Phe Asn Leu Met Ala Ser Glu Met Ile Ala Leu Asn Arg
          85          90          95

Ser Leu Pro Asp Val Arg Leu Glu Gly Cys Lys Thr Lys Val Tyr Pro
          100          105          110

Asp Asn Leu Pro Thr Thr Ser Val Val Ile Val Phe His Asn Glu Ala
          115          120          125

Trp Ser Thr Leu Leu Arg Thr Val His Ser Val Ile Asn Arg Ser Pro
          130          135          140

Arg His Met Leu Glu Glu Ile Val Leu Val Asp Asp Ala Ser Glu Arg
145          150          155          160

Asp Phe Leu Lys Arg Pro Leu Glu Ser Tyr Val Lys Lys Leu Lys Val
          165          170          175

Pro Val His Val Ile Arg Met Glu Gln Arg Ser Gly Leu Ile Arg Ala
          180          185          190

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

Asp Ala His Cys Glu Cys Thr Val Gly Trp Leu Glu Pro Leu Leu Ala
210          215          220

Arg Ile Lys His Asp Arg Lys Thr Val Val Cys Pro Ile Ile Asp Val
225          230          235          240

Ile Ser Asp Asp Thr Phe Glu Tyr Met Ala Gly Ser Asp Met Thr Tyr
          245          250          255

Gly Gly Phe Asn Trp Lys Leu Asn Phe Arg Trp Tyr Pro Val Pro Gln
          260          265          270

Arg Glu Met Asp Arg Arg Lys Gly Asp Arg Thr Leu Pro Val Arg Thr
          275          280          285

Pro Thr Met Ala Gly Gly Leu Phe Ser Ile Asp Arg Asp Tyr Phe Gln
          290          295          300

Glu Ile Gly Thr Tyr Asp Ala Gly Met Asp Ile Trp Gly Gly Glu Asn
305          310          315          320

Leu Glu Ile Ser Phe Arg Ile Trp Gln Cys Gly Gly Thr Leu Glu Ile
          325          330          335

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FIGURE 16 (CONT'D.)

Val	Thr	Cys	Ser	His	Val	Gly	His	Val	Phe	Arg	Lys	Ala	Thr	Pro	Tyr	340	345	350	
Thr	Phe	Pro	Gly	Gly	Thr	Gly	Gln	Ile	Ile	Asn	Lys	Asn	Asn	Arg	Arg	355	360	365	
Leu	Ala	Glu	Val	Trp	Met	Asp	Glu	Phe	Lys	Asn	Phe	Phe	Tyr	Ile	Ile	370	375	380	
Ser	Pro	Gly	Val	Thr	Lys	Val	Asp	Tyr	Gly	Asp	Ile	Ser	Ser	Arg	Leu	385	390	395	400
Gly	Leu	Arg	His	Lys	Leu	Gln	Cys	Arg	Pro	Phe	Ser	Trp	Tyr	Leu	Glu	405	410	415	
Asn	Ile	Tyr	Pro	Asp	Ser	Gln	Ile	Pro	Arg	His	Tyr	Phe	Ser	Leu	Gly	420	425	430	
Glu	Ile	Arg	Asn	Val	Glu	Thr	Asn	Gln	Cys	Leu	Asp	Asn	Met	Ala	Arg	435	440	445	
Lys	Glu	Asn	Glu	Lys	Val	Gly	Ile	Phe	Asn	Cys	His	Gly	Met	Gly	Gly	450	455	460	
Asn	Gln	Val	Phe	Ser	Tyr	Thr	Ala	Asn	Lys	Glu	Ile	Arg	Thr	Asp	Asp	465	470	475	480
Leu	Cys	Leu	Asp	Val	Ser	Lys	Leu	Asn	Gly	Pro	Val	Thr	Met	Leu	Lys	485	490	495	
Cys	His	His	Leu	Lys	Gly	Asn	Gln	Leu	Trp	Glu	Tyr	Asp	Pro	Val	Lys	500	505	510	
Leu	Thr	Leu	Gln	His	Val	Asn	Ser	Asn	Gln	Cys	Leu	Asp	Lys	Ala	Thr	515	520	525	
Asp	Glu	Asp	Ser	Gln	Val	Pro	Ser	Ile	Arg	Asp	Cys	Ser	Gly	Ser	Arg	530	535	540	
Ser	Gln	Gln	Trp	Leu	Leu	Arg	Asn	Val	Thr	Leu	Pro	Glu	Ile	Phe		545	550	555	

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FIGURE 17

(SEQ ID NO:19)

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Asp Pro Ser Pro Ala Leu Glu Pro Val Gln Lys Pro His Glu Gly Pro
1      5      10      15
Gly Glu Met Gly Lys Pro Val Val Ile Pro Lys Glu Asp Gln Glu Lys
20      25      30
Met Lys Glu Met Phe Lys Ile Asn Gln Phe Asn Leu Met Ala Ser Glu
35      40      45
Met Ile Ala Leu Asn Arg Ser Leu Pro Asp Val Arg Leu Glu Gly Cys
50      55      60
Lys Thr Lys Val Tyr Pro Asp Asn Leu Pro Thr Thr Ser Val Val Ile
65      70      75      80
Val Phe His Asn Glu Ala Trp Ser Thr Leu Leu Arg Thr Val His Ser
85      90      95
Val Ile Asn Arg Ser Pro Arg His Met Leu Glu Glu Ile Val Leu Val
100     105     110
Asp Asp Ala Ser Glu Arg Asp Phe Leu Lys Arg Pro Leu Glu Ser Tyr
115     120     125
Val Lys Lys Leu Lys Val Pro Val His Val Ile Arg Met Glu Gln Arg
130     135     140
Ser Gly Leu Ile Arg Ala Arg Leu Lys Gly Ala Ala Val Ser Lys Gly
145     150     155     160
Gln Val Ile Thr Phe Leu Asp Ala His Cys Glu Cys Thr Val Gly Trp
165     170     175
Leu Glu Pro Leu Leu Ala Arg Ile Lys His Asp Arg Lys Thr Val Val
180     185     190
Cys Pro Ile Ile Asp Val Ile Ser Asp Asp Thr Phe Glu Tyr Met Ala
195     200     205
Gly Ser Asp Met Thr Tyr Gly Gly Phe Asn Trp Lys Leu Asn Phe Arg
210     215     220
Trp Tyr Pro Val Pro Gln Arg Glu Met Asp Arg Arg Lys Gly Asp Arg
225     230     235     240
Thr Leu Pro Val Arg Thr Pro Thr Met Ala Gly Gly Leu Phe Ser Ile
245     250     255
Asp Arg Asp Tyr Phe Gln Glu Ile Gly Thr Tyr Asp Ala Gly Met Asp
260     265     270
Ile Trp Gly Gly Glu Asn Leu Glu Ile Ser Phe Arg Ile Trp Gln Cys
275     280     285
Gly Gly Thr Leu Glu Ile Val Thr Cys Ser His Val Gly His Val Phe
290     295     300
Arg Lys Ala Thr Pro Tyr Thr Phe Pro Gly Gly Thr Gly Gln Ile Ile
305     310     315     320
Asn Lys Asn Asn Arg Arg Leu Ala Glu Val Trp Met Asp Glu Phe Lys
325     330     335
Asn Phe Phe Tyr Ile Ile Ser Pro Gly Val Thr Lys Val Asp Tyr Gly
340     345     350

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FIGURE 17 (CONT'D.)

Asp	Ile	Ser	Ser	Arg	Leu	Gly	Leu	Arg	His	Lys	Leu	Gln	Cys	Arg	Pro	355	360	365	
Phe	Ser	Trp	Tyr	Leu	Glu	Asn	Ile	Tyr	Pro	Asp	Ser	Gln	Ile	Pro	Arg	370	375	380	
His	Tyr	Phe	Ser	Leu	Gly	Glu	Ile	Arg	Asn	Val	Glu	Thr	Asn	Gln	Cys	385	390	395	400
Leu	Asp	Asn	Met	Ala	Arg	Lys	Glu	Asn	Glu	Lys	Val	Gly	Ile	Phe	Asn	405	410	415	
Cys	His	Gly	Met	Gly	Gly	Asn	Gln	Val	Phe	Ser	Tyr	Thr	Ala	Asn	Lys	420	425	430	
Glu	Ile	Arg	Thr	Asp	Asp	Leu	Cys	Leu	Asp	Val	Ser	Lys	Leu	Asn	Gly	435	440	445	
Pro	Val	Thr	Met	Leu	Lys	Cys	His	His	Leu	Lys	Gly	Asn	Gln	Leu	Trp	450	455	460	
Glu	Tyr	Asp	Pro	Val	Lys	Leu	Thr	Leu	Gln	His	Val	Asn	Ser	Asn	Gln	465	470	475	480
Cys	Leu	Asp	Lys	Ala	Thr	Asp	Glu	Asp	Ser	Gln	Val	Pro	Ser	Ile	Arg	485	490	495	
Asp	Cys	Ser	Gly	Ser	Arg	Ser	Gln	Gln	Trp	Leu	Leu	Arg	Asn	Val	Thr	500	505	510	
Leu	Pro	Glu	Ile	Phe												515			