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(54) Title: N-ACETYLGLUCOSAMINYLTRANSFERASE V CODING SEQUENCES

(57) **Abrégé/Abstract:**

The present invention provides a substantially purified UDP-N-acetylglucosamine:α-6-D-mannoside β-1,6-N-acetylglucosaminyltransferase (GlcNAc T-V; EC 2:4.1.155) and antibodies which specifically bind GlcNAc T-V. The present invention also provides polynucleotide sequences and oligonucleotide probes capable of specifically hybridizing to nucleic acid sequences which encode GlcNAc T-V, and cDNA and genomic clones encoding GlcNAc T-V, as well as nucleotide sequences encoding GlcNAc T-V, as specifically exemplified by a GlcNAc T-V coding sequence from rat.



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(21) International Application Number: PCT/US93/06155 (22) International Filing Date: 29 June 1993 (29.06.93) (30) Priority data: 07/905,795 29 June 1992 (29.06.92) US 08/016,863 10 February 1993 (10.02.93) US (71) Applicants (for all designated States except US): UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; Boyd Graduate Studies Research Center, DW Brooks Drive, University of Georgia, Athens, GA 30602 (US). AMGEN, INC. [US/US]; 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US). (72) Inventors; and (75) Inventors/Applicants (for US only) : PIERCE, J., Michael [US/US]; 325 Snapfinger Drive, Athens, GA 30605 (US). SHOREIBAH, Mohamed, G. [US/US]; Route 2, Box 193, Comer, GA 30629 (US). ADLER, Beverly, S. [US/US]; 206 Anne Court, Newbury Park, CA 91320 (US). FREGIEN, Nevis, L. [US/US]; 1314 Southwest 18th Street, Miami, FL 33145 (US).		(74) Agents: GREENLEE, Lorraine, L. et al.; Greenlee and Winner, 5370 Manhattan Circle, Suite 201, Boulder, CO 80303 (US). (81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: N-ACETYLGLUCOSAMINYLTRANSFERASE V CODING SEQUENCES (57) Abstract The present invention provides a substantially purified UDP-N-acetylglucosamine:α-6-D-mannoside β-1,6-N-acetylglucosaminyltransferase (GlcNAc T-V; EC 2.4.1.155) and antibodies which specifically bind GlcNAc T-V. The present invention also provides polynucleotide sequences and oligonucleotide probes capable of specifically hybridizing to nucleic acid sequences which encode GlcNAc T-V, and cDNA and genomic clones encoding GlcNAc T-V, as well as nucleotide sequences encoding GlcNAc T-V, as specifically exemplified by a GlcNAc T-V coding sequence from rat.		

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N-ACETYLGLUCOSAMINYLTRANSFERASE V CODING SEQUENCES

Field of the Invention

The field of this invention is the area of protein glycosylation, specifically the area of the particular enzyme, UDP N-acetylglucosaminyltransferase V, involved in the expression of the $\beta(1,6)$ branch structure found in tri- and tetraantennary N-linked oligosaccharides. The field relates to purified active enzyme, the amino acid sequence of the rat enzyme protein, genes encoding active enzyme and cell lines genetically engineered to express a nucleotide sequence encoding active enzyme.

Background of the Invention

UDP-N-acetylglucosamine: α -6-D-mannoside β -1,6-N-acetylglucosaminyltransferase V (GlcNAc T-V; EC 2.4.1.155) is the Golgi enzyme responsible for the synthesis of the $\beta(1,6)$ branch structure of tri- and tetraantennary N-linked oligosaccharides. For brevity, this enzyme is abbreviated GlcNAc T-V herein. GlcNAc T-V activity has been found in many mammalian tissues and cell types.

Altered glycosylation of membrane glycoproteins and glycolipids is observed in mammalian cells transformed with diverse tumor viruses, carcinogens, or transfection with certain oncogenes. In some cases, there is a quantitative increase in a particular substituent, e.g., sialylation. In other instances, there is the reappearance of an oligosaccharide structure in the tumor which is normally only found in fetal tissue; for instance, certain Lewis histo-blood group antigens have been detected in adenocarcinomas.

Qualitative differences in oligosaccharides may also be observed in certain transformed cells. BHK fibroblasts transformed with polyoma virus or with Rous sarcoma virus display more highly branched complex N-linked oligosaccharides than do the corresponding normal cells. The expression of the β -1,6 branch structure ($-[\text{GlcNAc}-\beta(1,6)\text{Man}-\alpha(1,6)\text{Man}]$ -) found in tri- and tetraantennary N-linked oligosaccharides is increased in the transformed cells. This has been correlated with a 2 to 3-fold increase in the specific activity of GlcNAc T-V. Transformation of murine cells with polyoma viruses, adenovirus, tumorigenic DNA and either the ras or the fps/fes oncogenes also resulted in increased GlcNAc T-V activity. By contrast, several other glycosyl transferases involved in N-linked glycosylation are unchanged in the transformed cells. The mechanism for the increased specific activity of GlcNAc T-V in transformed cells is not known.

The increase in the $\beta(1,6)$ branching of the cell surface-bound oligosaccharides has been associated, at least in some cases, with capacity for metastasis. Increased levels of β -1,6 branching over the level in normal tissue has been observed for some human breast tumor tissues.

The purification of the rat kidney GlcNAc T-V has been published (Shoreibah et al. (1992) J. Biol. Chem. 267:2920-2927).

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Summary of the Invention

An object of this invention is the N-acetylglucosaminyltransferase V enzyme and DNA sequences encoding a protein having this enzymatic activity. Substantially pure and/or recombinantly-produced GlcNAc T-V will be useful in in vitro, and recombinant host cells expressing a GlcNAc T-V coding sequence will be useful for in vivo glycosylation reactions.

Provided herein are genomic and cDNA sequences encoding glcNAc T-V, amino acid sequences of GlcNAc T-V enzymes, and recombinant host cells genetically engineered to express a sequence encoding an active GlcNAc T-V enzyme.

Also provided by this invention are polyclonal and monoclonal antibodies specific for rat kidney GlcNAc T-V. These antibodies will also bind to and be useful for detection and isolation of GlcNAc T-V from other mammalian sources. It is understood that the molecular weight, kinetic parameters and primary amino acid sequence of GlcNAc T-V from a source other than rat kidney may vary from those characteristics disclosed herein for the rat kidney enzyme.

Another object of this invention is GlcNAc T-V produced by recombinant DNA technology in prokaryotic or eukaryotic host cells. Disclosed herein this invention is the complete amino acid sequence for GlcNAc T-V from rat and a complete nucleotide sequence encoding GlcNAc T-V from rat. Exemplary methods of producing recombinant active GlcNAc T-V by recombinant DNA technology are disclosed herein. The exemplified amino acid sequence and the nucleotide sequence encoding GlcNAc T-V, and subsequences within, as understood in the art, will be useful for isolating GlcNAc T-V coding sequences from a wide range of species and for producing useful quantities of GlcNAc T-V by recombinant DNA technology.

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Further objects of this invention are cDNA clones encoding GlcNAc T-V and genomic clones encoding GlcNAc T-V. Antibodies raised against rat kidney GlcNAc T-V or to conjugated peptides derived therefrom, for example, from analysis of the amino acid sequence of GlcNAc T-V disclosed herein, using art known methods for analysis and conjugation, can be used to detect expression of GlcNAc T-V from sources other than rat kidney by virtue of cross-reactivity with those other GlcNAc T-V enzymes; alternatively, these antibodies can be used to screen cDNA expression libraries. Similarly, the degenerate oligonucleotide probes and/or the coding sequence and/or the ampimer sequences of the present invention can be used to screen genomic or cDNA libraries constructed using nucleic acids from mammalian sources other than rat kidney, or these can be used to prepare primers to amplify sequences encoding GlcNAc T-V from mRNA populations prepared from rat kidney or from other mammalian sources. The cDNA and/or genomic sequences encoding GlcNAc T-V will be useful in directing the recombinant expression of GlcNAc T-V.

Further objects of this invention are nucleotide sequences encoding rat GlcNAc T-V, and nucleotide sequences encoding GlcNAc T-V from other vertebrate, preferably mammalian, sources, including cDNA and genomic sequences. The nucleotide sequence encoding rat GlcNAc T-V is provided herein as SEQ ID NO:15, from an ATG translation start codon beginning at nucleotide 299 through a translation stop codon ending at nucleotide 2521. The skilled artisan recognizes that there will be more than one nucleotide sequence capable of encoding the same amino acid sequence due to the degeneracy of the genetic code. These sequences, and sequence variants thereof which encode functionally equivalent GlcNAc T-V, can be used to express GlcNAc T-V in a desired recombinant host cell. The GlcNAc T-V coding sequences from other vertebrate species, preferably from mammals, will be highly homologous at the nucleotide sequence level to the exemplified rat GlcNAc T-V coding sequence disclosed herein. Functionally equivalent

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5 GlcNAc T-V coding sequences with at least 70%, preferably at least 80%, more preferably at least 90%, nucleotide sequence homology to the exemplified rat GlcNAc T-V coding sequence can be identified and isolated from cDNA libraries prepared from
10 MRNA sources other than rat cells, using well-known DNA-DNA hybridization technology and the exemplified rat GlcNAc T-V coding sequence provided herein. Also contemplated are genomic clones encoding GlcNAc T-V, which clones comprise the natural regulatory sequences. It is understood that any
15 intron sequences in genomic clones encoding GlcNAc T-V are not to be included in sequence comparisons to the exemplified full-length rat coding sequence.

20 Additional objects of this invention are DNA molecules containing a first nucleotide sequence encoding an enzymatically active GlcNAc T-V and a second nucleotide sequence not found associated with the GlcNAc T-V coding sequence in nature, termed an exogenous nucleotide sequence herein. Preferably the first nucleotide sequence encodes a polypeptide sequence with GlcNAc T-V activity, said
25 polypeptide having an amino acid sequence as given in SEQ ID NO:16.

30 Still further objects of the invention are cells genetically engineered to contain a DNA molecule containing a first nucleotide sequence encoding an enzymatically active GlcNAc T-V and a second nucleotide sequence not found associated with the GlcNAc T-V coding sequence in nature. Mammalian cells are preferred for recombinant expression of GlcNAc T-V coding sequences. Particularly preferred are COS-7 cells and CHO (Chinese Hamster Ovary) cells. The exemplified rat GlcNAc T-V amino acid sequence is particularly preferred, preferably encoded by the exemplified nucleotide coding sequence as in SEQ ID NO:15 from nucleotide 299 through nucleotide 2521.

Brief Description of the Drawings

Figure 1 is a reproduction of an ethidium bromide-stained agarose gel, showing the reaction products of the results of PCR amplification of GlcNAc T-V coding sequences. Lane 1 contains molecular weight standards (123 ladder); Lanes 2 and 7 were the results of reactions containing mouse lymphoma cell line BW5147 CDNA from total RNA as a template; Lanes 3 and 8 were the results of reactions containing mouse lymphoma cell line BW5147 cDNA from poly(A)+ RNA as template; Lanes 4 and 9 were the results of reactions containing rat mammary tumor cell line MAT C1 cDNA from total RNA as template; Lanes 5 and 10 were the results of reactions containing rat mammary tumor cell line MAT C1 cDNA from poly(A)+ RNA as template; and Lanes 6 and 11 were the results for reactions without added template. The reactions run in Lanes 2-6 were carried out with Primer 1 (SEQ ID NO:5) and Antiprimer 2 (SEQ ID NO:8) as the primers for PCR. In the reactions run in Lanes 7-11 were carried out with Primer 2 (SEQ ID NO:7) and Antiprimer 1 (SEQ ID NO:6).

Figure 2 illustrates autoradiograms resulting from Southern hybridizations using radiolabeled 200 ampimer (PCR product) prepared using rat mammary tumor cell line MAT C1 cDNA from poly(A)+ RNA as template and Primer 1 (SEQ ID NO:5) and Antiprimer 2 (SEQ ID NO:8) as primers. Figure 2A shows the results for BglII-digestion, Fig. 2B the results for NcoI/XhaI digestion, Fig. 2C for NcoI digestion and Fig. 2D for BamHI/BglII digestion. In each panel, lane 1 contains digested MAT C1 genomic DNA and lane 2 contains digested rat liver genomic DNA.

Figure 3 is a reproduction of an ethidium bromide-stained agarose gel, showing the products obtained after PCR amplification of Rat 1-EJ library cDNA sequences. Lane 1 contains molecular weight standards (Molecular Weight Marker II, Boehringer Mannheim, Indianapolis, IN); Lane 2 contains molecular weight standards (Molecular Weight Marker VII,

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Boehringer Mannheim); Lane 3 contains an aliquot of PCR reaction products resulting from amplification of Rat 1-EJ cDNA using primer T7:476-30 (SEQ ID NO: 11) and primer B:474-16 (SEQ ID NO: 10).

5 Figure 4 is a reproduction of an autoradiogram resulting from the Southern hybridization of the DNA transferred from the gel illustrated in Figure 3 in reverse orientation after it was probed with primer A:474-14 (SEQ ID NO: 9).

10 Figure 5 is a reproduction of an ethidium bromide-stained agarose gel, showing the products obtained after PCR amplification of an approximately 2.1 kb PCR product that was visualized in the autoradiogram in Figure 4. Lane 1 contains molecular weight standards (Molecular Weight Marker VII, 15
Boehringer Mannheim); Lane 2 contains the PCR products obtained after amplification of the approximately 2.1 kb PCR fragment with primer T7:476-30 (SEQ ID NO: 11) and primer 485-26: (SEQ ID NO: 12); Lane 3 contains molecular weight standards (Molecular Weight Marker II, Bohringer Mannheim).

20 Figure 6 presents the sequences of partial GlcNAc T-V coding sequences, termed amplimer sequences herein, from mouse (SEQ ID NO:17), rat (SEQ ID NO:18), and human (SEQ ID NO:19). Colons (:) indicate identical bases. There appear to be 13 differences between mouse and rat and two differences between mouse and human, as determined from comparison of the 25
available sequences.

Detailed Description of the Invention

30 In general, the terminology used herein is standard, as understood by those of ordinary skill in the fields of molecular biology, biochemistry, protein chemistry, and cell biology. For added clarity, certain terms are defined herein. Standard abbreviations are used; these abbreviations are consistent with those used and approved by scientific journals

in the field (e.g., Journal of Biological Chemistry, Science, Nature, etc.).

Methods used herein are either specifically referenced or are sufficiently well known as to be available in at least one of several readily accessible published collections of methodologies (See, e.g., Sambrook et al. (1989) Molecular Cloning, A Laboratory Manual (2nd ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, Innis et al. (1990) PCR Protocols: A Guide to Methods and Applications, Academic Press, New York, New York, and references cited therein).

Complementary DNA (cDNA) synthesis involves the in vitro synthesis of a double stranded DNA sequence by enzymatic reverse transcription of mRNA isolated from donor cells. In the present invention, polyadenylated RNA is prepared from rat 1-EJ cultured cells (described in Peles et al. (1992) Cell 69:205-216). Rat 1-EJ cells are Rat 1 fibroblasts which have been transfected with the human EJ gene, an activated Harvey ras gene, which is believed to elevated expression levels for GlcNAc T-V. cDNA molecules and/or libraries can be used for isolating a DNA sequence encoding a selected protein when the entire amino acid sequence of that protein is not known. Isolating a gene from a cDNA library is made much easier when at least a partial amino acid sequence is known, and is further facilitated when a complete coding sequence from at least one species is known. Procedures for the preparation of cDNA sequences in plasmid libraries derived from the reverse transcription of mRNA are well-known to the art.

The polymerase chain reaction (PCR) provides a powerful alternative to cDNA cloning for the amplification of sequences encoding a selected protein when at least a partial sequence of the selected protein is known. A degenerate oligonucleotide sequence is prepared according to the complement of the sequence encoding the partial amino acid

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sequence, and this degenerate oligonucleotide (i.e., a family of sequences) is used to prime PCR synthesis using cDNA derived from polyadenylated RNA as template. Further oligonucleotides for priming PCR are derived from unique (i.e., known) nucleotide sequences.

Expression refers to the transcription and translation of a structural gene (coding sequence) so that a protein having the biological activity of GlcNAc T-V is synthesized.

The term expression control sequence refers to a DNA sequence that controls and regulates the transcription and translation of another DNA sequence (i.e., a coding sequence). A coding sequence is operatively linked to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that coding sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

As used herein, an exogenous nucleotide sequence is one which is not in nature covalently linked to a particular nucleotide sequence, e.g., a GlcNAc T-V coding sequence. Examples of exogenous nucleotide sequences include, but are not limited to, plasmid vector sequences, expression control sequences not naturally associated with particular GlcNAc T-V coding sequences, and viral vector sequences.

Similarly, as used herein an exogenous gene is one which does not naturally occur in a particular recombinant host cell but has been introduced in using genetic engineering

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techniques well known in the art. An exogenous gene as used herein can comprise a GlcNAc T-V coding sequence expressed under the control of an expression control sequence not associated in nature with said coding sequence.

5 Another feature of this invention is the expression of the sequences encoding GlcNAc T-V. As is well-known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to
10 transform an appropriate host cell.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, nonchromosomal and synthetic DNA
15 sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., Escherichia coli plasmids ColE1, pCR1, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAs, e.g., M13 derivatives, the numerous derivatives of phage λ , e.g., λ gt11, and other phage DNA; yeast
20 plasmids derived from the 2 μ circle; vectors useful in eukaryotic cells, such as insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; baculovirus derivatives; and the
25 like. For mammalian cells there are a number of well-known expression vectors available to the art.

Any of a wide variety of expression control sequences may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include,
30 for example, the early and late promotes of SV40 or adenovirus for expression in mammalian cells, the lac system, the trp system, the TAC or TRC system, the major operator and promoter regions of phage λ , the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or phosphatase

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(e.g., pho5), the promoters of the yeast α -mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. The skilled artisan understands which expression control sequences are appropriate to particular vectors and host cells.

A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well-known eukaryotic and prokaryotic hosts, such as strains of E. coli, Pseudomonas, Bacillus, Streptomyces, fungi such as yeasts, and animal cells, such as CHO, R1.1, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS-7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in culture.

It is understood that not all combinations of vector, expression control sequence and host cell will function equally well to express the DNA sequences of this invention. However, one skilled in the art will be able to select the proper vector, expression control sequence, and host cell combination without undue experimentation to accomplish the desired expression without departing from the scope of this invention.

In selecting a suitable expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the promoter, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, e.g., with regard to potential secondary structure. Suitable unicellular hosts will be selected by consideration of factors including compatibility with the chosen vector, secretion characteristics, ability to fold proteins correctly, and fermentation requirements, as well as any toxicity to the host of the product encoded by the DNA sequences to be expressed,

and the ease of purification of the expression products. The practitioner will be able to select the appropriate host cells and expression mechanisms for a particular purpose.

N-acetylglucosaminyltransferase V (GlcNAc T-V) denotes the enzyme UDP-N-acetylglucosamine: α -6-D-mannoside β (1,6)-N-acetylglucosaminyltransferase (EC 2.4.1.155). This enzyme is responsible for the synthesis of β -1,6 branch structure (-[GlcNAc- β -(1,6)Man- α (1,6)Man]-) found in both tri- and tetra-antennary N-linked oligosaccharides.

Several strategies are available for the isolation and purification of recombinant GlcNAc T-V after expression in a recombinant host system. One method involves expressing the proteins in bacterial cells, lysing the cells, and purifying the protein by conventional means. Alternatively, one can engineer the DNA sequences for secretion from cells. See Example 11 and/or Colley et al. (1989) J. Biol. Chem. 264:17619-17622, which reference describes purifying a sialyltransferase by engineering the cleavable signal peptide of human gamma-interferon onto the DNA sequence for the transferase. Larsen et al. (1990) Proc. Natl. Acad. Sci. USA 87:6674-6678, fused the DNA sequence for protein A to the amino-terminal end of a fucosyl transferase gene and expressed it as an excreted fusion protein. In such a construction, one can optionally remove the transmembrane region from near the 3' end of the coding sequence. After secretion the proteins are purified from the medium. Similar strategies are available for bacterial expression systems.

It is understood by those skilled in the art that the exemplified rat GlcNAc T-V coding sequence, provided herein in SEQ ID NO:15 from nucleotide 299 through nucleotide 2521, is representative of GlcNAc T-V from other vertebrate sources, especially of other mammalian sources, including humans. The coding sequence for rat GlcNAc T-V provided herein is suitable for use in preparing or deriving PCR primers for identifying

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and/or amplifying sequences encoding human or other animal GlcNAc T-V, and/or for use as hybridization probes to identify clones encoding human or rat, other mammalian or other vertebrate GlcNAc T-V in appropriate genomic or cDNA libraries.

The techniques for the purification of the rat kidney GlcNAc T-V disclosed herein will be understood to be applicable to the purification of human or other GlcNAc T-V to a level comparable to that of rat kidney GlcNAc T-V. The skilled artisan recognizes that routine modifications of the procedures disclosed herein may provide improved results in isolating nonexemplified GlcNAc T-V enzymes.

Species other than rat, including mouse and human, contain genes encoding proteins which catalyze the same enzymatic reaction as rat GlcNAc T-V, which genes have significant nucleic acid sequence homology to the rat sequence encoding GlcNAc T-V. One can isolate these homologous genes using the DNA sequences of this invention as probes or primers under standard hybridization conditions. This invention specifically contemplates and encompasses such sequences.

A comparison of limited nucleotide sequence data (about 160-180 bases of "amplimer" sequence) from within the coding sequences of rat (SEQ ID NO:17), mouse (SEQ ID NO:18), and human (SEQ ID NO:19), obtained by PCR amplification of mRNA using primers disclosed herein, revealed significant sequence conservation; at least about 90% nucleotide sequence homology was observed in rat-mouse and rat-human comparisons (See Fig. 6). Thus, GlcNAc T-V coding sequences from vertebrate sources will have significant sequence homology to the exemplified rat GlcNAc T-V coding sequence provided herein. The ordinary skilled artisan can utilize the exemplified sequence or portions thereof preferably at least 25-30 bases in length, in hybridization probes to identify cDNA (or genomic) clones encoding GlcNAc T-V, where there is at least 70% sequence

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homology to the probe sequence using appropriate art-known hybridization techniques. The skilled artisan understands that the capacity of a cloned cDNA to encode functional GlcNAc T-V enzyme can be readily tested as taught herein (See Example 11).

Hybridization conditions appropriate for detecting various extents of nucleotide sequence homology between probe and target sequences and theoretical and practical consideration are given, for example in B.D. Hames and S.J. Higgins (1985) Nucleic Acid Hybridization, IRL Press, Oxford, and in Sambrook et al. (1989) supra. Under particular hybridization conditions the DNA sequences of this invention will hybridize to other DNA sequences having sufficient homology, including homologous sequences from different species. It is understood in the art that the stringency of hybridization conditions is a factor in the degree of homology required for hybridization. The skilled artisan knows how to manipulate the hybridization conditions so that the stringency of hybridization is at the desired level (high, medium, low). If attempts to identify and isolate the GlcNAc T-V gene from another mammalian source fail using high stringency conditions, the skilled artisan will understand how to decrease the stringency of the hybridization conditions so that a sequence with a lower degree of sequence homology will hybridize to the sequence used as a probe. The choice of the length and sequence of the probe is readily understood by the skilled artisan.

When a cDNA library is used as a source of GlcNAc T-V coding sequences, the skilled artisan will take steps to insure that the library is of high quality, i.e., that rare mRNAs will be represented and that large mRNAs (larger than about 3 kb) will be present as full length cDNA clones. If the artisan uses one of the commercially available or otherwise accessible cDNA libraries, he will choose one that

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meets the criteria taught herein. Providing for rare and/or large message representation is within the skill of the art.

5 The DNA sequences of this invention refer to DNA sequences prepared or isolated using recombinant DNA techniques. These include cDNA sequences, sequences isolated using PCR, DNA sequences isolated from their native genome, and synthetic DNA sequences. As used herein, this term is not intended to encompass naturally-occurring chromosomes or genomes. Sequences derived from the GlcNAc T-V gene can be used in studying the regulation of GlcNAc T-V expression in normal cells, in transformed cells and in metastatic tumor cells, and can be used in designing mechanisms, e.g., via antisense RNA or DNA, for inhibiting metastasis of tumor cells. These sequences can also be used to direct recombinant synthesis of GlcNAc T-V.

15 Expression of recombinant DNA molecules according to this invention may involve post-translational modification of a resultant polypeptide by the host cell. For example, in mammalian cells expression might include, among other things, glycosylation, lipidation or phosphorylation of a polypeptide, or proteolytic cleavage of a signal sequence to produce a "mature" protein. Accordingly, as used herein, the term "GlcNAc T-V" encompasses full-length polypeptides and modifications or derivatives thereof, such as glycosylated versions of such polypeptides, mature proteins, polypeptides retaining a signal peptide, truncated polypeptides having comparable biological activity, and the like. Expression of GlcNAc T-V in eukaryotic cell lines expressing biologically active glycoproteins will allow efficient branch structure initiation directed by GlcNAc T-V, where desired.

20 Rat kidney is useful as a source of GlcNAc T-V for purification because of the commercial availability of relatively large quantities of the tissue. The purification and relevant techniques of rat kidney GlcNAc T-V is described

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5 in Shoreibah et al. (1992) supra and in Examples 1-5. The data are summarized in Table 1. A survey of mouse, hamster and rat tissues had revealed that kidney was one of the most abundant sources of the enzyme in these rodents. The substantially pure GlcNAc T-V from rat kidney migrates predominantly as a doublet of 69 and 75 kDa on 10% SDS-polyacrylamide gels. This enzyme preparation is stable in the presence of 20% glycerol for several months when stored at 4°C.

TABLE 1

PURIFICATION OF RAT KIDNEY N-ACETYLGLUCOSAMINYLTTRANSFERASE V

Results described below are based on a preparation of the enzyme from 300 g of frozen rat kidneys.

Step	Volume ml	Protein mg	Total Activity nmol/h	Specific Activity nmol/(mg·h)	Yield %	Purification -fold
Rat kidney acetone powder Triton X-100 extract	3,300	13,900	2,221	0.16	100	1
UDP-hexanolamine-Sepharose™	96	38.0	889	23.2	40	145
Inhibitor-BSA-Sepharose™	6	0.0078	568	73,000	26	450,000

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To confirm that the two major SDS-PAGE protein bands (69 and 75 kDa) resulting from the two column purification scheme comprised GlcNAc T-V, an aliquot of the purified enzyme preparation was re-chromatographed on a 1 ml UDP-hexanolamine-agarose column. The bound material was eluted using several stepwise elutions of the ligand UDP, instead of the single concentration of NaCl, as used in the first chromatographic step. Almost no activity was detected in either the fractions eluted using a UDP concentration of 10 or 20 mM. Fifty mM UDP displaced the majority of the GlcNAc T-V activity from the column. A small peak was eluted using 50 mM UDP plus 150 mM NaCl. As judged by the silver staining pattern, rechromatography did not result in further increases in purity of the GlcNAc T-V. Similar results were obtained when a sample material resulting from the two column purification scheme were re-chromatographed on the inhibitor-BSA affinity column.

Once the GlcNAc T-V was substantially purified, the assay conditions were optimized. Enzymatic activity was stabilized and enhanced by the inclusion of 20% glycerol and 0.5 mg/ml IgG. The optimal pH range for the substantially pure GlcNAc T-V was 6.5 to 7.0; optimal Triton X-100 concentration was in the range of about 1.0 to about 1.5%. Enzyme activity was maximal at about 0.2 M NaCl, and was inhibited at higher salt concentrations. Divalent cations had a minimal effect on apparent enzyme activity when added as MnCl_2 , CaCl_2 or MgCl_2 , and the addition of 20 mM EDTA did not appear to be inhibitory.

Using the optimized assay conditions, kinetic parameters were determined for the substantially pure GlcNAc T-V enzyme. The apparent K_m for the oligosaccharide acceptor (BGlcNAc(1,2) α Man(1,6) β Man-O-(CH₂)₈COOCH₃) was 87 μM , and the apparent K_m for UDP-GlcNAc was 11.0 mM. The apparent V_{max} for the sugar nucleotide was 18.8 $\mu\text{mol}/(\text{mg}\cdot\text{min})$.

For amino acid sequence analysis, the enzyme was further purified by preparative SDS-PAGE using an Applied Biosystems High Performance Electrophoresis Apparatus (Applied Biosystems, Foster City, California). The fractions containing enzyme were pooled and concentrated. The enzyme protein was then precipitated by ethanol addition and lowering the temperature to (-20°C). The precipitate was collected by centrifugation, washed and dried.

NH₂-terminal amino acid sequencing was attempted, but the results indicated that the N-terminus of the protein was blocked. A sample of the substantially purified rat kidney GlcNAc T-V was digested using immobilized trypsin, separated from the immobilized trypsin and the peptides within the digest were then separated by reverse phase HPLC using a 2.1 x 150 mm VYDAC C18 column, eluted with a gradient of acetonitrile. Four peptide peaks were chosen for gas phase sequencing. The results were as follows:

Peak #34 AsnThrAspPhePheIleGlyLysProThrLeuArg
(SEQ ID NO:1)

Peak #49 AlaIleLeuAsnGlnLysIleGluProTyrMetProTyrGluPheThr
(SEQ ID NO:2)

Peak #28 ValLeuAspSerPheGlyThrGluProGluPheAsn
(SEQ ID NO:3)

Peak #61 SerAspPro[Cys]TyrAla[Asp]Tyr[Glu]Val
(SEQ ID NO:4)

Bracketed amino acid residues were assigned with some uncertainty. The amino acid sequences obtained from the four peaks were searched within the Swiss Protein Data Bank and deduced degenerate coding sequences were searched in the Genbank database. No significantly homologous sequences were found.

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The determination of a partial amino acid sequence for GlcNAc T-V allows the production of sets of degenerate oligonucleotide probes or primers, thus, enabling the cloning of the corresponding cDNA and genomic clones. Those oligonucleotides can also be used to study the transcriptional and/or translational mechanisms which control the level of expression of the gene encoding GlcNAc T-V.

From the amino acid sequences for the internal peptides corresponding to peaks 34 and 49, corresponding degenerate oligonucleotides were designed for use as primers for PCR amplification of cDNA sequences encoding GlcNAc T-V. Abbreviations for nucleotides used herein are in accordance with IUPAC practice: A is adenosine; C is cytosine; G is guanosine; T is thymidine; I is inosine; R is A or G; Y is C or T; H is A or C or T; D is G or A or T; and N is A or C or G or T. The degenerate 29 base oligonucleotide designed from the sequence of first ten amino acids of the Peak 34 peptide is presented as Primer 1 (SEQ ID NO:5). The antisense counterpart (SEQ ID NO:6) of Primer 1, termed AntiPrimer 1 herein, will be useful as a primer in the PCR amplification of sequences encoding GlcNAc T-V present within polyadenylated mRNA populations, prepared from cells including, but not limited to, rat kidney, mouse lymphoma BW5147 cells and ascites-grown rat mammary gland tumor MAT C1 cells.

Primer 1: AAYACIGAYTTYTTYATHGGIAARCCNAC (SEQ ID NO:5)

AntiPrimer 1: GTIGGYTTICCDATRAARAARTCIGTRTT (SEQ ID NO:6)
(antisense)

A second degenerate 29 base oligonucleotide was designed using the sequence of the last ten amino acids of the peptide corresponding to Peak 49:

Primer 2: ATHGARCCITAYATGCCITAYGARTTYAC (SEQ ID NO:7)

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AntiPrimer 2: TCRTAIGGCATRTAIGGYTCDATYTTYTG (SEQ ID NO:8)
(antisense)

The antisense primers given above can also be used to amplify mRNA encoding GlcNAc T-V in polymerase chain reactions. Other oligonucleotide primers and "antiprimers" may be designed using the peptide sequences and/or GlcNAc T-V sequences disclosed herein by one of ordinary skill in the art for use in priming PCR synthesis of GlcNAc T-V coding sequences.

The sequences of the antisense primers (AntiPrimers 1 and 2; SEQ ID NO:6 and SEQ ID NO:8) are complementary to those of the corresponding Primers 1 and 2, respectively (SEQ ID NO:5 and SEQ ID NO:7). Either the sense or the antisense primers, or preferably the PCR amplification product of Primer 1 and AntiPrimer 2, can be used as hybridization probes or as PCR primers for screening a rat kidney cDNA library, a rat genomic library or mouse libraries for clones encoding GlcNAc T-V. The primers and antisense primers in appropriate combination can be used to prime PCR reactions using cDNA prepared, for example, from rat kidney cell poly(A)+ RNA. Sequences amplifiable with these primers and antisense primers in PCR reactions will be those encoding portions of GlcNAc T-V.

For PCR amplification of sequences encoding GlcNAc T-V, Primer 1 and AntiPrimer 2 cDNA were used to prime PCR-directed DNA synthesis. The combination of Primer 2 (SEQ ID NO:7) and AntiPrimer 1 (SEQ ID NO:6) did not yield an amplification product from either cell line. Using cDNA prepared from poly(A)+ RNA from either the rat mammary tumor line MAT C1 or from the mouse lymphoma cell line BW5147 with Primer 1 and AntiPrimer 2, an amplification product of about 200 bp was obtained, as shown in Fig. 1. The rat amplimer DNA sequence is given in Fig. 6 and SEQ ID NO:18. Background signal was reduced substantially by using 55°C rather than 50°C as the annealing temperature in the PCR reactions. The results also

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indicate a high degree of homology between the GlcNAc T-V coding sequences in mouse and rat. Thus, the primer/antiprimer sequences disclosed herein will be useful in identifying GlcNAc T-V genes and coding sequences of sources other than rat.

The ampimer made by PCR with cDNA from MAT C1 poly(A)+ RNA as template and Primer 1 (SEQ ID NO:5) and AntiPrimer 2 (SEQ ID NO:8) was ³²P-labeled for use as a hybridization probe. Rat MAT C1 genomic DNA and rat liver genomic DNA were digested in separate restriction endonuclease reactions, the fragments were separated in parallel using agarose gel electrophoresis, blotted to support and DNA-DNA hybridization was carried out under standard hybridization conditions of low stringency. Hybridization patterns were consistent with a single genetic locus encoding GlcNAc T-V in each. Figure 2 illustrates the autoradiogram obtained for Southern hybridization with rat mammary tumor cell line MAT C1 and for rat liver genomic DNA. With BglII, BamHI/BglII and NcoI digestion, the size of the unique hybridizing genomic band is between 2 and 10 kbp. With NcoI/XbaI digestion, the size of the hybridizing band is between roughly 6 and 9 kb. Routine experimentation will allow size estimation with more precision. The roughly 180 bp ampimer used in this experiment can be used to screen cDNA or genomic libraries to identify GlcNAc T-V sequences. Standard "walking" experiments can be performed to obtain the sequences which flank the hybridizing fragment(s) after cloning of that fragment so that the entire gene can be isolated.

Labeled oligonucleotides having sequences of Primers 1 and 2 (SEQ ID NO: 5 and 7) or AntiPrimers 1 and 2 (SEQ ID NO:6 and SEQ ID NO:8), or preferably the PCR amplification product (ampimer) made using Primer 1 and AntiPrimer 2 as primers, can be successfully used as hybridization probes for screening cDNA libraries prepared from sources including mouse lymphoma BW5147 cells, mouse 3T3 cells and ascites-grown rat mammary gland MAT-C1 cells for sequences encoding GlcNAc T-V.

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When a HindIII/BglII restriction fragment from within the coding region of a partial mouse cDNA clone was used as a hybridization probe in a Northern blot of rat kidney mRNA size separated by agarose gel electrophoresis, a band of about 7 kb, along with apparent degradation products, was displayed. Thus, the size of the GlcNAc T-V mRNA is large, and care must be taken in preparing (or in choosing) a cDNA library from which to isolate a full length GlcNAc T-V coding sequence.

Examples 7-10 describe the steps in the successful identification and cloning of the rat GlcNAc T-V coding sequence using a PCR-cDNA strategy. In other experiments, an amplicon of about 170-200 bases was prepared by PCR. This amplicon was used to screen a mouse cDNA library, and a partial clone of about 1.7 kb was isolated. Sequence analysis revealed that the long open reading frame did not contain a start codon, and about 300 amino acids were determined by the open reading frame. A series of PCR amplification and screening steps were carried out using plasmid DNA prepared from pools of cDNA clones from subsets of a cDNA library prepared from Rat 1-EJ cell mRNA (See Examples 7-8).

A rat cDNA clone of about 4.8 kb, carrying the full length GlcNAc T-V coding sequence was isolated. A portion of the cDNA was sequenced; the resulting DNA sequence is presented in SEQ ID NO:15. The coding sequence extends from an ATG start codon beginning at nucleotide 299 through a stop codon ending at nucleotide 2521. Six potential N-glycosylation sites occur at asparagine residues at amino acid numbers 109, 114, 117, 333, 430, and 446. A putative transmembrane domain encompasses amino acids 14-30. Approximately 300 bp of 5' untranslated sequence is presented, followed by 2220 bp of coding sequence and about 100 bp of the 3' untranslated region.

The deduced amino acid sequence is given in SEQ ID NO:16. The predicted molecular weight (84,561) of the encoded GlcNAc

T-V is larger than the protein bands observed in SDS-PAGE gels. When GlcNAc T-V is purified from rat kidney by in the presence of a cocktail of protease inhibitors in vast excess, a band of about 95 kDa, in addition to the 69 and 75 kDa
5 bands, is observed. When a radioactive photoaffinity active site label was used to tag active enzyme, all three bands were labeled. These observations suggest that the 75 and 69 kDa bands represent proteolytic fragments of the larger protein. The 95 kDa band is likely to represent a glycosylated form of
10 the polypeptide encoded in SEQ ID NO:15. Six potential sites for N-linked glycosylation were identified: Asn residues at amino acid positions 109, 114, 117, 333, 432, and 446 in SEQ ID NO:16. A putative transmembrane domain, extending from amino acids 14-30, was identified by hydrophobicity analysis
15 using Kyte and Doolittle methodology. This proposed transmembrane domain is characteristic of type II membrane proteins, and is similar to other enzymes of the lumen of the Golgi apparatus.

Within the deduced amino acid sequence of rat GlcNAc T-V
20 (SEQ ID NO:16), the sequences corresponding to the Peak #s 34, 49 and 28 peptide sequences (SEQ ID NOS:1-3) were located at amino acids 546-557, 592-607, and 375-386, respectively. The amino acid sequence of Peak #61 (SEQ ID NO:4) occurs at amino acids 168-177 in SEQ ID NO:16. The identities of the cysteine
25 and aspartate residues are confirmed, and the amino acid at the ninth position in SEQ ID NO:4 was deduced to be glycine rather than glutamate, based on SEQ ID NO:15.

It is well-known in the biological arts that certain amino acid substitutions can be made within a protein
30 without affecting the functioning of that protein. Preferably such substitutions are of amino acids similar in size and/or charge properties. For example, Dayhoff et al. (1978) in Atlas of Protein Sequence and Structure, Volume 5, Supplement 3, Chapter 22, pages 345-352,
35 provides frequency tables for amino acid substitutions

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which can be employed as a measure of amino acid similarity. Dayhoff et al.'s frequency tables are based on comparisons of amino acid sequences for proteins having the same function from a variety of evolutionarily different sources.

5 Two mouse GlcNAc T-V partial clones have been sequenced. The deduced partial mouse sequence was compared to corresponding rat GlcNAc T-V amino acids 387-740 as in SEQ ID NOS:15-16. From the preliminary sequences, the only deduced
10 amino acid difference in the available mouse sequence was at the position corresponding to amino acid 679 of rat. The rat deduced amino acid is isoleucine whereas the mouse amino acid is threonine. A comparison of the available nucleotide sequences over the coding region revealed that there was about 96% sequence homology; a comparison of 103 available bases of
15 rat sequence with mouse sequence in the 3' noncoding region revealed about 88% sequence homology. Thus, the coding and the noncoding sequences (at the 3' end) are highly conserved between mouse and rat, especially within the coding region, where nucleotide differences are in all but one case silent.

20 The 4.8 kb cDNA insert determined by partial DNA sequence analysis to contain an apparently full length GlcNAc T-V coding sequence was subcloned into the pJT-2 expression vector and electroporated into COS-7 cells (See Example 11). After 3 or 4 days incubation after the electroporation, the
25 transfected cells were harvested, frozen and subsequently assayed for GlcNAc T-V activity. Parallel preparations of cells transfected with pJT-2 without insert DNA served as controls. It was estimated that about 3% of the cells were effectively electroporated. The data in Table 2 indicate that
30 the cloned rat cDNA fragment encodes a functional GlcNAc T-V enzyme.

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Table 2. GlcNAc T-V Activity in the Transient Expression Assay

Sample	Hrs incubation after electroporation	Specific activity (pmol/mg*hr)
COS-7 (pJT-2)	68	38
	92	65
COS-7 (pJT-2-TV)	68	624
	92	499

It will be a matter of routine experimentation for the ordinary skilled artisan to use the DNA sequence information presented herein to optimize GlcNAc T-V expression in a particular expression vector and cell line for a desired purpose. A cell line genetically engineered to contain and stably express a GlcNAc T-V coding sequence will be useful for the recombinant expression of protein products with the characteristic glycosylation dependent on GlcNAc T-V modification of glycoproteins. Any means known to the art can be used to introduce an expressible GlcNAc T-V coding sequence into a cell to produce a recombinant host cell, i.e., to genetically engineer such a recombinant host cell. Recombinant host cell lines which express high levels of GlcNAc T-V will be useful as sources for the purification of GlcNAc T-V, e.g., for studies of inhibitors of GlcNAc T-V activity for preventing or slowing metastasis of tumors. The coding sequence of GlcNAc T-V will be useful in preparing an antisense construct specific for GlcNAc T-V for inhibiting GlcNAc T-V expression where that is desired, for example, in metastasizing tumor cells.

The following examples are provided for illustrative purposes as well as for enablement. These examples are not

intended to limit the scope of the invention. The examples use many techniques well known and accessible to those skilled in the arts of molecular biology and biochemistry. It will be readily apparent to the skilled artisan that
5 modifications of the methods disclosed herein may be made, and that there will be DNA sequence modifications which can be made with the maintenance of the desired result. It will be readily apparent to one of ordinary skill in the art that the nucleotide sequences and amino acid sequences disclosed herein
10 make it unnecessary to repeat many of the examples to practice the invention.

EXAMPLES

Example 1. Preparation of UDP-Hexanolamine-Agarose

UDP-hexanolamine was prepared and linked to CNBr-
15 activated SEPHAROSE™ 4B according to the procedure in Barker et al. (1972) J. Biol. Chem. 247:7135-7147. The substitution of the SEPHAROSE™ (Pharmacia, Piscataway, N.J.; agarose) at a level of 14 μ moles of UDP-hexanolamine per ml of settled gel is critical; substitution levels of 6 and 9 μ moles per ml gave
20 significantly lower activity yields.

Example 2. Purification of GlcNAc T-V from Rat Kidney

Frozen rat kidneys were purchased from Pel-Freez Biological, Inc. (Rogers, Arkansas).

Three hundred g of frozen rat kidneys were homogenized in
25 3 liters of cold acetone in a Waring blender at 4°C. All subsequent steps were also performed at 4°C unless otherwise noted. The acetone-insoluble material was collected on

Whatman filter paper no. 4. The acetone insoluble material was re-homogenized in acetone and refiltered. The resulting powder was stirred in 1.8 liters Buffer A (0.1 M sodium acetate (pH 6.0), 0.2 M NaCl, 0.01 M EDTA) for 30 min. The residue was collected by centrifugation for 15 min at 7100 x g. The pellet was again extracted with Buffer A and centrifuged again.

The resulting pellet was then stirred in 2 liters water and collected by centrifugation. To the washed residue was then added the following protease inhibitors: 0.1 mM PMSF, 0.05 mg/ml aprotonin, 0.5 mg/ml soybean trypsin inhibitor, 0.5 μ g/ml leupeptin, and 1 μ g/ml pepstatin. This mixture was then homogenized in 1 liter Buffer B (0.01 M Tris-HCl (pH 7.8), 0.4 M KCl.

The resulting homogenate was brought to 1% Triton X-100 (w/v) and stirred 30 min. The suspension was centrifuged for 20 min at 7100 x g to give the first extract (the supernatant). The pellet was twice again homogenized, solubilized with Triton X-100, and clarified by centrifugation to yield the second and third extracts.

The three extracts were pooled and dialyzed against 20 liters Buffer C (50 mM MES pH 6.5, 0.2% (w/v) Triton X-100, 5 mM EDTA, 0.05% sodium azide over a 72 hr period with a single change of dialysis buffer. The resulting dialysate was clarified by centrifugation and then assayed for protein concentration and enzymatic activity.

In the first affinity chromatography step, 3 l of acetone powder Triton extract was applied to a 1.2 x 7 cm column of UDP-hexanolamine Sepharose pre-equilibrated with Buffer C. The column was then washed with about 400 ml Buffer C. The column was then eluted with Buffer C plus 0.5 M NaCl. Fractions were collected and assayed for GlcNAc T-V activity.

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Pooled fractions (about 100 ml) eluted from the UDP-hexanolamine SEPHAROSE column were dialyzed against Buffer C. The dialyzate was brought to 1 mM UDP-GlcNAc and 20% glycerol and was loaded on a 1.2 x 3 cm column of inhibitor-BSA-Sepharose pre-equilibrated with Buffer D (50 mM MES pH 6.5, 0.1% Triton X-100, 20% glycerol, 0.05% sodium azide. The column was then washed with 20 ml Buffer D without UDP-GlcNAc. Finally the column was stopped, brought to room temperature, and then eluted with the inclusion of 500 mM NaCl in Buffer D in which the pH had been adjusted to 8.0. Fractions were collected and assayed for GlcNAc T-V activity.

An aliquot (0.1 ml) of the pooled active fractions from the inhibitor-BSA affinity column was then dialyzed against Buffer C and applied to a 0.4 x 8 cm column of UDP-hexanolamine Sepharose pre-equilibrated with Buffer D. The column was then eluted with buffers comprising increasing amounts of UDP in Buffer D: 10 mM UDP, 20 mM UDP, 50 mM UDP, 50 mM UDP with 120 mM NaCl and finally 100 mM UDP with 150 mM NaCl. Fractions were collected and assayed for GlcNAc T-V activity. (For this purification step either Buffer D can be used or 50 mM sodium cacodylate pH 6.5, 0.1% Triton X-100, 20% glycerol, 0.05% sodium azide can be used, with incremental increases in NaCl concentration for elution as described for Buffer D.)

Equal volumes from each set of fractions were concentrated under reduced pressure and elevated temperature with a Speed Vac. SDS-polyacrylamide gel electrophoresis of various fractions was carried out on 10% SDS-polyacrylamide gels after the concentrated samples were boiled in 1 X gel sample buffer to reduce and denature the proteins (Laemmli (1970) Nature 227:680-685). The gels were silver-stained as described in Morrissey (1981) Anal. Biochem. 117:307-310 in order to visualize the material.

Example 3. Assay of GlcNAc T-V Activity

A typical radiochemical assay for determining activity during purification contained the following reagents which were dried in vacuo in a 1.5 ml conical centrifuge tube: 2 mM
5 ADP (pyrophosphatase inhibitor), 2.5 mM β -methylGlcNAc (β -hexosaminidase inhibitor), 10^6 cpm UDP-[6- ^{3} H]-GlcNAc (10 cpm/pmol) and 1 mM of the synthetic acceptor (β -D-GlcNAc)-(1,2)- α -D-Man-(1,6)- β -D-Man-O-(CH₂)₈CO₂Me in a total volume of 10 microliters.

10 To initiate the reaction, 0.01 ml of sample, in a buffer containing 50 mM MES pH 6.0, 0.1% Surfact-Amps (Triton) X-100 (Pierce, Rockford, Illinois), was added to the dried reagents and incubated at 37°C for several hrs.

To terminate the assay, 0.5 ml water was added to each
15 tube, vortexed thoroughly, and the contents of the tubes were centrifuged. The supernatant was then loaded onto a pellicular C18 Sep-Pak™ column (Millipore, Bedford, Massachusetts) activated with methanol and pre-equilibrated with water. The columns were washed with 200 ml water to
20 remove water-soluble radioactivity resulting from unreacted substrate and degradation products. The radiolabeled product of the GlcNAc T-V reaction was then eluted with a 0-100% step gradient of methanol, and radioactivity was quantitated by liquid scintillation counting. All assays were conducted in
25 duplicate, and the results were averaged. In tabulating the results for Table I, assays were done in at least two separate experiments and averaged. The variation between the values derived from duplicates or from separate experiments did not exceed $\pm 10\%$ and typically were less than $\pm 2\%$ of the averaged
30 values.

Radiolabeled product was then separated from the unreacted acceptor and radiolabeled UDP-GlcNAc by virtue of the hydrophobic moiety using C-18 chromatography.

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Once the GlcNAc T-V protein was purified, the parameters in the assay were optimized: 20% glycerol, near physiological levels of NaCl (about 200 mM), 0.5 mg/ml IgG, pH about 6.5 - 7.0, and Triton X-100 concentration about 1.0-1.5%.

5 GlcNAc T-V protein was measured using the enzyme-linked immunosorbent assay described in Crawley et al. (1990) Analytical Biochem 185:112-117. The ELISA uses unlabeled UDP-GlcNAc and a trisaccharide acceptor (β -D-GlcNAc)-(1,2)- α -D-Man-(1,6)- β -O-Man-D-(CH₂)₈CO₂Me coupled to BSA. This assay
10 relied on the use of a polyclonal antibody specific for the tetrasaccharide-BSA product of the GlcNAc T-V reaction. Due to the extreme sensitivity of the ELISA, column fractions
15 column fractions containing an inhibitory amount of NaCl, for example, could be assayed without prior dialysis by simply diluting the samples. Standard calibration curves were generated in each assay and absorbance (or relative activity) was correlated to a specific activity by comparison to values obtained for a sample of known GlcNAc activity, as measured in the radiochemical assay.

20 Example 4. Measurement of Small Amounts of Protein

The BCA protein assay (Pierce, Rockford, Illinois) was adapted for use in a microtiter plate format using standard polystyrene 96 well plates (Pierce, Rockford, Illinois) to assay column fractions for protein content during
25 purifications. BSA served as the standard protein.

Example 5. Preparation of Inhibitors, Acceptors, Substrates and Affinity Adsorbents

UDP hexanamine was synthesized and linked to CNBr-activated agarose support (SEPHAROSE 4B) as described in
30 Barker et al. (1972) J. Biol. Chem. 247:7135-7147. The concentration of the ligand relative to the support was 14 μ moles per ml of settled gel.

The deoxy oligosaccharide inhibitor of GlcNAc T-V activity (n-octyl 6-O-[2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-6-deoxy-α-D-mannopyranosyl]-β-D-glucopyranoside) was synthesized as described in Palcic et al. (1990) J. Biol. Chem. 265:6759-6769, and used in assays.

A similar GlcNAc T-V oligosaccharide inhibitor (BGlcNAc(1,2) 6-deoxy-α-Man(1,6) BMan-O-(CH₂)₈COOCH₃ was coupled to BSA according to the method of Pinto et al. (1983) Carbohydr. Res. 124:313-318 for use as an affinity chromatography ligand. The ligand in the column is an active site inhibitor which mimics the natural oligosaccharide acceptor of GlcNAc T-V, but contains a hydrogen in place of the reactive 6'-hydroxyl. The inhibitor oligosaccharide (4.1 mg) was converted to the acyl azide as a 25 mM solution in DMF (dimethyl formamide) at -15°C and then 222.2 mg BSA (Sigma Chemical Co., St. Louis, Missouri) was added in 2 ml of 0.35 M KHCO₃ and 0.07 M Na₂B₄O₇ (pH 9.0). The resulting solution was held at 4 C for 24 h, and then extensively dialyzed against distilled water on a Amicon PM-10 ultrafiltration membrane (Amicon, Inc., Division of WR Grace, Danvers, Massachusetts). The dialysate was then lyophilized, and redissolved. Protein content was measured using the Bradford assay (Bradford (1976) Analyt. Biochem. 72:248-254) with BSA as a standard. Carbohydrate content was measured using the phenol-sulfuric acid method (Dubois et al. (1956) Analyt. Chem. 28:350-356). The results indicated that 13 oligosaccharide molecules were incorporated per molecule of BSA (67% coupling).

The coupling of 3.6 mg of the inhibitor-protein complex to 3 ml of periodate-oxidized agarose (SEPHADEX CL-6B, Pharmacia, Piscataway, New Jersey) was carried out as described in Stults et al. (1989) Analyt. Biochem. 180:114-119 with NH₂(CH)₂OH-HCl as the final blocking reagent. A coupling of 34% of the oligosaccharide-BSA complex to the agarose gave a final incorporation of 0.07 μmol of ligand oligosaccharide

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per ml of settled gel as estimated by the Bradford protein assay.

Trisaccharide oligosaccharide acceptors and their syntheses are described in Palcic et al. (1990) supra; Pierce et al. (1987) Biochem. Biophys. Res. Commun. 146:679-684; Arango et al. (1988) J. Cell. Biochem. 37:225-231; and Srivastava et al. (1988) Carbohydr. Res. 179:137-161.

Example 6. Production of Antibodies specific for GlcNAc T-V

GlcNAc T-V is precipitated from storage buffer by adding 3 volumes of absolute ethanol and left to stand for 30 min at 4°C. The precipitated protein is collected by centrifugation (10,000 x G for 10 min), resuspended in 0.3 ml of Buffer D, and mixed with 1.0 ml of Freund's complete adjuvant. The resulting emulsion is administered to two rabbits by injecting intradermally in the back with 50-75 µl/site or about 75 µg protein per site. Each rabbit receives booster injections of 150 µg per dose, prepared in the same way, 14 days after the initial dose, and each rabbit receives 75 µg at 21, 34, 57 and 64 days after the initial injection. 10-20 ml of blood is collected from an ear vein of each rabbit at weekly intervals, and serum is prepared and stored at -20°C. Relative levels of antibody specific for GlcNAc T-V are estimated by determining the amount of serum required to inhibit 50% of the activity in the assay using the artificial substrate as acceptor. Serum samples with the highest activity are pooled.

Monoclonal antibodies specific for rat kidney GlcNAc T-V are prepared according to standard procedures (e.g., Campbell (1984) Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology (Burdon and van Knippenberg, eds.) Vol. 13, Elsevier, Amsterdam; Harlow and Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) after immunization of mice with purified rat kidney GlcNAc T-V.

A. Rat 1-EJ cDNA Library Construction

20 B. Design and Construction of Oligonucleotides

25 Primer A:474-14 GGGCCGATGAAGACTTCTGCG (SEQ ID NO: 9)
(antisense)

Primer B:474-16 GGGCTACTTCCTCTCGGTTATTGAG (SEQ ID NO:
10)
(antisense)

In addition, an oligonucleotide was designed using the T7 promoter sequence of the cloning vector pSPORT-1.

Primer T7:476-30 GCTCTAATACGACTCACTATAGG (SEQ ID NO: 11)
5 (sense)

C. PCR Amplification of Rat 1-EJ cDNA Library Sequences

An aliquot of plasmid DNA from each pool of the Rat 1-EJ cDNA library was combined to form a Rat 1-EJ cDNA library DNA mixture (Rat 1-EJ cDNA pool). PCR was carried out on the Rat
10 1-EJ cDNA pool using primers T7:476-30 (SEQ ID NO: 11) and B:474-16 (SEQ ID NO: 10). The T7 sequence of pSPORT-1 lies upstream from the 5' SalI cloning site used in the cDNA synthesis. Therefore, PCR priming using the oligonucleotide T7:476-30 (SEQ ID NO: 11) synthesizes an ampimer covering
15 the extreme 5' end of the cDNA and extending in the direction of the 3' end of the coding sequence. The PCR product extends into the coding sequence to the primer B:474-16 (SEQ ID NO: 10) which lies within the approximately 200 bp ampimer.

PCR was carried out using a GeneAmp™ DNA Amplification
20 Kit (Perkin Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions. Briefly, a 100 µl reaction consisted of the following:

	8	µl	MgCl ₂	25	mM
	10	µl	10X PCR buffer		
25	70.8	µl	sterile H ₂ O		
	2	µl	dGTP	10	mM
	2	µl	dATP	10	mM
	2	µl	dTTP	10	mM
	2	µl	dCTP	10	mM
30	1	µl	T7:476-30 primer	15	µM
	1	µl	B:474-16 primer	15	µM
	500	ng	Rat 1-EJ cDNA library pool		DNA

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The reaction mix was overlaid with mineral oil (Sigma, St. Louis, MO) and placed in a DNA thermal cycler (Perkin Elmer Cetus). Taq polymerase (0.5 μ l, 2.5 U) was added in a hot start procedure and the thermal cycler was programmed as follows:

1 min	94°C	40 cycles
1 min	59°C	
2 min	72°C	
10 min	72°C	
soak	4°C	

An aliquot of the reaction products was analyzed by agarose gel electrophoresis (0.8% agarose in Tris Borate EDTA buffer (TBE) containing ethidium bromide) and the gel was photographed (Figure 7). One major band at approximately 1200 bp and several smaller minor species were visible on the ethidium bromide-stained gel.

D. Southern Hybridization of PCR Products

After PCR, products from Example 7, Part C, were separated by agarose gel electrophoresis and analyzed by a standard Southern blot procedure. Briefly, the gel was denatured by soaking in 1.5 M NaCl, 0.5 N NaOH for 30 min. The gel was then neutralized by soaking in 1.5 M NaCl, 0.5 M Tris-HCL (pH 7.5) for 30 minutes. The DNA in the gel was transferred to nitrocellulose by capillary action in 10X SSC overnight. After transfer, the nitrocellulose was rinsed in 6X SSC, air dried and crosslinked in a UV Stratalinker (Stratagene, La Jolla, CA).

The nitrocellulose was prehybridized, hybridized and probed using an Enhanced Chemiluminescence 3' Oligolabelling and Detection System kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Prehybridization was carried out at 50°C for 30 min.

Hybridization was carried out for about one and a half hours

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at 50°C with approximately 8 ng/ml of oligonucleotide probe A:
474-14 (SEQ ID NO: 9).

After hybridization, the nitrocellulose was washed twice
in 5X SSC, 0.1% SDS at room temperature for 5 min each time.
Then the nitrocellulose was washed twice in 1X SSC, 0.1% SDS
at 50°C for 15 min each time. Horse Radish Peroxidase
Antibody development and ECL detection were carried out
according to kit instructions.

The nitrocellulose was exposed to x-ray film at room
temperature for 20 minutes. Autoradiography of the
nitrocellulose revealed a single band of approximately 2.1 kb
(Figure 8). This specific, but rare, PCR product was not
visible on the ethidium bromide-stained gel (Figure 2).

E. Amplification of Specific PCR Product

Since the specific 2.1 kb PCR product described in
Example 7, Part D was present in such minute quantities that
it could only be detected by autoradiography, it was amplified
by PCR. First, the 2.1 kb PCR product was isolated by cutting
a region of an agarose gel in which the specific DNA was
expected to have migrated. The DNA was eluted from the gel
using an S&S Elu-Quik DNA Purification Kit (Schleicher &
Schuell, Keene, NH) following the manufacturer's directions.
A PCR reaction was carried out on an aliquot of the eluted DNA
utilizing primer T7: 476-30 (SEQ ID NO: 11) at the 5' end.
The 3' primer was as follows:

485-26 GGGTACGTGTGAATGATATCCAGGTAG (SEQ ID NO: 12)
(antisense)

This oligonucleotide sequence lies approximately 350 bp
upstream from the 3' end of the 2.1 kb PCR fragment. This
sequence was elucidated by sequencing a partial mouse cDNA

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which was isolated by screening a mouse lymphoma BW 5147 library with the approximately 200 bp PCR amplimer sequence.

A 100 μ l PCR reaction using the eluted 2.1 kb PCR fragment as template was prepared as follows:

8 μ l $MgCl_2$ 25 mM
10 μ l 10X PCR buffer
61.5 μ l sterile H_2O
2 μ l dGTP 10 mM
2 μ l dATP 10 mM
2 μ l dTTP 10 mM
2 μ l dCTP 10 mM
1 μ l T7: 476-30 primer 15 μ M
1 μ l 485-26 primer 15 μ M
10 μ l eluted 2.1 kb PCR fragment

The reaction mix was treated as described in Example 7, Part C and the thermal cycler was programmed as follows:

94°C	30 sec
60°C	1 min
72°C	1 min

40 cycles

72°C 10 min
4°C soak

An aliquot of the reaction products was analyzed by agarose gel electrophoresis (0.8% agarose in TBE containing ethidium bromide) and the gel was photographed (Figure 4). Analysis of the ethidium bromide-stained gel revealed a single DNA band of approximately 1.8 kb.

F. DNA Sequence Analysis

The approximately 1.8 kb PCR product described in Example 7, Part E was sequenced using Taq DyeDioxy Terminator cycle sequencing kits (Applied Biosystems, Inc., Foster City, CA) and an automated DNA sequencer (Applied Biosystems 373A)

following the manufacturer's instructions. The PCR fragment was sequenced after it was passed over a Centricon™-100 unit (Amicon, Beverly, MA) and washed with sterile water. In some instances, sequences were derived after the PCR fragment was
5 subcloned into a pUC13 vector (Promega, Madison, WI). Nucleotide sequencing was carried out using synthetic oligonucleotides as primers.

Sequencing of approximately 1750 bp of the PCR fragment and analysis of all possible reading frames revealed overlap
10 with the partial mouse BW 5147 cDNA sequence. The partial mouse cDNA contained 3' untranslated sequence as well as an open reading frame of approximately 885 bases which would code for approximately 295 amino acids, but no start codon. The sequencing of the PCR fragment extended the open reading frame
15 coding region by an additional approximately 445 amino acid residues and located the methionine specifying ATG start codon. In addition, approximately 300 bp of 5' untranslated region was identified in the PCR fragment.

Alternatively, cDNA clones encoding GlcNAc T-V can be
20 isolated using the following strategy.

Total RNA is prepared in parallel isolations from rat kidney tissue, according to standard procedures, and from mouse lymphoma BW5147 cells and from ascites-grown rat mammary gland MAT-C1 cells, as described in Sambrook et al. (eds.)
25 (1989) supra. ATCC T1B47 is a clone (BW5147.3) of the BW5147 cell line adapted into culture (J. Natl. Cancer Inst. (1973) 51:883; J. Immunol. (1973) 110:1470). MAT C1 cells are described in Carraway et al. (1976) J. Biol. Chem. 251:6173-6178. The Poly(A)+ fraction of the total RNA is prepared by
30 chromatography over Oligo(dT) cellulose chromatography as described in Sambrook et al. (eds.) (1989) supra. Polyadenylated mRNA encoding GlcNAc T-V is included within the Poly(A)+ RNA thus prepared.

5 cDNA libraries are prepared using the poly(A)+ RNA prepared from rat kidney, mouse lymphoma BW5147 cells, and MAT-B1 cell total RNA according to the procedure of Sambrook et al. (eds.) (1989) supra. Cloning of the cDNA population into a suitable vector (such as λ gt11) is done according to standard protocols. (See, e.g., Huynh et al. (1985) in DNA Cloning, a Practical Approach, Vol. 1 (Glover, D.M., ed.), IRL Press, Washington, D.C., pp. 49-78).

10 Commercially-available cDNA libraries (e.g., rat kidney cDNA library, Clontech Laboratories, Palo Alto, California) can also be screened for GlcNAc T-V clones.

15 The cDNA libraries are screened for sequences encoding GlcNAc T-V by plaque hybridization under low stringency conditions using the approximately 200 bp amplicon radiolabeled by random hexamer labelling as described in Sambrook et al. (eds.) (1989) supra. Clones specifically hybridizing the amplicon sequence are selected for further analysis (restriction endonuclease digestion, nucleotide sequence determination).

20 Genomic clones encoding GlcNAc T-V can be identified from a rat (or mouse or other mammal) genomic library using Primer 1 (SEQ ID NO:5) or Primer 2 (SEQ ID NO:7) e.g., or Primers 1 and 2 in combination, or the amplicon where PCR synthesized as above was primed with Primer 1 (SEQ ID NO: 5) and AntiPrimer 2 (SEQ ID NO:8) to identify appropriate genomic sequences.

30 From the clones analyzed it is possible to reconstruct the entire coding sequence of GlcNAc T-V. If a full-length coding sequence is not reconstructed, further primers can be designed using sequences near the ends of the sequenced region for use in the RACE procedure (Rapid Amplification of cDNA Ends) as described in Frohman et al. (1988) Proc. Natl. Acad. Sci. USA 85:8998-9002. Where the entire gene is desired,

genomic libraries can be screened, and "walking" procedures known in the art are used to extend in both directions.

Example 8. Cloning of a Rat cDNA Sequence Encoding GlcNAc T-V

A. Southern Hybridization of Rat 1-EJ Library Pools

5 Nitrocellulose filters, containing NotI-linearized plasmid DNA from each of the individual 43 pools of the Rat 1-EJ cDNA library, were probed in order to identify which pool(s) contained a full-length GlcNAc T-V cDNA. A cDNA probe was derived from the partial mouse cDNA coding region and was
10 obtained as a HindIII/PstI fragment starting approximately 855 bp down stream from the Rat 1-EJ PCR fragment ATG sequence and extending approximately 650 bp toward the 3' end of the sequence.

15 The nitrocellulose filters were incubated with prehybridization solution at 42°C as described in Sambrook et al., (eds.) (1989) supra. Hybridization was then carried out in an overnight incubation using an approximately 650 bp mouse cDNA probe which was labeled with [α^{32} P]-dCTP using a
20 Multiprime DNA Labelling System kit (Amersham). The nitrocellulose was then washed and the filters were exposed to X-ray film with an intensifying screen at -80°C overnight. Autoradiography of the filters revealed 4 positive pools among the 43 screened.

B. PCR Analysis of Rat 1-EJ Library Pools

25 PCR was carried out using template DNA from each of the 4 positive Rat 1-EJ cDNA library pools identified in Example 8, Part A in order to determine which pool contained a full-length cDNA. The reactions were carried out as described in Example 7, Part C, except that the following primers were
30 used:

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Primer 501-16 CCCGTCGACGAGAGCCAAGGGAATGGTAC (SEQ ID NO: 13)
(sense)

Primer 496-2 CCCAGCAGGTACAGAGATGTG (SEQ ID NO: 14)
(antisense)

Primer 501-16 (SEQ ID NO: 13) was determined by sequencing the Rat 1-EJ PCR fragment to hybridize in the 5' untranslated region approximately 15 to 35 bases upstream from the ATG start codon. Primer 496-2 (SEQ ID NO: 14) was determined by sequencing to hybridize within the coding region about 900 bases downstream from the ATG start codon. Therefore, PCR with these two primers gives a predicted product of about 900 bp in length covering the 5' end of the coding region. The thermal cycle was programmed as follows:

94°C	30 sec
55°C	1 min
72°C	1 min

40 cycles

72°C 10 min
4°C soak

An aliquot of the reaction mixture was separated by agarose gel electrophoresis as described in Example 7, Part C. Analysis of the ethidium bromide-stained gel indicated two of the four pools gave the correct size band (about 900 bp). This information, together with the size of the bands obtained by Southern hybridization of the Rat 1-EJ cDNA library pools (Example 8, Part A) revealed that one pool could contain a full-length GlcNAc T-V cDNA.

C. Colony Hybridization Procedures for Screening a Rat 1-EJ cDNA Library Pool

Transformed E. coli from the glycerol stock of the one pool of the Rat 1-EJ cDNA library identified in Example 8, Part B, above, were spread out at a density of approximately 4,500 colonies per 10 x 10 cm plate on nutrient plates

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containing 50 µg/ml Ampicillin. Nitrocellulose filters were used to lift the colonies off the plate. The filters (colony side up) were treated by serially placing them over a piece of Whatman 3 MM paper saturated with each of the following:

1. 1.5 M NaCl, 0.5 N NaOH for 10 min
2. 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.5) for 5 min
3. 2X SSC for 5 min

The filters were then air dried and crosslinked by UV irradiation. The filters were then subjected to digestion with Proteinase K by incubating in a solution containing 0.2% SDS, 100 mM Tris-HCl (pH 8.5), 50 mM NaCl, 10 mM EDTA (pH 8) and 50 µg/ml proteinase K at 55°C for 30 min. The filters were then transferred to a solution containing 5X SSC, 0.5% SDS and 1 mM EDTA (pH 8) and incubated at 55°C for 30 min. Prehybridization, hybridization and subsequent treatments were carried out using an ECL 3' Oligolabelling and Detection System kit (Amersham) with the following conditions:

1. prehybridization was carried out at 53°C for approximately 2 hours.
2. hybridization was carried out at 53°C in an overnight incubation using primer 501-16 (SEQ ID NO: 13) at approximately 7 ng/ml.

After hybridization, the filters were washed as described in Example 7, Part D. After ECL detection, the filters were exposed to X-ray film at room temperature for 4 minutes.

Among the 36,000 colonies screened, 24 individual colonies and mixtures of colonies were picked for further analysis by PCR. PCR was carried out in the same manner as described in Example 8, Part B except that a 20 µl reaction volume was used and the template was obtained by touching a pipet tip to the bacterial plate and then dipping the pipet tip in the PCR mixture. After overlaying with mineral oil,

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the PCR tubes were incubated in the thermal cycler at 94°C for 4 min prior to adding 0.2 µl of Taq polymerase. The following temperature regime was applied:

5	94°C 30 sec	25 cycles
	53°C 1 min	
	72°C 1 min	
10	72°C 10 min	
	4°C soak	

An aliquot of the reaction mixture was separated by agarose gel electrophoresis as described in Example 7, Part C. Analysis of the ethidium bromide-stained gel revealed three positives among the 24 mixtures examined.

15 The 3 positive mixtures were replated and probed with primer 496-2 (SEQ ID NO: 14) as described above. Prehybridization and hybridization, according to instructions in the ECL 3' Labelling and Detection System kit, were carried out at 53°C for 30 min and approximately 2 hours
20 respectively. Washes were as described above and autoradiography was carried out for 20 min at room temperature. Analysis of the X-ray film revealed one positive among approximately 600 colonies screened. This colony was confirmed by PCR analysis with primers 501-16 (SEQ ID NO: 13)
25 and 496-2 (SEQ ID NO: 14) as described above except the reaction volumes were 50 µl.

30 The one positive colony mixture from above was replated at low density and probed with primer 496-2 (SEQ ID NO: 14) as described above except prehybridization and hybridization were carried out at 55°C. Filters were exposed to X-ray film for 2 min revealing 7 positives among approximately 300 colonies screened.

D. Sequencing Analysis of Rat 1-EJ cDNA

Plasmid DNA was isolated from 4 of the final positive colonies described in Example 8, Part C. Restriction enzyme analysis revealed that the plasmids each contained an approximately 4.8 kb cDNA insert. Nucleotide sequence
5 analysis of one of the plasmids was carried out using the procedures described in Example 7, Part F. Results are shown in SEQ ID NO:15.

In SEQ ID NO:15, the initial DNA sequence designates the sense strand of approximately 300 bases in what appears to
10 comprise the 5' untranslated region preceding the translated portion of the rat GlcNAc T-V cDNA. The sequence immediately following is seen to code for the amino acid sequence of rat GlcNAc T-V. This region spans 2220 bases and codes for 740 amino acids and a stop codon (TAG). The subsequent sequence
15 appears to be an untranslated 3' region of the rat GlcNAc T-V cDNA. By restriction mapping analysis of the plasmid DNA, this 3' untranslated region of the cDNA appears to be approximately 2300 bases in length. Only the first approximately 100 bases of the 3' untranslated region are
20 presented in SEQ ID NO:15.

SEQ ID NO:16 thus provides the primary structure (amino acid sequence) of rat GlcNAc T-V as including 740 specified amino acid residues (estimated M.W.= 84,561). There are six sites for possible N-linked glycosylation of the mature rat
25 GlcNAc T-V polypeptide.

Example 9. Southern Hybridizations

Appropriate amounts of rat mammary tumor genomic DNA and rat liver genomic DNA were digested in parallel reactions with restriction enzymes (BglII, NcoI, and NcoI/XhaI and
30 BamHI/BglII) according to the instructions of the suppliers. Restriction fragments were then separated by agarose gel electrophoresis (1.0% agarose, Tris-Acetate-EDTA buffer).

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The gels were then stained with ethidium bromide, excess stain was removed by soaking in TAE buffer, and the gels were photographed. The DNA in the gels was then depurinated by soaking in 0.25 N HCl for 10 min with agitation.

Prior to transfer to nitrocellulose, the DNA was denatured by soaking the gels in 0.5 N NaOH, 1.5 M NaCl for 30 min. The nitrocellulose was soaked in double distilled water for 20-30 min, and then in 10 x SSC for 20-30 min. The gel was rinsed with double distilled water and the base was neutralized by soaking the gel in 0.5 M Tris-HCl (pH 7.4), 3 M NaCl for 30 min.

The DNA bands in the treated gel were then blotted to the nitrocellulose by capillary transfer in 10 x SSC overnight at room temperature. The positions of the wells and the orientation of the gel were marked on the nitrocellulose with a #1 pencil.

The nitrocellulose sheet was then rinsed in 4 x SSC, air dried for 30 min, and baked in a vacuum oven at 80°C for 2 hr (until thoroughly dried).

The nitrocellulose was washed with prehybridization solution for 4 hr at 42°C. Hybridization was then carried out in an overnight incubation using an approximately 200 bp amplicon probe which was random-hexamer labeled with [α -³²P]-CTP (See Sambrook et al. (eds.) (1989) supra). The approximately 200 bp amplicon was made in a Taq polymerase reaction with Primer 1 (SEQ ID NO:5) and AntiPrimer 2 (SEQ ID NO:8) as described herein. The nitrocellulose was then washed twice with 2 x SSC, 0.2% SDS at 50°C for 30 min each time.

The hybridized nitrocellulose was then placed on X-ray film with an intensifying screen and held overnight at -80 C to expose the film.

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Example 10. Isolation of Partial Mouse and Human sequences
for Glc NAc T-V by PCR

PCR was carried out according to standard methods to determine whether Primers 1 and 2 could amplify a specific product from two cell lines (mouse lymphoma BW5147 and rat mammary tumor Mat C1 cells).

Total RNA and poly(A)+RNA was isolated from each cell line, and used as to generate cDNA using reverse transcriptase. These cDNA preparations served as template in parallel PCR reactions as follows:

10-50 ng template cDNA
5 μ l 10 x Taq buffer (Mg-free)
3 μ l 25 mM MgCl₂
1 μ l dNTP mix (10 mM each)
1 μ l 30 μ M Primer 1
1 μ l 30 μ M Primer 2
38 μ l sterile water
0.5 μ l Taq polymerase

Each reaction was overlaid with oil and then placed in a thermal cycler apparatus with the following temperature regime:

5 min 94°C

1 min 94°C

1 min 55°C

2 min 72°C

35-41 cycles

10 min 72°C

The reaction products were then separated by agarose gel electrophoresis (2% agarose).

Example 11.

A. Transient Expression of Rat GlcNAc T-V in COS-7 Cells

The entire approximately 4.8 kb cDNA insert from one rat GlcNAc T-V clone described in Example 8, Part D was ligated into an SalI- and NotI-digested pJT-2 plasmid expression vector (Wen et al. (1992) Cell 69:559-572). COS-7 cells (CRL 1651, American Type Culture Collection, Rockville, MD) were transfected with the pJT-2 plasmid alone or with pJT-2 plasmid containing the rat GlcNAc T-V cDNA insert by electroporation as follows: 4×10^6 cells in 0.8 ml of DMEM (Dulbecco's Modified Minimal Medium, Gibco, Grand Island, NY) and 7.5% FBS (Fetal Bovine Serum, Bocknek, Ltd.) were transferred to a 0.4 cm cuvette and mixed with 10 μ g of plasmid DNA in 10 μ l of water. Electroporation was performed at room temperature at 1600 volts and 25 μ F using a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA) with the pulse controller unit set at 200 ohms (Wen et al. (1988) supra). The cells were then diluted into approximately 40 ml of DMEM, 7.5% FBS and transferred to 100 mm culture dishes. After a 17 hr incubation at 37°C, the medium was replaced and incubation continued for an additional 51 hr or 75 hr.

B. Preparation of COS-7 Cells for GlcNAc T-V Activity Assay

The medium from each COS-7 plasmid transfected plate was removed and the cells were rinsed with phosphate-buffered saline (PBS). Cell scrapers were used to collect the cells, which were placed in tubes, diluted with PBS and centrifuged to pellet the cells. After the PBS had been aspirated, the cell pellet was subjected to quick freezing by immersion of the tube in liquid nitrogen. The cells were kept frozen on dry ice until resuspended in buffer for analysis by radiochemical assay and ELISA.

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C. Assay of GlcNAc T-V Activity

Cell pellets were resuspended in 20 μ l MES (pH 6.0) 150 mM NaCl buffer and disrupted by sonication. The protein content of each extract was determined as described in Example 4. GlcNAc T-V activity was then determined in radiochemical and ELISA assays.

The radiochemical assay uses a synthetic trisaccharide acceptor molecule (Srivastava et al. (1988) supra; Pierce et al. (1987) supra; Arango and Pierce (1988) supra; Palcic et al. (1988) Glycoconjugate J. 5:49-63; Pierce and Arango (1986) J. Biol. Chem. 261:10772-10277; Crawely et al. (1990) Anal. Biochem. 185:112-117). A typical assay mixture contains the following reagents dried under vacuum in a 1.5 ml centrifuge tube: 10⁶ cpm of UDP-[³H]-GlcNAc (25 cpm/pmol), and 1 mM of the synthetic acceptor in a total volume of 0.01 ml. To initiate the reaction, 0.01 ml of cell extract, typically containing about 30 μ g protein, in a buffer containing 50 mM MES (pH 6.0) and 1% Surfact-Amps (Triton) X-100, was added to the assay tube and incubated at 37 C several hours (e.g., about 7 hrs). To terminate the assay, 0.5 ml H₂O was added to each tube, vortexed to mix thoroughly, and then contents of the tubes were centrifuged. Radiolabeled product was separated from unincorporated substrate by virtue of its hydrophobic moiety by C-18 chromatography. Each supernatant was then loaded onto a pellicular C-18 Sep Pak column which had previously been activated with methanol and pre-equilibrated with water. The column was then washed with 200 ml H₂O to remove water-soluble radioactivity resulting from unreacted substrate and breakdown products. The radiolabeled product was then eluted with 100% methanol, and radioactivity was measured by liquid scintillation counting. All assays were conducted at least in duplicate for two time points and the results were averaged. The variation between the values from duplicate assays did not exceed plus or minus 5%, and

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typically were less than plus or minus 2% of the averaged value.

The ELISA assay for GlcNAc T-V activity allows the detection of femtomole amounts of assay product, and the assay range covers a 10^6 -fold range of GlcNAc T-V activity. This assay utilizes unlabeled sugar nucleotide, the trisaccharide acceptor coupled to bovine serum albumin (BSA), and a rabbit polyclonal antibody specific for the tetrasaccharide-BSA product of the reaction. In order to determine GlcNAc T-V activity, standard calibration curves must be generated in each assay using known amounts of GlcNAc T-V, as measured in the radiochemical assay, and then absorbance in a test sample must be correlated with a particular specific activity by comparison to the standard curve.

An alternate approach to demonstrate that the full-length cDNA clone isolated does encode GlcNAc T-V, the coding sequence is fused to the N-terminal Protein A coding sequence as described in Larsen et al. (1989) Proc. Natl. Acad. Sci. USA 86:8227-8231. The resultant recombinant plasmid is then introduced into mammalian cells such that cells which have incorporated the cDNA sequences survive in culture. Because the fusion protein contains the N-terminal sequences of Protein A, the fusion protein is directed to the secretion pathway and released from the cells. After removal of the cells by centrifugation, the culture medium is assayed for GlcNAc T-V activity as described herein. A portion of the cell-free medium is chromatographed over an IgG column to which the N-terminal Protein A sequences bind, causing GlcNAc T-V activity to be retained on the column.

A second alternative approach for confirming that the cDNA isolated does encode GlcNAc T-V is to insert the complete cDNA into a vector under the control of regulatory sequences which will allow expression in the chosen mammalian host

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cells. The host cell chosen is a GlcNAc T-V-deficient variant of the mouse lymphoma BW5147 cell line, which variant is PHA 2.1; this variant cell line is described in Cummings et al. (1982) J. Biol. Chem. 257:13421-13427. An alternative GlcNAc T-V-deficient cell line is the Lec4 variant of CHO cells, described by Stanley, P. (1983) Methods Enzymol. 96:157-184. Both variant cells lines were selected for growth in the presence of the cytotoxic lectin L-phyto-hemagglutinin, which binds to the galactosylated product of GlcNAc T-V. Expression of the cDNA sequences encoding the GlcNAc T-V restores GlcNAc T-V activity and lectin sensitivity to these variant cell lines.

The use of any one or more of the foregoing approaches provides confirmation that GlcNAc T-V is cloned as cDNA.

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SEQUENCE LISTING

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(ii) TITLE OF INVENTION: N-Acetylglucosaminyltransferase V
Coding Sequences

(iii) NUMBER OF SEQUENCES: 19

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(A) APPLICATION NUMBER:
(B) FILING DATE: 29-JUNE-1993
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/905,795
(B) FILING DATE: 29-JUN-1992

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/016,863
(B) FILING DATE: 10-FEB-1993

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(C) REFERENCE/DOCKET NUMBER: 34-92B PCT

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(A) TELEPHONE: (303)499-8080
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

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

Asn Thr Asp Phe Phe Ile Gly Lys Pro Thr Leu Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

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

Ala Ile Leu Asn Gln Lys Ile Glu Pro Tyr Met Pro Tyr Glu Phe
1 5 10 15
Thr

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

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

Val Leu Asp Ser Phe Gly Thr Glu Pro Glu Phe Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

(A) NAME/KEY: Region

(B) LOCATION: 1..10

(D) OTHER INFORMATION: /label= UNCERTAIN
/note= "AMINO ACIDS AT POSITIONS 4, 7 AND 9 WERE
IDENTIFIED WITH UNCERTAINTY"

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

Ser	Asp	Pro	Cys	Tyr	Ala	Asp	Tyr	Glu	Val
1				5				10	

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: misc_difference

(B) LOCATION: at positions 6 and 21

(D) OTHER INFORMATION: /standard_name= "N IS INOSINE
AT POSITIONS 6 AND 21"

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

AAAYACNGAYT TYTTYATHGG NAARCCNAC

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

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(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: misc_difference
(B) LOCATION: at positions 3, 9 and 24
(D) OTHER INFORMATION: /standard_name= "N IS INOSINE
AT POSITIONS 3, 9, 24

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

GTNGGYTTNC CDATRAARAA RTCNGTRTT

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

(ix) FEATURES

- (A) NAME/KEY: misc_difference
(B) LOCATION: at positions 9 and 18
(D) OTHER INFORMATION: /standard_name= "N IS INOSINE
AT POSITIONS 9, 18

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

ATHGARCCNT AYATGCCNTA YGARTTYAC

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

(ix) FEATURES

- (A) NAME/KEY: misc_difference
(B) LOCATION: at positions 3 and 15
(D) OTHER INFORMATION: /standard_name= "N IS INOSINE
AT POSITIONS 3, 15

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

TCRTANGGCA TRTANGGYTC DATYTTYTG

29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

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

GGGCCGATGA AGACTTCTGC G

21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

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

GGGCTACTTC CTCTCGGTTA TTGAG

25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

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

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

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

GCTCTAATAC GACTCACTAT AGG

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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

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

GGGTACGTGT GAATGATATC CAGGTAG

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

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

CCCGTCGACG AGAGCCAAG GGAATGGTAC

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

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

CCCAGCAGGT ACAGAGATGTG

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2624 base pairs

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 299..2521

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

	TGACCCCGCT CCTGGCTGTG CCTGGGACCC CAGTTCCCAG GAGCACGGTT	50
	GCAGGAGAGT GACCCCGACT GCTACTGATG GTGCTTCTGC TGCTCCTCTA	100
	CTAGCAGGAG TGACTCCTAC CCAGAAGTGG ACTTGGAGGA GGGTCCGTTA	150
	GACCATCAGA ATGGAAGCCC GACAAGCAAG TCAGCTGACT CAGGAACCAG	200
15	AGTGAGGGCC ACGCACTCTC CGCCCCAGCC TGCACCATGA ACTTGCCTTC	250
	CCCTTCTGCT TGTTGAGAGC CAAGGGAATG GTACATTACT AGAGAGAG	298
	ATG GCT TTC TTT TCT CCC TGG AAG TTG TCC TCT CAG AAG CTG	340
	Met Ala Phe Phe Ser Pro Trp Lys Leu Ser Ser Gln Lys Leu	
	1 5 10	
20	GGC TTT TTC TTG GTG ACT TTT GGC TTC ATA TGG GGG ATG ATG	382
	Gly Phe Phe Leu Val Thr Phe Gly Phe Ile Trp Gly Met Met	
	15 20 25	
	CTT CTA CAC TTC ACC ATC CAG CAG CGA ACT CAG CCT GAG AGC	424
	Leu Leu His Phe Thr Ile Gln Gln Arg Thr Gln Pro Glu Ser	
25	30 35 40	
	AGC TCC ATG TTG CGG GAG CAA ATC CTT GAC CTC AGC AAA AGG	466
	Ser Ser Met Leu Arg Glu Gln Ile Leu Asp Leu Ser Lys Arg	
	45 50 55	
	TAC ATT AAG GCA CTG GCA GAA GAG AAC AGG AAC GTG GTG GAT	508
30	Tyr Ile Lys Ala Leu Ala Glu Glu Asn Arg Asn Val Val Asp	
	60 65 70	
	GGC CCG TAT GCC GGT GTC ATG ACA GCC TAT GAT CTG AAG AAA	550
	Gly Pro Tyr Ala Gly Val Met Thr Ala Tyr Asp Leu Lys Lys	
	75 80	

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		ACG	CTC	GCC	GTG	CTG	CTG	GAT	AAC	ATC	TTG	CAG	CGC	ATC	GGC	592
		Thr	Leu	Ala	Val	Leu	Leu	Asp	Asn	Ile	Leu	Gln	Arg	Ile	Gly	
		85					90					95				
5		AAG	CTG	GAG	TCC	AAG	GTG	GAC	AAT	CTT	GTC	AAC	GGC	ACA	GGA	634
		Lys	Leu	Glu	Ser	Lys	Val	Asp	Asn	Leu	Val	Asn	Gly	Thr	Gly	
			100					105					110			
		GCG	AAT	TCT	ACC	AAC	TCC	ACC	ACG	GCT	GTC	CCC	AGC	TTG	GTG	648
		Ala	Asn	Ser	Thr	Asn	Ser	Thr	Thr	Ala	Val	Pro	Ser	Leu	Val	
				115					120					125		
10		TCA	CTG	GAG	AAA	ATT	AAT	GTG	GCA	GAT	ATC	ATT	AAT	GGA	GTT	718
		Ser	Leu	Glu	Lys	Ile	Asn	Val	Ala	Asp	Ile	Ile	Asn	Gly	Val	
					130					135					140	
15		CAA	GAA	AAA	TGT	GTA	TTG	CCT	CCT	ATG	GAT	GGC	TAC	CCC	CAC	760
		Gln	Glu	Lys	Cys	Val	Leu	Pro	Pro	Met	Asp	Gly	Tyr	Pro	His	
						145					150					
		TGC	GAG	GGG	AAA	ATC	AAG	TGG	ATG	AAA	GAC	ATG	TGG	CGG	TCA	802
		Cys	Glu	Gly	Lys	Ile	Lys	Trp	Met	Lys	Asp	Met	Trp	Arg	Ser	
		155					160					165				
20		GAC	CCC	TGC	TAC	GCA	GAC	TAT	GGA	GTG	GAC	GGG	ACC	TCC	TGC	844
		Asp	Pro	Cys	Tyr	Ala	Asp	Tyr	Gly	Val	Asp	Gly	Thr	Ser	Cys	
			170					175					180			
		TCC	TTT	TTT	ATT	TAC	CTC	AGT	GAG	GTT	GAA	AAT	TGG	TGT	CCT	886
		Ser	Phe	Phe	Ile	Tyr	Leu	Ser	Glu	Val	Glu	Asn	Trp	Cys	Pro	
25				185					190					195		
		CGT	TTA	CCT	TGG	AGA	GCA	AAA	AAT	CCC	TAT	GAA	GAA	GCT	GAC	928
		Arg	Leu	Pro	Trp	Arg	Ala	Lys	Asn	Pro	Tyr	Glu	Glu	Ala	Asp	
				200						205					210	
30		CAT	AAC	TCA	TTG	GCA	GAA	ATC	CGC	ACG	GAT	TTT	AAC	ATT	CTC	970
		His	Asn	Ser	Leu	Ala	Glu	Ile	Arg	Thr	Asp	Phe	Asn	Ile	Leu	
						215					220					
		TAC	GGC	ATG	ATG	AAG	AAG	CAT	GAG	GAG	TTC	CGG	TGG	ATG	AGA	1012
		Tyr	Gly	Met	Met	Lys	Lys	His	Glu	Glu	Phe	Arg	Trp	Met	Arg	
		225					230					235				
35		CTT	CGG	ATC	CGG	CGA	ATG	GCT	GAT	GCA	TGG	ATC	CAA	GCA	ATC	1054
		Leu	Arg	Ile	Arg	Arg	Met	Ala	Asp	Ala	Trp	Ile	Gln	Ala	Ile	
			240					245					250			
		AAG	TCT	CTG	GCA	GAG	AAA	CAA	AAC	CTA	GAG	AAG	AGG	AAA	CGG	1096
		Lys	Ser	Leu	Ala	Glu	Lys	Gln	Asn	Leu	Glu	Lys	Arg	Lys	Arg	
40				255					260					265		
		AAG	AAA	ATC	CTT	GTT	CAC	CTG	GGG	CTC	CTG	ACC	AAG	GAA	TCA	1138
		Lys	Lys	Ile	Leu	Val	His	Leu	Gly	Leu	Leu	Thr	Lys	Glu	Ser	
					270					275					280	

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	GGC	TTC	AAG	ATT	GCA	GAG	ACA	GCA	TTC	AGC	GGT	GGC	CCT	CTC	1180
	Gly	Phe	Lys	Ile	Ala	Glu	Thr	Ala	Phe	Ser	Gly	Gly	Pro	Leu	
					285					290					
5	GGC	GAG	CTC	GTT	CAG	TGG	AGT	GAC	TTA	ATC	ACA	TCT	CTG	TAC	1222
	Gly	Glu	Leu	Val	Gln	Trp	Ser	Asp	Leu	Ile	Thr	Ser	Leu	Tyr	
	295					300					305				
	CTG	CTG	GGC	CAT	GAC	ATC	CGC	ATC	TCA	GCC	TCG	CTG	GCT	GAG	1264
	Leu	Leu	Gly	His	Asp	Ile	Arg	Ile	Ser	Ala	Ser	Leu	Ala	Glu	
		310					315					320			
10	CTC	AAG	GAG	ATT	ATG	AAG	AAG	GTT	GTT	GGA	AAC	CGG	TCT	GGC	1306
	Leu	Lys	Glu	Ile	Met	Lys	Lys	Val	Val	Gly	Asn	Arg	Ser	Gly	
			325					330					335		
	TGT	CCA	ACT	GTA	GGA	GAC	AGA	ATC	GTT	GAG	CTT	ATT	TAT	ATC	1348
	Cys	Pro	Thr	Val	Gly	Asp	Arg	Ile	Val	Glu	Leu	Ile	Tyr	Ile	
15				340					345					350	
	GAT	ATT	GTG	GGA	CTT	GCT	CAA	TTC	AAG	AAA	ACG	CTA	GGA	CCA	1390
	Asp	Ile	Val	Gly	Leu	Ala	Gln	Phe	Lys	Lys	Thr	Leu	Gly	Pro	
				355						360					
20	TCC	TGG	GTT	CAT	TAC	CAG	TGC	ATG	CTC	CGG	GTG	CTG	GAC	TCC	1432
	Ser	Trp	Val	His	Tyr	Gln	Cys	Met	Leu	Arg	Val	Leu	Asp	Ser	
	365					370					375				
	TTT	GGA	ACA	GAA	CCT	GAG	TTC	AAT	CAC	GCA	AGT	TAC	GCC	CAG	1474
	Phe	Gly	Thr	Glu	Pro	Glu	Phe	Asn	His	Ala	Ser	Tyr	Ala	Gln	
25		380					385					390			
	TCG	AAA	GGC	CAC	AAG	ACC	CCC	TGG	GGA	AAG	TGG	AAT	CTG	AAC	1516
	Ser	Lys	Gly	His	Lys	Thr	Pro	Trp	Gly	Lys	Trp	Asn	Leu	Asn	
			395					400					405		
30	CCG	CAA	CAG	TTT	TAC	ACC	ATG	TTC	CCT	CAT	ACC	CCA	GAC	AAC	1558
	Pro	Gln	Gln	Phe	Tyr	Thr	Met	Phe	Pro	His	Thr	Pro	Asp	Asn	
				410					415					420	
	AGC	TTT	CTG	GGC	TTC	GTG	GTC	GAG	CAG	CAC	CTG	AAC	TCC	AGC	1600
	Ser	Phe	Leu	Gly	Phe	Val	Val	Glu	Gln	His	Leu	Asn	Ser	Ser	
35					425					430					
	GAC	ATC	CAC	CAC	ATT	AAC	GAG	ATC	AAA	AGG	CAG	AAC	CAG	TCC	1642
	Asp	Ile	His	His	Ile	Asn	Glu	Ile	Lys	Arg	Gln	Asn	Gln	Ser	
	435					440					445				
	CTT	GTG	TAT	GGC	AAA	GTG	GAT	AGT	TTC	TGG	AAG	AAT	AAG	AAG	1684
	Leu	Val	Tyr	Gly	Lys	Val	Asp	Ser	Phe	Trp	Lys	Asn	Lys	Lys	
40							450					460			
	ATC	TAC	TTG	GAC	ATC	ATT	CAC	ACG	TAC	ATG	GAA	GTG	CAC	GCC	1726
	Ile	Tyr	Leu	Asp	Ile	Ile	His	Thr	Tyr	Met	Glu	Val	His	Ala	
45				465				470					475		

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	ACT	GTT	TAC	GGC	TCC	AGT	ACC	AAG	AAC	ATC	CCC	AGT	TAC	GTG	1768
	Thr	Val	Tyr	Gly	Ser	Ser	Thr	Lys	Asn	Ile	Pro	Ser	Tyr	Val	
				480					485					490	
5	AAA	AAC	CAT	GGC	ATT	CTC	AGC	GGC	CGT	GAC	CTA	CAG	TTT	CTT	1810
	Lys	Asn	His	Gly	Ile	Leu	Ser	Gly	Arg	Asp	Leu	Gln	Phe	Leu	
					495					500					
	CTC	CGG	GAA	ACC	AAG	CTT	TTT	GTT	GGG	CTT	GGA	TTC	CCT	TAT	1852
	Leu	Arg	Glu	Thr	Lys	Leu	Phe	Val	Gly	Leu	Gly	Phe	Pro	Tyr	
	505					510					515				
10	GAA	GGT	CCA	GCT	CCC	CTG	GAA	GCC	ATC	GCG	AAT	GGA	TGT	GCT	1894
	Glu	Gly	Pro	Ala	Pro	Leu	Glu	Ala	Ile	Ala	Asn	Gly	Cys	Ala	
		520					525					530			
	TTC	CTG	AAC	CCC	AAG	TTC	AAC	CCT	CCT	AAA	AGC	AGC	AAA	AAC	1936
	Phe	Leu	Asn	Pro	Lys	Phe	Asn	Pro	Pro	Lys	Ser	Ser	Lys	Asn	
15			535					540					545		
	ACA	GAC	TTC	TTC	ATT	GGC	AAG	CCA	ACA	CTG	AGA	GAG	CTC	ACA	1978
	Thr	Asp	Phe	Phe	Ile	Gly	Lys	Pro	Thr	Leu	Arg	Glu	Leu	Thr	
				550					555					560	
20	TCC	CAG	CAC	CCG	TAC	GCA	GAA	GTC	TTC	ATC	GGC	CGG	CCA	CAC	2020
	Ser	Gln	His	Pro	Tyr	Ala	Glu	Val	Phe	Ile	Gly	Arg	Pro	His	
					565					570					
	GTC	TGG	ACC	GTG	GAC	CTC	AAT	AAC	CGA	GAG	GAA	GTA	GAA	GAC	2062
	Val	Trp	Thr	Val	Asp	Leu	Asn	Asn	Arg	Glu	Glu	Val	Glu	Asp	
	575					580					585				
25	GCA	GTA	AAA	GCC	ATC	TTA	AAC	CAG	AAG	ATT	GAG	CCG	TAT	ATG	2104
	Ala	Val	Lys	Ala	Ile	Leu	Asn	Gln	Lys	Ile	Glu	Pro	Tyr	Met	
		590					595					600			
	CCA	TAT	GAG	TTC	ACA	TGT	GAA	GGC	ATG	CTG	CAG	AGA	ATC	AAC	2146
	Pro	Tyr	Glu	Phe	Thr	Cys	Glu	Gly	Met	Leu	Gln	Arg	Ile	Asn	
30			605					610					615		
	GCT	TTC	ATC	GAG	AAA	CAG	GAC	TTC	TGC	CAC	GGC	CAA	GTG	ATG	2188
	Ala	Phe	Ile	Glu	Lys	Gln	Asp	Phe	Cys	His	Gly	Gln	Val	Met	
				620					625					630	
35	TGG	CCG	CCC	CTT	AGC	GCC	CTG	CAG	GTG	AAG	CTG	GCT	GAG	CCC	2230
	Trp	Pro	Pro	Leu	Ser	Ala	Leu	Gln	Val	Lys	Leu	Ala	Glu	Pro	
					635					640					
	GGG	CAG	TCC	TGC	AAA	CAG	GTG	TGC	CAG	GAG	AGC	CAG	CTC	ATC	2272
	Gly	Gln	Ser	Cys	Lys	Gln	Val	Cys	Gln	Glu	Ser	Gln	Leu	Ile	
40						650					655				
	TGC	GAG	CCG	TCC	TTC	TTC	CAG	CAC	CTC	AAC	AAG	GAA	AAG	GAC	2314
	Cys	Glu	Pro	Ser	Phe	Phe	Gln	His	Leu	Asn	Lys	Glu	Lys	Asp	
		660					665					670			

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

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Asp Leu Lys Lys Thr Leu Ala Val Leu Leu Asp Asn Ile Leu Gln Arg
85 90 95

Ile Gly Lys Leu Glu Ser Lys Val Asp Asn Leu Val Asn Gly Thr Gly
100 105 110

5 Ala Asn Ser Thr Asn Ser Thr Thr Ala Val Pro Ser Leu Val Ser Leu
115 120 125

Glu Lys Ile Asn Val Ala Asp Ile Ile Asn Gly Val Gln Glu Lys Cys
130 135 140

10 Val Leu Pro Pro Met Asp Gly Tyr Pro His Cys Glu Gly Lys Ile Lys
145 150 155 160

Trp Met Lys Asp Met Trp Arg Ser Asp Pro Cys Tyr Ala Asp Tyr Gly
165 170 175

Val Asp Gly Thr Ser Cys Ser Phe Phe Ile Tyr Leu Ser Glu Val Glu
180 185 190

15 Asn Trp Cys Pro Arg Leu Pro Trp Arg Ala Lys Asn Pro Tyr Glu Glu
195 200 205

Ala Asp His Asn Ser Leu Ala Glu Ile Arg Thr Asp Phe Asn Ile Leu
210 215 220

20 Tyr Gly Met Met Lys Lys His Glu Glu Phe Arg Trp Met Arg Leu Arg
225 230 235 240

Ile Arg Arg Met Ala Asp Ala Trp Ile Gln Ala Ile Lys Ser Leu Ala
245 250 255

Glu Lys Gln Asn Leu Glu Lys Arg Lys Arg Lys Lys Ile Leu Val His
260 265 270

25 Leu Gly Leu Leu Thr Lys Glu Ser Gly Phe Lys Ile Ala Glu Thr Ala
275 280 285

Phe Ser Gly Gly Pro Leu Gly Glu Leu Val Gln Trp Ser Asp Leu Ile
290 295 300

30 Thr Ser Leu Tyr Leu Leu Gly His Asp Ile Arg Ile Ser Ala Ser Leu
305 310 315 320

Ala Glu Leu Lys Glu Ile Met Lys Lys Val Val Gly Asn Arg Ser Gly
325 330 335

Cys Pro Thr Val Gly Asp Arg Ile Val Glu Leu Ile Tyr Ile Asp Ile
340 345 350

35 Val Gly Leu Ala Gln Phe Lys Lys Thr Leu Gly Pro Ser Trp Val His
355 360 365

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Tyr Gln Cys Met Leu Arg Val Leu Asp Ser Phe Gly Thr Glu Pro Glu
370 375 380

Phe Asn His Ala Ser Tyr Ala Gln Ser Lys Gly His Lys Thr Pro Trp
385 390 395 400

5 Gly Lys Trp Asn Leu Asn Pro Gln Gln Phe Tyr Thr Met Phe Pro His
405 410 415

Thr Pro Asp Asn Ser Phe Leu Gly Phe Val Val Glu Gln His Leu Asn
420 425 430

10 Ser Ser Asp Ile His His Ile Asn Glu Ile Lys Arg Gln Asn Gln Ser
435 440 445

Leu Val Tyr Gly Lys Val Asp Ser Phe Trp Lys Asn Lys Lys Ile Tyr
450 455 460

Leu Asp Ile Ile His Thr Tyr Met Glu Val His Ala Thr Val Tyr Gly
465 470 475 480

15 Ser Ser Thr Lys Asn Ile Pro Ser Tyr Val Lys Asn His Gly Ile Leu
485 490 495

Ser Gly Arg Asp Leu Gln Phe Leu Leu Arg Glu Thr Lys Leu Phe Val
500 505 510

20 Gly Leu Gly Phe Pro Tyr Glu Gly Pro Ala Pro Leu Glu Ala Ile Ala
515 520 525

Asn Gly Cys Ala Phe Leu Asn Pro Lys Phe Asn Pro Pro Lys Ser Ser
530 535 540

Lys Asn Thr Asp Phe Phe Ile Gly Lys Pro Thr Leu Arg Glu Leu Thr
545 550 555 560

25 Ser Gln His Pro Tyr Ala Glu Val Phe Ile Gly Arg Pro His Val Trp
565 570 575

Thr Val Asp Leu Asn Asn Arg Glu Glu Val Glu Asp Ala Val Lys Ala
580 585 590

30 Ile Leu Asn Gln Lys Ile Glu Pro Tyr Met Pro Tyr Glu Phe Thr Cys
595 600 605

Glu Gly Met Leu Gln Arg Ile Asn Ala Phe Ile Glu Lys Gln Asp Phe
610 615 620

Cys His Gly Gln Val Met Trp Pro Pro Leu Ser Ala Leu Gln Val Lys
625 630 635 640

35 Leu Ala Glu Pro Gly Gln Ser Cys Lys Gln Val Cys Gln Glu Ser Gln
645 650 655

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Leu Ile Cys Glu Pro Ser Phe Phe Gln His Leu Asn Lys Glu Lys Asp
660 665 670

Leu Leu Lys Tyr Lys Val Ile Cys Gln Ser Ser Glu Leu Tyr Lys Asp
675 680 685

Ile Leu Val Pro Ser Phe Tyr Pro Lys Ser Lys His Cys Val Phe Gln
690 695 700

Gly Asp Leu Leu Leu Phe Ser Cys Ala Gly Ala His Pro Thr His Gln
705 710 715 720

Arg Ile Cys Pro Cys Arg Asp Phe Ile Lys Gly Gln Val Ala Leu Cys
725 730 735

Lys Asp Cys Leu
740

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 178 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

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

AATACGGATT TCTTCATCGG GAAGCCTACA CTGAGAGAGC TGACATCCCA 50
GCATCCTTAC GCAGAAGTCT TCATCGGCCG GCCACACGTC TGGACTGTGG 100
ATCTCAATAA CCGAGAGGAA GTAGAAGATG CAGTAAAAGC CATCTTAAAC 150
CAGAAGATTG AGCCCTATAT GCCCTACG 178

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 179 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

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

AACACGGACT TTTTATCGG GAAGCCTACA CTGAGAGAGC TCACATCCCA 50
GCACCCGTAC GCAGAAGTCT TCATCGGCCG GCCACACGTC TGGACCGTGG 100
ACCTCAATAA CCGAGAGGAA GTAGAAGACG CAGTAAAAGC CATCTTAAAC 150
CAAAAAATTG AACCCTACAT GCCCTACGA 179

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 166 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (other nucleic acid)

(iii) HYPOTHETICAL: NO

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

AACACGGATT TCTTCATCGG GAAACCCACA CTGAGAGAGC TGACATCCCA 50
GCATCCTTAC GCAGAAGTCT TCATCGGCCG GCCACACGTC TGGACTGTGG 100
ATCTCAATAA CCGAGAGGAA GTAGAAGATG CAGTAAAAGC CATCTTAAAC 150
CAAAGATTG AGCCCT 166

WE CLAIM:

1. An isolated DNA molecule comprising a nucleotide sequence encoding a polypeptide having N-acetylglucosaminyl transferase V activity, said nucleotide sequence having
5 at least 70% identity with a nucleotide sequence as given in SEQ ID NO:15, from nucleotide 299 to nucleotide 2521.
2. The DNA molecule of claim 1 wherein said sequence encodes a mammalian GlcNAc T-V.
3. The DNA molecule of claim 2 wherein said nucleotide
10 sequence encodes rat GlcNAc T-V.
4. The DNA molecule of claim 3 wherein said nucleotide sequence encodes a polypeptide having an amino acid sequence as given in SEQ ID NO:16.
5. The DNA molecule of claim 4 wherein said nucleotide
15 sequence is as given in SEQ ID NO:15, from nucleotide 299 to nucleotide 2521.
6. A DNA molecule comprising the DNA sequence of claim 1 and further comprising an exogenous nucleotide sequence.
7. The DNA molecule of claim 6 wherein said exogenous
20 nucleotide sequence is an expression vector.
8. A transformed or transfected host cell comprising the DNA sequence of claim 1.
9. The transformed or transfected cell of claim 8 wherein said cell is a bacterial cell.
- 25 10. The transformed or transfected cell of claim 9 wherein said bacterial cell is *Escherichia coli*.

11. The transformed or transfected cell of claim 8, wherein said cell is a mammalian cell.

12. The transformed or transfected cell of claim 8, wherein said nucleotide sequence encodes rat GlcNAc T-V.

5 13. The transformed or transfected cell of claim 12, wherein said nucleotide sequence encodes an amino acid sequence as given in SEQ ID NO:16.

10 14. The transformed or transfected cell of claim 13, wherein said nucleotide sequence is as given in SEQ ID NO:15, from transformed or transfected nucleotide 299 to nucleotide 2521.

15. The transformed or transfected cell of claim 14 wherein said mammalian cell is derived from the COS-7 cell line.

15 16. A polypeptide having N-acetylglucosaminyltransferase T-V activity and encoded by the nucleotide sequence of claim 1, with the proviso that the polypeptide is not SEQ ID NO:16.

20 17. A method of producing a polypeptide having N-Acetylglucosaminyl transferase V activity, said method comprising the steps of:

25 (a) operably linking a nucleotide sequence encoding a polypeptide having GlcNAc T-V activity to an expression control sequence to form a GlcNAc T-V expression cassette, said nucleotide sequence having at least 70% nucleotide sequence identity with a sequence as given in SEQ ID NO:15, from nucleotide 299 to nucleotide 2521;

(b) transforming or transfecting a cell to contain the GlcNAc T-V expression cassette of step (b) to form a GlcNAc T-V recombinant cell;

(c) culturing the GlcNAc T-V transformed or transfected cell of step (b) under conditions appropriate for expression of said GlcNAc T-V expression cassette whereby said nucleotide sequence directs the expression of a polypeptide having GlcNAc T-V activity.

18. The method of claim 17 wherein said nucleotide sequence encodes a mammalian GlcNAc T-V.

19. The method of claim 18 wherein said nucleotide sequence encodes a rat GlcNAc T-V.

20. The method of claim 19 wherein said nucleotide sequence encodes a polypeptide having GlcNAc T-V activity and having an amino acid sequence as given in SEQ ID NO:16.

21. The method of claim 20 wherein said nucleotide sequence is as given in SEQ ID NO:15, from nucleotide 299 to nucleotide 2521.

22. A DNA sequence for use in producing a polypeptide having GlcNAc T-V activity, said sequence comprising:

(a) a DNA sequence encoding a polypeptide comprising an amino acid sequence as given in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:16;

(b) a DNA sequence comprising SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:18 or SEQ ID NO:19; or

- (c) a DNA sequence capable of hybridizing to a DNA sequence as given in SEQ ID NO:15, and which sequence encodes a polypeptide having GlcNAc T-V activity.

FIG. 1

1 2 3 4 5 6 7 8 9 10 11

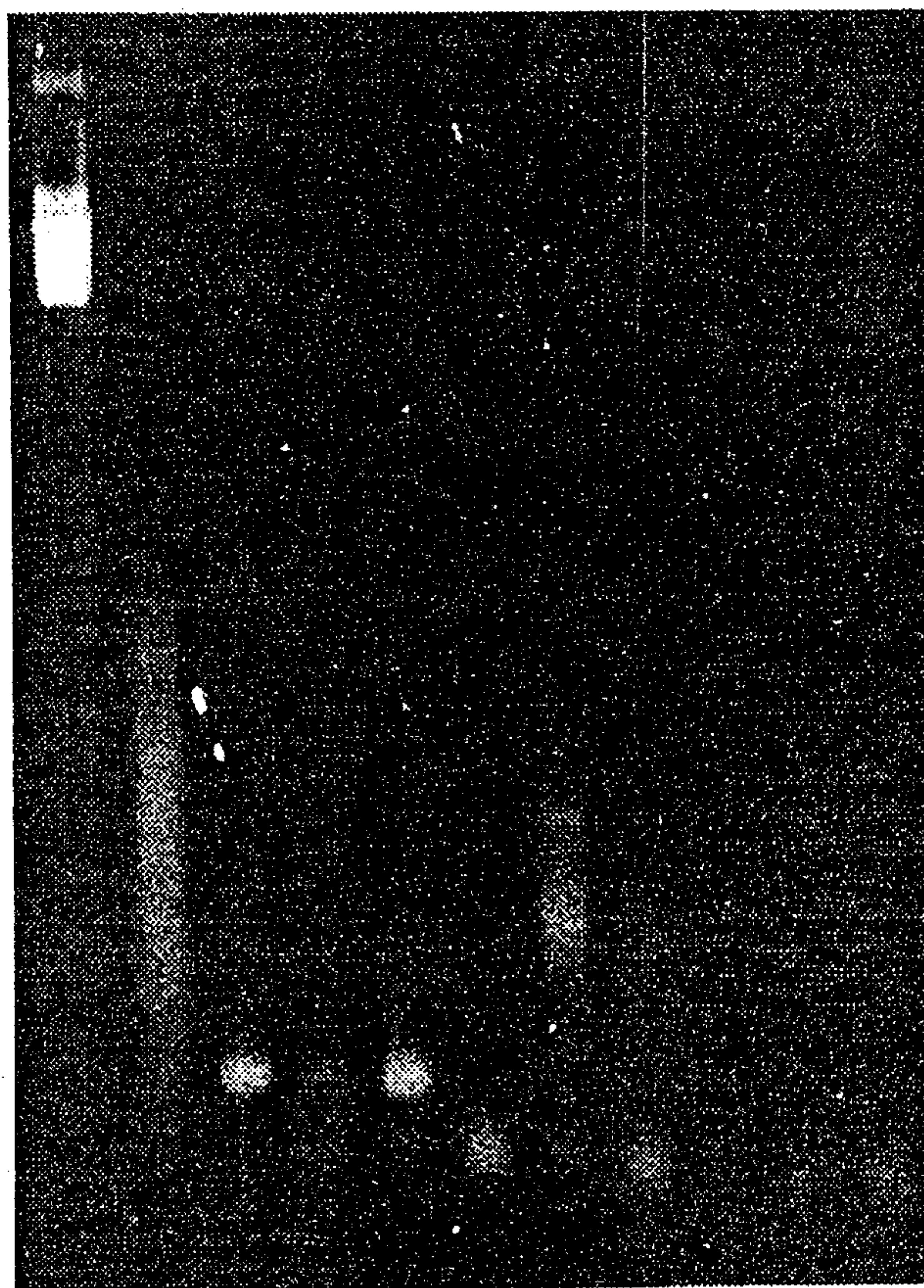


FIG. 2A

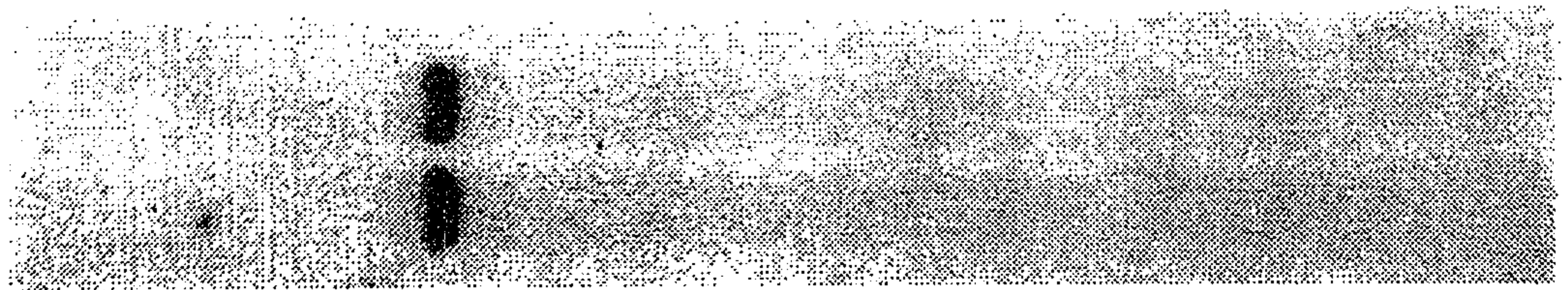


FIG. 2B

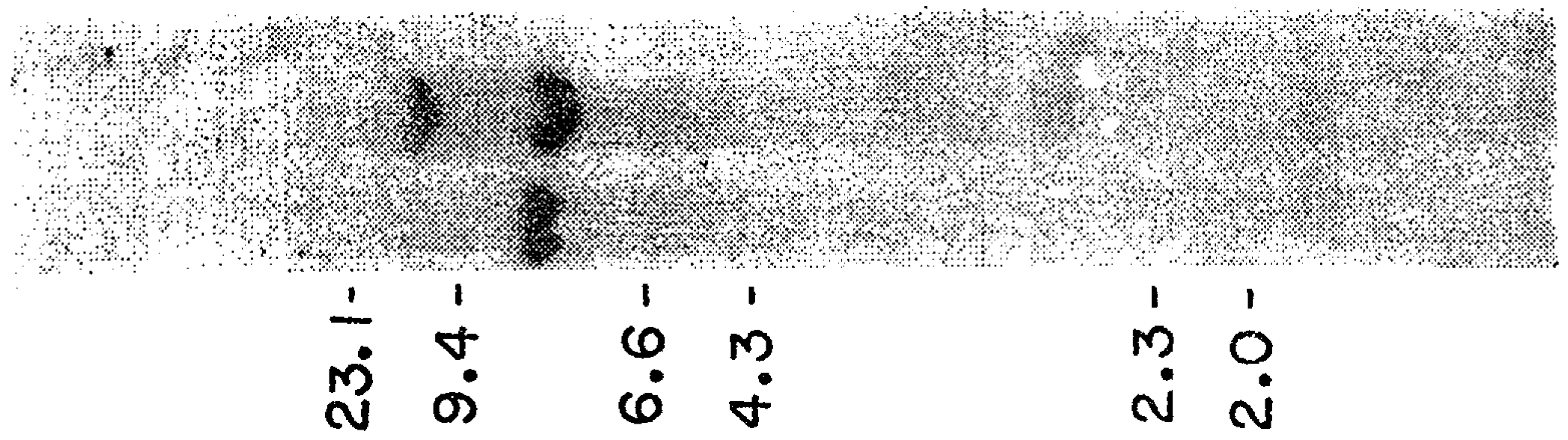


FIG. 2C

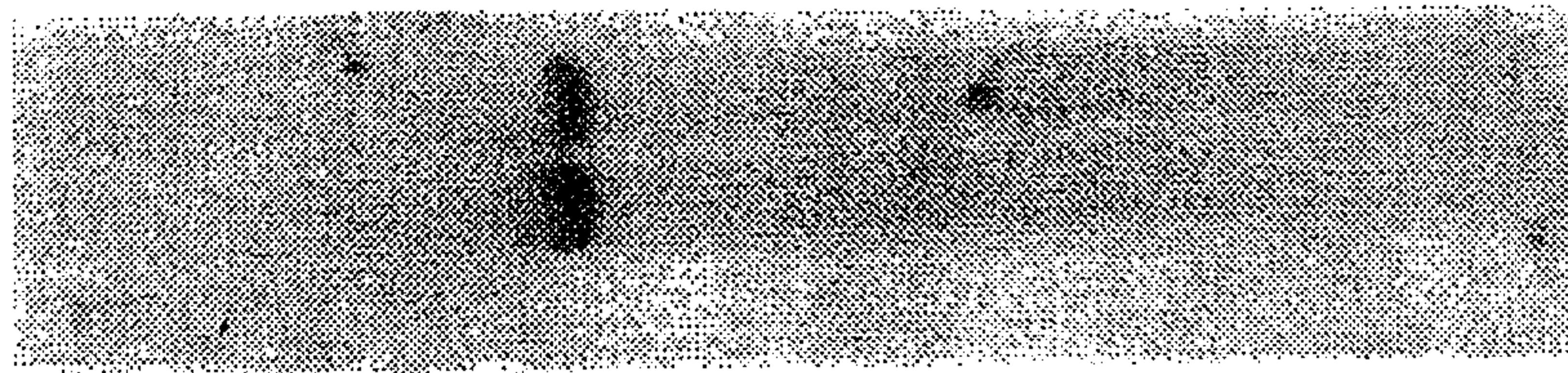
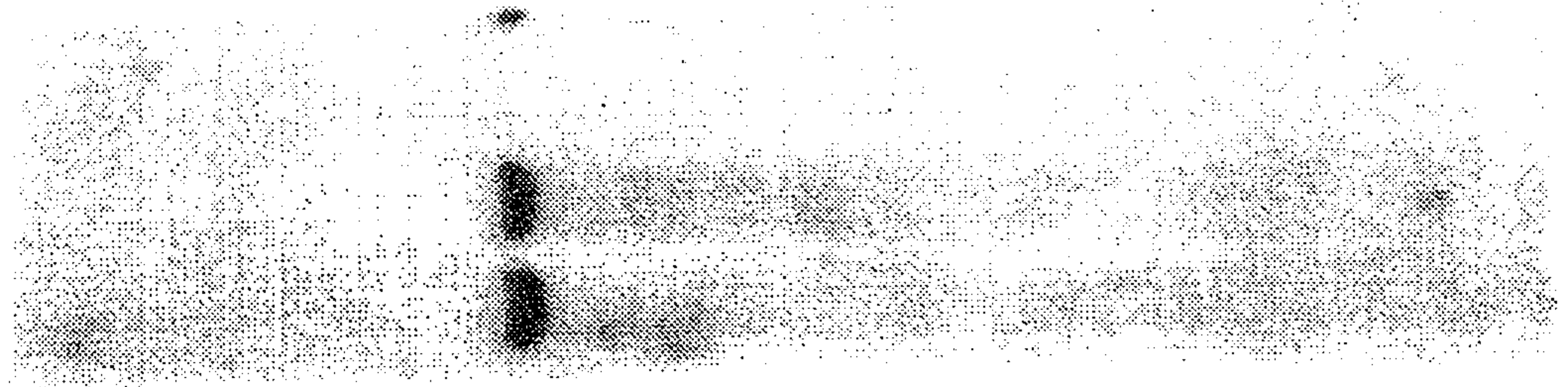


FIG. 2D



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FIG. 3

1 2 3

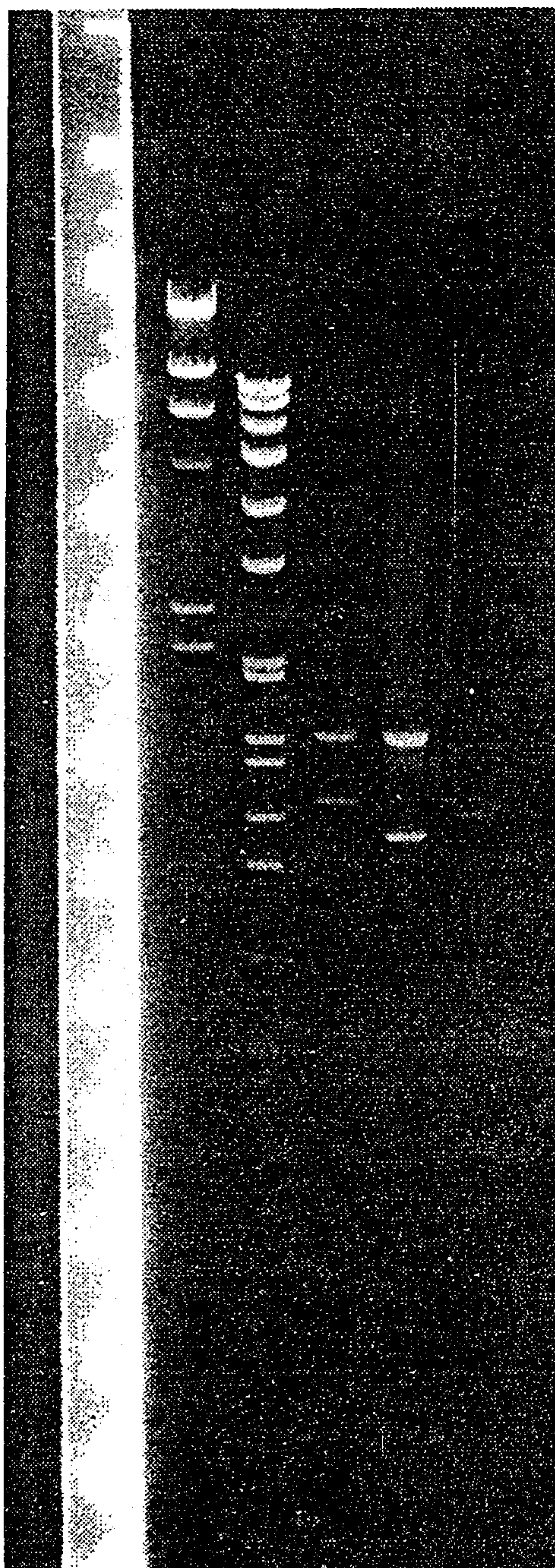
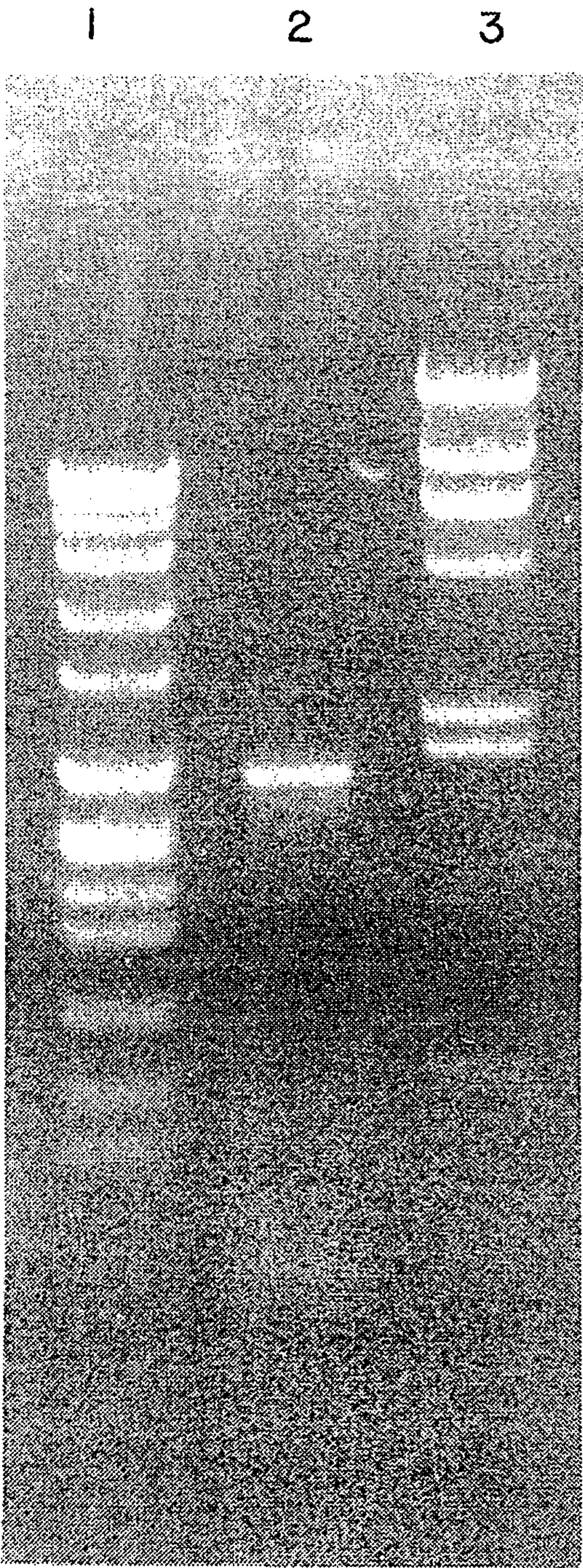


FIG. 4

3 2 1

FIG. 5



1
60
Mouse AATACGGATTCTTCATCGGGAAGCCCTACACTGAGAGAGCTGACATCCAGCATCCTTAC
::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
rat AACACGGACTTTTATCGGGAAGCCCTACACTGAGAGAGCTCACATCCAGCACCCGTAC
::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
Human AACACGGATTCTTCATCGGGAACCACACTGAGAGAGCTGACATCCAGCATCCTTAC

61
120
Mouse GCAGAAGTCTTCATCGGCCGGCCACACGTCTGGACTGTGGATCTCAATAACCGAGAGGAA
::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
rat GCAGAAGTCTTCATCGGCCGGCCACACGTCTGGACCGTGACCTCAATAACCGAGAGGAA
::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
Human GCAGAAGTCTTCATCGGCCGGCCACACGTCTGGACTGTGGATCTCAATAACCGAGAGGAA

121
180
Mouse GTAGAAGATGCAGTAAAAGCCATCTTAAC CAGAAGATTGAGCCCCTATATGCCCTACG
::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
rat GTAGAAGACGCAGTAAAAGCCATCTTAAC CAAAAAATTGAACCTACATGCCCTACGA
::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: ::: :::
Human GTAGAAGATGCAGTAAAAGCCATCTTAAC CAAAAGATTGAGCCCT-----

சென்னை