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A novel second isoenzyme of the human UDP-*N*-acetylglucosamine:α1,3-D-mannoside β1,4-*N*-acetylglucosaminyltransferase family: cDNA cloning, expression, and chromosomal assignment

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Abbreviations: **GnT, *N*-acetylglucosaminyltransferase; GnT-IV, UDP-*N*-acetylglucosamine:α1,3-D-mannoside β1,4-*N*-acetylglucosaminyltransferase; GnT-I, UDP-*N*-acetylglucosamine:α1,3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase; GnT-III, UDP-*N*-acetylglucosamine:β1,4-D-mannoside β1,4-*N*-acetylglucosaminyltransferase; GnT-V, UDP-*N*-acetylglucosamine:α1,6-D-mannoside β1,6-*N*-acetylglucosaminyltransferase; GnT-VI, UDP-*N*-acetylglucosamine:α1,6-D-mannoside β1,4-*N*-acetylglucosaminyltransferase; EST, expressed sequence tag; RT-PCR, polymerase chain reaction following reverse transcription of RNA; 5′-RACE, 5′-rapid amplification of cDNA ends; PCR, polymerase chain reaction; ORF, open reading frame.**

We isolated a novel cDNA encoding a second isoenzyme of UDP-*N*-acetylglucosamine:α1,3-D-mannoside β1,4-*N*-acetylglucosaminyltransferase (GnT-IV; EC 2.4.1.145). The nucleotide and deduced amino acid sequences of the cDNA were homologous to those of the previously cloned human GnT-IV cDNA (63% and 62% identity, respectively). The new cDNA is also confirmed to express GnT-IV activity, suggesting that two isoenzymes of human GnT-IV exist. Although genomic Southern analysis suggested that both genes exist in many mammalian species and the chicken, northern analysis revealed that both genes are expressed in different ways in human tissues. This is the first report concerning the gene family of an *N*-acetylglucosaminyltransferase in mammals.

Introduction

UDP-*N*-Acetylglucosamine:α1,3-D-mannoside β1,4-*N*-acetylglucosaminyltransferase (GnT-IV; EC 2.4.1.145) catalyzes the transfer of GlcNAc from UDP-GlcNAc in β1-4 linkage to Manα1-3Manβ1-4GlcNAc arm of R-Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4 GlcNAcβ1-4GlcNAcβ1-Asn [1,2]. This enzymatic activity has been detected in numerous mammalian species and the chicken [1–8]. A major role of GnT-IV is to produce tri- and tetra- antennary structures of complex type *N*-glycans [1,2,9], which exist in human α1-acid glycoprotein [10], hen ovomucoid [11], human erythropoietin [12], human γ-glutamyltranspeptidase [13], human CD45 [14], lamb and dog (Na, K)-ATPase [15], human CD52 [16], and human intercellular adhesion molecule-1 [17]. However, tri- and tetra-antennary structures occur less frequently than biantennary structures. In addition, a novel hybrid type *N*-glycan structure containing GlcNAcβ1-2(GlcNAcβ1-4)Manα1-3 arm also exists in hen ovalbumin [18]. These observations suggest that the expression and/or substrate specificity of GnT-IV are strictly regulated similar to those of GnT-V [1,5,19,20].

Differences in antenna number of complex *N*-glycans may have significant influences on clearance of bioactive glycoproteins from the serum. Human erythropoietin (hEPO) containing tetraantennary oligosaccharides has a higher *in vivo* biological activity than hEPO with biantennary oligosaccharides [21]. Tetraantennary *N*-glycans prevent rapid clearance from the circulation by the kidney, thereby maintaining higher levels of hEPO in the plasma [22]. Also, branching of *N*-glycans greatly affects the binding of several mammalian lectins, which are involved in

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clearing glycoproteins from serum and in regulating cell-cell interactions. The mouse C-type macrophage lectin [23] and the sialic acid-specific lectin CD22β expressed by B cells [24] bind to terminal sugar residues on tetraantennary *N*-glycans stronger than those on biantennary oligosaccharides. The hepatocyte asialoglycoprotein receptor binds three terminal galactose residues on a certain triantennary structure with the highest affinity [25]. Moreover, endothelial E-selectin preferentially binds to tetraantennary *N*-glycans having the 3-sialyl di-Lewis X structure on the GlcNAcβ1-4 branch synthesized by GnT-IV [26]. Previous studies also showed that the metastatic potency of tumor cells increases with an increase in synthesis of the GlcNAcβ1-6 branch by GnT-V [27,28].

The level of tri- and tetra- antennary *N*-glycans on cells changes during oncogenesis [3,13,28–33], embryo development [7,34,35], and lymphocyte activation [36,37]. Structural analyses of *N*-glycans on γ-glutamyltranspeptidase in hepatocarciogenesis [13] and on chorionic gonadotropin in choriocarcinogenesis [32,33] suggested that these disorders upregulate GnT-IV activity. GnT-IV and -V activity is also modulated during development, suggesting that these enzymes affect cell adhesion [7,35]. Additionally, it has been reported that 1α,25-dihydroxyvitamine D3 induces GnT-IV activity in the promyelocytic cell line HL-60 [4]. Besides these observations, little is known about how GnT-IV activity is regulated at the molecular level.

A glycoprotein having GnT-IV activity was purified to homogeneity from bovine small intestine by Oguri *et al.* [8]. A cDNA for this enzyme has also been cloned from bovine small intestine [38] and another cDNA from human liver [39]. These GnT-IV cDNAs do not have any noticeable homology with GnT-I [40–42], -II [43,44], -III [45,46], -V [47,48], C2GnT [49], IGnT [50] and iGnT [51]. In contrast, we discovered five homologous EST clones which have significant homology to human GnT-IV. We then isolated a 1.6 kb human cDNA, which shares high homology to the previously cloned human GnT-IV cDNA [39] and examined its enzymatic function by transient expression in COS7 cells. Only GnT-IV activity was observed, demonstrating that this cDNA encodes a novel second isoenzyme of human GnT-IV family. We designate this second isoenzyme as GnT-IVb and the previously cloned isoenzyme as GnT-IVa. This is the first identification of isoenzymes for any GnT.

Materials and methods

Isolation of partial cDNA fragments of human GnT-IVb by RT-PCR and 5′-RACE

Human cDNA sequences homologous to human GnT-IVa cDNA [39] were surveyed in the GenBank database by BLAST [52]. Based on cDNA sequences of five homologous EST clones, we synthesized four sets of sense and antisense primers, 5′-ACGATTGTGCAACAGTTCAAGCGT-3′ (2-1F) and 5′-GGGAGAACTCCAGGATCATCCAGT-3′ (1-1R), 5′-ACGATTGTGCAACAGTTCAAGCGT-3′ (2-45F) and 5′- GGGAGAACTCCAGGATCATCCAGT-3′ (2-43R), 5′-ACGATTGTGCAACAGTTCAAGCGT-3′ (2-2F) and 5′-GGGAGAACTCCAGGATCATCCAGT-3′ (2-1R), 5′-ACGATTGTGCAACAGTT CAAGCGT-3′ (2-3F) and 5′-GGGAGAACTCCAGGATCATCCAGT-3′ (2-8R). Using human lung total RNA (Clontech) as a template, we amplified a series of partial cDNA fragments by Access RT-PCR system (Promega). The RT-PCR was carried out for 40 cycles (30 sec at 94 °C, 1 min at 60 °C, and 2 min at 68 °C) after reverse transcription for 45 min at 48 °C and initial denaturation for 2 min at 94 °C on a Gene Amp PCR System 9600 (Perkin Elmer).

The 5′-RACE was performed using Human Lung 5′-RACE-Ready cDNA (Clontech) according to the manufacturer's instruction. An anchor primer and a primer of 5′-ATGACCGAGTCCTCCTTCTCCTGC-3′ (2-5R) were used in the primary amplification. The reaction was carried out for 35 cycles (45 sec at 94 °C, 45 sec at 55 °C, and 2 min at 72 °C). The secondary amplification was performed by using a nested second primer of 5′-ATGCCCATCAC-CACCGACACTCCG-3′ (2-3R) and the same cycling parameters.

The amplified fragments were cloned into pCR-Script Amp SK(+) (Stratagene) or pUC18 (Pharmacia) and sequenced. DNA sequencing was carried out using the DyePrimer Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer) and an ABI PRISM 377 DNA sequencer (Perkin Elmer).

Construction of the expression plasmid pHGT4-2 and its expression in COS7 cells

The putative open reading frame of human GnT-IVb was constructed by joining two RT-PCR fragments. Two pairs of sense and antisense primers, 5′-TTCTCGAGGAGAT-GAGGCTCCGCAATGGC-3′ (2-4F) and 5′-TGCTCCT-TCCGCAGCGCCTGCTTT-3′ (2-9R), and 5′-CGAA-GCACTGTGACCGGCAGAAAG-3′ (2-5F) and 5′-AATC TAGAAATGTGGGCTTCAGGGCTGGC-3′ (2-10R) were used in the same RT-PCR reaction as described above. The resulting two fragments were ligated at a *Bam* HI site (Fig. 1) and introduced into an expression vector pSVL (Pharmacia) by using *Xho* I and *Xba* I sites. The expression construct pHGT4-2 was transfected into COS7 cells (RIKEN Cell Bank, Tsukuba Science City, Japan) by electroporation as described previously [53]. Cells were cultured for 3 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and disrupted in 100 μl of 50 mM Tris-HCl (pH 7.5), 2 mM $MnCl_2$, and 1 mM phenylmethylsulfonyl fluoride by sonication. The supernatant was obtained by centrifuging the lysates at 2,000 × g for 5 min at 4 °C.

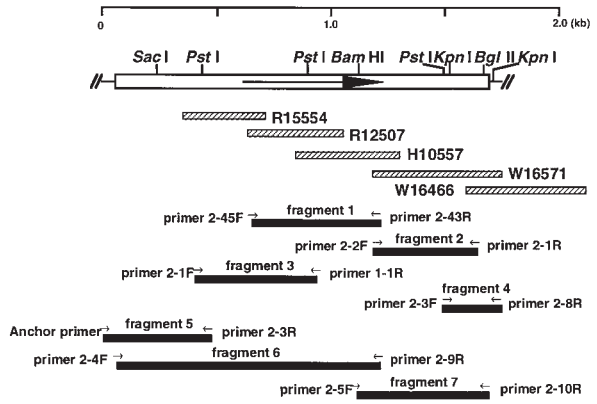

Figure 1. Schematic representation of human GnT-IVb cDNA clones. An open box and thick lines represent the protein coding region of the GnT-IVb and the noncoding regions, respectively. A bold arrow indicates the direction of transcription. Boxes with oblique lines and closed boxes indicate the EST clones and the RT-PCR fragments, respectively. Thin and small arrows represent the position and direction of primers.

Assay for GnT-III, -IV, and -V activity

The GnT-III, -IV and -V activity of cell lysates was measured simultaneously by reversed phase HPLC method using GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3) Manβ1-4 GlcNAcβ1-4GlcNAcβ1-2-aminopyridine(PA), or $Gn_2(2',2)$ core-PA, as an acceptor [8]. The protein concentration of the lysate was determined by Protein assay kit I (Bio-Rad) using bovine γ-globulin as a standard protein.

Northern and Southern hybridizations

Northern blots containing poly (A^+) RNA from various human tissues and cancer cell lines were purchased from Clontech, and hybridized with a ^{32}P-labeled DNA probe specific for human GnT-IVa or -IVb in Rapid hybridization buffer (Amersham) at 65 °C followed by washing twice in 2 × SSPE/0.1% SDS and 0.1 × SSPE/0.1% SDS at 65 °C, respectively [54]. The probe specific for human GnT-IVa was prepared as described previously [32]. The probe specific for human GnT-IVb was prepared using the PCR Radioactive Labeling System (Life Technologies) with [α-^{32}P]dCTP (Amersham), the sense primer 5′-ACAACCCTCAGTCAGACAAGGAGG-3′ (2-3F), the antisense primer 5′-GGTACCCTCAGAAGCCCGCAGCTT-3′ (2-6R), and pHGT4-2 as a template. The PCR reaction was performed with an initial denaturation for 10 min at 94 °C followed by 30 cycles (30 sec at 94 °C, 75 sec at 60 °C, and 2 min at 72 °C) on a Zymo Reactor II (Atto, Tokyo Japan). The blots were also rehybridized using a probe derived from human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene [55]. The probe was prepared by PCR as described previously [39]. A Southern blot containing genomic DNA from various species was obtained from Clontech, and hybridized with the human GnT-IVa or -IVb specific probe in Rapid hybridization buffer at 65 °C. The blot was washed twice in 2 × SSPE/0.1% SDS at 65 °C.

Isolation of the human GnT-IVb genomic clone and chromosomal mapping

A partial genomic fragment encoding human GnT-IVb was amplified by PCR using a sense primer 2-3F, an antisense primer 2-1R, and human genomic DNA (Clontech). Following initial denaturation for 5 min at 95 °C, PCR was carried out for 35 cycles (15 sec at 94 °C, 15 sec at 55 °C, and 20 sec at 72 °C) on a Gene Amp PCR System 9600. The amplified 247 bp fragment was cloned into the pCR-Script Amp SK(+) cloning vector and sequenced. Using the same primer set, Genome Systems screened a human genomic P1 plasmid library by PCR and performed fluorescent *in situ* hybridization. Purified DNA from one of the isolated P1 clones, clone F357, was labeled with digoxigenin-dUTP by nick translation. The labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemagglutunin-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextransulfate and 2 × SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluorescein isothiocyanate-labeled anti-digoxigenin antibody followed by counterstaining with 4,6-diamino-2-phenylindole.

Results

Isolation of human GnT-IVb cDNA

A BLAST search [52] of human cDNA sequences homologous to human GnT-IVa [39] uncovered five human EST clones (accession numbers R15554, R12507, H10557, W16571, and W16466), whose functions have not been identified. Although these partial cDNA clones overlapped one another (Fig. 1), no long ORF was detected. To confirm that the sequences were correct, four fragments (fragments 1 to 4 in Fig. 1) were amplified by RT-PCR and sequenced. These cDNA sequences exhibited a long reading frame of 1299 bp, whose amino acid sequence is highly homologous to the middle part of human GnT-IVa [39]. However, the potential initiation codon of the ORF was not included in these cDNAs. Therefore, the remaining 5′-region of the cDNA was amplified from human lung RNA by a modified 5′-RACE method. Since the products from the 5′-RACE were shown to be heterogeneous in agarose gel electrophoresis, they were cloned into pUC18 using *Eco*RI and *Pst*I. These enzymes target the anchor sequence and just upstream of the primer h2-3R, respectively. A 0.4 kb 5′-RACE fragment completed the GnT-IVb ORF by overlapping with the fragment 3 (Fig. 1). The nucleotide sequences of these five amplified fragments were assembled and shown in Fig. 2. This human GnT-IVb cDNA

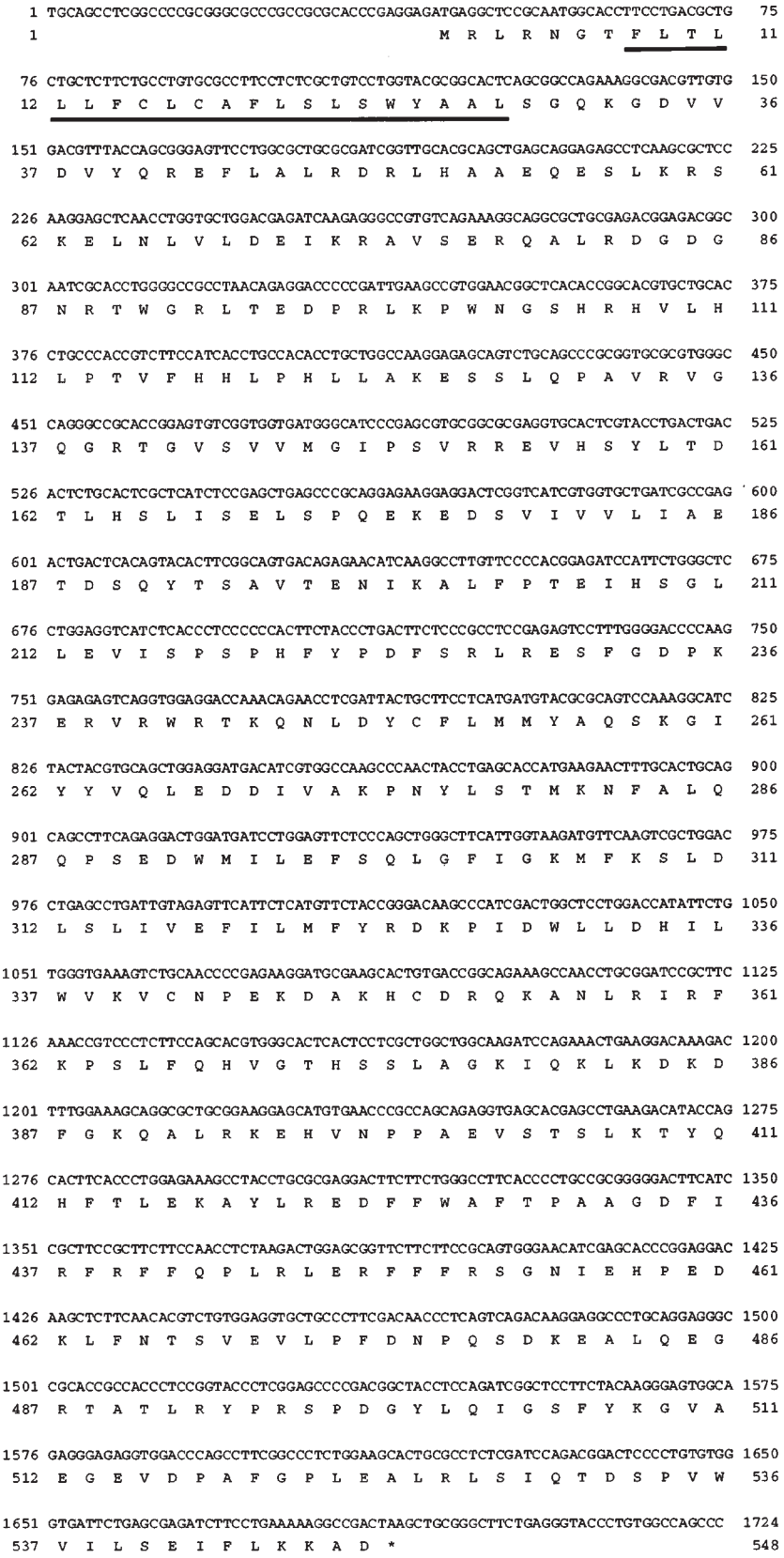

Figure 2. Nucleotide and deduced amino acid sequences of human GnT-IVb. Underline indicates the putative transmembrane domain.

sequence contains a 1644 bp ORF, which is 63% homologous to the coding region of human GnT-IVa cDNA [39].

Expression of GnT-IVb in COS7 cells

A transient expression experiment was carried out to determine the enzymatic function of the GnT-IVb cDNA. The putative coding region was amplified from human lung RNA by RT-PCR and cloned into the mammalian expression vector pSVL. The plasmid, pHGT4-2, which expresses the putative coding region was transfected into COS7 cells by electroporation. The lysates of transfectants were assayed for GnT-III, -IV, and -V activity using a oligosaccharide Gn_2(2′,2)core-PA as an acceptor. As shown in Table 1, GnT-IV activity of the transfectant with pHGT4-2 was 9.2-fold higher than the control transfectant, while neither GnT-III nor -V activity changed. The lysate of the transfectant with pHGT4-2 also did not exhibit GnT-VI activity (data not shown). These data demonstrate that the cloned cDNA encodes a new human GnT-IV. Then, we conclude that human GnT-IV has at least two isoenzymes. We designate the previously cloned GnT-IV as GnT-IVa [38,39] and the newly cloned GnT-IV as GnT-IVb.

Deduced amino acid sequence of human GnT-IVb

The human GnT-IVb was predicted to encode a protein of 548 amino acids with a calculated molecular mass of 63,195. As shown in Fig. 2, the deduced amino acid sequence contains four potential *N*-glycosylation sites at Asn5, Asn87, Asn103, and Asn465, having the consensus sequence Asn-Xaa-Ser/Thr (Xaa≠Pro) [56]. Hydrophobicity analysis [57] predicted that this protein has a type II membrane topology similar to other Golgi glycosyltransferases [58–60] (Fig. 3b). A putative transmembrane domain is amino acids 8–28 which follows a short cytoplasmic tail of 7 amino acids at the N-terminus (Fig. 2).

A comparison of the deduced amino acid sequences between human GnT-IVa [39] and -IVb is shown in Fig. 3A. The overall identity between them was 62%, however, particularly conserved regions were detected at amino acids 1–7 and 118–473 of human GnT-IVb. These regions exist in the putative cytoplasmic domain and the catalytic domain, respectively. One (Asn465) of four potential *N*-glycosylation sites and three (Cys250, Cys341, and Cys350) of five cysteine residues in GnT-IVb were detected at the corresponding positions in GnT-IVa (Fig. 3A). Hydrophobicity analyses indicate that GnT-IVa and -IVb have a similar structure (Fig. 3B). Notably, these enzymes do not exhibit structural similarity with other Golgi GnTs [40–51,60] beyond being type II membrane proteins.

Northern analysis of Human GnT-IVb mRNA in human tissues and cancer cell lines

The expression of human GnT-IVb mRNA in various tissues was analyzed by northern hybridization using a human GnT-IVb specific probe. As shown in Fig. 4, a single strong band of 2.7 kb was detected in all twenty-three normal tissues and eight cancer cell lines tested. No significant difference in the expression level was observed in normal tissues in comparison with the expression of G3PDH. Each cancer cell line also expressed a similar level of GnT-IVb mRNA. These results indicate that human GnT-IVb is constitutively expressed and is unaffected by oncogenesis. This expression pattern of GnT-IVb mRNA was significantly different from that of GnT-IVa mRNA (Fig. 4)

Distribution of GnT-IVb gene in other species

To determine if the GnT-IVb gene exists in other species, we probed a Southern blot containing human, monkey, rat, mouse, dog, cow, rabbit, and yeast genomic DNA with a GnT-IVb specific probe (Fig. 5). Under a high stringency hybridization condition, a single strongly hybridizing DNA fragment was detected in human, monkey, rat, mouse, cow, and chicken. A smear was observed for dog, suggesting a possibility that the probe sequence may be homologous to the dog repetitive sequence. No signal was detectable for rabbit and yeast under the condition used. These results suggest that the GnT-IVb gene exists in many mammalian species and the chicken. Furthermore, the GnT-IVa specific probe gave a different signal for all animals in the same

Table 1. GnT-IV activity in COS7 cells transiently expressing human lung GnT-IVb cDNA. The mean and deviation were determined by two independent transfections.

	specific activity (pmol/hour/mg protein)		
plasmid	*GnT-III*	*GnT-IV*	*GnT-V*
pSVL (vector)	U.D.[b]	1,037	239
pHGT4-1 (hGnT-IVa)[a]	U.D.[b]	25,370 ± 3,582	171 ± 1
pHGT4-2 (hGnT-IVb)	U.D.[b]	9,527 ± 1,498	168 ± 2

[a] Ref.32.
[b] U.D., undetectable.

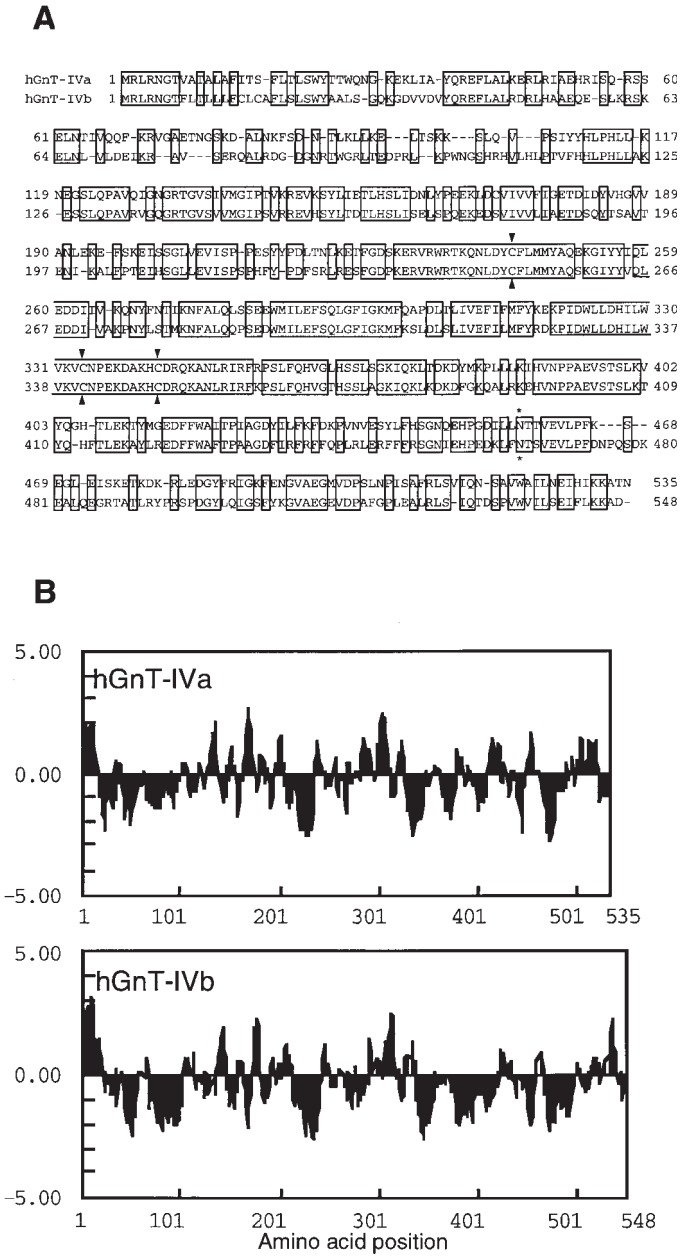

Figure 3. Comparison of human GnT-IVa and -IVb proteins. A, Deduced amino acid sequences of human GnT-IVa [39] and -IVb. Human GnT-IVa and -IVb are presented in upper and bottom lines, respectively. Arrowheads and asterisks indicate conserved cysteine residues and potential *N*-glycosylation sites (Asn-X-Ser/Thr) [56], respectively. B, Hydrophobicity profiles of human GnT-IVa and -IVb. Profiles were made by the method of Kyte and Doolittle [57].

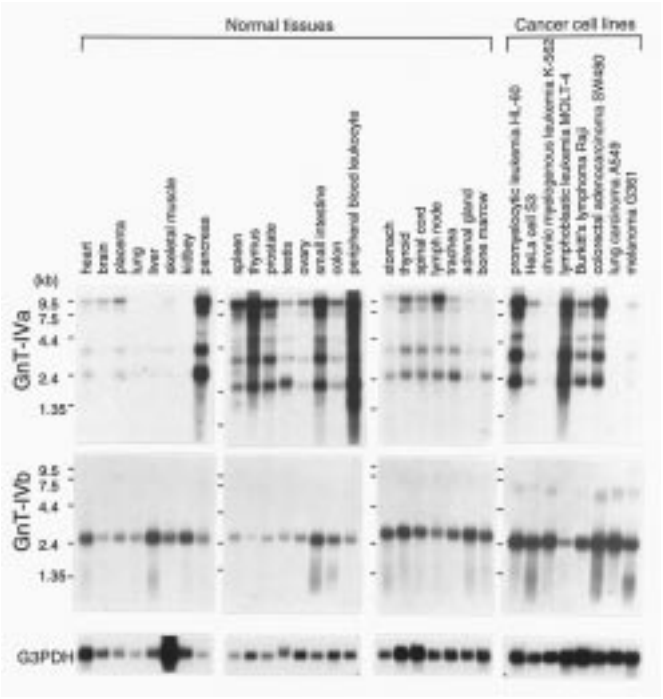

Figure 4. Northern analysis of GnT-IVb mRNA expression in human tissues and cancer cell lines. A ^{32}P-labeled GnT-IVb specific probe was hybridized to Human Multiple Tissue Northern blots (Human MTN, Human MTN II, Human MTN III, and Human Cancer cell line MTN) (middle panel). The blots were also probed with ^{32}P-labeled human cDNA fragments of GnT-IVa and G3PDH (top and lower panels, respectively). The sizes of RNA marker bands are indicated on the left.

blot, suggesting that many mammalian species and the chicken have both GnT-IVa and -IVb genes.

Chromosomal localization of human GnT-IVb gene

A genomic PCR using primers h2-3F and h2-1R amplified a 247 bp human GnT-IVb DNA containing an intron of 69 bp. We obtained a P1 plasmid clone, F357, by PCR screening of human P1 library with the same primers. We then used this clone as a probe to determine the precise chromosomal location of human GnT-IVb gene by fluorescence *in situ* hybridization analysis. The initial experiment produced a specific labeling of the terminus of the long arm of a group B chromosome. This chromosome was believed to be chromosome 5 on the basis of size, morphology, and banding pattern. The following co-hybridization employed the same probe and a probe specific for 5q21. This experiment demonstrated that clone F357 is mapped to the terminus of the long arm of chromosome 5 corresponding to 5q35 (Fig. 6). Sixty-nine of eighty metaphase cells exhibited the same specific labeling.

Discussion

We searched for cDNA sequences homologous to human GnT-IVa [39], and found five human EST clones whose functions were unknown. Based on their cDNA sequences, we isolated a cDNA encoding a new GnT-IV. Although other GnTs including GnT-I [40–42], -II [43,44], -III [45,46], -V[47,48], C2GnT [49], IGnT [50], iGnT [51], and β4GnT from *Lymnaea stagnalis* [61] have been cloned, no isoen-

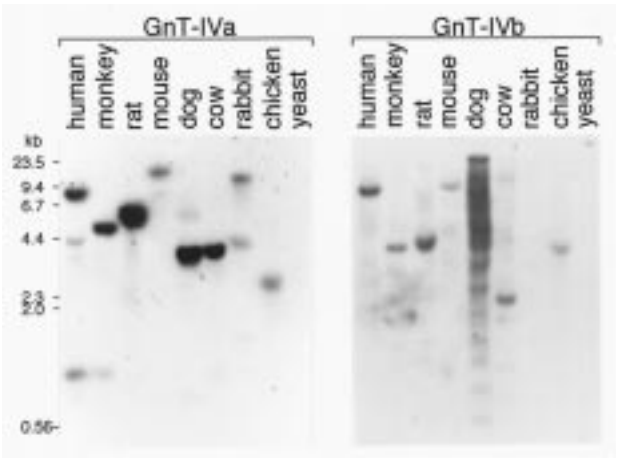

Figure 5. Existence of the GnT-IVb gene in numerous species. A blot containing *Eco*RI digests of genomic DNA from human, monkey, mouse, rat, dog, cow, rabbit, and yeast was probed with a ^{32}P-labeled probe specific to GnT-IVa (left panel) or GnT-IVb (right panel). The sizes of DNA marker bands are indicated on the left.

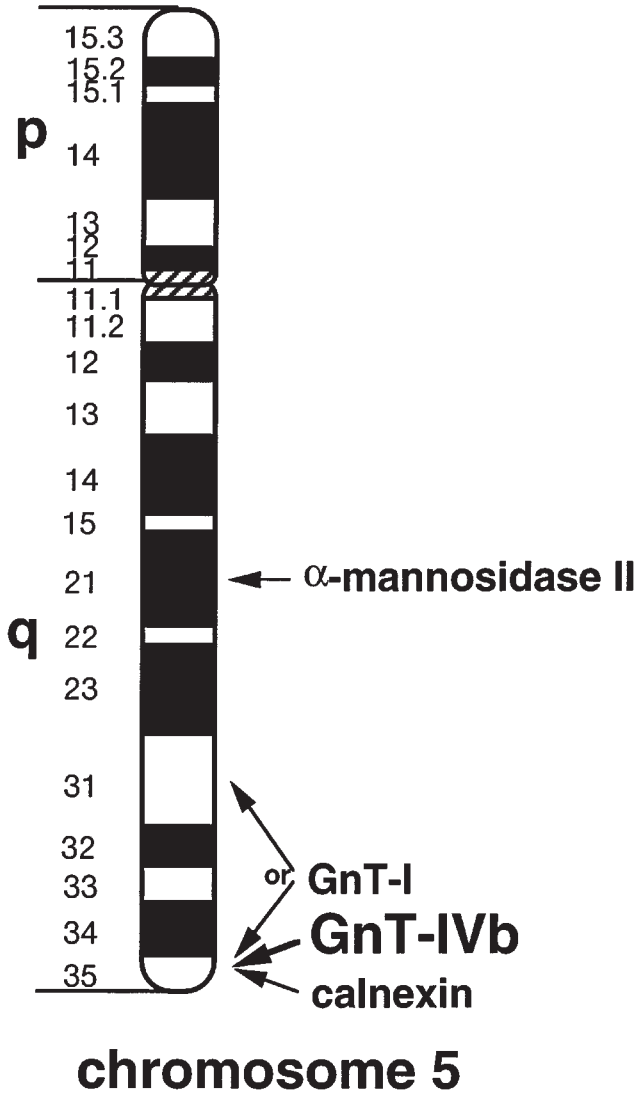

Figure 6. Chromosomal assignment of the human GnT-IVb gene by *in situ* hybridization. Location of the human GnT-IVb gene on chromosome 5 is schematically indicated by the bold arrow. Gene locations of α-mannosidase-II [64], calnexin [65], and GnT-I [42,44] are also represented by thin arrows.

zymes have been identified. Therefore, we are the first to identify isoenzymes of GnTs, specifically GnT-IVa and -IVb. Furthermore, Southern analysis suggested that this new isoenzyme, GnT-IVb, exists in many species such as monkey, mouse, rat, cow, and chicken as well as GnT-IVa (Fig. 5). We have also cloned the mouse GnT-IVb cDNA (unpublished observation).

In complex type *N*-glycans, it is known that there are three different GlcNAc β1-4 linkages to the trimannosyl core and that each linkage is catalyzed by GnT-III, -IV, and -VI, respectively [9]. While cDNAs encoding GnT-III [45,46] and -IVa [39] have been cloned, GnT-VI has not. Since a distinct homology has been reported between two different β1-6 *N*-acetylglucosaminyltransferases, C2GnT [49] and IGnT [50], we suspected that our new cDNA encoded a human GnT-VI. However, no GnT-VI activity was detected in the lysate of COS7 cells transfected with pHGT4-2 (data not shown).

Human GnT-IVb has 62% identity to human GnT-IVa [39], and highly conserved sequences between two isoenzymes were found in the putative cytoplasmic domain and the putative catalytic domain (Fig. 3A). The hydrophobicity profile (Fig. 3B) and secondary structure prediction indicated that the two human GnT-IV isoenzymes have a high structural similarity. Moreover, three cysteine residues and a putative *N*-glycosylation site in the catalytic domain are conserved (Fig. 3A). High conservation of the catalytic domain suggests that a highly specified structure is required to recognize UDP-GlcNAc and acceptor oligosaccharides. The three conserved cysteine residues of GnT-IV may be required for forming intramolecular disulfide bonds. These cysteine residues may also bind to a donor sugar nucleotide since cysteine residues of human β1,4-galactosyltransferase [62] and human α1,3- fucosyltransferases [63] have this function. However, the importance of the conserved *N*-glycan in these GnT-IV isoenzymes is not clear. In bovine GnT-IVa, it has been shown that the corresponding *N*-glycosylation site at Asn458 is not necessary for its folding and enzymatic activity [38].

Amino acid sequences in the transmembrane domain, stem domain, and C-terminal region of human GnT-IVa and -IVb are not conserved. The putative stem domain and the putative catalytic domain of GnT-IVb is 9 and 5 amino acids longer than those of GnT-IVa, respectively [39] (Fig. 3A). The differences in the transmembrane and the C-terminal regions may affect a specific function of each GnT-

IV. The topology of transmembrane domains may influence the cellular localization of these isoenzymes. It is also possible that the difference in C-terminal regions of GnT-IVa and -IVb affect their precise substrate specificity for glycoproteins and oligosaccharides.

Expression patterns of GnT-IVa and -IVb mRNA in normal human tissues are significantly different (Fig. 4). GnT-IVb mRNA is similarly expressed in many tissues whereas GnT-IVa mRNA is abundant in specific tissues such as lymphoid tissues [39]. In addition, GnT-IVb mRNA exists as a single transcript of 2.7 kb while multiple GnT-IVa transcript exist [39]. It is known that GnT-IV activity varies between tissues [5,8], and the differing enzymatic activity in tissues correlates with expression levels of GnT-IVa mRNA but not GnT-IVb mRNA [39]. We then hypothesize that GnT -IVb provides basal enzymatic activity in tissues and that the expression of GnT-IVa is regulated to augment levels of GnT-IV activity. Similarly, expression levels of GnT-IVb mRNA in cancer cell lines are fairly constant (Fig. 4), whereas expression of GnT-IVa mRNA in these cell lines varied [39]. Therefore, oncogenesis may regulate expression of GnT-IVa more often than that of GnT-IVb.

We demonstrated that the GnT-IVb gene exists at 5q35 (Fig. 6) whereas the GnT-IVa gene exists at 2q12 [39]. GnT-IVb cDNA exhibits relatively low nucleotide sequence homology with GnT-IVa (62%), and the G+C content of GnT-IVb (60%) is also significantly different from that of GnT-IVa (38%) [39]. Collectively, these data imply that a primordial gene was duplicated a long time ago. As shown in Fig. 6, the locus of GnT-IVb gene is very close to that of GnT-I gene (5q31 or 5q35) [42,44]. This is interesting because GnT-IV can only transfer GlcNAc to oligosaccharide substrates containing GlcNAcβ1-2 branch synthesized by GnT-I [2,8]. Similarly, the GnT-IVa and -V genes have been mapped on the same chromosome 2 [48]. It has been reported that GnT-IVa prefers to transfer GlcNAc to *N*-glycans containing GlcNAcβ1-6 branch made by GnT-V [2,8]. Co-localization may have provided a selective advantage during evolution. Among other glycosylation relating genes, α-mannosidase II (5q21) [64] and calnexin (5q35) [65] co-localize on the same chromosome 5 (Fig. 6).

The identification of GnT-IVb uncovers a new biosynthetic pathway which generates branching structures in *N*-glycans. It is particularly interesting why two isoenzymes of GnT-IV exist. Presumably, each isoenzyme serves a different purpose *in vivo.* Future investigations will examine substrate specificity, regulation of expression, and protein structure of GnT-IVa and -IVb to elucidate the biological function of each isoenzyme.

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