Tissue-specific Regulation of Mouse Core 2 β-1,6-N-Acetylglucosaminytransferase*

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Mouse kidney β-1,6-GlcNAc-transferase (GNT) is the key enzyme for the synthesis of a glycosphingolipid (Galβ1–4(Fucα1–3)GlcNAcβ1–6Galβ1–3GalNAcβ1–3Galα1–4Galβ1–4Glcβ1-ceramide) that contains the Leα trisaccharide epitope at its nonreducing terminus. The expression of this glycolipid in the kidney is polymorphic; it is expressed in BALB/c but not DBA/2 mice; and a single autosomal gene (Gsl5) is responsible for this polymorphism. We report here the cDNA sequence that encodes the kidney GNT of BALB/c mice, which possess a wild-type Gsl5 gene. The deduced amino acid sequence exhibits 84% identity to that of human core 2 β-1,6-GlcNAc-transferase. The GNT mRNA is expressed abundantly in the kidney, but it was not detected in other BALB/c organs or in the kidneys of DBA/2 mice by Northern blot analysis. In addition, we were able to clone and sequence another homologous cDNA from the submandibular gland. The two sequences differ only in their 5′-untranslated region. The submandibular gland type of cDNA was detected in various organs of DBA/2 mice by reverse transcription-polymerase chain reaction, which indicates that the submandibular gland type is ubiquitous and that its expression is not regulated by the Gsl5 gene. Results obtained using the long accurate synthesis polymerase chain reaction method indicate that the GNT gene is ~45 kilobases long, and the order of the exons from the 5′-end is exon 1 of the kidney type, exon 1 of the ubiquitous type, exon 2, and exon 3. Exons 2 and 3 are present in both transcripts, and the translated region is in exon 3. These data suggest that the expression of GNT is regulated by an alternative splicing mechanism and also probably by tissue-specific enhancers and that Gsl5 regulates the expression of GNT only in the kidney.

Carbohydrate chains of cell-surface glycoconjugates play important roles in cell-cell and cell-matrix communication (1). The diversity of the carbohydrate structures provides a basis for cell-specific recognition. The expression of carbohydrate chains is highly regulated and changes during embryogenesis, differentiation, and oncogenic transformation (2). The regulatory process may be mediated by many different gene products, including glycosyltransferases (3, 4), transcription factors (5–10), and nucleotide sugar transporters (11–14), kinases and phosphatases that act on transferases (15, 16), and other genes. The objective of our studies is to understand the genetic basis and mechanisms that regulate the expression of carbohydrates.

We have identified an autosomal mouse gene (Gsl5) that controls the expression of GlcNAcβ1–6Galβ1–3GalNAcβ1–3Glcβ1-Cer and its elongated glycolipids by regulating β-1,6-GlcNAc-transferase (GNT) activity in the kidney (17). DBA/2 mice are not able to express detectable levels of GNT activity or the glycolipids containing GlcNAcβ1–6Galβ1–3GalNAcβ1–3Glcβ1-Cer as a core structure because of a defect of the Gsl5 gene. To elucidate the role of Gsl5, the mouse kidney enzyme was purified, and its substrate specificity was characterized (18). The results indicated that the enzyme uses Galβ1–3GlcNAc1 derivative as a good substrate, in addition to the actual glycolipid substrate, Galβ1–3GlcNAcβ1–3Glcβ1-Cer. A partial sequence of the purified enzyme spanning 35 amino acid residues exhibited 83% homology to the reported human core 2 β-1,6-GlcNAc-transferase (core 2 Gnt) (19). Core 2 Gnt catalyzes the transfer of β-GlcNAc to the core 1 structure, Galβ1–3GlcNAcα1-Ser/Thr on glycoproteins, and creates the core 2 structure, GlcNAcβ1–6Galβ1–3GalNAcα1-Ser/Thr. The formation of the core 2 structure is essential for further elongation of carbohydrate chains of O-glycans. It is known that human elongated core 2 structures carry Leα, sialyl-Leα, and sialyl-LeX epitopes at the terminus of poly-N-acetylgalactosamine core chains (20) and that the expression of core 2 structures changes in activated human T cells (21), human leukemic cells (22), and T cells of individuals who have Wiskott-Aldrich syndrome (23). Therefore, core 2 Gnt plays a critical role in regulating the expression of O-glycan structures and in cellular physiology.

The polymorphism of the Gsl5 gene provides an excellent model to analyze the regulation of core 2 Gnt. We report here cDNA cloning of two forms of the mouse core 2 Gnt gene and analysis of its genomic organization and chromosomal location.

EXPERIMENTAL PROCEDURES

Materials—All reagents were molecular biology grade. [α-32P]dCTP and [γ-32P]ATP were purchased from NEN Life Science Products.

Preparation of RNA—Total RNA was prepared with ISOGEN reagents (NipponGene Co. Ltd., Tokyo) containing phenol and guanidine isothiocyanate. Male BALB/c and DBA/2 mice (6–8 weeks of age; ob-

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tained from SLC (Shizuoka, Japan) were killed under ether anesthesia. Freshly obtained organs (~200 mg) were cut into small pieces and homogenized with 1 ml of ISOGEN. After adding 0.2 ml of chloroform and mixing vigorously, tubes were centrifuged at 15,000 rpm for 15 min at 4 °C. The transparent upper phase was transferred to another tube, and the precipitate was dissolved in 1 ml of 40% ethanol and was dried. After standing at room temperature for 5–10 min, the tubes were centrifuged again at 15,000 rpm for 10 min at 4 °C. Precipitates were washed once with 1 ml of 75% ethanol, centrifuged again, and then dried briefly. Total RNAs obtained were dissolved in diethyl pyrocarbonate-treated water. Poly(A)-RNA was selected with an Oligotex dT30 (Takara, Tokyo, Japan) according to the manufacturer’s instruction using 1 µg of poly(A)-RNA as a template.

**Primer Extension**—To elucidate the 5′ terminus of RNA, primer extension was performed. A synthetic oligonucleotide primer that was complementary to the sequence from positions 46 to 75 of kidney cDNA was labeled at the 5′-end with [32P]dCTP and a Random labeling kit (Version 2, Pharmacia). The probe used was a PCR-amplified 494-bp cDNA fragment containing the 404-nt 3′-untranslated region and polyadenylation signal (AATAAA) at nucleotide 2021, there might be 100 µg/ml yeast RNA at 42 °C for 2 h. Hybridization was carried out at 42 °C for 16–18 h in the prehybridization solution containing a labeled probe at 3 × 106 cpm/ml. Membranes were washed twice at room temperature in 2 × SSC (1 × SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0) and 0.1% SDS and twice at 65 °C in 1 × SSC and 0.1% SDS. Membranes were analyzed by a BAS2000 bioimage analyzer (Fuji Photo Film Co. Ltd., Tokyo).

The probe was used in a PCR-amplified 494-bp cDNA fragment corresponding to the coding region. It was labeled by the random primer method using [α-32P]dCTP and a Random labeling kit (Version 2, TaKaRa). The probe was hybridized with a Nick column (Pharmacia Biotech Inc.).

**Isolation of BALB/c Mouse Genomic Clones**—A BALB/c mouse genomic library was purchased from CLONTECH. The library was grown in Escherichia coli K802 cells at a density of 3 × 108 plaques/plate (9.5 × 13.5 cm). Plaques were transferred to nitrocellulose membranes (BAS5, Schleicher & Schull), and 1.8 × 108 plaques were screened with one of three 32P-labeled probes, the 494-bp fragment described above or oligonucleotides complementary to kidney or submandibular gland type-specific sequences. Hybridization was performed at 42 °C overnight, and the membranes were washed with 1 × SSC and 0.1% SDS at 65 °C. They were then exposed to x-ray film for 2 days at ~80 °C. Three clones were identified with the 494-bp GNT probe, and one clone each with the oligonucleotide probe for kidney- or submandibular gland-specific fragment sequences. These clones were digested with restriction enzymes and subcloned into the pBluescript II SK− vector.

**LA-PCR**—We used an LA-PCR kit (Version 2, Takara) according to the manufacturer’s protocol. Genomic DNA (200 ng; as a template), oligonucleotide primers (10 pmol each), and LA Taq DNA polymerase (2.5 units) were contained in a 50-µl reaction mixture. Amplification was carried out by one cycle of 94 °C for 1 min, 14 cycles of 98 °C for 25 s and 68 °C for 20 min, and 16 cycles of 98 °C for 25 s, 68 °C for 20–24 min (increasing 1 s/cycle), and 72 °C for 10 min. The reaction mixture was concentrated by ethanol precipitation and then subjected to 0.4% agarose gel electrophoresis (Seakem Gold, FMC Corp. BioProducts).

**RESULTS**

**cDNA Cloning of Mouse Kidney GNT**—We cloned a mouse kidney cDNA of 2047 nucleotides (nt) by using PCR and the 5′- and 3′-RACE methods. As shown in Fig. 1, the amino acid sequence deduced from the cDNA consists of 428 residues with a calculated molecular mass of 49.8 kDa and contains amino acid sequences identical to those of four peptides obtained from the purified enzyme (18). The amino acid sequence also consists of a short N terminus, a hydrophobic transmembrane portion, and a long catalytic domain, indicating the structure of a typical type 2 membrane protein. There are two possible N-glycosylation sites. The sequence exhibits 84% homology to that of human core 2 GNT, suggesting that kidney GNT is a mouse homologue of core 2 GNT.

The cDNA of 2047 nt contains a 5′-untranslated region of 356 nt. Data obtained from the primer extension assay, using kidney mRNA, indicate that the start site is 75 nt upstream from the primer (Fig. 2), which confirms the identification of the 5′ terminus of kidney cDNA shown in Fig. 1.

Northern blotting of kidney RNA with a 494-bp probe (dashed line in Fig. 1) that is part of the open reading frame (ORF) demonstrates that there are two major transcripts, 5.4 and 2.2 kb, in BALB/c mice (Fig. 3). Although the cloned cDNA contains the 404-nucleotide 3′-untranslated region and polyadenylation signal (AATAAA) at nucleotide 2021, there might be another polyadenylation signal farther downstream. Other tissues, including the submandibular gland, liver, spleen, thymus, lung, and brain of both BALB/c and DBA/2 mice, did not give signals on the Northern blot. It should be noted that no signal was detected in the kidney tissue of DBA/2 mice, which suggests that the Gsl5 gene regulates transcription of the GNT gene or that a mutated Gsl5 gene produces an unstable mRNA.

If the core 2 and extended core 2 structures have important biological functions, how do DBA/2 mice compensate for the absence of the enzyme? To answer this question, we looked at cDNAs obtained from the submandibular gland of wild-type mice that express the Gsl5 gene, although signals were not detected on the Northern blot with the 494-bp probe. A cDNA was amplified by 5′-RACE from poly(A)-RNA of the submandibular gland of BALB/c mice. Submandibular gland cDNA is 2024 nt long, which is 23 nt shorter than kidney cDNA. As shown in Fig. 4, the major difference between two sequences is in the length of their 5′-untranslated regions. 80 nt in kidney cDNA and 62 nt in submandibular gland cDNA. Another difference is that the 3′-untranslated region of submandibular gland cDNA is 5 nt shorter than that of kidney cDNA. Aside from these differences, both sequences are identical and have the same ORF. These results suggest the existence of tissue-specific promoters and alternative splicing.
pression of the two transcripts was investigated by RT-PCR with sets of primers that were specific for either kidney or submandibular gland cDNA. As shown in Fig. 5, the kidney type of transcript was highly expressed in BALB/c kidney, but not in DBA/2 kidney, and no signals were detected in other organs of BALB/c and DBA/2 mice. In contrast, the submandibular gland type of cDNA was detected also in the kidney, liver, stomach, spleen, lung, and brain of both mouse strains. These results indicate that the two transcripts are produced by independent transcriptional regulation and that DBA/2 mice have a defect only in the expression of the kidney transcript.

**Fig. 1.** cDNA and deduced amino acid sequence of kidney β-1,6-GlcNAc-transferase. The sequences of four peptides obtained from the purified enzyme are indicated by underlining, and the transmembrane portion is indicated by double underlining. The sequence indicated by the dashed line was used as the probe for Northern and Southern blot analyses. The arrow underlines the oligonucleotide primer used for the primer extension assay. Vertical arrowheads indicate splice sites. The open box and asterisks indicate a polyadenylation signal and putative N-glycosylation site, respectively.

**Fig. 2.** Primer extension analysis. Primer extension was performed with 5 μg of poly(A)⁺ RNA isolated from BALB/c mouse kidney (K lane) as described under “Experimental Procedures.” The product is indicated by an arrowhead. The A, C, G, and T lanes are standard sequence ladders.

**Fig. 3.** Northern blot of β-1,6-GlcNAc-transferase mRNAs from BALB/c and DBA/2 mouse kidney. Two μg of poly(A)⁺ RNA were electrophoresed, blotted, and hybridized with a 32P-labeled 494-bp probe that includes the coding sequence of β-1,6-GlcNAc-transferase and is indicated in Fig. 1.
Genomic Organization—We isolated five clones by genomic screening. Three clones (m4, m7, and m20) were identified by a 494-bp probe specific for a part of the ORF. Clone m42 was identified by a probe specific for a kidney 5'-untranslated region, and clone m32 hybridized with a probe specific for a submandibular gland 5'-untranslated region. Restriction mapping indicated that clones m4, m7, and m20 overlap each other and that clones m42 and m32 are unique. We used LA-PCR to determine the order of the 5'-untranslated regions of the kidney (exon 1) and submandibular gland (exon 1) genes. As shown in Fig. 6A, LA-PCR was performed with a combination of sense primers for the 5'-flanking sequences specific to the kidney type (1s) and submandibular gland type (1's), an antisense primer for a common sequence (2a), and other combinations of sense (1s and 1's) and antisense (1a and 1'a) primers. Fig. 6B displays the results of the LA-PCR analysis. A 17-kb product was obtained using primers 1s and 1'a, and a 25-kb product using primers 1's and 2a, but no products were obtained with reverse combinations of these primers. Although we could not obtain the product using primers 1s and 2a, probably because the distance was too large, we concluded that the kidney-type exon 1 is located 17 kb 5'-upstream of the submandibular gland-type exon 1 and that the common sequence is located 42 kb downstream of exon 1. The genomic DNA from DBA/2 mice, which do not express a kidney-type transcript, gave the same LA-PCR products as that from BALB/c mice (Fig. 6A). We confirmed that nested PCR with these LA-PCR products as templates gave bands with estimated sizes. These results suggest that a deletion or rearrangement detectable by LA-PCR does not occur in DBA/2 mice.

Comparing the sequences of cDNA and genomic DNA, we found that the common sequence was contained in two exons separated by a 2.8-kb intron. The exon/intron boundaries are indicated in the legend of Fig. 6A, and splice-junction sequences are in accord with the GT-AG rule (26). Overall, the data indicate that the GNT gene contains at least four exons spanning over 45 kb and that the coding sequence is in exon 3, as shown in Fig. 6A.

GNT Gene Mapping—Genomic Southern blotting indicates that there is only one copy of the gene per haplotype (data not shown). A restriction fragment length polymorphism analysis was performed using EcoRI to map the GNT gene. BALB/c and AKR/J mice, both of which express GlcNAcβ1–6(Galβ1–3)GalNAcβ1–3Gb3Cer and its extended glycolipids in the kidney, exhibit a 2.7-kb EcoRI fragment of the GNT gene. DBA/2 mice, which do not express those glycolipids, exhibit a 6.2-kb EcoRI fragment. Using EcoRI digests, the strain distribution pattern of the GNT gene was analyzed in recombinant inbred strains of AKR and DBA/2 mice (AKXD recombinant inbred strains). Table I demonstrates that the strain distribution pattern of Gsl5 is identical to that of GNT and that the GNT gene is located between the CD5 and Rln gene loci on mouse chromosome 19. The Gsl5 gene responsible for the expression of the glycolipids and the GNT activity in the kidney was not separated from the GNT gene by the recombinant inbred strains we used. The results described above suggest that the Gsl5 gene...
might encode for a trans-acting transcription factor or might be the promoter region of the GNT gene.

**DISCUSSION**

We have isolated two cDNAs from the kidney and submandibular gland that encode mouse core 2 β-1,6-GlcNAc-transferase. The two cDNAs have identical ORFs and an untranslated exon 2 and differ only in the first exon, which suggests the utilization of alternative promoters and alternative splicing. The submandibular gland type is expressed in many tissues and is ubiquitous, but its expression was so low that we hardly detected it by Northern blot analysis. The other cDNA is found only in the kidney, where it is expressed abundantly and is apparently regulated by a kidney-specific promoter.

DBA/2 mice express the ubiquitous type of mRNA in their kidneys. Using kidney microsomes as a source of enzyme and Galβ1–3GalNAcβ1–3Gb3Cer or Galβ1–3GalNAcα1 derivative as a substrate (18), we detected a very low level of GNT activity, barely above background. These results indicate that the Gsl5 gene is responsible for the expression of high levels of GNT mRNA and protein, in contrast to the low activity of the ubiquitous type of GNT enzyme. Therefore, to characterize the ubiquitous enzyme, further studies are required, including optimization of the conditions for enzyme assay. In addition, we plan to determine whether the activity of the ubiquitous GNT enzyme in DBA/2 kidney is sufficient to produce core 2 and elongated core 2 structures in glycoproteins. The specificity of the enzymes produced by the kidney-type and ubiquitous type mRNAs should be the same because both cDNAs encode the same ORF, unless tissue-specific post-translational modification is involved.

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**Fig. 6.** A, genomic organization of the mouse β-1,6-GlcNAc-transferase gene. Open boxes are exons. E1, exon 1; E1', exon 1'; E2, exon 2; E3, exon 3. E1'-1 and E1'-2 indicate exons of the third transcript, whose cDNA was cloned by Warren (see Footnote 2) from MDAY-Dw33 cells that were established from a DBA/2 mouse. Details are described under “Discussion.” Sequences of splice donor sites are GCAUG/guaagu in the exon 1/intron 1 junction, UCCAG/guaac in the exon 1'/intron 2 junction, AACUG/guag in the exon 1'/exon 4 junction, and AAACU/guaaa in the exon 2/intron 5 junction. Those of acceptor sites are ucuauuuuacag/GCUGC in the intron 4/exon 2 junction and uguccugugcag/UGACA in the intron 5/exon 3 junction. These splice-junction sequences are in accord with the GT-AG rule (26). sub.gl., submandibular gland. B, LA-PCR products for determination of the order of and distance between exons. LA-PCR was achieved between the specific primers of 30 nucleotide length shown in A.
The deduced amino acid sequence of GNT shares 84% homology with that of human core 2 β-1,6-GlcNAc-transferase (19), and the Southern blot indicates that the mouse has only one copy of the GNT gene. We have mapped the mouse GNT gene to chromosome 19, where it is closely linked to the Gsd5 gene. The human core 2 GntT gene was mapped to human chromosome 9q21 (27), and the human chromosome segment including 9q21 exhibits synteny with the mouse segment that includes the Gsd5 and GNT genes (28). These results suggest that the cloned GNT is the mouse homologue of human core 2 GntT, although it has not been demonstrated whether human core 2 GntT can take the glycolipid as a substrate or not. The genomic structures are different, however. Tissue-specific splicing occurs in mice, but not in humans, and a pseudogene is present in humans, but not in mice (29). These results suggest that tissue-specific alternative splicing and kidney-specific enhancement of GNT occurred in mice after the divergence of mice and humans. Encoding of the ORF in one exon is conserved in both species, however.

Tissue-specific alternative splicing has been reported for several glycosyltransferases, including α-2,3- and α-2,6-sialyltransferases (30, 31), β-1,4-Gal-transferase (7, 8), and GlcNAc-transferases I (32) and V (33). The 5'-flanking regions of the α-2,3-sialyltransferase isoforms contain heterogeneous transcriptional start sites and different transcriptional regulation sequences (9). Shaper and co-workers (10) reported that binding sites for Sp1 are present in the 5'-flanking region of the ubiquitous type of β-1,4-galactosyltransferase, and multiple binding sites, including AP2, in the 5'-flanking region of the tissue-specific enzyme. Vallet et al. (34) reported recently that activation of aldolase B in the proximal tubules of mouse kidney requires 5'-flanking region binding sites for hepatocyte nuclear factor 1α, CCAAT/enhancer-binding protein, and D site-binding protein. Igarashi et al. (35) also reported that the hepatocyte nuclear factor 1-binding site is necessary for kidney-specific expression of the Na+K+-Cl- cotransporter gene. Our preliminary sequence analysis suggests that the 5'-flanking regions of the two core 2 GntT transcripts in the BALB/c genome are quite different from each other. The ubiquitous type contains binding sites for Sp1, and the other contains a TATA box, a hepatocyte nuclear factor 1 site, and several other binding sites (data not shown). Analysis of the 5'-upstream sequences of the two BALB/c transcripts and comparison of the BALB/c and DBA/2 sequences are now in progress.

With regard to alternative splicing of the GNT gene in mice, another interesting cDNA has been reported by Warren. It was cloned from MDAY-Dw33 cells, which are derived from a lymphoma of a DBA/2 mouse. Its 5'-untranslated region (306 bp long) is completely different from that of our two cDNAs, and there are differences in seven nucleotides and two deduced amino acids in the open reading frame. These data indicate that the MDAY cDNA is another alternative splicing product. We searched for this 5'-untranslated segment in our genomic clones and found that a 205-bp segment containing the 3'-end (indicated as E'1-2 in Fig. 6A) was included in clone m20 and that the rest of the 101-bp segment at the 5'-side (indicated as E'1-1 in Fig. 6A) was located 5.5 kb upstream from exon 1'-2.

Thus, the genomic arrangement of these three transcripts is exon 1-exon 1'-exon 1'-1'-exon 1'-2-exon 2-exon 3. This scheme explains how the clone of Warren was derived from a DBA/2 mouse and suggests that there are at least three transcripts produced by alternative splicing and tissue-specific promoters. We tried to determine the transcriptional activity of the 5'-upstream sequence of kidney exon 1, ligated to a luciferase gene, in several cell lines derived from kidney, but we have not succeeded yet.

Granovskiy et al. (36) reported expression of core 2 GntT in post-implantation mouse embryos. They performed in situ RNA hybridization using a mouse core 2 GntT cDNA reported by Warren as a probe. The core 2 GntT message was detected in epithelial cells of most mucin-producing organs, such as kidney proximal tubules, intestinal villi, respiratory epithelium, pancreas, and cartilage. The probe they used detects three alternatively spliced transcripts and is not able to determine which type of transcripts are expressed in these tissues. Further studies are necessary to clarify whether the kidney-type mRNA is expressed in mouse embryos and which transcripts are present in DBA/2 embryos.

Understanding the mechanism of regulation of core 2 GntT is essential for studies of functions of core 2 and elongated core 2 carbohydrate chains. It is interesting to consider why alternative promoters are used to produce an identical transferase protein and whether there are any functional consequences of the absence of the kidney type of transferase in DBA/2 kidneys. The polymorphic expression of kidney-specific GNT in BALB/c and DBA/2 mice provides an excellent opportunity to study the regulation and function of this enzyme.

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