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Identification of a New 5′-Noncoding Exon Region and Promoter Activity in Human N-Acetylglucosaminyltransferase III Gene

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In a previous paper (Kim *et al.*, 1996a), the immediate 5′-flanking region and coding region of the human UDP-N-acetylglucosamine:-D-mannoside-1,4-N-acetylglucosaminyltransferase III (N-acetylglucosaminyltransferase-III; GnT-III) gene was reported, isolated and analyzed. Herein, we report on amplification of a new 5′-noncoding region of the GnT-III mRNA by single-strand ligation to single-stranded cDNA-PCR (5′-RACE PCR) using poly(A)+ RNA isolated from human fetal liver cells. A cDNA clone was obtained with 5′ sequences (96 bp) that diverged seven nucleotides upstream from the ATG (+1) start codon. A concensus splice junction sequence, TCTCCCGCAG, was found immediately 5′ to the position where the sequences of the cDNA diverged. The result suggested the presence of an intron in the 5′-noncoding region and that the cDNA was an incompletely reverse-transcribed cDNA product derived from an mRNA containing a new noncoding exon. When mRNA expression of GnT-III in various human tissues and cancer cell lines was examined, Northern blot analysis indicated high expression levels of GnT-III in human fetal kidney and brain tissues, as well as for a number of leukemia and lymphoma cancer cell lines. Promoter activities of the 5′-flanking regions of exon 1 and the new noncoding region were measured in a human hepatoma cell line, HepG2, by luciferase assays. The 5′-flanking region of exon 1 was the most active, whilst that of exon 2 was inactive.

Keywords: D-N-acetylglucosamine:-D-mannoside-1,4-N-acetylglucosaminyltransferase III, Expression, Promoter.

Introduction

Oligosaccharides of glycoproteins and glycolipids alter a wide variety of structures in mammalian cells (Rademacher *et al.*, 1988). These various alterations of the carbohydrate structures are known to associate with development, differentiation, and malignant transformation (Feizi, 1985; Hakomori, 1989). This suggests that cell surface oligosaccharides play a specific role in cell-to-cell interaction. Carbohydrate structures are mainly determined by genes encoding glycosyltransferases and glycosidases. Neoplastic transformation is also associated with changes in complex type N-linked oligosaccharides on the cell surface (Dennis and Lafelt, 1989). Among the alterations of oligosaccharide chains seen in malignant cells are increased branching on the trimannosyl core (Yamashita *et al.*, 1984), increased polylactosaminoglycan chain formation (Pierce and Arango, 1986), and increased sialylation (Warren *et al.*, 1972). The formation of the branches is governed by the activities of a set of N-acetylglucosaminyltransferase(GnT)s (GnT-I to GnT-VI) (Schachter, 1986). Until now, we have cloned several glycosyltransferases such as human GnT-III (Kim *et al.*, 1996a), human Gal 1,4GalNAc 2,3sialyltransferase (Kim *et al.*, 1996b), mouse Gal 1,3(4)GalNAc 2,3sialyltransferase (Kim *et al.*, 1997a), mouse Gal 1,4(3)GalNAc 2,3sialyltransferase (Kim *et al.*, 1997a), human 2,8sialyltransferase (Kim *et al.*, 1997b), mouse 2,8sialyltransferase (Lee *et al.*, 1998), and GnT-V (unpublished result).

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Among them, GnT-III catalyzes the addition of N-acetylglucosamine through a 1-4 linkage (bisecting N-acetylglucosamine) to the linked mannose of the trimannosyl core structure of N-linked oligosaccharides of glycoproteins. GnT-III activity was detected at high levels in a variety of tissues and cell lines (Brockhausen *et al.*, 1991; Ohno *et al.*, 1992; Kim *et al.*, 1996a). In some arguments, increased expression of the GnT-III was closely related to malignant transformation or oncogenesis. The biological significance of increased GnT-III expression has not yet been clearly understood. However it is known that elevated GnT-III activity might cause an imbalance in branching on the trimannosyl core by inhibiting further branching by GnT-II, IV, V, and 1-4GT (UDP-galactose: N-acetylglucosamine 1-4 galactosyltransferase) at the substrate level (Schachter, 1986). Therefore, the changes of GnT-III levels seem to play a key role in the alteration of N-glycan structures. Recently, the enzyme was purified from rat kidney and the cDNAs encoding rat and human GnT-III were cloned (Nishikawa *et al.*, 1992; Ihara *et al.*, 1993). Human gene GnT-III, coding for 531 amino acids, was mapped to chromosome 22q.13.1. Unlike the other glycosyltransferases, including GnT-V, which are composed of large numbers of exons and introns (Saito *et al.*, 1994), the whole coding region for GnT-III is believed to be included in a single exon (Ihara *et al.*, 1993; Kim *et al.*, 1996a). It was known that GnT-III expression is tissue-specific, being prominent in rat kidney (Nishikawa *et al.*, 1992), and it was dramatically increased in proportion to the enzyme activity in metastatic lesions or during hepatocarcinogenesis in LEC (Long-Evans with a cinnamon-like coat color) rats (Miyoshi *et al.*, 1993). Although the coding region of GnT-III has been cloned and sequenced, the precise structures of the 5′-noncoding regions and regulatory regions have not been characterized. Cloning and analysis of the genetic regulatory elements of this gene should yield important insights into the mechanisms that underlie the tissue- and stage-specific expression of GnT-III. Therefore, in order to characterize the region, we have isolated the immediate 5′-flanking region (1651 bp) of the GnT-III from a human placental genomic library in the previous reports (Kim *et al.*, 1995; 1996a).

In this paper, as a primary step towards elucidating the regulation mechanism of human GnT-III gene expression, we have tried to isolate and identify the new 5′-noncoding exon of the GnT-III gene using the 5′-RACE PCR method. Also, we describe that the GnT-III gene has a new 5′-noncoding exon region which was not yet reported, and also, the promoter activity of the genomic DNA fragment containing the 5′-flanking region, and the expression levels of the GnT-III gene in various cells.

Materials and Methods

Screening of human genomic DNA library To use a probe for the screening, human GnT-III cDNA was cloned from human fetal liver cDNA by PCR using primers designed from the sequence described previously (Kim *et al.*, 1996). This cDNA, which included the coding sequence, was digested with *Sac*I and electrophoresed on an agarose gel. The *Sac*I fragment (1.5 kb) was purified using a GeneClean kit (BIO 101, Vista, USA). The 1.5kb-*Sac*I cDNA fragments were labeled with [α-^{32}P] dCTP (~3000 Ci/mmol, Amersham, Richmond, USA) using the Random Primed labeling kit (Boehringer Mannheim, Mannheim, Germany), as described previously (Kim and Kim, 1996).

Using this cDNA fragment as a probe, approximately 6×10^5 phages from a human placental genomic library constructed in EMBL3 were screened to isolate a 5′-flanking region of the GnT-III gene (Sambrook *et al.*, 1989). Replica filters were hybridized at 42°C in a buffer containing 50% formamide, 5× SSPE (pH 7.4), 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA. After 20 h hybridization, the filters were sequentially washed 3 times at room temperature in 2× SSC/0.1% SDS, then further washed twice at 55°C in 1× SSC/0.1% SDS for 20 min. One of the hybridization-positive clones (HG12.5; Kim *et al.*, 1996a) containing a 12-kb insert was subjected to restriction map analysis and subcloning.

Recombinant DNA techniques Subcloning and restriction mapping of cDNA inserts and genomic DNA fragments were carried out as described by Sambrook *et al.* (1989). Both strands were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using a Sequenase 2.0 kit (United States Biochemical Co., Cleveland, USA). Purifications of phage, plasmid DNA, and cDNA inserts were carried out using standard methods (Sambrook *et al.*, 1989).

RNA isolation and amplification of the 5′-noncoding region (5′-RACE) Total RNA was extracted with guanidine thiocyanate, phenol, and chloroform according to Chomczynski and Sacchi (1987). Poly(A)+ RNA was isolated by two successive passages through an oligo(dT)-cellulose column (Aviv *et al.*, 1972). The 5′-noncoding region of GnT-III mRNA was amplified from human fetal liver poly(A)+ RNA by single-strand ligation to single-stranded cDNA-PCR using the 5′-Ampli FINDER rapid amplification of cDNA ends kit (Clontech, Palo Alto, USA). First strand cDNA was synthesized using a GnT-III primer, PE primer (5′-GGGGCATTGTTCCAGAAAAAG-3′, complementary to the sequence located 146–166 bp downstream from the translation initiation site). An anchor oligonucleotide (supplied with the kit) was ligated to the cDNA, which was then amplified twice by PCR using a primer complementary to the anchor and one of two GnT-III primers: KA1 primer (5′-AGAAGTGCAGGAAGGAGATGAGGC-3′, 47–70 bp downstream from the translation initiation site) or TSPA primer (5′-GGCCATACAGAACATGAGAAAGAGC-3′, 15–39 bp downstream from the translation initiation site). PCR products were then directly cloned in pBluescript SK(+) and sequenced.

Expression of GnT-III in various human cancer, normal, and fetal cells Promyelocytic leukemia HL-60, HeLa S3, chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361 cell lines were provided by the JCRB (Japanese Cancer Research Resources Bank) and GenBank, Genetic Resources Center, Korea Research

Institute of Bioscience and Biotechnology. Cells were cultured in DMEM or RPMI 1640 medium with 10% fetal calf serum and 150 mg/ml kanamycin. Human adult and fetal tissues were supplied by Prof. T.-W. Chung, Kyungpook University Hospital. Total RNAs were prepared from the confluent cells or tissues according to the method described by Chymczynski and Sacchi (1987). Northern blot analyses were performed as described previously (Sambrook *et al.* 1989; Kim *et al.*, 1998). The *Sac*I fragment of GnT-III cDNA (Kim *et al*, 1996a) was used as a probe for detection.

Construction of GnT-III-promoter-luciferase-gene fusion vector A 7.8-kb *Eco*RI–*Eco*RI fragment containing GnT-III exon-1 was isolated from pHG7.8 (Kim *et al.*, 1996a), digested by *Sac*I, and pGnTp16-1.6 was obtained by subcloning the 1.6-kb *Eco*RI–*Sac*I fragment into *Eco*RI–*Sac*I sites of the enhancer vector. The 0.9-kb *Xho*I–*Sac*I fragment was excised from the pGnTp16-1.6 and subcloned into the same *Xho*I–*Sac*I sites of the enhancer vector to construct the pGnTp16-0.9. A series of deletion plasmids of pGnTp16-1.2, pGnTp16-0.6, and pGnTp16-0.4, containing 0.4-kb, 1.0-kb, and 1.2-kb deletions, from the pGnTp16-1.6, respectively, were constructed.

Transfections of HepG2 cells with GnT-III-promoter-luciferase-gene fusion vector HepG2 cells were plated 24 h prior to transfection onto 35-mm dishes at a density of 1.7×10^6 cells/dish. DMEM (high glucose-supplemented) containing 10% fetal calf serum was used for the medium. Cells were transfected with 10 μg of plasmid DNA. A control vector containing Simian virus 40 (SV40) promoter and enhancer was used as the positive control, while an enhancer vector containing the SV40 enhancer but no promoter was used as the negative control. Transfected cells were cultured at 37°C in 3% CO_2 for 24 h, followed by 5% CO_2 for 24 h, and then used for the luciferase assays.

Luciferase assays Luciferase assays were performed with PicaGene. Cells were washed twice with phosphate buffer (4 mM NaH_2PO_4, 1.4 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl), and then 0.25 ml of PicaGene cell lysis solution was added. After a 15-min incubation at room temperature, cells were suspended, and cell debris was removed by centrifugation. 2 μl of cell lysate and 50 μl of luciferase substrate solution were mixed and the light intensity was measured using a luminometer (Bio-Rad Co., Richmond, USA). Protein concentration was measured by the Sigma protein assay kit.

Results and Discussion

We have previously identified the immediate 5′-flanking region from a human placental genomic library by plaque hybridization using the 1.5-kb *Sac*I cDNA fragment, corresponding to the coding region of the human GnT-III gene as a probe (Kim *et al.*, 1996a). One hybridization-positive clone containing a 12-kb insert was identified and mapped with restriction enzyme digestion. Of the two *Eco*RI fragments (7.8-kb and 4-kb) contained in the insert, only the 7.8-kb fragment hybridized to the 1.5-kb *Sac*I probe. Subsequently, a 4.5-kb *Eco*RI–*Hin*dIII subfragment was identified by Southern blotting and hybridization (data not shown). To identify whether the whole coding region for GnT-III is included in a single exon as suggested previously (Ihara *et al.*, 1993; Kim *et al.*, 1996a), we have isolated a 2.0-kb *Kpn*I–*Sac*I fragment from the 4.5-kb *Eco*RI–*Hin*dIII genomic fragment. DNA sequencing and restriction enzyme analysis showed that the full-length coding region for human GnT-III is included in this fragment (2.0-kb) and has the same sequence as that reported previously for the cDNA clone. We have analyzed a 2.0-kb *Eco*RI–*Sac*I fragment, including 1.7-kb of the 5′-noncoding region and 0.3-kb of the 5′-coding region from the human GnT-III, which was isolated from the 4.5-kb *Eco*RI–*Hin*dIII fragment.

Figure 1 shows the immediate 5′-flanking region of the human GnT-III gene (Kim *et al.*, 1996a) and nucleotide sequence of new 5′-noncoding exon which was first identified using 5′-RACE in this paper. According to the analysis of several putative promoter elements in the immediate 5′-flanking region of the human GnT-III gene, the region lacks a TATA-box and CCAAT-box and has eleven IRE consensus sequences, seven potential AP-2 binding sites, two SP1 consensus sequences (GC boxes), and two GRE sequences similar to the half-palindromic glucocorticoid-responsive element. Two possible sequences similar to the cAMP responsive regulatory element were also found in the region. These characteristics are typical of promoters for housekeeping genes. Moreover, in some genes (developmentally regulated genes, tissue-specific genes, and highly regulated genes) that have promoters lacking TATA boxes, binding of SP1 has been reported to play a critical role in transcription initiation (Pugh and Tjian, 1991). However, considering that the expression of GnT-III mRNA and enzyme activity are dramatically increased during hepatocarcinogenesis (Miyoshi *et al.*, 1993), it is notable that consensus recognition sequences for hepatocyte-specific transcription factor HNF-1 and HNF-4 (Lichtsteiner *et al.*, 1989) were not found in the 5′-flanking region (1651 bp) of the GnT-III gene. We also found that GnT-III transcripts were significantly increased in liver cancer tissue (data not shown). From these results, it is suggested that a new regulatory region of the human GnT-III gene may be present upstream from the immediate 5′-flanking region.

To isolate the other new regulatory region of the GnT-III gene, we amplified the 5′-noncoding region of the GnT-III mRNA by single-strand ligation to single-stranded cDNA-PCR using poly(A)+ RNA isolated from human fetal liver cells (Fig. 2). A cDNA clone was obtained with new 5′ sequences (96 bp) that diverged seven nucleotides upstream from the ATG (+1) start codon. A consensus splice junction sequence, TCTCCCGCAG, was found immediately 5′ to the position where the sequences of the cDNA diverged (Fig. 1). This result suggested the presence of an intron in the 5′-noncoding region and that the cDNA is an incompletely reverse-transcribed cDNA product derived from an mRNA containing a new exon.

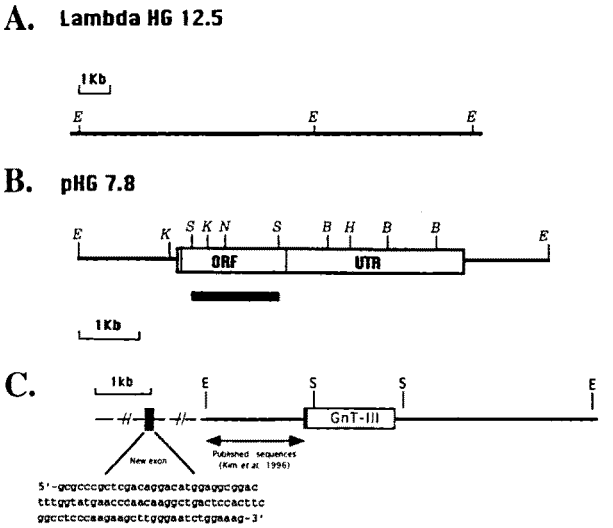

Fig. 1. Schematic representation of the human GnT-III gene and a new 5′-noncoding exon region, and sequence of 5′-flanking region. A. Physical map of Lambda HG12.5. B. Physical map of pHG7.8 carrying GnT-III ORF and 3′-UTR regions. C. Structure of a newly-isolated exon region and Exon-I. D. Sequence of intron-1 region of GnT-III gene. Exons are indicated by boxes (open box represents the coding sequences). The new exon was isolated by 5′-RACE using primers complementary to sequences within the coding region as described in Materials and Methods. E, *Eco*RI; S, *Sac*I; K, *Kpn*I; N, *Not*I; B, *Bam*HI restriction cutting sites. ORF, open reading frame; UTR, 3′-untranslated region. The nt sequence from −1651 to +94 is shown. Numbering is in relation to ATG (+1), which was previously proposed as a possible start codon (Kim *et al.*, 1996). Underlines indicate the putative promoter elements based on sequence comparison to known motifs. GRE, half-palindromic glucocorticoid response element (TGTCCT); recognition sites for CREB (CGTGACGA), AP-2 (GGCCTGGGGA), and SP1 (GGGCGG). The EMBL data library Accession No. is L48489.

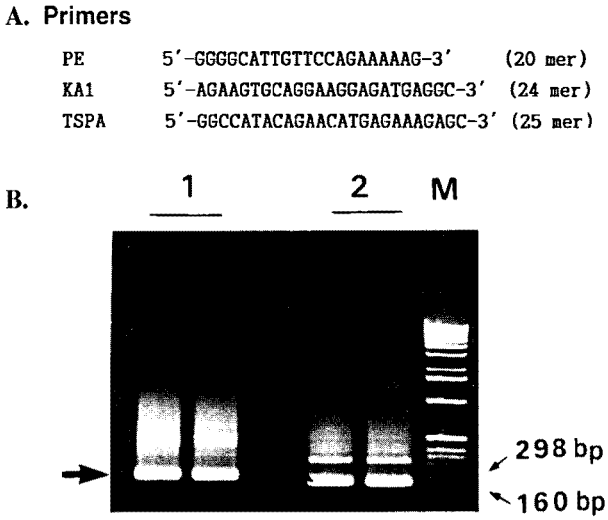

Fig. 2. PCR amplification of the 5′-ends of the human GnT-III gene using 5′-AmpliFINDER RACE kit. Human fetal liver poly (A)+ RNA was reverse-transcribed with PE primer, and the anchor was ligated to the cDNA. The anchor-ligated cDNA was amplified twice by PCR using the anchor and two primers; KA1 and TSPA. A. Primers used for secondary PCR. B. Agarose gel electrophoresis of secondary PCR products. Lane 1, PCR product generated using the anchor and KA1 primer; lane 2, PCR product generated using the anchor and TSPA primer; lane M, 1 kb DNA size markers. The arrow indicates the newly amplified 200 bp DNA fragment carrying 96 bp DNA fragment of the new exon region.

To examine the extent of GnT-III gene expression in human adult and fetal tissues, Northern blot analysis was performed. GnT-III mRNA was detected as a single band of 4.7 kb in fetal kidney and fetal brain tissues, and slightly in fetal liver tissue, but not in other tissues (Fig. 3). Although GnT-III was not detected in normal tissues, the gene was highly expressed only in the brain tissue (Fig. 4). To determine which types of human cancer cell lines express GnT-III, Northern blot analysis was likewise performed. GnT-III mRNAs were highly detected in cancer cell lines such as promyelocytic leukemia HL-60 and chronic myelogenous leukemia K-562; however, lymphoblastic leukemia MOLT-4 and Burkitt's lymphoma Raji cells showed low levels of expression of GnT-III mRNA.

To investigate the promoter activities of the flanking regions of the noncoding region and exon-1, luciferase assays were carried out. Because the noncoding region was isolated from the human fetal liver library, we used human hepatoblastoma HepG2, which has GnT-III activity (Kim *et al.*, 1997c), for luciferase assays. The flanking regions were subcloned upstream of the luciferase gene in the enhancer vector in which the luciferase gene without promoter, SV40 enhancer, and SV40 replication origin were inserted into the *E. coli* plasmid. The plasmids,

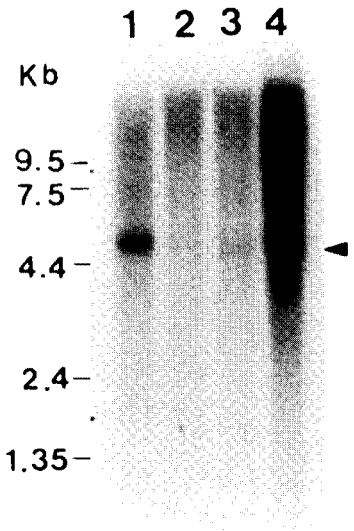

Fig. 3. Northern blot analysis for the GnT-III transcripts in various human fetal tissues. About 20 μg of poly(A)+ RNA was applied on each lane in 18% formaldehyde agarose gel and then hybridized as described in Materials and Methods. The *Sac*I fragment of GnT-III cDNA (Kim *et al.*, 1996a) was used as a probe for detection. Lane 1, brain; 2, lung; 3, liver; 4, kidney. The left lane indicates DNA size markers.

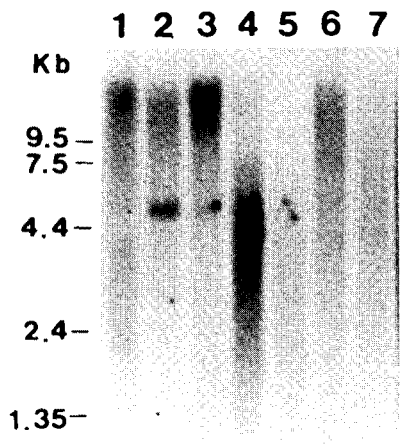

Fig. 4. Northern blot analysis for the GnT-III transcripts in various human normal tissues. About 20 μg of poly(A)+ RNA was applied on each lane in 18% formaldehyde agarose gel and then hybridized as described in Materials and Methods. The same probe as that described in Fig. 3 was used for detection. Lane 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney. The left lane indicates DNA size markers.

designated pGnTp16-1.6, pGnTp16-1.2, pGnTp16-0.9, pGnTp16-0.6, and pGnTp16-0.4, were transfected into HepG2 cells transiently and after 24 h the luciferase activities of the cell lysates were determined by measuring luminescence. As controls, the enhancer vector and the

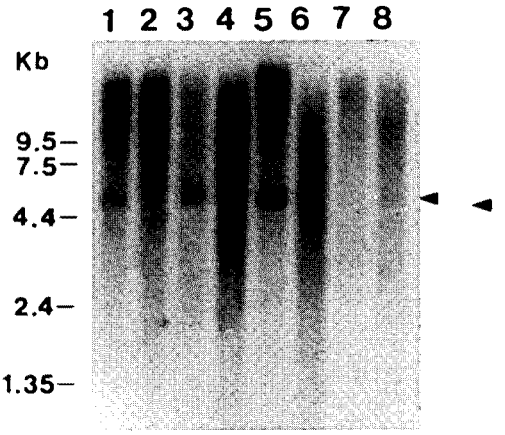

Fig. 5. Northern blot analysis for the GnT-III transcripts in various human cancer cells. About 20 μg of poly(A)+ RNA was applied on each lane in 18% formaldehyde agarose gel and then hybridized as described in Materials and Methods. The same probe as that described in Fig. 3 was used for detection. Lane 1, promyelocytic leukemia HL-60; 2, HeLa cell S3; 3, chronic myelogenous leukemia K-562; 4, lymphoblastic leukemia MOLT-4; 5, Burkitt's lymphoma Raji cell; 6, colorectal adenocarcinoma SW480; 7, lung carcinoma A549; 8, myeloma G361. The left lane indicates DNA size markers.

control vector were used in which the SV40 promoter is inserted into the upstream region of the luciferase gene of the enhancer vector. The high activity of luciferase was observed in cells transfected with pGnTp16-0.9 (Fig. 6), but no activity was observed with pGnTp16-1.6, pGnTp16-1.2, pGnTp16-0.6, or pGnTp16-0.4. Although pGnTp16-1.6 and pGnTp16-1.2 exhibited promoter activity, their activities were about four-times lower than that of pGnTp16-0.9. Moreover, the luciferase activities of pGnTp16-0.6 and pGnTp16-0.4 were twice as low as those of pGnTp16-1.6 and pGnTp16-1.2. These findings suggest that negative regulatory sequences lie between the *Eco*RI site at -1600 and the *Xho*I site at position -900 of the exon-1 promoter.

On the other hand, luciferase activities of cells transfected with the flanking region of the new exon region were not observed (data not shown), indicating that the 5′-flanking region of the new noncoding exon-2 does not work as a promoter in HepG2 cells. Two possible reasons can be given: there is an intron between the transcription-initiation site and the 5′-end of the noncoding exon-2, and the promoter for the noncoding exon-2 is located much further upstream, and this region is active in human fetal liver cells, but is inactive in HepG2 cells because of the differences in transcription machinery.

In summary, we have first identified the new 5′-noncoding exon region and determined the structure of the 5′-noncoding region of the human GnT-III gene. Furthermore, we have demonstrated that the gene

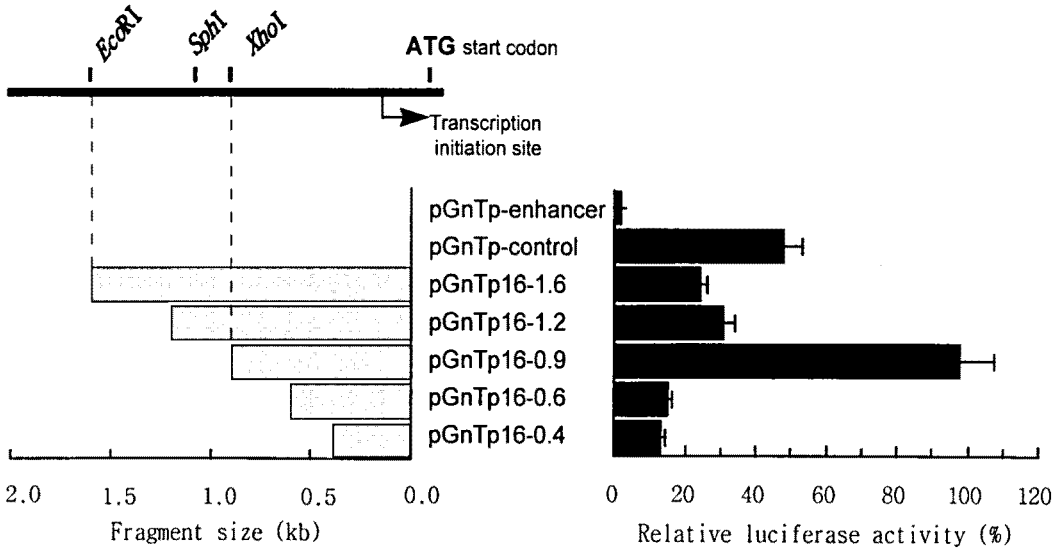

Fig. 6. Promoter activity of DNA fragments derived from each promoter. DNA fragments tested for promoter activity are shown by boxes on the left, and closed boxes on the right show the luciferase activity normalized by protein relative to the SV40 promoter of each construct in five separate experiments.

expression of GnT-III is controlled mainly by the exon-1 promoter in hepatoblastoma cells. This finding will make it possible to clarify the regulatory mechanism of GnT-III gene expression in liver malignancy. Further studies will be required to determine the transcriptional initiation site of GnT-III and the promoter function of the 5′-flanking region. Functional analysis of the 5′-flanking region and correlation with the regulation of the GnT-III promoter will provide further insight into the elucidation of mechanisms that control the expression of the GnT-III gene.

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