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Cloning and Expression of a Novel Galactoside β1,3-Glucuronyltransferase Involved in the Biosynthesis of HNK-1 Epitope*

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We isolated a cDNA encoding a novel glucuronyltransferase, designated GlcAT-D, involved in the biosynthesis of the HNK-1 carbohydrate epitope from rat embryo cDNA by the degenerate polymerase chain reaction method. The new cDNA sequence revealed an open reading frame coding for a protein of 324 amino acids with type II transmembrane protein topology. The amino acid sequence of GlcAT-D displayed 50.0% identity to rat GlcAT-P, which is involved in the biosynthesis of the HNK-1 epitope on glycoproteins. Expression of GlcAT-D in COS-7 cells resulted in the formation of the HNK-1 epitope on the cell surface. The enzyme expressed in COS-7 cells transferred a glucuronic acid (GlcA) not only to asialo-orosomucoid, a glycoprotein bearing terminal *N*-acetyllactosamine structure, but also to paragloboside (lacto-*N*-neotetraosylceramide), a precursor of the HNK-1 epitope on glycolipids. Furthermore, substrate specificity analysis using a soluble chimeric form of GlcAT-D revealed that GlcAT-D transfers a GlcA not only to Galβ1–4GlcNAcβ1–3Galβ1–4Glc-pyridylamine but also to Galβ1–3GlcNAcβ1–3Galβ1–4Glc-pyridylamine. Enzymatic hydrolysis and Smith degradation of the reaction product indicated that GlcAT-D transfers a GlcA through a β1,3-linkage to a terminal galactose. The GlcAT-D transcripts were detected in embryonic, postnatal, and adult rat brain. *In situ* hybridization analysis revealed that the expression pattern of GlcAT-D transcript in embryo is similar to that of GlcAT-P, but distinct expression of GlcAT-D was observed in the embryonic pallidum and retina. Regions that expressed GlcAT-D and/or GlcAT-P were always HNK-1-positive, indicating that both GlcATs are involved in the synthesis of the HNK-1 epitope *in vivo*.

HNK-1, also known as CD57, is recognized as an important epitope involved in neurogenesis. Expression of HNK-1 epitope is spatiotemporally regulated, and the epitope is found on migrating neural crest cells (1), odd-numbered rhombomeres (2), and myelinating Schwann cells in motor neurons (3). The HNK-1 epitope is a glycan expressed on glycoproteins, glycolipids, and proteoglycans (reviewed in Refs. 4 and 5). In particular, the epitope has been discovered on a series of cell adhesion molecules, such as myelin-associated glycoproteins (6), L1 (7), neural cell adhesion molecule (7), P0 (8), and transiently expressed axonal glycoprotein-1 (9). It is interesting that only a subpopulation of these molecules expresses HNK-1 epitope. The epitope has also been identified as a ligand for P0 (10), laminin (11), and L- and P-selectins (12). Moreover, it was demonstrated that HNK-1 antibody or isolated HNK-1 glycan interferes with cell-cell or cell-substrate interaction (13–15). These observations indicate that HNK-1 epitope plays a significant role in cell-cell and cell-matrix interaction.

Structures of HNK-1 epitope determined to date almost invariably carry HSO_3-3GlcAβ1–3Galβ1–4GlcNAc at nonreducing termini (16–19). The precursor of the epitope, Galβ1–4GlcNAc sequence, is commonly found on glycoproteins and glycolipids, but expression of HNK-1 epitope is spatially and temporally restricted. The key enzymes in the biosynthesis of HNK-1 are a β1,3-glucuronyltransferase, which transfers a GlcA to a terminal galactose, and a sulfotransferase, which adds a sulfate group to the GlcA. Recently, GlcAT-P, a glucuronyltransferase involved in the biosynthesis of HNK-1 on glycoprotein, was purified, and its cDNA was cloned (20, 21). Subsequently, a sulfotransferase that directs a final step of the biosynthesis of HNK-1 was cloned by an expression cloning strategy that involved cotransfection of GlcAT-P cDNA (22, 23). On the other hand, GlcAT-I, another glucuronyltransferase involved in the biosynthesis of the linkage region of proteoglycans (EC 2.4.1.135), was cloned by PCR[1] strategy based on motifs conserved in GlcAT-P with putative proteins in *Caenorhabditis elegans* and *Schistosoma mansoni* (24).

We examined the enzymatic features of glycosyltransferases, including β1,3-glucuronyltransferase, expressed in rat. We found β1,3-glucuronyltransferase activity to glycolipids as well as glycoproteins in rat embryonic brain, suggesting that this enzyme is a novel glucuronyltransferase. To clone cDNA of the novel enzyme, we used the RNA of rat embryonic day 13 (E13) brain as a template of reverse transcription-PCR and designed degenerate primers to the highly conserved regions found in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF106624.

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[1] The abbreviations used are: PCR, polymerase chain reaction; GlcA, D-glucuronic acid; HPLC, high performance liquid chromatography; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LNFP III, lacto-*N*-fucopentaose III; LNnDFH I, lacto-*N*-neodifucohexaose I; LNnT-Cer, lacto-*N*-neotetraosylceramide; GlcA-LNnT-Cer, glucuronylneolactotetraosylceramide; E*n*, embryonic day *n*; SD, Smith degradation; MES, 4-morpholineethanesulfonic acid; PA, pyridylamine; kb, kilobase(s).

the alignment of amino acid sequence of GlcAT-P with GlcAT-I. In this study, we describe the cDNA cloning of a new member of the glucuronyltransferase family, GlcAT-D. The expression of cDNA revealed that GlcAT-D is a glucuronyltransferase involved in the biosynthesis of HNK-1 epitope on both glycolipid and glycoprotein. We also demonstrate that GlcAT-D transfers a GlcA to a Gal residue of Galβ1–3/4GlcNAc by β1,3-linkage.

EXPERIMENTAL PROCEDURES

Materials—UDP-[^{14}C]GlcA (10.1 GBq/mol) was purchased from NEN Life Science Products. Unlabeled UDP-GlcA, CDP-choline, and GlcA were purchased from Sigma. Lacto-*N*-neotetraose (LNnT), lacto-*N*-tetraose (LNT), lacto-*N*-fucopentaose III (LNFP III), and lacto-*N*-neodifucohexaose I (LNnDFH I) were purchased from Seikagaku. Lacto-*N*-neotetraosylceramide (LNnT-Cer) and glucuronylneolactotetraosylceramide (GlcA-LNnT-Cer) were purchased from Dia-Iatron and Wako Pure Chemicals, respectively. Asialo-orosomucoid was prepared by mild acid hydrolysis (in 0.05 M H_2SO_4 at 80 °C for 1 h) of α_1-acid glycoprotein purchased from Sigma. β-Glucuronidases from bovine liver and *Helix pomatia* were purchased from Seikagaku and Sigma, respectively. α1,3/4-Fucosidase from *Streptomyces* sp. 142 and endo-β-galactosidase from *Escherichia freundii* were purchased from Seikagaku. Ceramide glycanase from leech and β-galactosidases from *Diplococcus pneumoniae* and bovine testes were purchased from Roche Molecular Biochemicals.

PCR-based Cloning of a New Glucuronyltransferase—Based on the amino acid sequence alignment of rat GlcAT-P (21) and human GlcAT-I (24), degenerate oligonucleotides to elements conserved in motifs II and IV were synthesized. The sequences of the 5′- and 3′-primers were 5′-TSGTSTAYTTYGCYGAYGAYGA-3′ and 5′-TTYTCNGTNCKNGTRTGCCANA-3′ (K = A + C, N = A + C + G + T, R = A + G, S = C + G, and Y = C + T), respectively. PCR was performed using first strand cDNA synthesized with total RNA of rat E13 brain. Thirty cycles (94 °C for 45 s, 50 °C for 45 s, and 72 °C for 90 s) were run using AmpliTaq Gold polymerase (PE Applied Biosystems). The PCR product of around 380 base pairs in length was subcloned into the pGEM-T Easy vector (Promega), and 10 clones were sequenced. Two of the 10 clones contained a novel putative glucuronyltransferase (GlcAT-D) fragment that was used as a probe for screening a rat E15 brain cDNA library (25). One positive clone, 58-2-2, was isolated and sequenced.

To obtain the entire coding sequence of GlcAT-D, rapid amplification of cDNA 5′-end was employed with 5′-AmpliFINDER RACE Kit (CLONTECH) using rat E15 brain total RNA as a template. The amplified cDNA of about 1.6 kilobase pairs in length was subcloned into pGEM-T Easy and sequenced. Several clones were sequenced to compensate for misreading by AmpliTaq Gold polymerase.

Northern Blot Analysis—Total RNA was extracted from embryo, newborn, and adult rat brain using ISOGEN reagent (Nippon gene). Equal amounts of total RNA (20 μg in each lane) were run on formaldehyde 1.2% agarose gel and transferred to a nitrocellulose membrane. Multiple tissue Northern blot of rat poly(A)$^+$ RNA was purchased from CLONTECH. The blots were hybridized overnight with ^{32}P-labeled *Eco*RI fragment of 58-2-2 at 42 °C in hybridization buffer. Then the filters were washed in 0.5× SSC plus 0.1% SDS for 1 h at 65 °C.

In Situ Hybridization—For the construction of riboprobes for *in situ* hybridization analysis, we obtained the cDNA fragment of GlcAT-D (nucleotide positions 285–1005) by digestion of 58-2-2 plasmid with *Eco*RI and subcloned into pBluescript II SK(−) (Stratagene). The GlcAT-P cDNA was obtained by reverse transcription-PCR in which the cDNA template was synthesized from the total RNA taken from E13 rat embryo brain. Oligonucleotides used to amplify the GlcAT-P cDNA (21) are 5′-AAACCTGCTGCCACAATGGGTAA-3′ (nucleotides −15 to 8) and 5′-ATGGGAGAGGATGGAAGCCAAGAT-3′ (nucleotides 1297 to 1274). The identity of the PCR product was confirmed by sequencing the subcloned fragment. The cDNA fragment of GlcAT-P was cloned into pBluescript II SK(−) (Stratagene). The antisense RNA probes were generated with T3 or T7 RNA polymerase. Corresponding sense probes were used to check the specificity of hybridization signals.

In situ hybridization with probes of GlcAT-D and GlcAT-P was performed as described previously (26) with slight modifications. Briefly, the head region of E13.5 Sprague-Dawley rat embryos was fixed in 4% paraformaldehyde in phosphate-buffered saline overnight at 4 °C. Cryosections, 14 μm in thickness, were thaw-mounted onto VECTABOND® (Vector Laboratories)-coated glass slides. After rehydration in phosphate-buffered saline with 0.1% Tween 20, the sections were postfixed in 4% paraformaldehyde for 20 min, treated with proteinase K (1 μg/ml) at 37 °C for 4 min, postfixed again in 4% paraformaldehyde for 20 min, and hybridized with digoxigenin-labeled probes (1 μg/ml) overnight at 65 °C. Then the sections were washed in 50% formamide, 5× SSC, and 1% SDS for 30 min at 65 °C, subsequently twice in 50% formamide and 2× SSC for 45 min at 65 °C, and finally in Tris-buffered saline containing 0.1% Tween 20. The slides were then immersed in blocking solution and incubated overnight at 4 °C with anti-digoxigenin Fab-alkaline phosphatase conjugate diluted to 1:2000 by blocking solution. The hybrids were visualized by the alkaline phosphatase reaction with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate.

Immunohistochemistry—Immunostaining patterns of HNK-1 antibody were examined basically as described previously (27, 28). Frozen sections were prepared as described above, rehydrated in Tris-buffered saline containing 0.1% Tween 20, and boiled in 0.01 M sodium citrate for antigen enhancement. After preincubation with the blocking solution for 30 min, the sections were incubated with Leu7 monoclonal antibody (Becton Dickinson) diluted to 1:25 by the blocking solution overnight at 4 °C. Biotinylated anti-mouse IgM (Zymed Laboratories Inc.) was used as a secondary antibody, and the sections were quenched with hydrogen peroxide. Immunoreactivity was detected using the ABC kit (Vector Laboratories) and the metal enhanced diaminobenzidine kit (Pierce).

Construction of Expression Vector Containing a Full-length GlcAT-D cDNA—A DNA fragment, containing the open reading frame of GlcAT-D, from nucleotide positions −34 to 1004 was amplified by PCR with cDNA reverse-transcribed from total RNA of rat E13 brain using a 5′-primer (5′-CCCAAGCTTGAGGGTGGTGTCCGAGACGCT-3′) containing a *Hin*dIII site and a 3′-primer (5′-GGAATTCCCTTCTCTCCTCAGCGGCTGCTC-3) containing an *Eco*RI site. After restriction enzyme digestion, the PCR fragment was subcloned into *Hin*dIII and *Eco*RI sites of pBluescript II SK(−) (Stratagene), yielding pBS-GlcAT-D. pBS-GlcAT-D was digested with *Hin*dIII and *Not*I and then cloned into *Hin*dIII and *Not*I sites of pCDM8, yielding pCDM8-GlcAT-D.

Construction of Soluble Form of GlcAT-D—A truncated form of GlcAT-D, lacking the first 35 amino acids of GlcAT-D, was amplified by PCR with pBS-GlcAT-D as a template using a 5′-primer (5′-GGAATTCCCGACCCTACTTCTCTCCGCATA-3′) containing an *Eco*RI site and the same 3′-primer as used in the amplification of the fragment containing the open reading frame of GlcAT-D. The PCR product was digested with *Eco*RI and cloned into the *Eco*RI site of pPROTA vector (29). A recombinant plasmid with the correct orientation, pPROTA-GlcAT-D, was used for expression.

Flow Cytometry Analysis—COS-7 cells (5×10^6 cells) were transfected with pCDM8-GlcAT-D or pCDM8 by electroporation using a Bio-Rad Gene Pulser at 300 V, 960 microfarad. After 2 days, the cells were harvested, stained with fluorescein isothiocyanate-conjugated anti-CD57 (Pharmingen), and analyzed on FACSCalibur (Becton Dickinson).

Assays of Glucuronyltransferase Activity to Glycoproteins and Glycolipids—COS-7 cells (1×10^6 cells) were transfected with one of the expression plasmids, pCDM8-GlcAT-D or pCDM8 (10 μg each) using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. After 3 days, the cells were harvested, washed with phosphate-buffered saline, homogenized in 100 μl of 0.3% Triton X-100, and used as an enzyme source.

Glucuronyltransferase activity to glycoproteins was measured in 0.1 M sodium cacodylate, pH 6.5, 100 μg of asialo-orosomucoid, 100 μM UDP-[^{14}C]GlcA, 5 mM CDP-choline, 10 mM GlcA, 10 mM $MnCl_2$, and 10 μl of the enzyme source (total volume, 30 μl). After incubation at 37 °C for 2 h, the reaction mixtures were spotted onto Whatman number 1 paper (2 × 2 cm). The papers were dipped in 10% (w/v) trichloroacetic acid and then rinsed twice with fresh 10% (w/v) trichloroacetic acid, once with ethanol/ethylether (2:1, v/v), and finally with ethylether. After being air-dried, the radioactivity on the papers was counted using a liquid scintillation counter.

When a glycolipid was used as the acceptor, glucuronyltransferase activity was measured in 0.1 M MES, pH 6.0, 6 μg of LNnT-Cer, 100 μM UDP-[^{14}C]GlcA, 5 mM CDP-choline, 10 mM GlcA, 10 mM $MnCl_2$, and 10 μl of the enzyme source (total volume, 30 μl). After incubation at 37 °C for 2 h, the reaction mixtures were applied to a 1cc Sep-Pak Vac Cartridge (Millipore) preactivated sequentially with methanol, chloroform/methanol (2:1) and then methanol again and subsequently equilibrated with water. The column was washed with 50 ml of water and eluted with 0.9 ml of methanol and 1.0 ml of chloroform/methanol (2:1). The eluant was evaporated by SpeedVac and resuspended with 30 μl of chloroform/methanol (2:1). The product was applied to a silica gel 60 high performance thin-layer chromatography plate (Merck) and devel-

oped with chloroform/methanol/0.5% $CaCl_2$ (55:45:10). The glycolipids were visualized with orcinol reagent, and the mobility was compared with that of GlcA-LNnT-Cer purchased from Wako Pure Chemicals. The position and radioactivity of the product were estimated with a Fujix BAS 2000 Bioimage Analyzer (Fuji Photo Film).

Preparation of Pyridylaminated Glycans—Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA, Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA, and Galβ1–4(Fucα1–3)GlcNAcβ1–3Galβ1–4Glc-PA were synthesized by pyridylamination of LNnT, LNT, and LNFP III, respectively, with a Palstation and pyridylamination reagent kit (Takara) according to the manufacturer's instructions. GlcAβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA was synthesized from GlcA-LNnT-Cer using 1 milliunit of ceramide glycanase from leech according to the method of Shimamura *et al.* (30). GlcNAcβ1–3Galβ1–4Glc-PA was prepared by β-galactosidase digestion (from *D. pneumoniae*, 5 milliunits in 100 μl of 50 mM sodium acetate buffer, pH 6.0, containing 0.5 mg/ml of bovine serum albumin at 37 °C for 2 h) of 50 nmol of Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA, following purification by gel filtration on a Superdex Peptide HR 10/30 column (Amersham Pharmacia Biotech). Fucα1–2Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA was prepared by pyridylamination of LNnDFH I, following α1,3/4-fucosidase digestion (from *Streptomyces* sp. 142, 20 microunits in 100 μl of 50 mM potassium phosphate buffer, pH 6.0, 37 °C, overnight) and purification by Superdex Peptide HR 10/30 column. Galβ1–4GlcNAcβ1–3Gal-PA and Galβ1–3GlcNAcβ1–3Gal-PA were synthesized by endo-β-galactosidase digestion (25 milliunits in 100 μl of 0.1 M sodium acetate buffer, pH 5.5, 37 °C, 48 h) of 100 μg of LNnT and LNT, respectively, following isolation of trisaccharides by gel filtration on a Superdex Peptide HR 10/30 column and pyridylamination. Galβ1–3GalNAcβ1–4Galβ1–4Glc-PA was purchased from Takara.

Assays of Glucuronyltransferase Activity to Pyridylaminated Glycans—For the expression of the soluble form of GlcAT-D, 10 μg of pPROTA-GlcAT-D or pPROTA plasmid was transfected to COS-7 as described above. After 3 days, 20 μl of the 50% suspension of IgG-Sepharose (Pharmacia) was added to 1 ml of culture medium and rotated overnight at 4 °C. The beads were washed twice with reaction buffer and used as an enzyme source.

Glucuronyltransferase activity to pyridylaminated glycans was measured in 0.1 M sodium cacodylate, pH 6.5, 0.1 mM pyridylaminated glycan, 1 mM UDP-GlcA, 5 mM CDP-choline, 10 mM GlcA, 20 mM $MnCl_2$, and 10 μl of the enzyme source (total volume, 30 μl). After incubation at 37 °C for 4–16 h, the reactions were stopped by boiling for 5 min. After centrifugation 10,000 × *g* for 5 min, 10 μl of the supernatant was subjected to HPLC analysis using PALPAK Type N column (Takara), equilibrated, and eluted with acetonitrile/200 mM acetic acid-triethylamine, pH 7.3 (55:45) at a flow rate of 1.0 ml/min at 40 °C. The elution profile was monitored with a fluorescence spectrophotometer (Shimadzu). The amounts of the products were determined from their fluorescence intensities using PA sugar chain 041 (Takara).

β-Glucuronidase Digestion of the Reaction Products—The products from the glucuronyltransferase reaction using Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA or Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA were isolated by HPLC using PALPAK Type N column in the conditions described above. The isolated products were digested with 1 milliunit of β-glucuronidase from bovine liver overnight at 37 °C or with 100 units of β-glucuronidase from *H. pomatia* for 2 days at 37 °C in a total volume of 50 μl of 0.1 M sodium acetate buffer, pH 5.0. The digests were analyzed by HPLC using the same PALPAK type N column as that noted above.

Smith Degradation of the Reaction Products—The products from the glucuronyltransferase reaction using Galβ1–4GlcNAcβ1–3Gal-PA or Galβ1–3GlcNAcβ1–3Gal-PA were isolated by HPLC using PALPAK Type N column eluted with acetonitrile/200 mM acetic acid-triethylamine, pH 7.3 (66:34). The isolated products (0.5 nmol each) and intact Galβ1–4GlcNAcβ1–3Gal-PA and Galβ1–3GlcNAcβ1–3Gal-PA (2.5 nmol each) were subjected to Smith degradation according to the method of Hase *et al*. (31) with slight modification. A pyridylaminated glycan was dissolved in 20 μl of 80 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.0, and left to stand at 4 °C for 48 h in the dark. The reaction was terminated by adding 5 μl of 20% ethylene glycol and kept at room temperature for 1 h. Aldehyde groups produced were reduced with 1 mg of sodium borohydride in 100 μl of 0.1 M borate buffer, pH 8.0, overnight at room temperature. After neutralizing with acetate, the product was desalted on a Superdex Peptide HR 10/30 column and concentrated. Mild acid hydrolysis of the products was carried out in 100 μl of 0.05 M H_2SO_4 at 80 °C for 1 h. After adding 10 μl of 1 M NaOH, the product was desalted on a Superdex Peptide HR 10/30 column and concentrated. Half an aliquot of the Smith degradation products thus obtained was digested with 25 milliunits of β-galactosidase (bovine testes) overnight at 37 °C in a total volume of 50 μl of 0.1 M sodium citrate phosphate buffer, pH 4.3, containing 10% glycerol and 1% bovine serum albumin. These products were analyzed by HPLC using PALPAK Type N column eluted with acetonitrile/200 mM acetic acid-triethylamine, pH 7.3 (75:25).

RESULTS

Cloning of a New Glucuronyltransferase—Our preliminary experiment of the enzymatic characterization of glycosyltransferases from rat embryo suggested that a glucuronyltransferase other than GlcAT-P, which is involved in the biosynthesis of HNK-1 epitope, may be expressed in the rat embryonic nervous system. To clone new members of the glucuronyltransferase gene family, we designed two degenerate primers to the sequences conserved in motifs II and IV of rat GlcAT-P and human GlcAT-I. The PCR using these primers with rat E13 brain cDNA as a template resulted in the amplification of a product around 380 base pairs in length. After subcloning the PCR product, we sequenced and characterized 10 individual clones: one clone was GlcAT-P; seven clones were putative rat GlcAT-I, a sequence of which had 93% identity with human GlcAT-I[2]; and the other two clones had sequences that were similar to but distinct from those of GlcAT-P and GlcAT-I, suggesting that these clones contained a novel glucuronyltransferase (GlcAT-D) fragment. Using the PCR fragment as a probe, we screened a rat E15 brain cDNA library and obtained one clone, 58-2-2. The sequence of 58-2-2 contained a putative stop codon but no in-frame ATG. To obtain the entire coding sequence of GlcAT-D, rapid amplification of cDNA 5′-end was employed. The nucleotide sequence of the overlapping cDNA fragments revealed that GlcAT-D has a single open reading frame consisting of 324 amino acids, with a molecular mass of 37,177 Da, and two potential *N*-glycosylation sites (Fig. 1*A*). The predicted translation initiation site conformed to Kozak's consensus sequence (32), and the upstream region contained an in-frame stop codon. Hydropathy analysis (Fig. 1*B*) indicated the presence of a potential transmembrane domain at the N-terminal region (from Ala-4 to Val-25), suggesting that the protein has type II transmembrane topology, which has been found in almost all glycosyltransferases cloned to date. The domain (from Asp-26 to Pro-81) next to the transmembrane region was characterized by its high proline content (20%; 11 of 56 amino acids), as seen in several other glycosyltransferases including rat GlcAT-P and human GlcAT-I. The putative catalytic domain (from Leu-80 to Val-324) showed a very high sequence identity with those of both rat GlcAT-P and human GlcAT-I (Fig. 2). A data base search indicated that a hypothetical protein of *Drosophila melanogaster* (accession number AL033125) has high homology with these three GlcATs in addition to those of *C. elegans* and *S. mansoni*, as pointed out by Terayama *et al.* (21).

Northern Blot Analysis—Of the adult rat tissues examined, GlcAT-D was expressed only in the brain (Fig. 3*A*). Three transcripts of 1.1 kb (major), 2.3 kb (minor), and 4.0 kb (faint) were detected in the adult whole brain (Fig. 3*A*), cerebral cortex, and cerebellum (Fig. 3*B*). In the postnatal (P1 and P7) cerebral cortex, the 1.1-kb transcript was more intense than the 2.3-kb one, whereas these two transcripts in the corresponding cerebellum showed almost equal intensity. In the E18 brain, the 1.1- and 2.3-kb transcripts showed similar intensity. A faint single 2.3-kb transcript was detected in the E13 brain.

Localization of HNK-1 Epitope and Gene Expression of GlcATs in Embryonic Brain—HNK-1 immunohistochemistry was performed on E13.5 rat brain sections. Intense staining

[2] Y. Shimoda, Y. Tajima, T. Nagase, K. Harii, N. Osumi, and Y. Sanai, unpublished data.

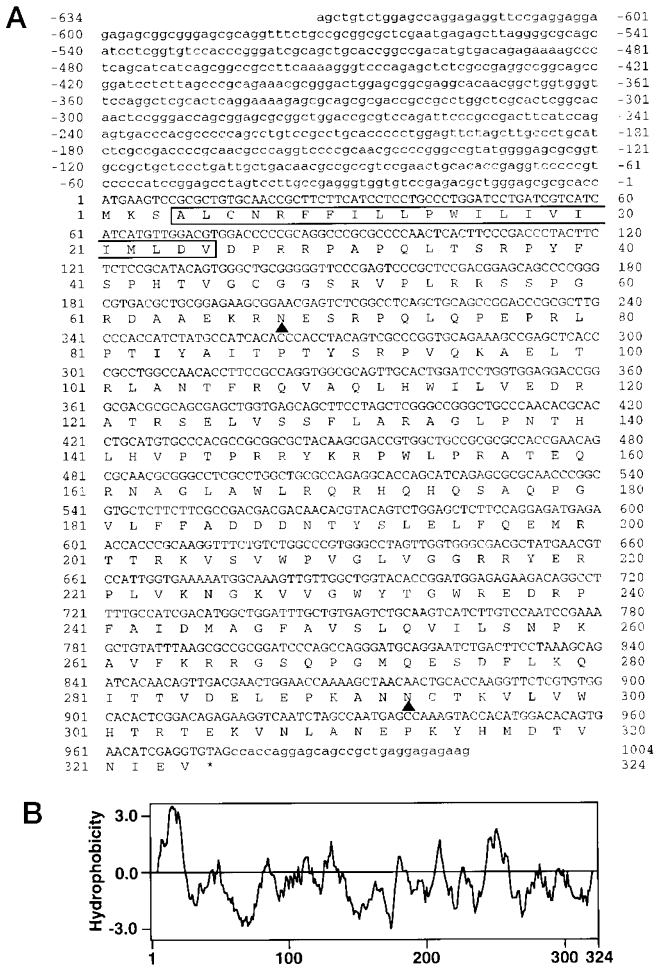

FIG. 1. *A*, nucleotide and deduced amino acid sequences of GlcAT-D. The putative transmembrane domain is *boxed*. Potential *N*-glycosylation sites are indicated by *arrowheads*. *B*, hydropathy analysis of GlcAT-D according to the method of Kyte and Doolittle (37).

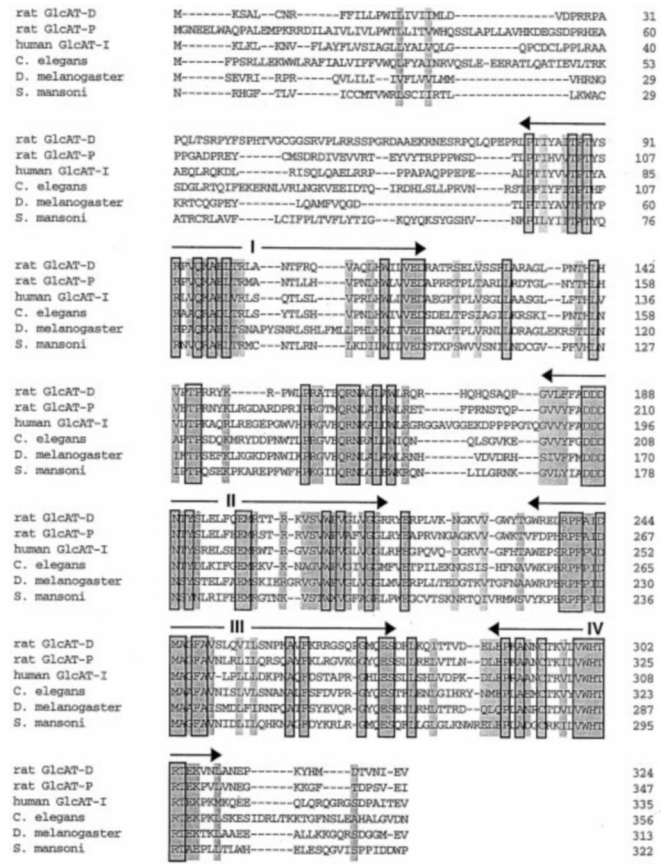

FIG. 2. **Sequence alignment of GlcAT-D with rat GlcAT-P (accession number D88035), human GlcAT-I (accession number AB009598), and putative proteins in *C. elegans* (accession number Z47358), *D. melanogaster* (accession number AL033125), and *S. mansoni* (accession number U30260).** *Boxes* and *shaded backgrounds* indicate identical and similar residues, respectively. Four highly conserved regions named motifs I–IV are indicated by *arrows*.

was observed in the outer layer (preplate) of the cerebral cortex, the lateral and medial ganglionic eminences, and the broad subventricular area of the basal ganglia. Specific staining was also detected in the retina and slightly in the lens (Fig. 4*A*).

Adjacent brain sections were used for *in situ* hybridization to detect the *in vivo* expression patterns of GlcAT-D and GlcAT-P. Expression of GlcAT-D was observed in the preplate of the cerebral cortex and ventral regions of the basal ganglia (Fig. 4*B*). A focal staining spot was also noted in the pallidal subventricular zone (*arrow* in Fig. 4*B*). GlcAT-P basically showed similar expression in the telencephalon (*arrowheads* in Fig. 4*C*), although no signal was observed in the pallidum (compare with *arrow* in Fig. 4*B*). In the eye primordium, GlcAT-D mRNA was detected in the retina, whereas GlcAT-P was very weakly expressed in the lens. The regions that expressed GlcAT-D and/or GlcAT-P were always HNK-1-positive, although transcripts of both GlcATs were below the detection level in the putative piriform cortex (*white arrowheads* in Fig. 4*C*).

Expression of GlcAT-D in COS-7 and Characterization as Glucuronyltransferase Involved in Biosynthesis of HNK-1 Epitope—To prove the enzymatic activity of the cDNA product, expression plasmid containing GlcAT-D cDNA was transfected into COS-7 cells. The cell homogenate was assayed for glucuronyltransferase activity using a glycoprotein and a glycolipid, both of which contained Galβ1–4GlcNAc sequences at nonreducing termini, as acceptor substrates. As shown in Table I, significant activity was detected using both glycoprotein and

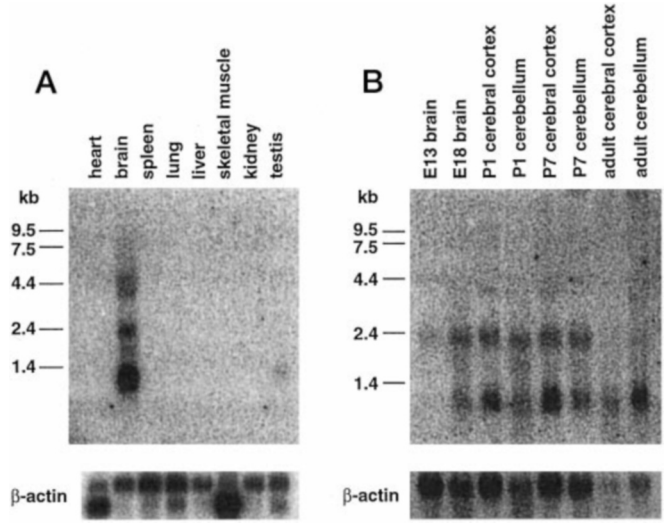

FIG. 3. **Northern blot analysis of GlcAT-D.** The blots of adult rat multiple tissue (*A*) and the blots of total RNA (20 μg/lane) prepared from embryo, postnatal, and adult rat brain (*B*) were hybridized with ^{32}P-labeled GlcAT-D cDNA followed by β-actin.

glycolipid acceptors. Cells transfected with pCDM8 vector showed much less activity. As shown in Fig. 5, the reaction product using a glycolipid, LNnT-Cer, as an acceptor was detected on high performance thin-layer chromatography at a position corresponding to GlcA-LNnT-Cer. Moreover, a signif-

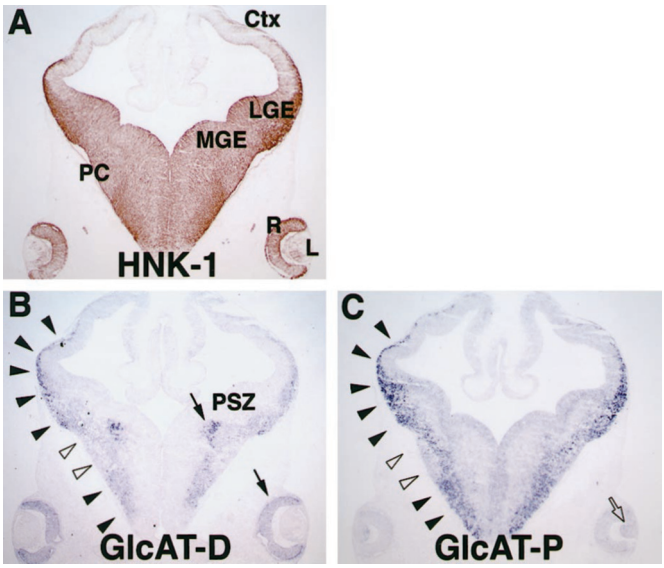

FIG. 4. **Immunohistochemistry of HNK-1 and *in situ* hybridization of GlcAT-D and GlcAT-P in E13.5 rat brain.** HNK-1 immunostaining (*A*) was observed in the preplate of the cortex (*Ctx*), the broad area of the basal ganglia, lens (*L*), and retina (*R*). HNK-1-positive regions totally included the areas where gene expression of GlcAT-D (*B*) and GlcAT-P (*C*) was observed. Transcripts of both genes were localized in the cerebral cortex and ventral surface of the basal ganglia (*arrowheads*), whereas only GlcAT-D transcripts was observed in the pallidal subventricular zone (*PSZ*) and the retina (*arrows*). Slight GlcAT-P expression was detected in the lens (*white arrow*). Note that transcripts of the both GlcATs were absent from the piriform cortex (*PC*; *white arrowheads*). *LGE*, lateral ganglionic eminence; *MGE*, medial ganglionic eminence.

TABLE I

Glucuronyltransferase activity of GlcAT-D expressed in COS-7 cells

The activities of cells transfected with pCDM8-GlcAT-D or pCDM8 vector were measured using asialo-orosomucoid (glycoprotein) or LNnT-Cer (glycolipid) as a GlcA acceptor.

Plasmid	Acceptor activity	
	Glycoprotein	Glycolipid
	pmol/h/mg protein	
pCDM8-GlcAT-D	210	89.4
pCDM8	0.2	1.2

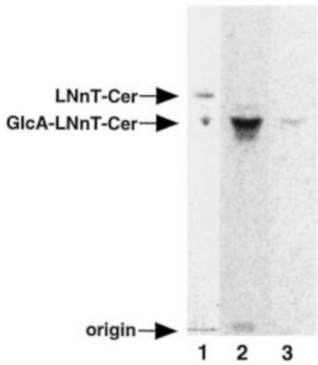

FIG. 5. **Glucuronyltransferase activity to LNnT-Cer in COS-7 cells transfected with GlcAT-D cDNA.** *Lane 1*, standard glycosphingolipids visualized with orcinol reagent. Glucuronyltransferase activity in COS-7 cells transiently transfected with pCDM8-GlcAT-D (*lane 2*) or pCDM8 vector (*lane 3*) was measured using UDP-[^{14}C]GlcA and LNnT-Cer as donor and acceptor substrate. The reaction products were visualized using a Fujix BAS 2000.

icant portion of COS-7 cells transfected with GlcAT-D cDNA were stained with anti-CD57, showing that these cells expressed HNK-1 epitope (Fig. 6). Cells transfected with pCDM8 vector did not express the epitope. These results are consistent

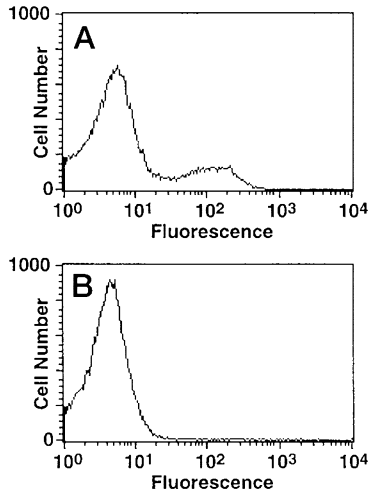

FIG. 6. **Flow cytometric analysis of HNK-1 expression in transiently transfected COS-7 cells.** pCDM8-GlcAT-D-transfected (*A*) or pCDM8-transfected (*B*) cells were incubated with fluorescein isothiocyanate-conjugated anti-CD57.

with those of Terayama *et al*. (21), who showed that COS-1 cells transfected with GlcAT-P cDNA expressed HNK-1 epitope. The results of expression in COS-7 indicate that GlcAT-D is a glucuronyltransferase involved in the biosynthesis of HNK-1 epitope.

Expression of a Soluble Form of GlcAT-D—To clarify the function of the cDNA product, a soluble form of GlcAT-D was generated by fusing the putative stem and catalytic domain of the protein to a secreted form of the protein A IgG-binding domain. The fused protein was expressed in COS-7 cells and absorbed on IgG-Sepharose beads from culture medium, and then the enzyme-bound beads were used as an enzyme source.

The glucuronyltransferase activity of the bound fusion protein was determined using a variety of pyridylaminated glycans as acceptor substrates. An aliquot of the reaction mixture was subjected to HPLC. The elution pattern is shown in Fig. 7. Compared with the elution pattern of the reaction mixture without UDP-GlcA (Fig. 7, *B* and *E*), the enzymatic products using Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA (Fig. 7*A*) and Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA (Fig. 7*D*) as acceptors were eluted at 13.7 and 12.9 min, respectively. The former was eluted at the same time as a synthetic standard, GlcAβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA. In contrast, no product was detected using the medium from mock transfected COS-7 cells (Fig. 7, *C* and *F*). As shown in Table II, Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA and Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA were good acceptors for GlcA, whereas no activity was detected using Galβ1–3GalNAcβ1–4Galβ1–4Glc-PA or α1,2- or α1,3-fucosylated or the agalacto-form of Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA as acceptors. These results confirm that the cloned cDNA encodes a glucuronyltransferase, which transfers a GlcA to a carbohydrate chain containing an unsubstituted Galβ1–4GlcNAc or Galβ1–3GlcNAc sequence.

Characterization as a Galactoside β1,3-Glucuronyltransferase—Reactions using pyridylaminated glycans as acceptors were performed on a large scale to identify the products, and those isolated by HPLC were subjected to further analyses.

Products using Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA and Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA as acceptors were subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and both showed molecular ions $[M + Na]^+$ at *m/z* 984 and $[M\text{-}H+2Na]^+$ at *m/z* 1006 (data not shown). These products were incubated with β-glucuronidase from bovine liver or *H. pomatia*, and then subjected to HPLC

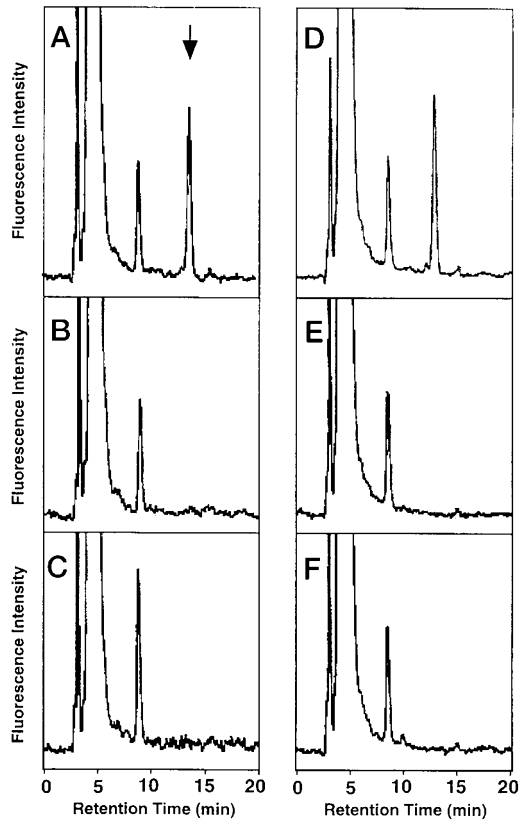

FIG. 7. **HPLC analysis of glucuronyltransferase reaction products.** The enzyme adsorbed to IgG-Sepharose from COS-7 transfected with pPROTA-GlcAT-D (*A*, *B*, *D*, and E) was incubated with Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA (*A* and *B*) or Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA (*D* and *E*) in the presence of UDP-GlcA (*A* and *D*) or in the absence of UDP-GlcA (*B* and *E*). The beads adsorbing the medium from pPROTA-transfected COS-7 were incubated with Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA (*C*) or Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA (*F*) in the presence of UDP-GlcA. The *arrow* indicates the elution position of the authentic GlcAβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA.

TABLE II
Acceptor specificity of the glucuronyltransferase absorbed on IgG-Sepharose beads

Acceptor	Activity
	pmol/h/ml medium
Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA	2.0
Galβ1–3GalNAcβ1–3Galβ1–4Glc-PA	1.9
Galβ1–3GalNAcβ1–4Galβ1–4Glc-PA	ND[a]
GlcNAcβ1–3Galβ1–4Glc-PA	ND
Galβ1–4(Fucα1–3)GlcNAcβ1–3Galβ1–4Glc-PA	ND
Fucα1–2Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA	ND

[a] ND, not detected (less than 0.1 pmol/h/ml medium).

analysis. The product from Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA was completely digested with β-glucuronidase from bovine liver (Fig. 8*A*), but the product from Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA was not digested by the same enzyme (data not shown). By contrast, β-glucuronidase from *H. pomatia* digested the product from Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA (Fig. 8*C*), as well as the product from Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA (data not shown). These results indicate that a GlcA is transferred to the Galβ1–4GlcNAc or Galβ1–3GlcNAc sequence through a β-linkage.

To prove whether a GlcA was transferred to the C-3 position of Gal of Galβ1–4GlcNAc and Galβ1–3GlcNAc sequence by Smith degradation, the glucuronyltransferase reactions using Galβ1–4GlcNAcβ1–3Gal-PA and Galβ1–3GlcNAcβ1–3Gal-PA as acceptors were performed on a large scale. The products

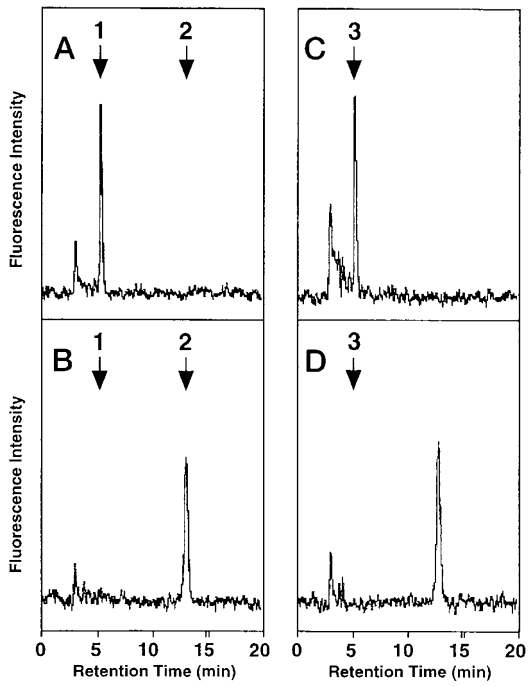

FIG. 8. **β-Glucuronidase digestion of glucuronyltransferase reaction products.** The glucuronyltransferase reaction products using Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA as an acceptor were incubated with (*A*) or without (*B*) β-glucuronidase from bovine liver. The products using Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA as an acceptor were incubated with (*C*) or without (*D*) β-glucuronidase from *H. pomatia*. The *arrows* indicate the elution positions of the authentic pyridylaminated glycans: *arrows 1*, Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA; *arrows 2*, GlcAβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA; *arrows 3*, Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA.

were detected in the reaction mixture using both trisaccharide-PAs as acceptors (data not shown), and isolated by HPLC for further analyses. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of both products showed a molecular ion $[M-H+2Na]^+$ at *m/z* 843. Smith degradation (SD) of the intact acceptors and isolated products was carried out as described under "Experimental Procedures," and the predicted products are shown in Fig. 9. The terminal galactoses of Galβ1–4GlcNAcβ1–3Gal-PA and Galβ1–3GlcNAcβ1–3Gal-PA are cleaved by periodate, and thus the SD products are both GlcNAcβ1–3-L-threosyl-PA (Fig. 9*A*). When the GlcA was transferred to the C-3 position of the nonreducing terminal galactose by GlcAT-D, as *O*-3-substituted Gal was not cleaved, the SD products of the enzyme products using Galβ1–4GlcNAcβ1–3Gal-PA and Galβ1–3GlcNAcβ1–3Gal-PA as acceptors were Galβ1–4GlcNAcβ1–3-L-threosyl-PA (Fig. 9*B*) and Galβ1–3GlcNAcβ1–3-L-threosyl-PA (Fig. 9*C*), respectively. When the GlcA was linked at any position other than the C-3 position of galactose, the galactose was cleaved, and thus the SD products were both GlcNAcβ1–3-L-threosyl-PA (Fig. 9*A*). Fig. 10 shows the HPLC analysis of the SD products. The SD products of the two acceptors were eluted at the same retention time (6.1 min, Fig. 10, *A* and *D*), whereas two species of the SD products of the enzyme reaction products were eluted at different times from those of the acceptors (Fig. 10, *B* and *E*) and eluted at the same time (6.1 min) after β-galactosidase digestion (Fig. 10, *C* and *F*). These results indicate that the SD products of the glucuronyltransferase reaction products have a β-linked Gal at their nonreducing termini and therefore that the C-3 position of Gal of the enzyme reaction products is substituted with a GlcA. In conclusion, the cloned GlcAT-D is characterized as a galactoside β1,3-glucuronyltransferase.

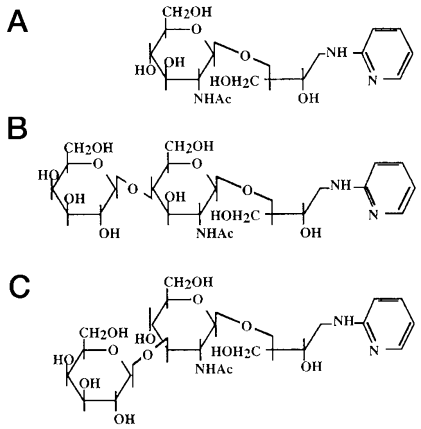

FIG. 9. **The predicted structures of Smith degradation products of the glucuronyltransferase reaction products.** The Smith degradation products of Galβ1–4GlcNAcβ1–3Gal-PA and Galβ1–3GlcNAcβ1–3Gal-PA are both GlcNAcβ1–3-L-threosyl-PA (*A*). The products of GlcAβ1–3Galβ1–4GlcNAcβ1–3Gal-PA and GlcAβ1–3Galβ1–3GlcNAcβ1–3Gal-PA are Galβ1–4GlcNAcβ1–3-L-threosyl-PA (*B*) and Galβ1–3GlcNAcβ1–3-L-threosyl-PA (*C*), respectively.

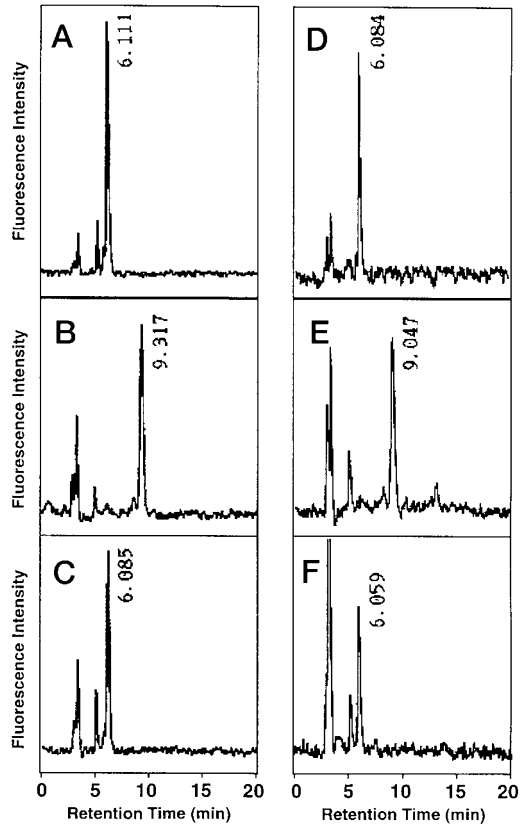

FIG. 10. **HPLC analysis of Smith degradation products.** Smith degradation was carried out as described under "Experimental Procedures." The Smith degradation products of Galβ1–4GlcNAcβ1–3Gal-PA (*A*) and Galβ1–3GlcNAcβ1–3Gal-PA (*D*) and the glucuronyltransferase reaction products of Galβ1–4GlcNAcβ1–3Gal-PA (*B*) and Galβ1–3GlcNAcβ1–3Gal-PA (*E*) were subjected to HPLC analysis using PALPAK type N column. After β-galactosidase digestion, the Smith degradation products of glucuronyltransferase reaction products of Galβ1–4GlcNAcβ1–3Gal-PA (*C*) and Galβ1–3GlcNAcβ1–3Gal-PA (*F*) were analyzed with the same HPLC column.

DISCUSSION

We cloned a new member of the glucuronyltransferase gene family that transfers a GlcA to the glycan chain terminated by a Gal residue. We named the cloned enzyme GlcAT-D, meaning a glucuronyltransferase with dual specificity for both glycolipid and glycoprotein acceptors as mentioned below. The GlcAT-D showed high homology with the two glucuronyltransferases cloned to date, rat GlcAT-P (21) and human GlcAT-I (24) (overall amino acid identity of 50.0 and 45.7%, respectively). These three enzymes may constitute a family of galactoside β1,3-glucuronyltransferases, which has high homology with hypothetical proteins of *D. melanogaster, C. elegans*, and *S. mansoni* (Fig. 2). In addition, a data base search suggested that more than five additional isoforms have been identified in *C. elegans* and that plant *Arabidopsis thaliana* and rice might have glucuronyltransferases homologous to this family (data not shown). The galactoside β1,3-glucuronyltransferase appears to be commonly conserved from plant, nematode, and insect to mammal.

The β-glucuronidase digestion and Smith degradation of the glucuronyltransferase reaction products demonstrated that the GlcAT-D catalyzed the formation of GlcAβ1–3Gal sequence, which is consistent with the determined structure of glycans containing the HNK-1 epitope, sulfated GlcAβ1–3Gal (16–19). For the first time in this cloned glucuronyltransferase family, the enzyme was confirmed to be a galactoside β1,3-glucuronyltransferase.

The GlcAT-D catalyzed the transfer of a GlcA to both glycoprotein and glycolipid containing Galβ1–4GlcNAc sequence at nonreducing termini. The transfer is assumed to form the precursor of HNK-1 epitope, which is used by HNK-1-sulfotransferase as an acceptor substrate. We observed that the COS-7 cells transfected with the cloned cDNA expressed HNK-1 epitope and that the sites *in situ* detected with the transcript were also stained with HNK-1 antibody. These results support the idea that this enzyme is a glucuronyltransferase involved in the biosynthesis of HNK-1 epitope.

The glucuronyltransferase activity involved in the biosynthesis of HNK-1 epitope has been studied using brain extracts from chick embryo (33), rat embryo (34), and postnatal rat (35, 36) as well as purified enzyme from postnatal rat forebrain (20). The present study showed that GlcAT-D acts on both glycoprotein and glycolipid acceptors *in vitro*, whereas GlcAT-P has been reported to be specific to glycoprotein acceptors (20, 35). It remains to be determined what these glucuronyltransferases use as acceptor substrates in the nervous system. Our study examined the substrate specificity as to the carbohydrate sequence using pyridylaminated glycans as acceptor substrates. The GlcAT-D transferred a GlcA to not only type 2 (Galβ1–4GlcNAc) but also type 1 (Galβ1–3GlcNAc) glycan chains, suggesting that the HNK-1 epitope expressed on type 1 glycan chain may occur, although the existence of such glycans has not previously been reported. This result is in conflict with those of previous studies, which showed that glucuronyltransferase from rat brain extract has no or little activity transferring a GlcA to a glycolipid containing type 1 glycan (34, 36). Because type 3 (Galβ1–3GalNAc) glycan did not serve as an acceptor of GlcAT-D, it remains to be examined whether the GlcAT-D can transfer a GlcA to Galβ1–3Gal, which is a good acceptor for GlcAT-I (24). In addition, the GlcAT-D did not transfer a GlcA to Fucα1–2Galβ1–4GlcNAc or Galβ1–4(Fucα1–3)GlcNAc, the product formed by α1,2- or α1,3-fucosyltransferase acting on type 2 glycan chain, suggesting that the glucuronyltransferase may also compete with these fucosyltransferases for acceptor substrates *in vivo*.

The Northern blot analysis showed that the ratio of the two major transcripts (1.1 and 2.3 kb) changes according to the developmental stage. Combined with the structure of the cloned cDNA, we speculated that the major initiation site of the transcription might differ between the early embryo and adult brain, although the structure of transcripts is yet to be analyzed.

In situ hybridization and immunohistochemistry on the developing brain revealed that regions that expressed GlcAT-D and/or GlcAT-P were always HNK-1-positive. This fact, along with the above-mentioned *in vitro* data, indicates that both GlcATs are involved in the synthesis of the HNK-1 epitope *in vivo*. The GlcAT-D expression pattern was slightly different from that of GlcAT-P. GlcAT-D transcripts were positive in the pallidum and retina where GlcAT-P expression was not specifically observed, suggesting different *in vivo* functions between the two GlcATs. GlcAT-D had catalytic activity on both glycoprotein and glycolipid acceptors in our *in vitro* assay, whereas GlcAT-P was reported to be active on only glycoprotein acceptors (20). This may reflect different localization patterns of the GlcATs, where the GlcAT-D is expressed in a wider region than GlcAT-P. It should be noticed that there were some HNK-1-positive areas where both GlcATs were negative. This observation suggests several possible mechanisms: 1) transcripts below the detection level by *in situ* hybridization, 2) existence of unknown GlcAT genes, or 3) proliferation or migration of HNK-1-positive neurons into transcript-negative areas. Further studies will reveal the mechanism of spatiotemporally restricted expression of HNK-1 epitope.

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