Molecular Cloning and Characterization of GalNAc 4-Sulfotransferase Expressed in Human Pituitary Gland

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We have previously cloned chondroitin-4-sulfotransferase (C4ST) cDNA from mouse brain. In this paper, we report cloning and characterization of GalNAc 4-sulfotransferase (GalNAc4ST), which transfers sulfate to position 4 of the nonreducing terminal GalNAc residue. The obtained cDNA contains a single open reading frame that predicts a type II transmembrane protein composed of 424 amino acid residues. Identity of the amino acid sequence between GalNAc4ST and human C4ST was 30%. When the cDNA was transfected into COS-7 cells, sulfotransferase activity toward carbonic anhydrase VI was overexpressed but no sulfotransferase activity toward chondroitin or desulfated dermatan sulfate was increased over the control. Sulfation of carbonic anhydrase VI by the recombinant GalNAc4ST occurred at position 4 of the GalNAc residue of N-linked oligosaccharides. The recombinant GalNAc4ST transferred sulfite to position 4 of GalNAc residue of p-nitrophenyl GalNAc, indicating that this sulfotransferase transfers sulfate to position 4 at the nonreducing terminal GalNAc residue. Dot blot analysis showed that the message of GalNAc4ST was expressed strongly in the human pituitary, suggesting that the cloned GalNAc4ST may be involved in the synthesis of the nonreducing terminal GalNAc 4-sulfate residues found in the N-linked oligosaccharides of pituitary glycoprotein hormones.

Sulfated sugar chains are found not only in glycosaminoglycans but also in oligosaccharides of glycoproteins and glycolipids (1). Sulfate moieties attached to the sugar residues of glycosaminoglycans and oligosaccharides play key roles in various molecular and cellular interactions: binding of FGF2 to heparan sulfate (2, 3); interaction of L-selectin on the lymphocytes with L-selectin ligands on the endothelial cells of high endothelial venule (4–10); binding of HNK-1 epitope to the sulfoglucuronyl carbohydrate-binding protein (11); and rapid clearance of a pituitary glycoprotein hormone, lutropin, mediated by the interaction with a hepatic reticuloendothelial cell receptor (12, 13). Various sulfotransferases involved in the sulfation of glycosaminoglycans (14) and oligosaccharides (15–17) have been cloned. We have purified and cloned chondroitin-6-sulfotransferase (C6ST) (18, 19) and chondroitin-4-sulfotransferase (C4ST) (20, 21), which are involved in the sulfation of position 6 and position 4, respectively, of GalNAc residues of chondroitin. C6ST also transfers sulfate to position 6 of Gal residue of keratan sulfate and sialyl N-acetyllactosamine oligosaccharides (22, 23). We have cloned keratan sulfate Gal-6-sulfotransferase using the homology with C6ST. Keratan sulfate Gal6ST transfers sulfate to position 6 of the Gal residue of keratan sulfate and sialyl N-acetyllactosamine oligosaccharides but not to GalNAc residue of chondroitin (24, 25). Several GlcNAc-6-sulfotransferases, which are involved in the synthesis of 6-sulfo-sialyl Lewis x, have been cloned from the family genes including C6ST and keratan sulfate Gal6ST (9, 10, 26). On the other hand, C4ST showed significant homology with HNK-1 sulfotransferase (21, 27), which transfers sulfate to position 3 of nonreducing terminal GlcA residue and is responsible for the synthesis of the HNK-1 epitope (16, 17). These observations suggest that, in some cases, sulfotransferases involved in the sulfation of glycosaminoglycans and sulfotransferases involved in the sulfation of oligosaccharides of glycoproteins may be included in a common gene family.

Nonreducing terminal GalNAc 4-sulfate residue is present in oligosaccharides attached to pituitary glycoprotein hormones (lutropin, follitropin, and thyrotropin) (28–30), pro-opiomelanocortin (31), and carbonic anhydrase VI of submaxillary gland (32), and was shown to play an important role in a pulsatile appearance of lutropin in the blood through the binding to the hepatic receptor for the sulfated GalNAc residue (12, 33). GalNAc 4-sulfotransferase (GalNAc4ST) that transfers sulfate to...
Cloning of GalNAc 4-Sulfotransferase

EXPERIMENTAL PROCEDURES

Materials—The following commercial materials were used: H$_2$SO$_4$, was from PerkinElmer Life Sciences; chondroitinase ACI, chondroitinase ABC, dermatan sulfate (pig skin), Δ-Di-0S, Δ-Di-4S, Δ-Di-di-$S_3$, and Δ-Di-di-$S_6$ were from Seikagaku Corp., Tokyo; Partisil SAX-10 was from Whatman; GalNAc 4-sulfate, GalNAc 6-sulfate, GalNAc 4,6-bissulfate, GlcNAc 6-sulfate, and GlcNAc 3-sulfate were from Sigma; recombinant F was from Roche Molecular Biochemicals; Hildao Superdex 30 HR 16/60, Fast Desalting Column HR 10/10 were from Amersham Pharmacia Biotech. Hiload Superdex 30 HR 16/60, Fast Desalting Column HR 10/10 were from Amersham Pharmacia Biotech.

Preparation of Carbonic Anhydrase VI from Bovine Submaxillary Gland—Carbonic anhydrase VI was purified from bovine submaxillary gland as described previously (32). All operations were carried out at 4°C. 200 g of the freshly excised glands, which were obtained from a local slaughterhouse under the help of a veterinary, Dr. A. Mabuchi, were put through a meat grinder and homogenized by a Polytron homogenizer with 50 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM ethylenediamine tetraacetate (EDTA). The homogenate was centrifuged at 10,000 × g, and the supernatant was filtered through two layers of cotton cloth and then precipitated with an equal volume of a saturated ammonium sulfate solution for 1 h. The solution was centrifuged at 10,000 × g, and the precipitate was resuspended with 60 ml of 0.1 M NH$_4$HCO$_3$ and dialyzed against 4 changes of 1.5 liter of 0.1 M NH$_4$HCO$_3$. After centrifuging the diastase at 100,000 × g for 60 min, one-half of the solution (50 ml) was passed over 5 ml of p-aminomethylbenzene sulfonamide-garose (Sigma) followed by washing with 150 ml each of 0.1 M NH$_4$HCO$_3$ and 0.2 M NaI in 0.1 M NH$_4$HCO$_3$. The column was eluted in 40 ml of 0.4 M NaI in 0.1 M NH$_4$HCO$_3$, and the fractions containing protein and carbonic anhydrase activity were pooled and dialyzed against 300 ml of 25 mM Tris-HCl, pH 7.4. This affinity chromatography was repeated once. The diastase was bound to a 15-ml DEAE-Sepacel column equilibrated with 25 mM Tris-HCl, pH 7.4, for 60 min, washing in 200 ml of 25 mM NaCl in 25 mM Tris-HCl, pH 7.4, and elution in 100 ml of 200 mM NaCl in 25 mM Tris-HCl, pH 7.4. The fractions containing protein and carbonic anhydrase activity were pooled and dialyzed against 25 mM Tris-HCl, pH 7.4. Carbonic anhydrase activity was determined by the method using phenol red (40). Through the purification, 20 mg of carbonic anhydrase was obtained.

Polymerase Chain Reaction and Preparation of a Probe for Screening—When the sequence of mouse C4ST was used for the homology search, we found a human expressed sequence-tagged cDNA clone (accession number AC005615). Examination of the sequence of the cDNA indicated the presence of the nucleotide sequences corresponding to putative PAPS binding motifs (5’-PSB and 3’-PB) found in every sulfotransferases; therefore, we predicted that this cDNA would encode a novel sulfotransferase with the substrate specificity similar to that of C4ST. We designed oligonucleotide primers for PCR from the sequence of the clone to amplify a DNA fragment, which was used as a probe for screening COS-7 cDNA library. The primers CGACC/CAGGTTAC/TTCTTGCA and GATGTCGGGCTTTGAGCCG, respectively. The PCR reaction was carried out in a final volume of 50 μl containing 50 pmol each of the oligonucleotide primers, 1 μl of human brain cDNA solution (OriGene Technologies), 0.2 μM each of four deoxynucleoside triphosphates, 1.5 unit of AmpliTaq polymerase (PerkinElmer Life Sciences). Amplification was carried out by 40 cycles of 94°C for 45 s, 44°C for 1.5 min, and 72°C for 1 min. Reaction products were subjected to electrophoresis and the amplified DNA band (416 base pairs) was recovered from the gel. The radioactive probe for screening of the cDNA library was prepared from the PCR product by the random oligonucleotide-primed labeling method (41) using [α-32P]dCTP (Amersham Pharmacia Biotech) and a DNA random labeling kit (Takara Shuzo).

Screening of agt 11 Library—Approximately 4 × 109 plaques from the human fetal brain cDNA library (CLONTECH) were screened. Hybond N+ nylon membrane (Amersham Pharmacia Biotech) replicas of the plaques from the agt 11 cDNA library were fixed by the alkaline fixation method recommended by the manufacturer, prehybridized in a solution containing 50% formamide, 5 × SSPE, 5 × Denhardt’s solution, 0.5% SDS, 0.04 mg/ml denatured salmon sperm DNA for 5.5 h at 42°C. Hybridization was carried out in the same solution containing 32P-labeled probe for 16 h at 42°C. The filters were washed at 55°C in 1 × SSPE, 0.1% SDS, and subsequently in 0.1 × SSPE, 0.1% SDS, and positive clones were detected by autoradiography.

DNA Sequence Analysis—DNA from agt 11 positive clones were isolated and cut with EcoRI, which excised the cDNA insert. The fragments were inserted into pBluescript II vector (Stratagene). The complete nucleotide sequence was determined by the dideoxy chain termination method using a DNA sequencer (Applied Biosystem Model 373A). DNA sequences were compiled and analyzed using the MacVector computer programs (Oxford Molecular Group PLC).

Construction of pFLAGGalNAc4ST and Transient Expression of GalNAc4ST cDNA in COS-7 Cells—A DNA fragment which codes for full open reading frame was amplified by PCR using human GalNAc4ST cDNA as a template. The 5′ and 3′ primers were CGACCGTATGACCGTCGACCGAT and CAGAAGGTTTACGAGCGTGGGGAT, respectively. The PCR reaction was carried out in a final volume of 50 μl containing 50 pmol each of the oligonucleotide primers, 1 μl of human brain cDNA solution (OriGene Technologies), 0.2 μM each of four deoxynucleoside triphosphates, 1.5 unit of AmpliTaq polymerase (PerkinElmer Life Sciences). Amplification was carried out by 40 cycles of 94°C for 45 s, 57°C for 1.5 min, and 72°C for 1 min. The PCR product was digested with EcoRI and HindIII, and subcloned into these sites of pFLAG-CMV-2 plasmid (Kodak, New Haven, CT). COS-7 cells (obtained from Riken Cell Bank, Tsukuba, Japan) were plated in 100-mm culture dishes at a density of 8 × 10$^5$/dish. Volume of the medium was 25 ml. The medium used was Dulbecco’s modified Eagle’s medium containing penicillin (100 units/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum (Life Technologies, Inc.), and cells were grown at 37°C in 5% CO2, 95% air. When the cell density reached 3 × 10$^5$/dish, the medium was replaced with 5 ml of 10% dimethyl sulfoxide in phosphate-buffered saline. After the cells were left at room temperature for 2 min, the dimethyl sulfoxide solution was aspirated and 25 ml of Dulbecco’s modified Eagle’s medium containing 10% FCS and 10% FBS was added. After incubation for 60–65 h, the cells were washed with Dulbecco’s modified Eagle’s medium alone, and the recombinant protein was produced in the conditioned media as assayed by the method described previously (42). The standard reaction mixture contained 50 mM imidazole-HCl, pH 6.8, 0.0025% proteinate chloride, 2 mM dithiothreitol, 25 mM (as gluconic acid) chondroitin, 50 pmol of [γ-32P]SIPAPS (about 5.0 × 10$^5$ cpm), and enzyme in a final volume of 50 μl. For determination of the activity toward desulfated dermatan sulfate, chon-
droitin was replaced with 25 nmol (as galactosamine) of desulfated dermatan sulfate and the amount of protamine chloride was increased to 0.02%. The reaction mixtures were incubated at 37 °C for 20 min and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, 35S-labeled glycosaminoglycans were isolated by the precipitation with ethanol followed by gel chromatography with a Fast Desalting Column as described previously and radioactivity was determined. For determining the incorporation into position 4 and position 6 of GalNAc residues, 35S-labeled chondroitin and 35S-labeled desulfated dermatan sulfate were digested with chondroitinase ACII and chondroitinase ABC, respectively. The resulting unsaturated disaccharides (DDi-4S and DDi-6S) were separated with paper chromatography, and their radioactivities were measured.

Assay of GalNAc4ST Activity—GalNAc4ST activity was assayed using carbonic anhydrase VI as an acceptor by the method described previously (34) with slight modification. The standard reaction mixture contained 15 mM imidazole-HCl, pH 7.2, 6 mM Mg(CH3COO)2, 40 mM 2-mercaptoethanol, 1% Triton X-100, 10 mM NaF, 0.1 mM 5'-AMP, 13% glycerol, 10 μg of the purified carbonic anhydrase VI, 50 pmol of [35S]PAPS (about 5.0 × 10^5 cpm), and enzyme in a final volume of 50 μl. The reaction mixtures were incubated at 37 °C for 60 min to degrade excess amounts of [35S]PAPS. After the mixtures were spotted on Toyo No. 51A filter paper, the filter paper was developed with a solvent described below until the solvent front reached the edge of the paper. The dried paper strips were cut into 1.25-cm segments, which were analyzed for radioactivity by liquid scintillation counting.

Dot blot hybridization—Human Multiple Tissue Expression Array was prehybridized in ExpresHyb solution (CLONTECH) at 68 °C. Hybridization was carried out in the same solution containing 32P-labeled probe for 1 h at 68 °C. The radioactive probe was prepared from the cDNA fragment excised from the pBluescript II plasmid with EcoRI by the random oligonucleotide-primed labeling method using [α-32P]dCTP and a DNA random labeling kit (Takara Shuzo). The filters were washed at room temperature in 2× SSC, 0.1% SDS, and subsequently in 0.1× SSC, 0.1% SDS at 50 °C. The membrane was exposed to x-ray film at −80 °C with an intensifying screen.

SDS-Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis of proteins in SDS was carried out on 10% polyacrylamide gels as described (43). Protein bands were detected by Coomasie Brilliant Blue. 35S radioactivity was detected by autoradiography after the gel was dried.
Protein was precipitated with 10% trichloroacetic acid and analyzed by the methods recommended by the manufacturer. After digestion, the precipitates were washed with acetone and digested with recombinant N-glycosidase F (Roche Molecular Biochemicals) by the methods recommended by the manufacturer. After digestion, the protein was precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE. Oligosaccharides released by N-glycosidase F digestion were recovered from the supernatant of 10% trichloroacetic acid.

Superdex 30 Chromatography, Paper Electrophoresis, Paper Chromatography, and HPLC—A Superdex 30 16/60 column was equilibrated with 0.2 M NH₄HCO₃ and run at a flow rate of 1 ml/min. One-ml fractions were collected. Paper electrophoresis was carried out on Whatman No. 53 paper (25 cm × 75 cm) in pyridine/acetate/water (10:10:80, by volume) at 30 V/cm for 40 min. Paper chromatography was performed on Toyo No. 51A paper (20 cm × 50 cm) using a solvent system, 1-butanol, acetic acid, 1 M NH₃ (2:3:1, by volume). The dried paper strips after paper electrophoresis or paper chromatography were cut into 1.5-cm segments, which were analyzed for radioactivity by liquid scintillation counting. Separation of GalNAc4ST(4SO₄) was carried out by HPLC using a Whatman Partisil 10-SAX column (4.6 mm × 25 cm) equilibrated with 10 mM KH₂PO₄. The column was developed with 10 mM KH₂PO₄ for 10 min followed by a linear gradient from 10 to 450 mM KH₂PO₄ as indicated in Fig. 5. Fractions (0.5 ml) were collected at a flow rate of 1 ml/min and a column temperature of 40 °C.

RESULTS
cDNA and Predicted Protein Sequence of the GalNAc4ST—When approximately 4 × 10⁸ plaques of a human fetal brain cDNA library were screened using a probe, which was prepared by PCR using human brain cDNA as a template and primer oligonucleotides designed from the sequence of a human expressed sequence-tagged cDNA clone (accession number AC005615), two cDNA clones (2.2 and 1.3 kilobase pairs) were isolated. One of these clones (2.2 kilobase pairs) was found to contain whole open reading frame. The nucleotide sequence of the GalNAc4ST cDNA and the predicted amino acid sequence are shown in Fig. 1A. A single open reading frame predicts a protein of 424 amino acid residues with four potential N-linked glycosylation sites. Putative PAPS-binding domains (5'-PSB and 3'-PB) were well conserved. Homology sequences of 5'-PB were not found. Comparison of the coding sequence of human GalNAc4ST with that of human C4ST (45) has revealed that there are 30% identity on the amino acid level (Fig. 2). Homology in the amino acid sequence between the two proteins was observed in the carboxyl-terminal side of the molecules. Especially, amino acid sequences of 5'-PSB and 3'-PB were well conserved. Homology of the N-terminal region was rather poor.

Expression of GalNAc4ST cDNA in COS-7 Cells—COS-7 cells were transfected with the pFLAGGalNAc4ST, a recombinant plasmid containing the isolated cDNA in the mammalian system, 1-butanol, acetic acid, 1 M NH₃ (2:3:1, by volume). The dried paper strips after paper electrophoresis or paper chromatography were then analyzed for location of any transmembrane domain, a hydropathy plot was generated from the translated sequence. Analysis of the plot revealed one prominent hydrophobic segment in the amino-terminal region, 22 residues in length, that extends from amino acid residues 10 to 31 (Fig. 1B).

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expression vector pFLAG-CMV-2. The transfected cells were extracted with a buffer containing 0.5% Triton X-100 and centrifuged. Activities of sulfotransferase was determined using chondroitin, desulfated dermatan sulfate, or carbonic anhydrase VI as acceptors. In contrast, no expression of the sulfotransferase activity was observed when carbonic anhydrase VI was incubated with the recombinant GalNAc4ST, was pooled and used for further analysis. D, peak 5 fraction from C was separated by paper chromatography for 20 h. The arrows indicate the position of standard sugars detected by silver nitrate staining: a, ΔDi-0S; b, GalNAc(4SO₄); c, ΔDi-6S; d, ΔDi-4S; e, GalNAc(6SO₄); f, GalNAc(3SO₄); g, GalNAc(4SO₄); h, GalNAc(3SO₄); i, GalNAc(4SO₄); j, GalNAc(3SO₄); k, GalNAc(4SO₄); l, GalNAc(3SO₄); m, GalNAc(4SO₄); n, GalNAc(3SO₄); o, GalNAc(4SO₄); p, GalNAc(3SO₄); q, GalNAc(4SO₄); r, GalNAc(3SO₄); s, GalNAc(4SO₄); t, GalNAc(3SO₄); u, GalNAc(4SO₄); v, GalNAc(3SO₄); w, GalNAc(4SO₄); x, GalNAc(3SO₄); y, GalNAc(4SO₄); z, GalNAc(3SO₄).

Analysis of Sulfated Carbonic Anhydrase VI—When ³⁵S-labeled carbonic anhydrase VI was separated with SDS-PAGE, the radioactivity was coincided with the protein band visualized with Coomassie Blue (Fig. 4, lane 1 and 3) The radioactivity, however, was completely removed after N-glycosidase F digestion (Fig. 4, lane 2 and 4), indicating that ³⁵SO₄ was transferred to N-linked oligosaccharides of carbonic anhydrase VI. The ³⁵S-labeled N-linked oligosaccharides released from the ³⁵S-labeled carbonic anhydrase VI with N-glycosidase F digestion were identified with Superdex 30 chromatography (Fig. 5A). The ³⁵S-labeled oligosaccharide fractions were hardly obtained when carbonic anhydrase VI was incubated with the extracts from COS-7 cells transfected with vector alone (control extract) (open circle in Fig. 5A). The ³⁵S-labeled oligosaccharides were subjected to mild acid hydrolysis (40 mM HCl, 100 °C, 120 min) and separated with the Superdex 30 column again (Fig. 5B). The ³⁵S radioactivity was detected in the position of GalNAc(4SO₄), inorganic sulfate and larger molecules, which were thought to be partially degraded oligosaccharides. The fractions corresponding to GalNAc(4SO₄) and inorganic sulfate (indicated by a horizontal bar in Fig. 5B) were combined. After removal of inorganic sulfate by paper electrophoresis, the materials which behaved together with GalNAc(4SO₄) from the paper and used for further analysis. B, peak 2 fraction from A was separated by paper electrophoresis. Peak 3 (indicated by a horizontal bar) was eluted from the paper and subjected to mild acid hydrolysis (40 mM HCl, 100 °C, 60 min). C, after the mild acid hydrolysis, peak 3 from B was separated by paper electrophoresis. Peak 6 represents inorganic sulfate. Peak 5 (indicated by a horizontal bar), which was observed only when pNP-GalNAc was incubated with recombinant GalNAc4ST, was pooled and used for further analysis. D, peak 5 fraction from C was separated by paper chromatography for 20 h. The arrows indicate the position of standard sugars detected by silver nitrate staining: a, ΔDi-0S; b, GalNAc(4SO₄); c, ΔDi-6S; d, ΔDi-4S; e, GalNAc(6SO₄); f, GalNAc(3SO₄); g, GalNAc(4SO₄); h, GalNAc(3SO₄); i, GalNAc(4SO₄); j, GalNAc(3SO₄); k, GalNAc(4SO₄); l, GalNAc(3SO₄); m, GalNAc(4SO₄); n, GalNAc(3SO₄); o, GalNAc(4SO₄); p, GalNAc(3SO₄); q, GalNAc(4SO₄); r, GalNAc(3SO₄); s, GalNAc(4SO₄); t, GalNAc(3SO₄); u, GalNAc(4SO₄); v, GalNAc(3SO₄); w, GalNAc(4SO₄); x, GalNAc(3SO₄); y, GalNAc(4SO₄); z, GalNAc(3SO₄).
Cloning of GalNAc 4-Sulfotransferase

The GalNAc4ST activity was determined as described under “Experimental Procedures” except that the concentration of carbonic anhydrase VI was varied. The inset represents the double reciprocal plot, in which the concentration of carbonic anhydrase VI was calculated on the assumption that molecular weight of carbonic anhydrase VI is 41,000.

Analysis of Sulfated p-Nitrophenyl GalNAc—It was reported that N-linked oligosaccharides attached to carbonic anhydrase VI contained GalNAcβ-4GlcNAc sequence at the nonreducing terminal (32); therefore, it is most likely that $^{35}$SO$_4$ was transferred to GalNAc residue at the nonreducing terminal. To demonstrate that the recombinant GalNAc4ST could transfer sulfate to nonreducing terminal GalNAc residue, we tested the possibility that p-nitrophenyl-β-D-GalNAc (pNP-GalNAc) could serve as acceptor for GalNAc4ST, since pNP-GalNAc was reported to inhibit GalNAc4ST activity (34). After pNP-GalNAc was incubated with the recombinant GalNAc4ST together with $[^{35}]$S]PAPS, the reaction products were separated with paper chromatography. A radioactive peak migrating near the solvent front was observed (peak 2 in Fig. 6A). This peak was also observed when pNP-GalNAc was incubated with the control extract. The radioactive materials contained in peak 2 (indicated by a horizontal bar in Fig. 6A) were eluted from the paper and separated with paper electrophoresis (Fig. 6B). Two peaks (peaks 3 and 4 in Fig. 6B) were observed when pNP-GalNAc was incubated with the control extract. When peak 3 in Fig. 6B (indicated by a horizontal bar) was eluted, subjected to mild acid hydrolysis (40 mM HCl, 100°C, 60 min) and separated again with paper electrophoresis, a radioactive peak (peak 5 in Fig. 6C), which migrated slowly than inorganic sulfate (peak 6 in Fig. 6C), was observed when pNP-GalNAc was incubated with the recombinant GalNAc4ST. A small peak was observed slightly ahead of peak 5, but this peak was not examined further. Peak 5 was not detected at all when pNP-GalNAc was incubated with the control extract. The mild acid hydrolysis of peak 4 in Fig. 6B resulted in complete release of inorganic sulfate even when pNP-GalNAc was incubated with the recombinant GalNAc4ST (data not shown). When peak 5 was recovered and separated with paper chromatography, the 35S radioactivity was detected in two peaks (Fig. 6D). One of the two peaks (peak 7 in Fig. 6D) migrated to the position of GalNAc(6SO$_4$)$_3$, and was clearly separated from GalNAc(6SO$_4$)$_2$ and GalNAc(6SO$_4$)$_1$. The faster migrating peak (peak 8 in Fig. 6D) seemed to contain sulfated pNP-GalNAc which remained intact during the mild acid hydrolysis. These observations clearly indicate that GalNAc4ST transfers sulfate to position 4 of nonreducing terminal GalNAc residue. Both the recombinant GalNAc4ST and the control extracts catalyzed the formation of 35S-labeled material that was degraded completely by the mild acid hydrolysis (peak 4 in Fig. 6B). Since this acid-
labile $^{35}$S-labeled material was formed when $p$-nitrophenol was used as acceptor, and was migrated together with $p$-nitrophe-nyl sulfate in paper chromatography and paper electrophoresis (data not shown), this material appears to be $p$-nitrophenyl sulfate. $p$-Nitrophenyl sulfate might be formed by the sulfation of contaminating $p$-nitrophenol in $p$NP-GalNAc with endoge-nous cytosol sulfotransferase. Unlike $p$NP-GalNAc, no sulfated GlcNAc was obtained when $p$NP-GlcNAc was used as acceptor, although acid-labile $^{35}$S-labeled material was formed (Fig. 7). These results suggest that GalNAc4ST may not transfer sulfate to nonreducing terminal GlcNAc residue.

**Properties of GalNAc4ST**—The pH optimum for the recombinant GalNAc4ST was around 7.2 (Fig. 8A). The recombinant GalNAc4ST was stimulated with 2-mercaptoethanol (Fig. 8B) and protamine chloride (Fig. 8C). These properties were similar to those of the GalNAc4ST preparation from the bovine pituitary (34). The $K_m$ for carbonic anhydrase VI was 10 $\mu$M on the assumption that molecular weight of the purified carbonic anhydrase VI is 41,000 (Fig. 9). This value is similar to the $K_m$ for GalNAc$\beta$$\alpha$1–4GlcNAc$\beta$$\alpha$1–2Man$\beta$$\alpha$-O-(CH$_2$)$_2$-COOCH$_3$ of the pituitary GalNAc4ST (34).

**Dot Blot Analysis**—Dot blot analysis using Human Multiple Tissue Expression Array (CLONTECH) showed that GalNAc4ST was expressed in various brain tissues and placenta; the strongest expression was observed in the pituitary gland (Fig. 10).

**DISCUSSION**

We have cloned GalNAc4ST from a fetal brain library as a protein showing sequence homology with C4ST. GalNAc4ST shared several properties with C4ST: 1) both sulfotransferases were type II transmembrane proteins having four potential $N$-glycosylation sites. 2) Amino acid sequences of the putative PAPS-binding domains, especially 3$\beta$PB, of these sulfotran-sferases were highly conserved. 3) 2-Mercaptoethanol and protamine chloride activated both sulfotransferases. 4) Both sulfotransferases transferred sulfate to position 4 of GalNAc residue. However, expression pattern in various human tissues were quite different; human C4ST was expressed strongly in peripheral blood leukocytes (27, 45) and colorectal adenocarcinoma (45), whereas expression of GalNAc4ST was detected in the various brain-related tissues and placenta. The strongest expression of GalNAc4ST was observed in the pituitary gland, suggesting that the cloned GalNAc4ST might participate in the biosynthesis of nonreducing terminal GalNAc(4SO$_4$) residue found in $N$-linked oligosaccharides of pituitary hormones. As observed in C4ST, GalNAc4ST also contains Cys in the 5$\beta$PSB domain, and was activated with 2-mercaptoethanol, suggesting...
that the Cys residue in 5'-PSB may be relevant to the stimulation of GalNAc4ST and C4ST by 2-mercaptoethanol.

Although both C4ST and GalNAc4ST transfer sulfate to position 4 of GalNAc residue, a clear difference in the recognition of the neighboring sugar residue was observed between these sulfotransferases. GalNAcNAc residues in the repeating disaccharide units of chondroitin, GalNAcβ1–4GlcA, acted as acceptor for C4ST, but did not serve as acceptor for GalNAc4ST. On the other hand, GalNAcNAc residues in the nonreducing terminal GalNAcβ1–4GlcNAc sequence present in N-linked oligosaccharides of carbonic anhydrase VI did not serve as acceptor for C4ST. It has been reported that isoforms of a glycosaminoglycan sulfotransferase transferred sulfate to the same position of the same sugar residue, but showed difference in the recognition of the structure of the neighboring sugar residue. Both 30-ST-1 and 30-ST-2 transferred sulfate to position 3 of GlcN(SO4)2, but 30-ST-1 required GlcA at the nonreducing side, whereas 30-ST-2 required IdoA(2SO4) or GlcA(2SO4) (47, 48). HS6ST-1, -2, and -3 transferred sulfate to position 6 of GlcN(SO4)2 of heparan sulfate, but each isoform showed the different specificity toward the isomeric hexuronic acid adjacent to the targeted N-sulfoglucosamine; HS6ST-1 appeared to prefer iduronosyl N-sulfoglucosamine unit, while HS6ST-2 had the different substrate preference depending upon the concentration of substrate and HS6ST-3 acted on either substrate (49). To understand the substrate specificity, it will be required to establish the three-dimensional interaction between each sulfotransferase and acceptor substrates.

Nonreducing terminal GalNAc(4SO4)b1–4GlcNAc sequence found in pituitary hormones has been implicated in the pulsatil characteristic of the circulating hormone levels through binding to the receptor for sulfated GalNAcβ1–4GlcNAc termini expressed by hepatic endothelial cells and Kupffer cells (12, 33). A pituitary sulfotransferase responsible for the 4-O-sulfation of terminal GalNAcNAc residue was characterized using GalNAcβ1–4GlcNAcβ1–2Manα(1→2)Manα(1→6)Manα(1→4)GlcAβ1–4GlcAβ1–3Galβ1–3Galβ1–4GlcNAc (GGnMCO) as an acceptor (34), and the sulfotransferase with the same substrate specificity as that of the putative sulfotransferase was purified from bovine submaxillary gland (35). The recombinant GalNAc4ST expressed in COS-7 cells from the cDNA shared several properties with the purified GalNAc4ST. The pH optimum of both the recombinant GalNAc4ST and the purified GalNAc4ST fell between 7.0 and 7.5, and both the sulfotransferases were activated with 2-mercaptoethanol and protamine chloride. In contrast, molecular size of the purified GalNAc4ST was quite different from that of the recombinant GalNAc4ST; molecular size of the purified GalNAc4ST was 128 kDa on SDS-PAGE (35), whereas molecular mass of the recombinant GalNAc4ST calculated from the cDNA was 48,831. Such a discrepancy in molecular size may be explained by a hypothetical that the Cys residue in 5'-PSB may be relevant to the stimulation of GalNAc4ST and C4ST by 2-mercaptoethanol.

Human genomic clones (accession numbers AC010510, AC007205, and AC005615) located on chromosome 19q13.1 were found to contain nucleotide sequences identical to the sequence of GalNAc4ST cDNA. From these genomic clones, GalNAc4ST gene was found to be composed of at least four exons, A, exons are indicated by boxes and introns are indicated by lines. Closed boxes represent the coding sequence and open boxes indicate 5'- and 3'-untranslated sequence. The lateral lengths of boxes and lines are roughly proportional to the number of nucleotides. ATG, TGA, and poly(A) indicate initiation codon, termination codon, and the presumptive polyadenylation signal, respectively. B, nucleotide sequences of the exon-intron junctions. Nucleotide sequences of exon and intron were indicated by uppercase and lowercase, respectively. Numbers under the sequences represent the nucleotide number indicated in Fig. 1A.

FIG. 11. Putative genomic organization of GalNAc4ST gene. The pH optimum of both the recombinant GalNAc4ST and the purified GalNAc4ST fell between 7.0 and 7.5, and both the sulfotransferases were activated with 2-mercaptoethanol and protamine chloride. In contrast, molecular size of the purified GalNAc4ST was different from that of the recombinant GalNAc4ST; molecular size of the purified GalNAc4ST was 128 kDa on SDS-PAGE (35), whereas molecular mass of the recombinant GalNAc4ST calculated from the cDNA was 48,831. Such a discrepancy in molecular size may be explained by a hypothetical that the Cys residue in 5'-PSB may be relevant to the stimulation of GalNAc4ST and C4ST by 2-mercaptoethanol.
exon, suggesting that the GalNAc4ST cDNA did not contain the sequence of 5'-terminal region of the first exon. PAPS-binding domains (5'-PSB and 3'-PB) were both present in the fourth exons.

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