J. Biochem. **134**, 111–120 (2003)
DOI: 10.1093/jb/mvg118

Mouse *N*-Acetylgalactosamine 4-Sulfotransferases-1 and -2. Molecular Cloning, Expression, Chromosomal Mapping and Detection of Their Activity with GalNAcβ1-4GlcNAcβ1-octyl

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Received April 20, 2003; accepted May 8, 2003

***N*-Acetylgalactosamine 4-sulfotransferase (GalNAc4ST) transfers sulfate to position 4 of nonreducing terminal GalNAc residues. We previously cloned human GalNAc4ST-1 cDNA. In this paper, we report the cloning, characterization and chromosomal mapping of mouse GalNAc4ST-1 and GalNAc4ST-2. Mouse GalNAc4ST-1 and GalNAc4ST-2 contain single open reading frames that predict type II transmembrane proteins composed of 417 and 413 amino acid residues, respectively. The amino acid sequence identity between the two isoforms is 49%. When the cDNA was transfected to COS-7 cells, sulfotransferase activities toward carbonic anhydrase VI and GalNAcβ1-4GlcNAcβ1-octyl were overexpressed, but the sulfotransferase activity toward chondroitin showed no increase over the control level. Northern blot analysis showed that the 2.4 kb messages of GalNAc4ST-1 and GalNAc4ST-2 were strongly expressed in the kidney, where both of the human isoforms were hardly expressed. Reverse transcription-PCR analysis showed that, unlike human GalNAc4ST-1, the expression of mouse GalNAc4ST-1 in the pituitary gland was only marginal, while that of GalNAc4ST-2 in the pituitary gland was as high as that in the kidney. These results suggest that the functions of the two GalNAc4ST isoforms may differ between human and mouse. By fluorescence *in situ* hybridization, the GalNAc4ST-1 and GalNAc4ST-2 genes were localized to mouse chromosome 7B3 distal–B5 proximal and chromosome 18A2 distal–B1 proximal, respectively.**

Key words: chromosomal mapping, cloning, GalNAc 4-sulfotransferase, kidney, mouse.

Abbreviations: GalNAc4ST, *N*-acetylgalactosamine 4-sulfotransferase; C4ST, chondroitin 4-sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; GalNAc, *N*-acetyl-D-galactosamine; ΔDi-4S, 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; EST, expressed sequence-tagged.

Sulfated glycoproteins and glycolipids have been reported to play important roles in maintaining normal animal life: homing of lymphocytes *via* interaction of L-selectin and L-selectin ligands (*1*, *2*); essential roles of the HNK-1 epitope in higher brain functions (*3*, *4*); rapid clearance of a pituitary glycoprotein hormone, lutropin, mediated by interaction with a hepatic reticuloendothelial cell receptor (*5*, *6*); and myelin functions and spermatogenesis mediated by sulfatide and seminolipid, respectively (*7*).

A nonreducing terminal GalNAc 4-sulfate residue is present in oligosaccharides attached to pituitary glycoprotein hormones (lutropin, follitropin and thyrotropin) (*8–10*), pro-opiomelanocortin (*11*), carbonic anhydrase VI of submaxillary gland (*12*), Tamm-Horsfall glycoprotein (*13*, *14*), and urokinase (*15*). The nonreducing terminal GalNAc 4-sulfate residue present in lutropin was shown to play an important role in the clearance of this hormone in the blood through binding to the hepatic receptor for the sulfated GalNAc residue (*16*, *17*). However, the functions of the nonreducing terminal GalNAc 4-sulfate residue in the oligosaccharides attached to carbonic anhydrase VI, Tamm-Horsfall glycoprotein and urokinase remain unclear.

As a sulfotransferase exhibiting sequence homology to chondroitin 4-sulfotransferase (C4ST), we cloned *N*-acetylgalactosamine 4-sulfotransferase-1 (GalNAc4ST-1) from a human fetal brain library that transfers sulfate to the nonreducing terminal GalNAc residue attached to the *N*-linked oligosaccharides of carbonic anhydrase VI (*18*). The same sulfotransferase was cloned as a homologue of HNK-1 sulfotransferase (*19*, *20*). The message of GalNAc4ST-1 was expressed strongly in the pituitary (*18–20*), suggesting that GalNAc4ST-1 may be involved in the synthesis of the GalNAc 4-sulfate residue in an oligosac-

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charide attached to lutropin. Another isoform, GalNAc4ST-2, has been cloned (*20*, *21*). Human GalNAc4ST-2 was found to be expressed strongly in the trachea; however, the physiological acceptors in the trachea have not been identified. Human GalNAc4ST-2 has been shown to exhibit activity toward chondroitin as well as oligosaccharides containing the GalNAcβ1–4GlcNAc sequence at the nonreducing terminal (*21*). The activity toward chondroitin, however, has not been confirmed (*20*). To obtain the information on the differences in function and acceptor substrate specificity between GalNAc4ST-1 and GalNAc4ST-2, we cloned mouse GalNAc4ST-1 and GalNAc4ST-2, and compared the expression patterns of these mouse isoforms with the human counterparts. The expression patterns of mouse GalNAc4ST-1 and GalNAc4ST-2 were very different from those of the human counterparts; both mouse GalNAc4ST-1 and GalNAc4ST-2 were expressed strongly in the kidney.

MATERIALS AND METHODS

Materials—The following commercial materials were used: $H_2{}^{35}SO_4$ from Perkin-Elmer; chondroitinase ACII, ΔDi-6S, and ΔDi-4S from Seikagaku Corporation, Tokyo; Partisil SAX-10 from Whatman; GalNAc 4-sulfate, GalNAc 6-sulfate, GalNAc 4,6-bissulfate, GlcNAc 6-sulfate, and GlcNAc 3-sulfate from Sigma; and a Fast Desalting Column HR 10/10 from Amersham Pharmacia Biotech. [^{35}S]PAPS was prepared as described (*22*). GalNAc 3-sulfate was synthesized as described (*18*). Chondroitin was prepared from squid skin as previously described (*23*). Carbonic anhydrase VI was purified from bovine submaxillary gland as described previously (*18*)

Preparation of GalNAcβ1–4GlcNAcβ1-octyl and GalNAc(4SO$_4$) β1–4GlcNAcβ1-octyl—GalNAcβ1–4GlcNAcβ1-octyl and GalNAc(4SO$_4$)β1–4GlcNAcβ1-octyl were prepared as described below. The skeleton of GalNAcβ1–4GlcNAcβ1-octyl was constructed by fluoride activation glycosylation of 4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl fluoride with phenyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (*24*) in a bis(cyclopentadienyl)hafnium dichloride-$AgClO_4$ system, followed by the coupling of phenyl thioglycoside with octanol through the *N*-iodosuccinimide-trifluoromethanesulfonic acid promoted reaction. The phthalimido groups of octyl *O*-glycoside were converted to acetamido ones, and then the disaccharide obtained was deprotected by de-*O*-acetylation at the 4-position of the GalNAc residue and hydrogenation to give GalNAcβ1-4GlcNAcβ1-octyl. GalNAc(4SO$_4$)β1-4GlcNAcβ1-octyl was prepared by sulfation of the octyl *O*-glycoside having a hydroxy group at the 4-position of the GalNAc residue with sulfur trioxide, followed by Pd-catalyzed hydrogenation. The detailed preparation procedures will be reported elsewhere.

Octyl (2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1-4)-2-acetamido-2-deoxy-β-D-glucopyranoside; R_f = 0.15 (30% methanol-chloroform, double-development); ^{1}H NMR (399.65 MHz, CD_3OD) δ = 0.89 (t, 3H, J = 6.8 Hz, $OCH_2(CH_2)_6C\underline{H}_3$), 1.25–1.35 (m, 10H, $OCH_2(C\underline{H}_2)_6CH_3$), 1.49–1.54 (m, 2H, $OCH_2(C\underline{H}_2)_6CH_3$), 1.95 (s, 3H, $NHCOC\underline{H}_3$), 2.00 (s, 3H, $NHCOC\underline{H}_3$), 3.27–3.32 (m, 1H, H-5 or H-5′), 3.40–3.46 (m, 1H, $OC\underline{H}(H)(CH_2)_6CH_3$), 3.50–3.88 (m, 11H, H-2, H-3, H-4, H-6, H-3′, H-4′, H-6′, and H-5 or H-5′, $OCH(\underline{H})(CH_2)_6CH_3$), 3.97 (d, 1H, $J_{2',3'}$=10.6 Hz, $J_{1',2'}$=8.5 Hz, H-2′), 4.34 (d, 1H, $J_{1,2}$=8.3 Hz, H-1), and 4.45 (d, 1H, $J_{1',2'}$=8.5 Hz, H-1′); ^{13}C NMR (100.40 MHz, CD_3OD) δ=14.41 (q, $OCH_2(CH_2)_6\underline{C}H_3$), 22.95 (q, $NHCO\underline{C}H_3$), 23.08 (q, $NHCO\underline{C}H_3$), 23.73 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 27.12 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 30.49 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 30.65 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 30.75 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 33.02 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 54.32 (d, C-2′), 56.49 (d, C-2), 61.78 (t, C-6 or C-6′), 62.48 (t, C-6 or C-6′), 69.54 (d, C-4′), 70.70 (t, $O\underline{C}H_2(CH_2)_6CH_3$), 73.00 (d, C-3′), 74.32 (d, C-4), 76.41 (d, C-5 or C-5′), 77.18 (d, C-5 or C-5′), 81.40 (d, C-3), 102.84 (d, C-1), 103.49 (d, C-1′), 173.39 (s, $NH\underline{C}OCH_3$), and 174.05 (s, $NH\underline{C}OCH_3$).

Octyl (2-acetamido-2-deoxy-4-*O*-sulfonato-β-D-galactopyranosyl)-(1-4)-2-acetamido-2-deoxy-β-D-glucopyranoside sodium salt; R_f = 0.47 (50% methanol-chloroform); ^{1}H NMR (399.65 MHz, CD_3OD) δ=0.89 (t, 3H, J = 6.7 Hz, $OCH_2(CH_2)_6C\underline{H}_3$), 1.26–1.39 (m, 10H, $OCH_2(C\underline{H}_2)_6CH_3$), 1.49–1.56 (m, 2H, $OCH_2(C\underline{H}_2)_6CH_3$), 1.95 (s, 3H, $NHCOC\underline{H}_3$), 1.99 (s, 3H, $NHCOC\underline{H}_3$), 3.32–3.34 (m, 1H, H-5), 3.41–3.87 (m, 11H, H-2, H-3, H-4, H-6, H-3′, H-5′, H-6′, $OC\underline{H}_2(CH_2)_6CH_3$), 3.95 (d, 1H, $J_{2',3'}$ = 10.7 Hz, $J_{1',2'}$ = 8.3 Hz, H-2′), 4.33 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1), 4.49 (d, 1H, $J_{1',2'}$ = 8.3 Hz, H-1′), and 4.66 (d, 1H, $J_{3',4'}$ = 3.4 Hz, H-4′); ^{13}C NMR (100.40 MHz, CD_3OD) δ = 14.41 (q, $OCH_2(CH_2)_6\underline{C}H_3$), 22.96 (q, $NHCO\underline{C}H_3$), 23.07 (q, $NHCO\underline{C}H_3$), 23.73 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 27.12 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 30.48 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 30.64 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 30.75 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 33.02 (t, $OCH_2(\underline{C}H_2)_6CH_3$), 55.03 (d, C-2′), 56.42 (d, C-2), 61.79 (t, C-6 or C-6′), 62.22 (t, C-6 or C-6′), 70.70 (t, $O\underline{C}H_2(CH_2)_6CH_3$), 71.95 (d, C-3′), 74.32 (d, C-5′), 76.08 (d, C-4′), 76.26 (d, C-4), 76.37 (d, C-5), 81.75 (d, C-3), 102.81 (d, C-1), 103.44 (d, C-1′), 173.43 (s, $NH\underline{C}OCH_3$), and 173.95 (s, $NH\underline{C}OCH_3$).

Cloning of Mouse GalNAc4ST-1 and Mouse GalNAc4ST-2—Mouse GalNAc4ST-1 was cloned by screening of a mouse brain λgt 11 cDNA library (CLONTECH) using ^{32}P-labeled human GalNAc4ST-1 cDNA (*18*) as the probe. DNA from λgt 11 positive clones was isolated and cut with *Eco*RI, which excised the cDNA insert. The fragment was inserted into the pBluescript II KS$^-$ vector (Stratagene). When we searched the EST data base, we found a mouse cDNA clone (AK017407) that was homologous to human GalNAc4ST-2. This clone appeared to cover the whole open reading frame of mouse GalNAc4ST-2, but contained no hydrophobic transmembrane domain. It is possible that this clone might have been derived from alternatively spliced mRNA; therefore, we tried to isolate the cDNA by RT-PCR. Total RNA was prepared from adult C52/B mouse brains using TRIzol (Invitrogen) by the method recommended by the manufacturer. Synthesis of the first strand cDNA and amplification of DNA by PCR were carried out using a 5′ RACE system (Life Technology) according to the methods recommended by the manufacturer. An oligonucleotide primer, 4ST2-R1, TGGCTAGTTATTGCTTCCACAGCATA, which was synthesized according to the sequence of the cDNA clone, was used for the synthesis of the first strand cDNA. The PCR reaction was carried out using oligonucleotides 4ST2-F1, GGTGACTCTGTGGAATGACCTCCAAGA, and 4ST2-R2, CACAGCATAAGCTACAATGCCAAAACA, as primers, and the first strand cDNA as the template. Amplification was

carried out by 40 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 2 min. The second PCR was carried out using oligonucleotides 4ST2-F2, CGCTCTAGAATTCAGACATGAAGAGAGACA, and 4ST2-R3, CAGGGATCCCATACAGAGAATTGTAGGACA, as primers, and the first PCR reaction mixture as the template. At the 5′-ends of oligonucleotides 4ST2-F2 and 4ST2-R3, restriction enzyme recognition sites were introduced; a *Xba*I site for 4ST2-F2 and a *Bam*HI site for 4ST2-R3. Amplification was carried out by 40 cycles of 94°C for 20 s, 50°C for 30 s, and 72 °C for 2 min. The reaction products were subjected to agarose gel electrophoresis. The amplified DNA band was cut out and the DNA fragment was recovered from the gel, digested with *Xba*I and *Bam*HI, and then subcloned into these sites of pBluescript KS$^-$ (Stratagene). The complete nucleotide sequence was determined by the dideoxy chain termination method using a DNA sequencer (Applied Biosystem Model 373A).

Construction of pcDNAmGalNAc4ST-1 and pcDNAmGalNAc4ST-2, and Transient Expression in COS-7 Cells—The *Eco*RI fragment containing the 1703-bp cDNA was excised from the pBluescript plasmid containing mouse GalNAc4ST-1 and ligated into the *Eco*RI site of the pcDNA3 expression vector (Invitrogen) to construct pcDNAmGalNAc4ST-1. A DNA fragment that codes for the full open reading frame of mouse GalNAc4ST-2 was amplified by PCR using mouse GalNAc4ST-2 cDNA as a template, and oligonucleotide primers 4ST2-F3, CAGGGATCCACACAGTGGTCTGAAGTGGTC, and 4ST2-R4, CGCTCTAGAGGCCATCATCATTAAATCTATGTAG. At the 5′-ends of oligonucleotides 4ST2-F3 and 4ST2-R4, restriction enzyme recognition sites were introduced; a *Bam*HI site for 4ST2-F3 and a *Xba*I site for 4ST2-R4. The PCR reaction was carried out by 20 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min. The PCR product was digested with *Bam*HI and *Xba*I, and then subcloned into these sites of the pcDNA3 plasmid to construct pcDNAmGalNAc4ST-2. The transfection into COS-7 cells was performed by the DEAE-dextran method as described previously (*25*). The recombinant protein produced was extracted from the cells with a buffer comprising 10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5% Triton X-100, and 20% glycerol by gentle shaking on a rotatory shaker for 30 min at 4°C. The extract was centrifuged at 10,000 ×*g* for 10 min. The supernatant fraction was used for the experiments on the recombinant mouse GalNAc4ST-1 or mouse GalNAc4ST-2.

Assaying of Sulfotransferase Activity Toward Chondroitin—The sulfotransferase activity toward chondroitin was assayed by the method described previously (*26*). The standard reaction mixture contained 50 mM imidazole-HCl, pH 6.8, 0.0025% protamine chloride, 2 mM dithiothreitol, 25 nmol (as glucuronic acid) chondroitin, 50 pmol [^{35}S]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 20 min and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction had been stopped, ^{35}S-labeled glycosaminoglycans were isolated by precipitation with ethanol, followed by gel chromatography on the Fast Desalting Column as described previously (*27*), and then the radioactivity was determined.

Assaying of GalNAc4ST Activity—GalNAc4ST activity was assayed using carbonic anhydrase VI as the acceptor by the method described previously (*28*) with slight modification. The standard reaction mixture contained 15 mM imidazole-HCl, pH 7.2, 6 mM $Mg(CH_3COO)_2$, 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 [^{35}S]PAPS (about 5.0×10^5 cpm), and enzyme, in a final volume of 50 μl. The reaction mixtures were incubated at 28°C for 2 h. The reaction mixtures were placed in an ice bath and then injected into the Fast Desalting Column as described previously, and then the radioactivity in the void fraction was determined. GalNAc4ST activity was also assayed using GalNAcβ1-4GlcNAcβ1-octyl as the acceptor by the method described previously (*20*) with slight modification. The standard reaction mixture contained 50 mM imidazole-HCl, pH 6.8, 0.015% protamine, 40 mM 2-mercaptoethanol, 0.1% Triton-X 100, 10 mM NaF, 2 mM 5′-AMP, 25 nmol (as the sum of galactosamine and glucosamine) of GalNAcβ1-4GlcNAcβ1-octyl, 50 pmol [^{35}S]PAPS (about 5.0×10^5 cpm), and enzyme, in a final volume of 50 μl. The reaction mixtures were incubated at 28°C for 2 h. The reaction mixtures were immersed in a boiling water bath for 2 min and then diluted with 450 μl of 0.25 M ammonium formate, pH 4.0. Each diluted solution was passed through a Sep-Pak C-18 cartridge. After the cartridge had been washed with 3 ml of 0.25 M ammonium formate, pH 4.0, the adsorbed materials were eluted with 1 ml of 30% acetonitrile and then the radioactivity was determined.

Northern Blot Hybridization—Mouse Multiple Tissue Northern Blot Filters were prehybridized in ExpresHyb solution (CLONTECH) at 68°C. Hybridization was carried out in the same solution containing ^{32}P-labeled probe for 1 h at 68 °C. The radioactive probe was prepared from the cDNA fragments by the random oligonucleotide-primed labeling method using [α-^{32}P]dCTP and a DNA random labeling kit (Takara Shuzo). The cDNA fragment for mouse GalNAc4ST-1 was excised from the pBluescript II plasmid with *Eco*RI. The cDNA fragment for mouse GalNAc4ST-2 was prepared by PCR using mouse GalNAc4ST-2 cDNA as a template, and oligonucleotides 4ST2-F4, GAAGCGTCGGTCATTCCTTCA, and 4ST2-R5, GGTGAGCATCCAGCAAATAGTAC, as primers. The filters were washed at room temperature in 2 × SSC, 0.05% SDS, and subsequently in 0.1× SSC, 0.1% SDS at 50°C. The membranes were exposed to X-ray film at –80°C with an intensifying screen.

Semiquantitative RT-PCR Analysis—Total RNA was isolated from adult mouse brains as described above. RT-PCR was carried out using a SuperScript One-Step RT-PCR system (Invitrogen) by the method recommended by the manufacturer. Reverse transcription was carried out at 42°C for 30 min. The cycling conditions were as follows: 2 min at 94°C; 40 cycles of 15 s at 94°C, 30 s at 50°C and 1 min at 72°C; and one cycle of 2 min at 72°C. The following primers were used: for mouse GalNAc4ST-1, 4ST1-F, AACAGGAGCGCAAGCGTGTGA, and 4ST1-R, GGACATCCAACAGGTACTGGA, which amplify a 498 bp fragment corresponding to nucleotides 437–934 in the ORF of mouse GalNAc4ST-1; for mouse GalNAc4ST-2, 4ST2-F4 and 4ST2-R5, which amplify a 497 bp fragment

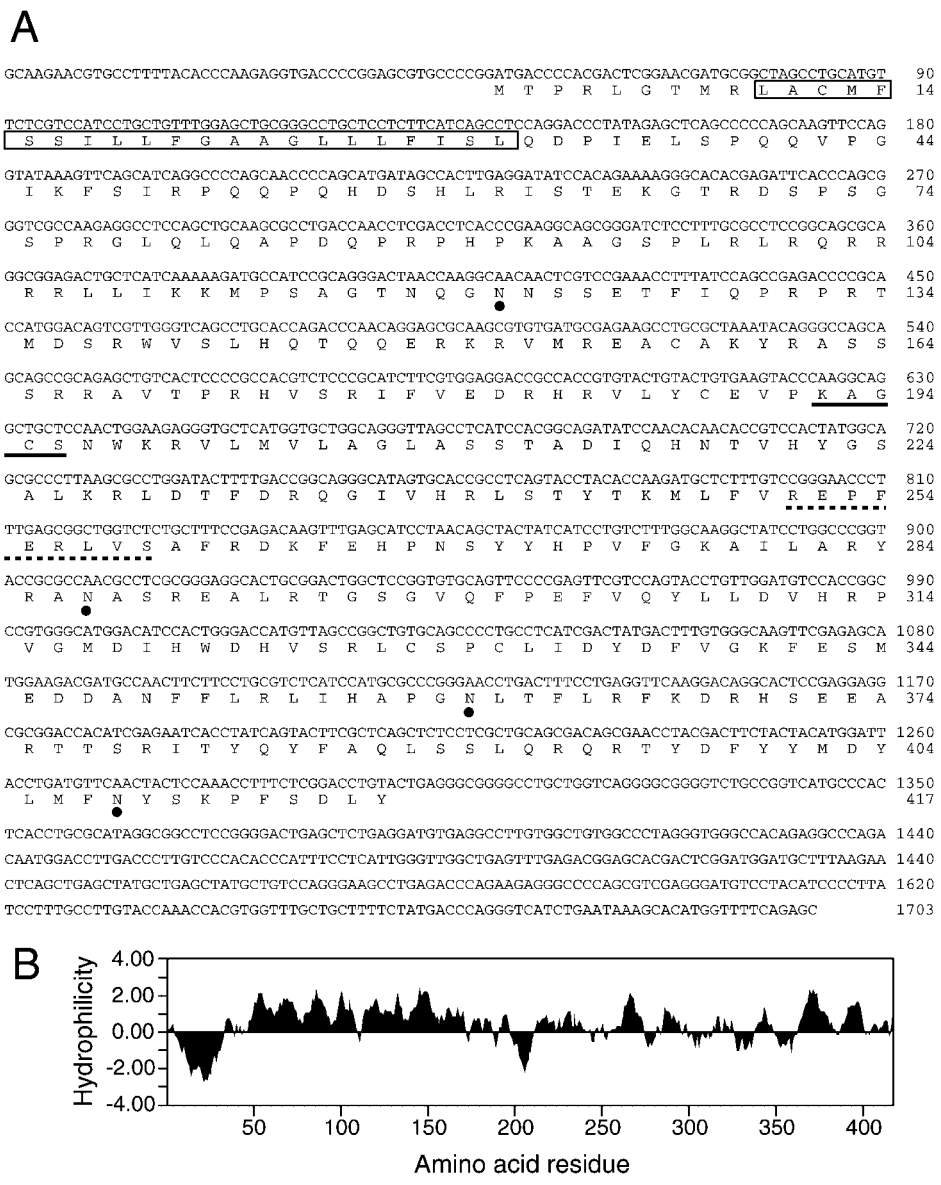

Fig. 1. **Nucleotide sequence of mouse GalNAc4ST-1, and predicted amino acid sequence.** (A) The predicted amino acid sequence is shown below the nucleotide sequence. Four potential *N*-linked glycosylation sites are indicated by dots. The putative transmembrane hydrophobic domain is boxed. The putative PAPS binding domains were underlined: solid line for 5′-PSB and broken line for 3′-PB. (B) Hydropathy plot of mouse GalNAc4ST-1. The hydropathy plot was calculated by the method of Kyte and Doolittle (*39*) with a window of 11 amino acids.

corresponding to nucleotides 438–934 in the ORF of mouse GalNAc4ST-2; and for β-actin, actin-F, GACCCAGATCATGTTTGAGAC and actin-R, ATGCCTGGGTACATGGTGGTA, which amplify a 570 bp fragment corresponding to nucleotides 357–926 in the ORF of mouse β-actin. The resulting amplified DNA was electrophoresed and visualized with ethidium bromide. To detect extremely weak expression, the second PCR reactions were carried out in a reaction mixture comprising 15 μl of the first RT-PCR reaction mixture and 1.5 units of EX Taq polymerase. The cylcing conditions were as follows: 2 min at 94°C; 40 cycles of 15 s at 94°C, 30 s at 50°C and 2 min at 72°C; and one cycle of 5 min at 72°C.

Assaying of Protein—Protein was determined by the method of Bradford (*29*) using bovine serum albumin as the standard. Protein assay reagent was obtained from BioRad.

Paper Electrophoresis and HPLC—Paper electrophoresis was carried out on Whatman No. 3 paper (2.5 cm × 57 cm) in pyridine/acetic acid/water (1:10:400, by volume, pH 4) at 30 V/cm for 40 min. Separation of GalNAc($4SO_4$) was carried out by HPLC on a Whatman Partisil 10-SAX column (4.6 mm × 25 cm) equilibrated with 5 mM KH_2PO_4. The column was developed with 5 mM KH_2PO_4 for 10 min, followed by a linear gradient of 5 to 500 mM KH_2PO_4, as indicated in Fig. 5. Fractions (0.5 ml) were collected at the flow rate of 1 ml/min and a column temperature of 40 °C.

Thin Layer Chromatography—Separation of the sulfated product formed from GalNAcβ1-4GlcNAcβ1-octyl was carried out by HPTLC on a HPTLC plate silica gel 60 (Merk). The plate was developed with the solvent system (chloroform:methnol = 4:1 by volume) eight times. The materials on the plate were detected by autoradiography and with sulfuric acid reagent.

Chromosome Preparation and In Situ Hybridization—The direct R-banding FISH method was used for chromosomal assignment of the GalNAc4ST-1 and GalNAc4ST-2 genes to mouse chromosomes. Preparation of R-banded chromosomes and FISH were performed as described by

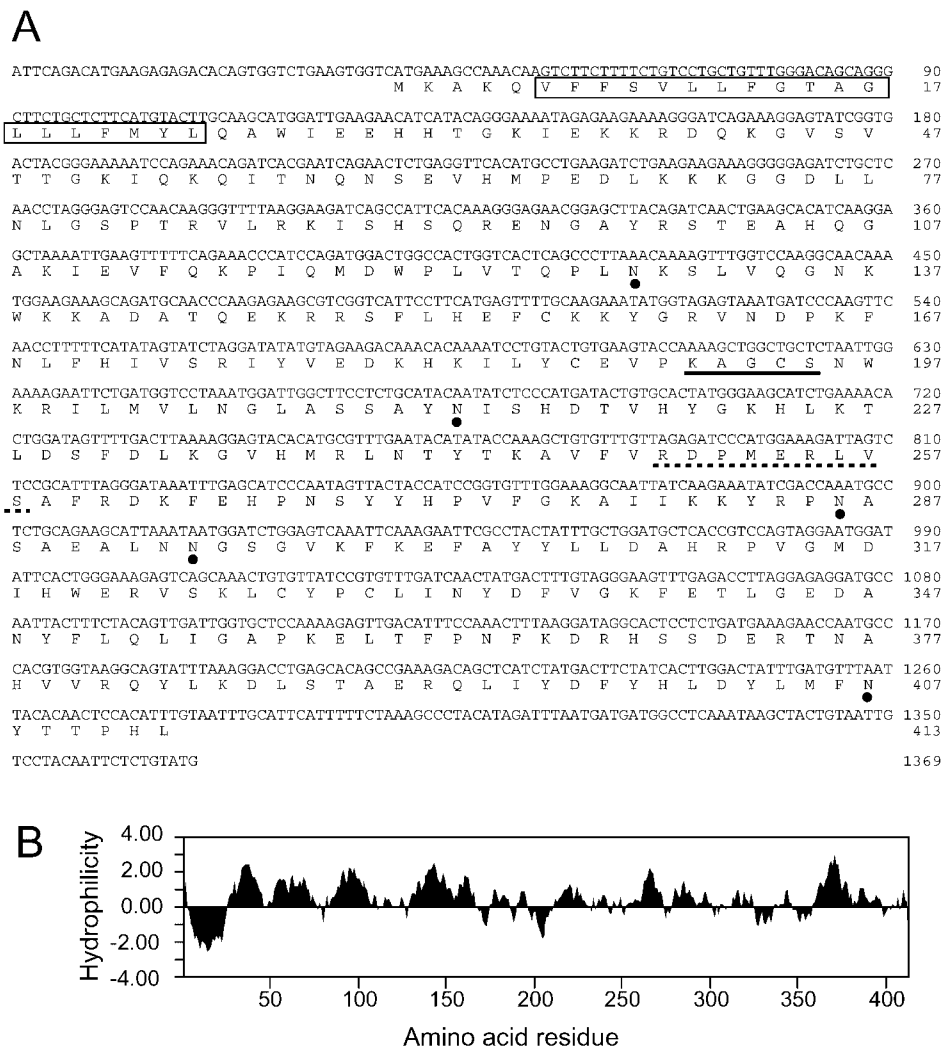

Fig. 2. **Nucleotide sequence of mouse GalNAc4ST-2, and predicted amino acid sequence.** (A) The predicted amino acid sequence is shown below the nucleotide sequence. Five potential *N*-linked glycosylation sites are indicated by dots. The putative transmembrane hydrophobic domain is boxed. The putative PAPS binding domains were underlined: solid line for 5′-PSB and broken line for 3′-PB. (B) Hydropathy plot of mouse GalNAc4ST-1. The hydropathy plot was calculated by the method of Kyte and Doolittle (*39*) with a window of 11 amino acids.

Matsuda *et al*. (*30*), and Matsuda and Chapman (*31*). Mitogen-stimulated splenocyte cultures were synchronized by thymidine blockage, and the incorporation of 5-bromodeoxyuridine during the late replication stage was performed for differential replication staining after the removal of excess thymidine. R-band staining was performed by exposure of chromosome slides to UV light after staining with Hoechst 33258. The chromosome slides were hardened at 65°C for 2h, denatured at 70°C in 70% formamide, 2× SSC, and then dehydrated in a 70–85–100% ethanol series at 4°C.

The mouse 1.7 kb GalNAc4ST-1 cDNA fragment inserted in pcDNA3 (Invitrogen) and the mouse 1.4 kb GalNAc4ST-2 cDNA fragment inserted in pBluescript KS (Stratagene) were labeled by nick translation with biotin-labeled 16-dUTP (Roche Diagnostics) following the manufacturer's protocol. The labeled DNA fragment was ethanol-precipitated with salmon sperm DNA and *E. coli* tRNA, and then denatured at 75°C for 10 min in 100% formamide. The denatured probe was mixed with an equal volume of the hybridization solution to obtain final concentrations of 50% formamide, 2× SSC, 10% dextran sulfate, and 2 μg/μl BSA (Roche Diagnostics). A 20 μl mixture containing 250 ng labeled DNA was put on a denatured slide, covered with parafilm, and then incubated overnight at 37°C. The slides were washed for 20 min in 50% formamide, 2× SSC at 37°C, and in 2× SSC and 1× SSC for 20 min each at room temperature. After rinsing in 4 × SSC, they were incubated under the coverslips with goat anti-biotin antibodies (Vector Laboratories) at 1:500 dilution in 1% BSA, 4× SSC for 1 h at 37°C. They were washed with 4× SSC, 0.1% Nonidet P-40 in 4× SSC, 4× SSC for 5 min each, and then stained with fluoresceinated donkey anti-goat IgG (Nordic Immunology) at 1:500 dilution for 1 h at 37°C. After washing with 4× SSC, 0.1% Nonidet P-40 in 4× SSC, 4× SSC for 10 min on a shaker, the slides were rinsed with 2× SSC and then stained with 0.75 μg/ml propidium iodide. FISH images were obtained under a Nikon fluorescence microscope using Nikon filter sets B-2A and UV-2A. Kodak Ektachrome ASA100 films were used for microphotography.

RESULTS

cDNA and Predicted Protein Sequences of Mouse GalNAc4ST-1 and GalNAc4ST-2—The nucleotide sequences of the mouse GalNAc4ST-1 and GalNAc4ST-2 cDNA, and the predicted amino acid sequences are shown in Figs. 1 and 2, respectively. A putative hydrophobic transmembrane domain was found in the amino-ter-

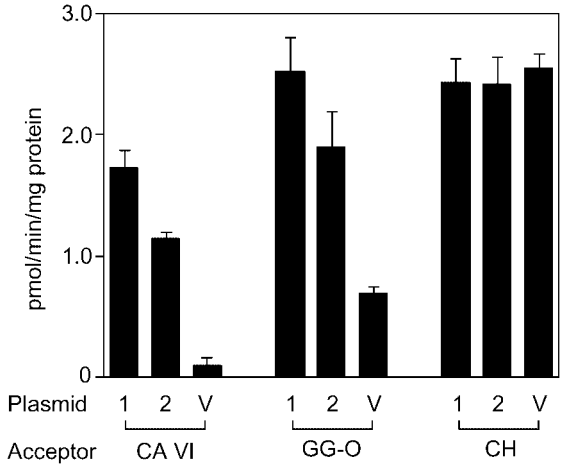

Fig. 3. **Overexpression of mouse GalNAc4ST-1 and mouse GalNAc4ST-2 in COS-7 cells.** COS-7 cells were transfected with pcDNAmGalNAc4ST-1 (1), pcDNAmGalNAc4ST-2 (2), or the vector alone (V). Sulfotransferase activity was determined using carbonic anhydrase VI (CA VI), GalNAcβ1-4GlcNAcβ1-octyl (GG-O), or chondroitin (CH). Incorporation of $^{35}SO_4$ into these substrates were determined by the method described under "MATERIALS AND METHODS." In the assay involving Sep-Pak C18 cartridges, the background value observed without the acceptor was about 0.78 pmol/min/mg protein. Bars represent means for triplicate cultures. S.D. is indicated by narrow lines.

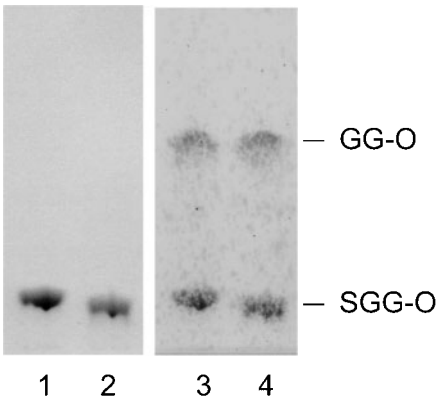

Fig. 4. **Incorporation of $^{35}SO_4$ into GalNAcβ1-4GlcNAcβ1-octyl.** GalNAcβ1-4GlcNAcβ1-octyl was incubated with the recombinant mouse GalNAc4ST-1 (1, 3) or GalNAc4ST-2 (2, 4) and [^{35}S]PAPS as described under "MATERIALS AND METHODS." The sulfated products were isolated with Sep-Pak C-18 cartridges as described under "MATERIALS AND METHODS", mixed with GalNAcβ1-4GlcNAcβ1-octyl and GalNAc($4SO_4$)β1-4GlcNAcβ1-octyl, and then separated by HPTLC. The radioactivity was detected by autoradiography (1, *2*). GalNAcβ1-4GlcNAcβ1-octyl (GG-O) and GalNAc($4SO_4$)β1-4GlcNAcβ1-octyl (SGG-O) were visualized with sulfuric acid reagent (*3*, *4*).

minal region, 22 residues in length, which covers amino acid residues 10–31 for GalNAc4ST-1, and 19 residues in length, which covers from amino acid residues 6–24 for GalNAc4ST-2. Putative PAPS binding sites (*32*) were present in both proteins: KAGCS (5′-PSB) and REPFERLVS (3′-PB) in GalNAc4ST-1, and KAGCS (5′-PSB), and RDPMERLVS (3′-PB) in GalNAc4ST-2.

Comparison of the coding sequences of mouse GalNAc4ST-1 and mouse GalNAc4ST-2 with those of human counterparts revealed that there is 85% identity between mouse GalNAc4ST-1 and human GalNAc4ST-1, and 73% identity between mouse GalNAc4ST-2 and human GalNAc4ST-2 at the amino acid level. The amino acid sequence identity between mouse GalNAc4ST-1 and mouse GalNAc4ST-2 was 49%. Four and five potential *N*-linked glycosylation sites were found in mouse GalNAc4ST-1 and mouse GalNAc4ST-2, respectively.

Expression of GalNAc4ST cDNA in COS-7 Cells—COS-7 cells were transfected with pcDNAmGalNAc4ST-1 or pcDNAmGalNAc4ST-2, recombinant plasmids containing mouse GalNAc4ST-1 or mouse GalNAc4ST-2 cDNA in the mammalian expression vector pcDNA3 (Invitrogen). The transfected cells were extracted with a buffer containing 0.5% Triton X-100 and then centrifuged. The sulfotransferase activity in the cellular extracts was determined using chondroitin, carbonic anhydrase VI or GalNAcβ1–4GlcNAcβ1-octyl as the acceptor (Fig. 3). Control experiments with the vector alone were also performed. As shown in Fig. 3, overexpression of the sulfotransferase activity was observed when carbonic anhydrase VI and GalNAcβ1-4GlcNAcβ1-octyl were used as the acceptors. In contrast, sulfotransferase activity was not overexpressed when chondroitin was used as the acceptor. The Km for carbonic anhydrase VI was 26 μM for mouse GalNAc4ST-1 and 85 μM for mouse GalNAc4ST-2.

Analysis of sulfated GalNAcβ1-4GlcNAcβ1-octyl—GalNAcβ1-4GlcNAcβ1-octyl was incubated with the recombinant mouse GalNAc4ST-1 or GalNAc4ST-2 together with [^{35}S]PAPS. The sulfated products were isolated with a Sep-Pak C-18 cartridge, and then separated by HPTLC. The ^{35}S-labeled products obtained on incubation with GalNAc4ST-1 or GalNAc4ST-2 both migrated to the position of GalNAc($4SO_4$)β1-4GlcNAcβ1-octyl (Fig. 4). When the sulfated GalNAcβ1-4GlcNAcβ1-octyl was subjected to mild acid hydrolysis (40 mM HCl, 100°C, 120 min) and the hydrolysate was analyzed by SAX-HPLC after removing inorganic sulfate by paper electrophoresis, the major radioactivity was detected at the position of GalNAc($4SO_4$) (Fig. 5). Minor peaks around 4.5 min and 10 min were previously observed when GalNAcβ1-4GlcNAcβ1-2Manα1-6Manβ1-octyl was used as the acceptor for human GalNAc4ST-1 and 2 (*20*), suggesting that these minor peaks may represent partially degraded products. These observations indicate that sulfate was transferred to position 4 of the GalNAc residue of GalNAcβ1-4GlcNAcβ1-octyl.

Northern Blot and RT-PCR Analysis—Northern blot analysis was performed using Mouse Multiple Tissue Northern Blots (CLONTECH), to which poly $(A)^+$ RNAs derived from various adult mouse tissues were applied (Fig. 6). The mouse GalNAc4ST-1 message of 2.4 kb was expressed strongly in the brain, and weakly in the kidney and lung (Fig. 6A). The expression pattern of mouse GalNAc4ST-1 was clearly distinct from that of mouse GalNAc4ST-2; the mouse GalNAc4ST-2 message of 2.4 kb was expressed mainly in the kidney (Fig. 6B). To determine the expression of mouse GalNAc4ST-1 and mouse GalNAc4ST-2 in the pituitary and other tissues, we carried out semiquantitative RT-PCR. As shown in Fig. 7B, under the conditions used here, the amounts of the

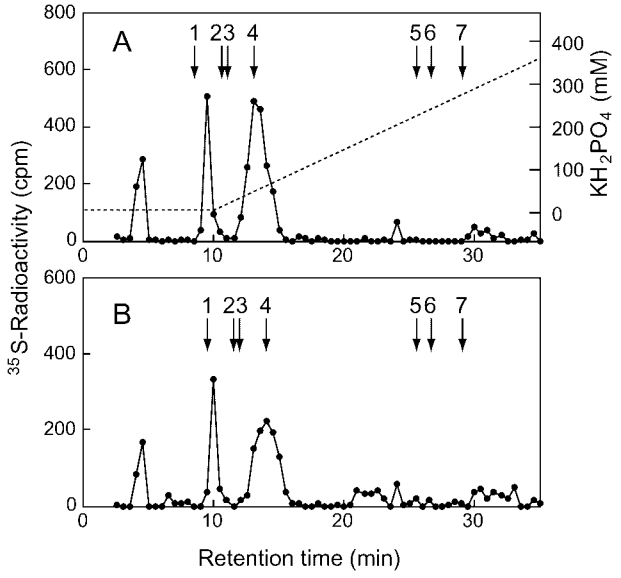

Fig. 5. **Identification of [^{35}S]GalNAc(4SO$_4$) in the mild acid hydrolysate of ^{35}S-labeled GalNAcβ1-4GlcNAcβ1-octyl.** GalNAcβ1-4GlcNAcβ1-octyl was incubated with the recombinant mouse GalNAc4ST-1 (A) or GalNAc4ST-2 (B) together with [^{35}S]PAPS as described under "MATERIALS AND METHODS". ^{35}S-Labeled GalNAcβ1-4GlcNAcβ1-octyl isolated with a Sep-Pak C-18 cartridge was subjected to mild acid hydrolysis (40 mM HCl, 100°C, 120 min). The hydrolysate was subjected to SAX-HPLC after removing inorganic sulfate by paper electrophoresis. The arrows indicate the elution positions of: 1, GlcNAc 3-sulfate and GalNAc 3-sulfate; 2, GalNAc 6-sulfate; 3, GlcNAc 6-sulfate; 4, GalNAc 4-sulfate; 5, ΔDi-6S; 6, ΔDi-4S; and 7, GalNAc 4,6-bissulfate.

amplified DNA were nearly proportional to the amounts of the templates. Mouse GalNAc4ST-1 was expressed in the brain, lung and kidney, as observed on the Northern blotting. Unlike that of human GalNAc4ST-1, the expression of mouse GalNAc4ST-1 was hardly detected in the pituitary gland in the first PCR (Fig. 7A). The expression of mouse GalNAc4ST-1 in the pituitary gland and pancreas was only detected after the second PCR (Fig. 7C). In

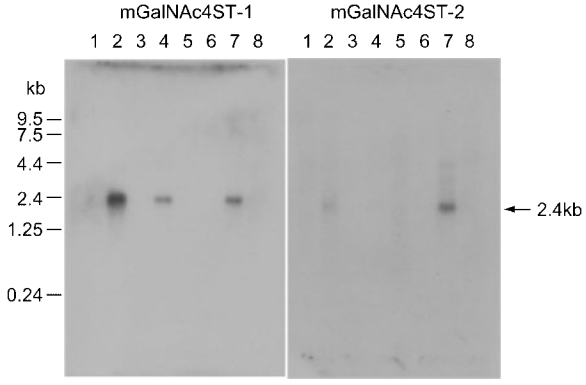

Fig. 6. **Northern blot analysis of mouse GalNAc4ST-1 and mouse GalNAc4ST-2 in various adult mouse tissues.** Northern blots with poly(A)$^+$RNA from heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and testis (lane 8) were hybridized with ^{32}P-labeled DNA probes for mouse GalNAc4ST-1 (left) and mouse GalNAc4ST-2 (right). The positions of the molecular size standards (kb) are indicated at the left.

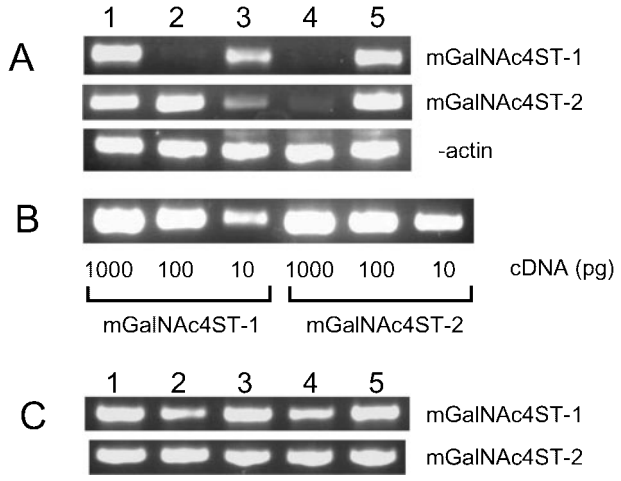

Fig. 7. **RT-PCR analysis of mouse GalNAc4ST-1 and mouse GalNAc4ST-2.** Semiquantitative RT-PCR was carried out as described under "MATERIALS AND METHODS". (A) The results of the first PCR. As a control, the expression of β-actin was compared. (B) Effects of the amounts of cDNA added to the reaction mixtures. Under the conditions used, the intensity of the amplified DNA bands was nearly proportional to the amount of the cDNA. (C) The results of the second PCR. Total RNAs from whole brain (lane 1), pituitary gland (lane 2), lung (lane 3), pancreas (lane 4), and kidney (lane 5) were used as templates.

contrast, mouse GalNAc4ST-2 was strongly expressed in the brain, pituitary gland and kidney, and weakly in the lung (Fig. 7A). The expression of mouse GalNAc4ST-2 in the pancreas was detected after the second PCR (Fig. 7C). The expression of both mouse GalNAc4ST-1 and mouse GalNAc4ST-2 could not be detected in the submaxillary gland or trachea even after the second PCR (data not shown).

Assignment of Mouse GalNAc4ST Genes by Fluorescence In Situ Hybridization—The mouse GalNAc4ST-1 and GalNAc4ST-2 genes were localized to mouse chromosomes by direct R-banding FISH using mouse cDNA fragments as probes (Fig. 8). The GalNAc4ST-1 gene was localized to the B3 distal–B5 proximal region of mouse chromosome 7. On the other hand, the GalNAc4ST-2 gene was localized to the A2 distal–B1 proximal region of mouse chromosome 18 (*33–35*, *30*). The genomic BLAST search gave nearly the same results; the mouse GalNAc4ST-1 and GalNAc4ST-2 genes were mapped to A3 of chromosome 7 and A1 of chromosome 18, respectively.

DISCUSSION

Human GalNAc4ST-1 has been reported to be expressed strongly in the pituitary gland and thought to be involved in the biosynthesis of the nonreducing terminal GalNAc(4SO$_4$) residues of oligosaccharides attached to pituitary glycoprotein hormones such as lutropin (*18–20*). In contrast, human GalNAc4ST-2 is expressed in the trachea (*20*, *21*). The acceptor substrates in the trachea, however, have not been identified. Unlike human GalNAc4ST-1, the expression of mouse GalNAc4ST-1 in the pituitary was much weaker than in the whole brain and kidney. In contrast, mouse GalNAc4ST-2 was

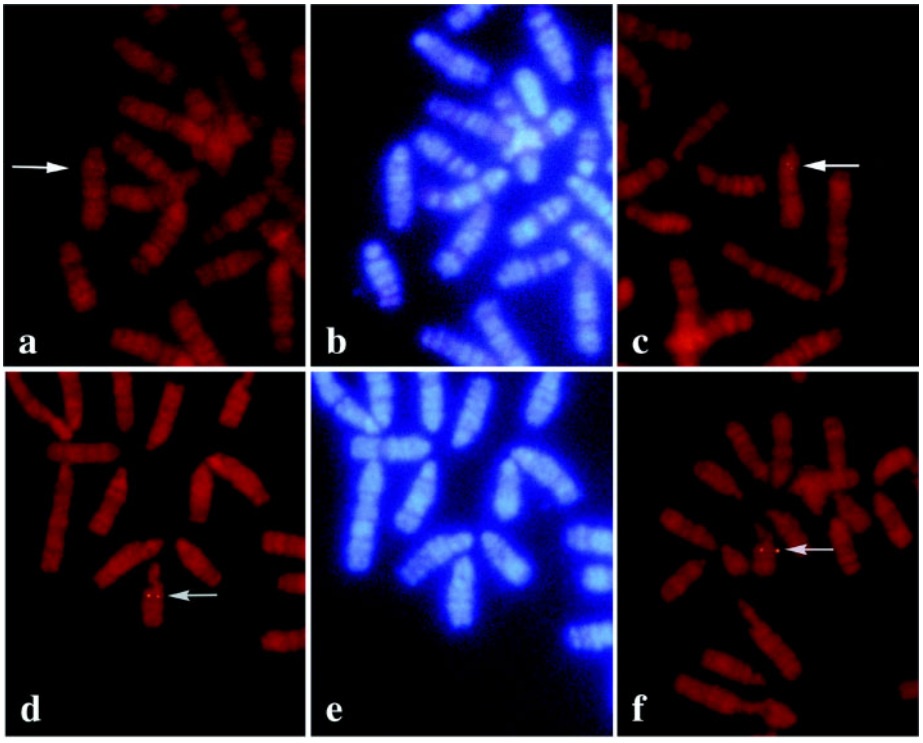

Fig. 8. **Chromosomal localization of the GalNAc4ST-1 (a–c) and GalNAc4ST-2 (d–f) genes to mouse R-banded chromosomes.** The mouse GalNAc4ST-1 and mouse GalNAc4ST-2 cDNA fragments were used as biotinylated probes. Arrows indicate the hybridization signals. The GalNAc4ST-1 and GalNAc4ST-2 genes were localized to mouse chromosome 7 B3 distal–B5 proximal and mouse chromosome 18 A2 distal–B1 proximal, respectively. The metaphase spreads were photographed with Nikon B-2A (a, c, d, f) and UV-2A (b, e) filters. R- and G-banded patterns are demonstrated in (a, c, d, f) and (b, e), respectively.

expressed strongly in the pituitary. The functional role of human GalNAc4ST-1 may be played by GalNAc4ST-2 in the mouse pituitary. The 2.4 kb messages of mouse GalNAc4ST-1 and GalNAc4ST-2 were strongly expressed in the kidney, where the messages of the human isoforms were hardly detected on Northern blot analysis. Tamm-Horsfall glycoprotein, which is one of the major components of the kidney, has been reported to have nonreducing terminal GalNAc($4SO_4$) residues (*13*, *14*). In the mouse kidney, GalNAc4ST-1 and GalNAc4ST-2 may be involved in the biosynthesis of Tamm-Horsfall glycoprotein. Tissue factor pathway inhibitor, which is expressed in 293 cells, has been reported to contain *N*-linked oligosaccharides terminating with the sequence GalNAc($4SO_4$)β1-4GlcNAcβ1-2Manα (*36*). The membrane fraction obtained from 293 cells was found to exhibit GalNAc4ST activity as well as GalNAc transferase activity (*36*). The 293 cell line was established from human embryonal kidney cells. These observations suggest that the functional role of GalNAc4ST in the adult mouse kidney may be related with the function of the human isoforms in the human fetal kidney. Although glycoproteins with nonreducing terminal GalNAc($4SO_4$) has been reported to be present in the bovine submaxillary gland (*12*, *37*), and GalNAc4ST has been purified from this tissue (*37*), the expression of both GalNAc4ST-1 and GalNAc4ST-2 was below the detectable level in the mouse submaxillary gland and trachea.

GalNAcβ1-4GlcNAcβ1-octyl served as a good acceptor for mouse GalNAc4ST-1 and GalNAc4ST-2, suggesting that the Man residue attached to the reducing side of GalNAcβ1-4GlcNAcβ1 may contribute little to the recognition of the acceptor substrate by GalNAc4ST. GalNAcβ1-4GlcNAcβ1-octyl appears to be a useful substrate for determining GalNAc4ST activity. We previously reported that *p*-nitrophenyl-βGalNAc served as an acceptor for human GalNAc4ST-1, but that *p*-nitrophenyl-βGlcNAc did not (*18*). GalNAc4ST may require a nonreducing terminal GalNAc residue flanked by a hydrophobic aglycon as the acceptor.

Comparison of the coding sequences of mouse GalNAc4ST-1 and GalNAc4ST-2 with that of mouse C4ST (*38*) revealed that there is 32% and 30% identity, respectively, at the amino acid level. According to the similarity in the amino acid sequence, these sulfotransferases exhibit similar substrate specificities; both GalNAc4STs and C4ST transfer sulfate to position 4 of GalNAc residues; nevertheless, the substrate specificities of GalNAc4STs and C4ST are clearly distinguishable in the recognition of structures neighboring the targeted GalNAc residues. Human GalNAc4ST-1 transfers sulfate to only nonreducing terminal GalNAc residues (*18–20*), whereas human GalNAc4ST-2 has been reported to be able to sulfate both nonreducing terminal GalNAc residues of oligosaccharides and internal GalNAc residues of chondroitin (*21*). However, the activity of human GalNAc4ST-2 toward chondroitin has not been confirmed (*20*). In the present study, we could not detect any sulfotransferase activity toward chondroitin for either mouse GalNAc4ST-1 or GalNAc4ST-2. Further investigation is required to conclude whether or not GalNAc4ST-2 is involved in the synthesis of chondroitin sulfate as well.

The localization of the mouse GalNAc4ST-1 and GalNAc4ST-2 genes determined by fluorescence *in situ* hybridization was nearly the same as the results obtained through the genomic BLAST search. Comparison of the genes flanking GalNAc4ST-1 or GalNAc4ST-2 between the two species showed fairly good agreement. On human chromosome 19 and mouse chromosome 7, the genes of low density lipoprotein receptor-related protein

3, solute carrier family 7, CCAAT/enhancer binding protein and glucose phosphate isomerase are present in the region flanking GalNAc4ST-1. On the other hand, on human chromosome 18 and mouse chromosome 18, aquaporin 4 and cadherin 2 are present in the region flanking GalNAc4ST-2.

We wish to thank Drs. A. Oohira, S. Aono, and R. Katoh-Semba, Department of Perinatology, Institute for Developmental Research, for preparation of the RNA from the mouse brain tissues. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas, No. 10178102, from the Ministry of Education, Science, Sports and Culture of Japan, and by special research funds from Seikagaku Corporation. The nucleotide sequence data reported in this paper have been submitte in the DDBJ/EMBL/GenBank data base under accession number AB106878 for GalNAc4ST-1 and accession number AB106879 for GalNAc4ST-2.

REFERENCES

1. Hemmerich, S. and Rosen, S.D. (2000) Carbohydrate sulfotransferases in lymphocyte homing. *Glycobiology* **10**, 849–856
2. Kannagi, R. (2002) Regulatory roles of carbohydrate ligands for selectins in the homing of lymphocytes. *Curr. Opin. Struct. Biol.* **12**, 599–608
3. Yamamoto, S., Oka, S., Inoue, M., Shimuta, M., Manabe, T., Takahashi, H., Miyamoto, M., Asano, M., Sakagami, J., Sudo, K., Iwakura, Y., Ono, K., and Kawasaki, T. (2002) Mice deficient in nervous system-specific carbohydrate epitope HNK-1 exhibit impaired synaptic plasticity and spatial learning. *J. Biol. Chem.* **277**, 27227–27231
4. Senn, C., Kutsche, M., Saghatelyan, A., Bosl, M.R., Lohler, J., Bartsch, U., Morellini, F., and Schachner, M. (2002) Mice deficient for the HNK-1 sulfotransferase show alterations in synaptic efficacy and spatial learning and memory. *Mol. Cell. Neurosci.* **20**, 712–729
5. Roseman, D.S. and Baenziger, J.U. (2000) Molecular basis of lutropin recognition by the mannose/GalNAc-4-SO_4 receptor. *Proc. Natl Acad. Sci. USA* **97**, 9949–9954
6. Mi, Y., Shapiro, S.D., and Baenziger, J.U. (2002) Regulation of lutropin circulatory half-life by the mannose/N-acetylgalactosamine-4-SO_4 receptor is critical for implantation in vivo. *J. Clin. Invest.* **109**, 269–276
7. Honke, K., Hirahara, Y., Dupree, J., Suzuki, K., Popko, B., Fukushima, K., Fukushima, J., Nagasawa. T., Yoshida, N., Wada, Y., and Taniguchi, N. (2002) Paranodal junction formation and spermatogenesis require sulfoglycolipids. *Proc. Natl Acad. Sci. USA* **99**, 4227–4232
8. Green, E.D., van Halbeek, H., Boime, I., and Baenziger, J.U. (1985) Structural elucidation of the disulfated oligosaccharide from bovine lutropin. *J. Biol. Chem.* **260**, 15623–15630
9. Green, E.D. and Baenziger, J.U. (1988) Asparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin. I. Structural elucidation of the sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormones. *J. Biol. Chem.* **263**, 25–35
10. Green, E.D. and Baenziger, J.U. (1988) Asparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin. II. Distributions of sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormones. *J. Biol. Chem.* **263**, 36–44
11. Skelton, T.P., Kumar, S., Smith, P.L., Beranek, M.C., and Baenziger, J.U. (1992) Pro-opiomelanocortin synthesized by corticotrophs bears asparagine-linked oligosaccharides terminating with SO_4-4GalNAc beta 1, 4GlcNAc beta 1, 2Man alpha. *J. Biol. Chem.* **267**, 12998–13006
12. Hooper, L.V., Beranek, M.C., Manzella, S.M., and Baenziger, J.U. (1995) Differential expression of GalNAc-4-sulfotransferase and GalNAc-transferase results in distinct glycoforms of carbonic anhydrase VI in parotid and submaxillary glands. *J. Biol. Chem.* **270**, 5985–5993
13. Hard, K., Van Zadelhoff, G., Moonen, P., Kamerling, J.P., and Vliegenthart, F.G. (1992) The Asn-linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male. Novel sulfated and novel N-acetylgalactosamine-containing N-linked carbohydrate chains. *Eur. J. Biochem.* **209**, 895–915
14. van Rooijen, J.J., Kamerling, J.P., and Vliegenthart, J.F. (1998) Sulfated di-, tri- and tetraantennary N-glycans in human Tamm-Horsfall glycoprotein. *Eur. J. Biochem.* **256**, 471–487
15. Bergwerff, A.A., Van Oostrum, J., Kamerling, J.P., and Vliegenthart, J.F. (1995) The major N-linked carbohydrate chains from human urokinase. The occurrence of 4-O-sulfated, (alpha 2–6)-sialylated or (alpha 1–3)-fucosylated N-acetylgalactosamine(beta 1–4)-N-acetylglucosamine elements. *Eur. J. Biochem.* **228**, 1009–1019
16. Fiete, D., Srivastava, V., Hindsgaul, O., and Baenziger, J.U. (1991) A hepatic reticuloendothelial cell receptor specific for SO_4-4GalNAc beta 1, 4GlcNAc beta 1, 2Man alpha that mediates rapid clearance of lutropin. *Cell* **67**, 1103–1110
17. Baenziger, J.U., Kumar, S., Brodbeck, R.M., Smith, P.L., and Beranek, M.C. (1992) Circulatory half-life but not interaction with the lutropin/chorionic gonadotropin receptor is modulated by sulfation of bovine lutropin oligosaccharides. *Proc. Natl Acad. Sci. USA* **89**, 334–338
18. Okuda, T., Mita, S., Yamauchi, S., Fukuta, M., Nakano, H., Sawada, T., and Habuchi, O. (2000) Molecular cloning and characterization of GalNAc 4-sulfotransferase expressed in human pituitary gland. *J. Biol. Chem.* **275**, 40605–40613
19. Xia, G., Evers, M.R., Kang, H.G., Schachner, M., and Baenziger, J.U. (2000) Molecular cloning and expression of the pituitary glycoprotein hormone N-acetylgalactosamine-4-O-sulfotransferase. *J. Biol. Chem.* **275**, 38402–938409
20. Hiraoka, N., Misra, A., Belot, F., Hindsgaul, O., and Fukuda, M. (2001) Molecular cloning and expression of two distinct human N-acetylgalactosamine 4-O-sulfotransferases that transfer sulfate to GalNAc beta 1→4GlcNAc beta 1→R in both N- and O-glycans. *Glycobiology* **11**, 495–504
21. Kang, H.G., Evers, M.R., Xia, G., Baenziger, J.U., and Schachner, M. (2001) Molecular cloning and expression of an N-acetylgalactosamine-4-O-sulfotransferase that transfers sulfate to terminal and non-terminal beta 1, 4-linked N-acetylgalactosamine. *J. Biol. Chem.* **276**, 10861–10869
22. Delfert, D.M. and Conrad, H.E. (1985) Preparation and high-performance liquid chromatography of 3′-phosphoadenosine-5′-phospho[^{35}S]sulfate with a predetermined specific activity. *Anal. Biochem.* **148**, 303–310
23. Habuchi, O. and Miyata, K. (1980) Stimulation of glycosaminoglycan sulfotransferase from chick embryo cartilage by basic proteins and polyamines. *Biochim. Biophys Acta* **616**, 208–217
24. Kanie, O., Ito, Y., and Ogawa, T. (1994) Orthogonal glycosylation strategy in oligosaccharide synthesis. *J. Amer. Chem. Soc.* **116**, 12073–12074
25. Fukuta, M., Uchimura, K., Nakashima, K., Kato, M., Kimata, K., Shinomura, T., and Habuchi, O. (1995) Molecular cloning and expression of chick chondrocyte chondroitin 6-sulfotransferase. *J. Biol. Chem.* **270**, 18575–18580
26. Yamauchi, S., Hirahara, Y., Usui, H., Takeda, Y., Hoshino, M., Fukuta, M., Kimura, J.H., and Habuchi, O. (1999) Purification and characterization of chondroitin 4-sulfotransferase from the culture medium of a rat chondrosarcoma cell line. *J. Biol. Chem.* **274**, 2456–2463
27. Habuchi, O., Matsui, Y., Kotoya, Y., Aoyama, Y., Yasuda, Y., and Noda, M. (1993) Purification of chondroitin 6-sulfotransferase secreted from cultured chick embryo chondrocytes. *J. Biol. Chem.* **268**, 21968–21974
28. Skelton, T.P., Hooper, L.V., Srivastava, V., Hindsgaul, O., and Baenziger, J.U. (1991) Characterization of a sulfotransferase responsible for the 4-O-sulfation of terminal beta-N-acetyl-D-

galactosamine on asparagine-linked oligosaccharides of glycoprotein hormones. *J. Biol. Chem.* **266**, 17142–17150
29. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
30. Matsuda, Y., Harada, Y.N., Natsuume-Sakai, S., Lee, K., Shiomi, T., and Chapman, V.M. (1992) Location of the mouse complement factor H gene (cfh) by FISH analysis and replication R-banding. *Cytogenet. Cell Genet.* **61**, 282–285
31. Matsuda, Y. and Chapman, V.M. (1995) Application of fluorescence in situ hybridization in genome analysis of the mouse. *Electrophoresis* **16**, 261–272
32. Kakuta, Y., Pedersen, L.G., Pedersen, L.C., and Negishi, M. (1998) Conserved structural motifs in the sulfotransferase family. *Trends Biochem Sci.* **23**, 129–310
33. Satoh, H., Yoshida, M.C., and Sasaki, M. (1989) High resolution chromosome banding in the Norway rat, Rattus norvegicus. *Cytogenet. Cell Genet.* **50**, 151–154
34. Evans, E.P. (1996) Standard G-banded karyotype in *Genetic Variants and Strains of the Laboratory Mouse* (Lyon, M.F., Rastan, S., and Brown, S.D.M., eds.) pp. 1446–1449, Oxford University Press, Oxford
35. Somssich, I.E. and Hameister, H. (1996) Standard karyotype of early replicating bands (RBG- banding) in *Genetic Variants and Strains of the Laboratory Mouse* (Lyon, M.F., Rastan, S., and Brown, S.D.M., eds.) pp. 1450–1451, Oxford University Press, Oxford
36. Smith, P.L., Skelton, T.P., Fiete, D., Dharmesh, S.M., Beranek, M.C., MacPhail, L., Broze, G.J. Jr, and Baenziger, J.U. (1992) The asparagine-linked oligosaccharides on tissue factor pathway inhibitor terminate with SO_4-4GalNAc beta 1, 4GlcNAc beta 1, 2 Man alpha. *J. Biol. Chem.* **267**, 19140–19146
37. Hooper, L.V., Hindsgaul, O., and Baenziger, J.U. (1995) Purification and characterization of the GalNAc-4-sulfotransferase responsible for sulfation of GalNAc beta 1, 4GlcNAc-bearing oligosaccharides. *J. Biol. Chem.* **270**, 16327–16332
38. Yamauchi, S., Mita, S., Matsubara, T., Fukuta, M., Habuchi, H., Kimata, K., and Habuchi, O. (2000) Molecular cloning and expression of chondroitin 4-sulfotransferase. *J. Biol. Chem.* **275**, 8975–8981
39. Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132