Human N-Acetylgalactosamine 4-Sulfate 6-O-Sulfotransferase cDNA Is Related to Human B Cell Recombination Activating Gene-associated Gene

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N-Acetylgalactosamine 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST) transfers sulfate from 3'-phosphoadenosine 5'-phosphosulfate to position 6 of N-acetylgalactosamine 4-sulfate (GalNAc(4SO4)) in chondroitin sulfate and dermatan sulfate. We have previously purified the enzyme to apparent homogeneity from the squid cartilage. We report here cloning and characterization of human GalNAc4S-6ST. The strategy for identification of human GalNAc4S-6ST consisted of: 1) determination of the amino acid sequences of peptides derived from the purified squid GalNAc4S-6ST, 2) amplification of squid DNA by polymerase chain reaction, and 3) homology search using the amino acid sequence deduced from the squid DNA. The human GalNAc4S-6ST cDNA contains a single open reading frame that predicts a type II transmembrane protein composed of 561 amino acid residues. The recombinant protein expressed from the human GalNAc4S-6ST cDNA transferred sulfate from 3'-phosphoadenosine 5'-phosphosulfate to position 6 of the nonreducing terminal and internal GalNAc(4SO4) residues contained in chondroitin sulfate A and dermatan sulfate. When a triasaccharide and a pentasaccharide having sulfate groups at position 4 of N-acetylgalactosamine residues were used as acceptors, only nonreducing terminal GalNAc(4SO4) residues were sulfated. The nucleotide sequence of the human GalNAc4S-6ST cDNA was nearly identical to the sequence of human B cell recombination activating gene-associated gene.

N-Acetylgalactosamine 4,6-bissulfate (GalNAc(4,6-bisSO4))

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank*///EBI Data Bank with accession numbers AB062423 (for human GalNAc4S-6ST) and AB062424 (for squid GalNAc4S-6ST).

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2 The abbreviations used are: GalNAc(4,6-bisSO4), 4,6-O-sulfo-N-acetylgalactosamine; GalNAc(4SO4), 4-O-sulfo-N-acetylgalactosamine; GalNAc4S-6ST, N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; HPLC, high performance liquid chromatography; CS-A, chondroitin sulfate A; CS-C, chondroitin sulfate C; CS-E, chondroitin sulfate E; DS, dermatan sulfate; CDSNS-heparin, completely desulfated N-resulfated heparin; ΔDi-6S, 2-acetamide-2-deoxy-3-O-(β-D-glucurono-4-ene)pyranosyluronic acid-6-O-sulfogalactose; ΔDi-4S, 2-acetamide-2-deoxy-3-O-(β-D-glucurono-4-ene)pyranosyluronic acid-4-O-sulfogalactose; ΔDi-diS, 2-acetamide-2-deoxy-3-O-(β-D-glucurono-4-ene)pyranosyluronic acid-6,4-di-O-sulfogalactose; ΔDi-diS-p, 2-acetamide-2-deoxy-3-O-(2-O-sulfogalactose; RAG, human B cell RAG-associated gene; PCR, polymerase chain reaction; SAX, strong anion exchange; 30-ST, glucosaminyl 3-O-sulfotransferase; PSB, phosphosulfate binding; PB, phosphate binding.

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Cloning of GalNAc 4-Sulfate 6-O-Sulfotransferase

EXPERIMENTAL PROCEDURES

Materials—The following commercial materials were used: H$_2$SO$_4$ was from PerkinElmer Life Sciences; chondroitinase ACII, chondroitina

nase ABC, chondro-6-sulfatase, CS-A (whale cartilage), CS-C (shark cartilage), DS (pig skin), heparan sulfate (bovine kidney), completely

acetylated. A nonsulfated trisaccharide and a nonsulfated pentasaccharide

has been shown in a previous paper (29). Molar ratios of the nonreducing terminal GalNAc(4SO$_4$) residue to the internal GalNAc(4SO$_4$) residue were determined by SAX-HPLC after chondroitinase ACII or ABC digestion as described previously (29). CS-A and DS (625 nmol as galactosamine) were digested with chondroitinase ACII and CS-C, respectively. The oligosaccharide materials (25 nmol as galactosamine for determination of DI-4S and 500 nmol for determination of GalNAc(4SO$_4$)) were subjected to SAX-HPLC together with Na$_2$SO$_4$ as an internal standard. GalNAc(4SO$_4$) and DI-4S were monitored by absorption at 210 nm (Fig. 5). At 210 nm, the observed ratio of (molecular absorption of monosaccharides/molecular absorption of unsaturated disaccharides) was 0.32 (29). From the elution profiles, molar ratios of GalNAc(4SO$_4$) to DI-4S (4,6-bissulfate) were calculated. A nonsulfated trisaccharide and a nonsulfated pentasaccharide were prepared from desulfated CS-A by digestion with testicular hyaluronidase and β-glucuronidase (34).

Determination of Amino Acid Sequence of the Purified Protein—The squid GalNAc4S-6ST was purified from the squid cartilage as described previously (29). A portion of the purified GalNAc4S-6ST (10 μg as protein) was subjected to SDS-polyacrylamide gel electrophoresis (10% gel) according to the method of Laemmli (35) after reduction and denaturation in loading buffer containing 5% (v/v) 2-mercaptoethanol. The polyacrylamide gel was stained with Coomassie Brilliant Blue, and the band of the 63-kDa protein was excised. The excised gel containing the protein was sent to Apro Science Co. Ltd. (Naruto, Japan) for the amino-terminal amino acid sequencing of peptides in the reverse phase HPLC after limited digestion of the protein with proteinase Lys-C.

Preparation of Poly(A)$^+$ RNA from the Squid Cartilage—Squid crani
cal cartilage was dissected, freed of soft tissues by wiping with cotton cloth, and put into liquid nitrogen. The frozen cartilage was ground to powder in a mortar in the presence of liquid nitrogen. The cartilage powder was placed in 10 volumes of an ice-cold guanidine thiocyanate solution and homogenized with a Polytron homogenizer. The homogene
te was centrifuged at 100,000 g for 30 min. The clear supernatant was used for isolation of total RNA by the guanidine thiocyanate procedure.

Oligonucleotides and Polymerase Chain Reaction—Degenerate oligo

nucleotide primers were designed as indicated in Fig. 1A. Two sense primers (5'-1 and 4a'-2) and two antisense primers (4a'-1 and 4a'-2) were prepared from the amino acid sequences of peptide 5 and peptide 4, respectively. The first strand of cDNA was synthesized by the reverse transcriptase reaction using poly(A)$^+$ RNA from the squid cartilage as a template and oligo(dT) or random oligonucleotide as a primer. The reverse transcriptase reaction mixture contained, in a final volume of 20 μl, 25 μg of poly(A)$^+$ RNA, 0.5 μg of random oligo(dT)$_{20}$ or dithiothreitol, 0.5 mM each of four deoxynucleoside triphosphates, 30 units of RNase inhibitor (Takara), and 400 units of reverse transcriptase (Superscript II, Life Technologies, Inc.). The reaction was carried out at 42 °C for 50 min. After the reaction was stopped by heating at 70 °C for 15 min, 0.5 unit of RNase H (Life Technologies, Inc.) was added and incubated for 20 min at 37 °C. The PCR reaction was carried out in a final volume of 25 μl containing 25 pmol each of the oligonucleotide primers (5'-1 and 4a'-2), 1 μl of the reverse transcriptase reaction mixture in which the first strand cDNA was synthesized, 0.2 μM each of four deoxynucleoside triphosphates, and 1.5 units of Taq polymerase (Qiagen). Amplification was carried out by 40 cycles of 94 °C for 45 s, 52 °C for 1.5 min, and 72 °C for 1.5 min. The following annealing temperature was used: 72 °C for the first cycles, was decreased to 68 °C for the next 19 cycles, and was decreased to 60 °C for the remaining cycles, and was decreased to 50 °C for the remaining cycles, and was decreased to 40 °C for the remaining cycles, and was decreased to 30 °C for the remaining cycles. The PCR products were separated by reverse phase HPLC after limited digestion of (Naruto, Japan) for the amino-terminal amino acid sequencing of liant Blue, and the band of the 63-kDa protein was excised. The and denaturation in loading buffer containing 5% (v/v) 2-mercapto-

nate was centrifuged at 100,000
TABLE I
Amino acid sequences of the peptides derived from the purified squid GalNAc4S-6ST

| Peptide no. | Amino acid sequence |
|------------|---------------------|
| Peptide 1  | ITSEASASTL          |
| Peptide 2  | LINKFEDW            |
| Peptide 3  | SGTDDVFRR          |
| Peptide 4  | FYQPHNRLVYK        |
| Peptide 5  | SLENFXXHRVTE       |

RESULTS

Identification of a Human cDNA Showing Sequence Homology to the Squid GalNAc4S-6ST—We obtained amino acid sequences of five peptides from the purified squid GalNAc4S-6ST (Table I). When primer 5s-1 and 5s-2 (for the nucleotide sequences of the primers, see Fig. 1A) designed from the amino acid sequences of peptides 5 and 4, respectively, were used in a PCR with poly(A) RNA from squid cartilage as a template, no amplification of DNA was observed (data not shown); however, a DNA fragment of about 380 base pairs was clearly amplified on the second PCR, in which the reaction mixture of the first PCR was used as a template and oligonucleotide 5s-2 and 4a-1 were used as primers (Fig. 1B, lane 1). When the amino acid sequence deduced from the nucleotide sequence of the amplified DNA was used for FASTA search, we found a human cDNA clone (accession no. AB011170) located to chromosome 10 that showed significant homology to the squid DNA. Comparison of the sequence of the human cDNA clone with the amplified squid DNA revealed ~40% identity and ~61% similarity at the amino acid level (Fig. 2). This clone contained an open reading frame that predicts a protein composed of 561 amino acids. Examining the amino acid sequence of this protein revealed the presence of a transmembrane domain of type II topology and a putative PAPS binding domain (PSB). A frame that predicts a protein composed of 561 amino acids was strictly the same as the sequence that appeared in the data base. Evidence that the human cDNA encodes a novel sulfotransferase was obtained by expressing it in COS-7 cells. COS-7 cells were transfected with the pcDNAGalNAc4S-6ST, a recombinant plasmid containing the cDNA in a mammalian expression vector, pcDNA3 (Invitrogen). The transfected cells were scraped at 67 h after transfection, extracted with gentle shaking in a buffer containing 0.5% Triton X-100, and centrifuged. Evidence that the human cDNA encodes a novel sulfotransferase was obtained by expressing it in COS-7 cells. DNA was used for the PCR experiment shown in B. To design oligonucleotide 5s-1 and 5s-2, we used codons for Cys at the position of unidentified amino acid residue. In Table II, the sulfotransferase activity toward various substrates was determined using CS-A or a trisaccharide having sulfate groups at position 4 of GalNAc residues as acceptors. As shown in Fig. 3, when the plasmid containing the human cDNA was used, the sulfotransferase activity toward the 4-sulfated trisaccharide was increased more than 5-fold over the control, although the sulfotransferase activity toward CS-A was increased only 2-fold. We hypothesized that this sulfotransferase might transfer sulfate predominantly to the nonreducing terminal sugar residue, because the 4-sulfated trisaccharide was a better acceptor than CS-A.

Substrate Specificity of the Recombinant Human Sulfotransferase—To determine the substrate specificity of the expressed enzyme, we prepared the affinity-purified protein from the extracts of COS-7 cells transfected with pFLAGGalNAc4S-6ST and the sulfotransferase activity was determined using various acceptors. As shown in Fig. 3, when the plasmid containing the human cDNA was used, the sulfotransferase activity toward the 4-sulfated trisaccharide was increased more than 5-fold over the control, although the sulfotransferase activity toward CS-A was increased only 2-fold. We hypothesized that this sulfotransferase might transfer sulfate predominantly to the nonreducing terminal sugar residue, because the 4-sulfated trisaccharide was a better acceptor than CS-A.

Expression of the Human cDNA Showing Homology to the Squid GalNAc4S-6ST in COS-7 Cells—The human cDNA isolated from human brain (accession no. AB111170, gene no. KIAA 0598) (40), which had been found in the GenBank database by FASTA search, was a generously gift from the Kazusa DNA Research Institute (Kisarazu, Japan). We confirmed that the nucleotide sequence of the open reading frame of this cDNA was identical to the sequence that appeared in the data base. Evidence that the human cDNA encodes a novel sulfotransferase was obtained by expressing it in COS-7 cells. COS-7 cells were transfected with the pcDNAGalNAc4S-6ST, a

Cloning of GalNAc 4-Sulfate 6-O-Sulfotransferase
Structural Analysis of the 35S-Labeled Glycosaminoglycans and 35S-Labeled Oligosaccharides—To determine the position of the sulfate group transferred to CS-A, we digested 35S-labeled CS-A with chondroitinase ACII, and analyzed the digestion products by Partisil-10 SAX-HPLC (Fig. 4). When the 35S-labeled CS-A was digested with chondroitinase ACII, the radioactivity was detected at the position of GalNAc(4,6-bisSO4) and H9004 Di-diSE (Fig. 4A). To determine which sulfate group of GalNAc(4,6-bisSO4) and H9004 Di-diSE had 35S radioactivity, the degradation products obtained after chondroitinase ACII were further digested with chondro-6-sulfatase, FIG. 2.

Table II

| Acceptors                      | Sulfotransferase activity (pmol/min/ml) |
|--------------------------------|----------------------------------------|
| CS-A                           | 33.7                                   |
| CS-C                           | 0.5                                    |
| CS-E                           | 0.5                                    |
| DS                             | 4.9                                    |
| Chondroitin                    | 2.5                                    |
| Keratan sulfate                | 0.3                                    |
| Heparan sulfate                | 0.0                                    |
| CDSNS-heparin                  | 0.0                                    |
| 4-Sulfated trisaccharide       | 95.2                                   |
| 4-Sulfated pentasaccharide     | 99.9                                   |
| Nonsulfated trisaccharide      | 0.0                                    |
| Nonsulfated pentasaccharide    | 0.0                                    |

COS-7 cells were transfected with pFLAGGalNAc4S-6ST or vector alone, and the affinity-purified protein was prepared as described under "Experimental Procedures." Sulfotransferase activities were assayed using various glycosaminoglycans and oligosaccharides as described under "Experimental Procedures." The activity of the affinity-purified fraction obtained from COS-7 cells transfected with the vector alone was less than 0.6 pmol/min/ml for any acceptor used and was subtracted from the individual activity.

**Fig. 2.** Sequence comparison of human GalNAc4S-6ST and squid GalNAc4S-6ST. The amino acid sequence deduced from the squid GalNAc4S-6ST cDNA is aligned under the amino acid sequence deduced from the human GalNAc4S-6ST cDNA. Amino acid sequences of four peptides obtained from the purified squid GalNAc4S-6ST (bold letters) are also aligned under the homologous sequences of human GalNAc4S-6ST. Asterisks and colons indicate that the predicted amino acid in the alignment is identical and similar, respectively, between the two sequences. Five potential N-linked glycosylation sites are indicated by dots. The putative PAPS binding domains, 5'-PSB and 3'-PB, are indicated by an underline and a double underline, respectively.

**Fig. 3.** Overexpression of human GalNAc4S-6ST in COS-7 cells. COS-7 cells were transfected with pcDNAGalNAc4S-6ST (G4–6ST), a plasmid containing the human GalNAc4S-6ST cDNA, or pcDNA3 alone (Vector), as described under "Experimental Procedures." Incorporations into CS-A (A) or 4-sulfated trisaccharide (B) were determined as described under "Experimental Procedures." Bars represent averages of triplicate cultures with S.D.

**Fig. 4.** HPLC separation of the degradation products obtained from 35S-labeled CS-A and 35S-labeled DS after digestion with chondroitinase ACII, chondroitinase ACII plus chondro-6-sulfatase or chondroitinase ABC plus chondro-6-sulfatase. The sulfotransferase reaction was carried out as described under "Experimental Procedures" using 25 nmol (as galactosamine) of CS-A or DS. The 35S-labeled CS-A was applied to the Partisil-10 SAX column after digestion with chondroitinase ACII (A) or chondroitinase ACII plus chondro-6-sulfatase (B). The 35S-labeled DS was applied to the Partisil-10 SAX column after digestion with chondroitinase ABC (C) or chondroitinase ABC plus chondro-6-sulfatase (D). Arrows indicate the elution position of: 1, ΔDi-0S; 2, GalNAc6SO4; 3, GalNAc4SO4; 4, ΔDi-6S; 5, ΔDi-4S; 6, GalNAc4,6-bisSO4; 7, SO42−; 8, ΔDi-diSO4; 9, ΔDi-diSO6.
and subjected to SAX-HPLC (Fig. 4B). After digestion with chondro-6-sulfatase, 35S radioactivity was shifted to the position of inorganic sulfate, indicating that 35SO4 was transferred to position 6 of GalNAc(4SO4) residues located at the nonreducing terminal and internal repeating units of CS-A at a nearly equal rate. On the other hand, 35S-labeled DS was hardly depolymerized by chondroitinase ACII digestion (data not shown). When the 35S-labeled DS was digested with chondroitinase ABC, a main radioactive peak was detected at the position of GalNAc(4,6-bisSO4) and a small peak was detected at the position of ΔDi-diS6; 5, ΔDi-4S. Small peaks whose retention times were not agreed with those of the standards were not identified.

The recombinant human GalNAc4S-6ST transferred sulfate to the 4-sulfated oligosaccharides efficiently (Table II). To determine the position of the sulfate group transferred to the 4-sulfated oligosaccharides, the 35S-labeled trisaccharide and pentasaccharide were digested with chondroitinase ACII and applied to SAX-HPLC (Fig. 6). In both the 35S-labeled oligosaccharides, most of the radioactivity appeared at the position of GalNAc(4,6-bisSO4) (Fig. 6, A and C). To establish the position to which 35SO4 was transferred, we digested the 35S-labeled trisaccharide and pentasaccharide with chondro-6-sulfatase after digestion with chondroitinase ACII, and subjected it to SAX-HPLC. The radioactivity of GalNAc(4SO4) to the total unsaturated disaccharide became 0.011 for CS-A and 0.021 for DS. When the chondroitinase ACII digests of CS-C was analyzed, the peak of 35S-Labeled trisaccharide (A and B) and 35S-labeled pentasaccharide (C and D) were digested by chondroitinase ACII (A and C) or chondroitinase ACII plus chondro-6-sulfatase (B and D), and separated with SAX-HPLC. The amount of 35S-labeled pentasaccharide contained in the reaction mixture for D was twice as much as the amount for C. Elution positions of the standard materials indicated by arrows are the same as those in Fig. 4.
We have identified the human GalNAc4S-6ST cDNA for the first time. The strategy for the identification of the human GalNAc4S-6ST cDNA consisted of: 1) determination of amino acid sequences of the peptides derived from the purified squid GalNAc4S-6ST, 2) amplification of the squid DNA by PCR using squid poly(A) RNA as a template and degenerate oligonucleotides as primers, and 3) FASTA search by the amino acid sequence deduced from the squid DNA fragment. Different lines of evidence indicated that the identified cDNA corresponds to the human counterpart of squid GalNAc4S-6ST previously purified from the squid cartilage: (a) the predicted amino acid sequence of the protein showed relatively high homology to the sequence deduced from the squid DNA; (b) the four of five peptides derived from the purified squid GalNAc4S-6ST nearly matched the human protein; (c) the expressed protein in COS-7 cells catalyzed the transfer of sulfate to position 6 of GalNAc(4SO\textsubscript{4}) residues of CS-A and DS; (d) as observed in the purified squid enzyme, human GalNAc4S-6ST absolutely requires the presence of 4-sulfate moiety on GalNAc residue for its activity, because nonsulfated oligosaccharides did not serve as acceptor for human GalNAc4S-6ST; and (e) the predicted protein contained five potential N-linked glycosylation sites, which fits with the observation that the purified squid GalNAc4S-6ST is an N-linked glycoprotein (29). The predicted protein showed low but significant homology to 30-ST family (41, 42). Comparison of the sequence of human GalNAc4S-6ST with human 30-ST-3A revealed $\sim 25\%$ identity and $\sim 40\%$ similarity at the amino acid level (Fig. 7). Relatively high homology was observed in the putative PAPS binding domains (5'-'PSB and 3'-'PB) and the carboxy-terminal region.

Although both human and squid GalNAc4S-6ST transfer sulfate to position 6 of GalNAc(4SO\textsubscript{4}) residue, a clear difference in the recognition of the targeted sugar residue is present between human and squid GalNAc4S-6ST. When CS-A was used as the acceptor, the squid GalNAc4S-6ST transferred sulfate mainly to position 6 of GalNAc(4SO\textsubscript{4}) residues in the repeating disaccharide units. In contrast, human GalNAc4S-6ST transferred sulfate to position 6 of GalNAc(4SO\textsubscript{4}) residue located at the nonreducing terminal and repeating disaccharide units at a nearly equal rate. The preference of human GalNAc4S-6ST to the nonreducing terminal GalNAc(4SO\textsubscript{4}) residues was much more evident when DS was used as the acceptor. The observed difference in the substrate specificity between human and squid GalNAc4S-6ST may be related not only to the difference in the amino acid sequence but also to the fact that the squid enzyme is the natively expressed enzyme, whereas the human enzyme is a recombinantly expressed protein. The rate of sulfation of DS by human GalNAc4S-6ST was much lower than the rate of sulfation of CS-A (Table II). The relatively poor acceptor activity of DS may be the result of the presence of iduronic acid residue but of the lower concentration of nonreducing terminal GalNAc(4SO\textsubscript{4}) residues than CS-A. CS-C hardly served as acceptor, because the proportion of GalNAc(4SO\textsubscript{4}) residue in the total repeating disaccharide units of CS was larger than that of CS-A. CS-C hardly served as acceptor, because the inability of CS-C as the acceptor may be attributed not only to the very low contents of nonreducing terminal GalNAc(4SO\textsubscript{4}) residue but also to the higher contents of GalNAc(6SO\textsubscript{4}) residue; GalNAc(6SO\textsubscript{4}) residue might inhibit the sulfation of the adjacent GalNAc(4SO\textsubscript{4}) residue. Further works using oligosaccharides with the defined structures are required to reveal the effects of GalNAc(6SO\textsubscript{4}) residue on the sulfation of both nonreducing terminal and internal GalNAc(4SO\textsubscript{4}).

Human GalNAc4S-6ST transferred sulfate to the 4-sulfated oligosaccharides more efficiently than CS-A. Human GalNAc4S-6ST transferred sulfate predominantly to position 6 of nonreducing terminal GalNAc(4SO\textsubscript{4}) residues when the 4-sulfated trisaccharide and 4-sulfated pentasaccharide were used as acceptors. Human GalNAc4S-6ST failed to transfer sulfate to the reducing terminal and internal GalNAc(4SO\textsubscript{4}) residues of these oligosaccharides. Unlike these oligosaccharides, the sulfate transfer to CS-A occurred at the internal GalNAc(4SO\textsubscript{4}) residues. Such discrepancy in the acceptor specificity of human GalNAc4S-6ST might be accounted for by a unique conformation of CS-A that is not feasible for the oligosaccharides.

Vertebral sulfotransferases capable of producing GalNAc(4,6-bisSO\textsubscript{4}) residues have been reported in quail oviduct (27), and human serum (28). These sulfotransferases mainly catalyze sulfation of position 6 of nonreducing terminal GalNAc(4SO\textsubscript{4}) residues. Such specificity is similar to that of human GalNAc4S-6ST. Human GalNAc4S-6ST may thus be involved in the formation of the nonreducing terminal GalNAc(4,6-bisSO\textsubscript{4}) residues found in chondroitin sulfate chains attached to thrombomodulin (3) or aggrecan (23). Human GalNAc4S-6ST may also be involved in the synthesis of CS-E contained in the granules of mast cells, because this enzyme could catalyze the sulfation of the internal GalNAc(4SO\textsubscript{4}) residues when IS-A was used as acceptor. However, the specificity of human GalNAc4S-6ST appears to be not
suitable for the synthesis of the glycosaminoglycan containing IdoAα1–3GalNAc(4,6-bisSO4) unit, which was found in rat glomeruli (43) and rat mesangial cells (44), because human GalNAc4S-6ST transfers sulfate mainly to the nonreducing terminal GalNAc(4SO4) residues when DS was used as acceptor. Inoue et al. (45) reported that the substrate specificity of the partially purified GalNAc4S-6ST from human serum depended on the pH of the reaction mixtures; the rate of sulfation of the internal GalNAc(4SO4) residues increased as the pH was lowered. It remains to be determined whether the activity of human GalNAc4S-6ST toward the internal GalNAc(4SO4) may also depend on the pH of the reaction mixtures. Alternatively, an isoform of human GalNAc4S-6ST that transfers sulfate mainly to the internal GalNAc(4SO4) residue might be present in the mast cells.

Surprisingly, the nucleotide sequence of the human GalNAc4S-6ST cDNA was nearly identical to the sequence of human B cell RAG-associated gene. The RAG1 and RAG2 play an important role in V(D)J recombination (46). hBRAG was cloned from Nalm-6 pre-B cell library as a cDNA that coexpressed closely with RAG1 mRNA (30). hBRAG gene was mapped to 10q26 (30). The expression pattern of hBRAG in human pro-B, pre-B, and mature B cell lines was closely related to that of RAG1. In human tissues, hBRAG is expressed in B cell-enriched tissues such as bone marrow and tonsil, but is not expressed in fetal or adult thymus. The product of hBRAG was shown to potentially involve in B cell-specific regulation of the expression of RAG1, because stable transfection of the complete hBRAG cDNA into a low RAG-expressing B cell variant increased levels of RAG1 transcripts, but not in a nonlymphoid cell line (30). Immunoblotting and immunoprecipitation with the antibody raised against hBRAG protein demonstrated that hBRAG protein is expressed at both the cell surface and intracellular location of B cells as a membrane-integrated glycoprotein (47). The nucleotide sequence of the coding region of hBRAG is 99% identical to that of human GalNAc4S-6ST, but amino acid sequences of these proteins are rather different from each other. Missense mutations are observed in the codons corresponding to amino acid residues 180, 239, 240, and 241. Deletion of a nucleotide corresponding to amino acid residue 181 results in a frameshift mutation. The reading frame is corrected by the insertion of a nucleotide at amino acid residue 181. Deletion of a nucleotide corresponding to amino acid residue 489 results in the appearance of a stop codon corresponding to amino acid residue 503. The hBRAG protein may not be a counterpart of the squid GalNAc4S-6ST because peptide 4 derived from the squid GalNAc4S-6ST did not match the hBRAG protein. Despite such alterations in the amino acid sequence, hBRAG protein still contains the PAPS binding domains that are characteristic to most of sulfotransferases so far cloned, suggesting that hBRAG protein may be a novel sulfotransferase whose substrate specificity is related to human GalNAc4S-6ST. It remains to be studied in future works whether formation of GalNAc(4,6-bisSO4) residues might be involved in the regulation of the expression of RAG in B cells. Another hBRAG cDNA (accession no. AB025341) was also isolated from a human fetal brain library, and the hBRAG gene was shown to consist of seven exons and six introns (24). The nucleotide sequence of the hBRAG cDNA isolated from the human fetal brain library was strictly the same as the sequence of the cDNA that was identified as human GalNAc4S-6ST in this paper. The observed difference in the nucleotide sequence between hBRAG isolated from Nalm-6 pre-B cell and human GalNAc4S-6ST suggests that tissue-specific variants of GalNAc4S-6ST might be present.
