Specificities of Three Distinct Human Chondroitin/Dermatan 
N-Acetylgalactosamine 4-O-Sulfotransferases Demonstrated 
Using Partially Desulfated Dermatan Sulfate as an Acceptor

IMPLICATION OF DIFFERENTIAL ROLES IN DERMATAN SULFATE BIOSYNTHESIS*

Received for publication, June 9, 2003, and in revised form, July 3, 2003
Published, JBC Papers in Press, July 7, 2003, DOI 10.1074/jbc.M306044200

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4-O-Sulfation of GalNAc is a high frequency modification of chondroitin sulfate and dermatan sulfate (DS), and three major GalNAc 4-O-sulfotransferases including dermatan 4-O-sulfotransferase-1 (D4ST-1) and chondroitin 4-O-sulfotransferases-1 and -2 (C4ST-1 and -2) have been identified. 4-O-Sulfation of GalNAc during DS biosynthesis had long been postulated to be a prerequisite for iduronic acid (IdoUA) formation by C5-epimerization of GlcUA. This hypothesis has recently been argued based on enzymological studies using microsomes that C5-epimerization precedes 4-O-sulfation, which was further supported by the specificity of the cloned D4ST-1 with predominant preference for IdoUA-GalNAc flanked by GlcUA-GalNAc over IdoUA-GalNAc flanked by IdoUA-GalNAc in exhaustively desulfated dermatan. Whereas the counterproposal explains the initial reactions, apparently it cannot rationalize the synthetic mechanisms of IdoUA-GalNAc in exhaustively desulfated dermatan. In this study, we examined detailed specificities of the three recombinant human 4-O-sulfotransferases using partially desulfated DS as an acceptor. Enzymatic analysis of the transferase reaction products showed that D4ST-1 far more efficiently transferred sulfate to GalNAc residues in -IdoUA-GalNAc than in -GlcUA-GalNAc-GlcUA- sequences. In contrast, C4ST-1 showed the opposite preference, and C4ST-2 used GalNAc residues in both sequences to comparable degrees, being consistent with its phylogenetic relations to D4ST-1 and C4ST-1. Structural analysis of the oligosaccharides, which were isolated after chondroitinase AC-I digestion of the 35S-labeled transferase reaction products, revealed for the first time that D4ST-1, as compared with C4ST-1 and C4ST-2, most efficiently utilized GalNAc residues located not only in the sequence -IdoUA-GalNAc-IdoUA- but also in -GlcUA-GalNAc-IdoUA-GalNAc-GlcUA-. The isolated oligosaccharide structures also suggest that 4-O-sulfation promotes subsequent 4-O-sulfation of GalNAc in the neighboring disaccharide unit.

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* This work was supported in part by the Scientific Research Promotion Fund of the Japan Private School Promotion Foundation, by Grants-in-aid for Encouragement of Young Scientists 14771299 (to T. M.) and Scientific Research (B) 13470493 (to K. S.), and by the Sasagawa Scientific Research Grant from the Japan Science Society (to T. M.) and Scientific Research (B) 13470493 (to K. S.).

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1 The abbreviations used are: DS, dermatan sulfate; CS, chondroitin sulfate; C4ST, chondroitin 4-O-sulfotransferase; HNK-1ST, HNK-1 sulfotransferase; GalNAcST, N-acetylgalactosamine 4-O-sulfotransferase; PAPS, 3’-phosphoadenosine 5’-phosphosulfate; ΔHexUA, 4,5-unstacked hexuronic acid or 4-deoxy-a-L-threo-hex-4-ene-pyrano-syluronic acid; ΔDi-0S, Δ14HexUA-1-3GalNAc-ΔDi-4S; Δ14HexUA-1-3GalNAc-ΔDi-6S, Δ14HexUA-1-3GalNAc-6-O-sulfate; ΔDi-diS, Δ14HexUA-2-O-sulfate; Δ14HexUA-1-3GalNAc-6,4-di-sulfate; ΔDi-diS, Δ14HexUA-2-O-sulfate; Δ14HexUA-1-3GalNAc-4,6-di-sulfate; EST, expressed sequence tag; HPLC, high performance liquid chromatography; DE MALDI-TOF, delayed extraction matrix-assisted laser desorption ionization time-of-flight; 4S, 4-O-sulfate; COSY, correlation spectroscopy; contig, group of overlapping clones.
see Refs. 25–27. 4-O-Sulfation of GalNAc residues is a typical modification found in CS/DS at higher frequency and was once postulated to be a prerequisite for the adjacent IdUA formation by glucuronyl C5-epimerase, and GalNAc 4-O-sulfotransferase(s) had been suggested to be a critical and rate-limiting factor during DS biosynthesis (28). However, it was recently demonstrated that human skin fibroblast microsomes exhibited GalNAc 4-O-sulfotransferase activity with a marked substrate preference to dermatan, chemically desulfated DS, rather than chondroitin (29), suggesting that C5-epimerization of GlcUA residues can precede 4-O-sulfation of GalNAc residues. Indeed, recent molecular cloning studies revealed that two chondroitin 4-O-sulfotransferases termed C4ST-1 and C4ST-2 also catalyzed the 4-O-sulfation of GalNAc in dermatan as well as in chondroitin (30–32). However, the detailed properties of C4STs and the precise spatial arrangement of IdUA residues and 4-O-sulfation sites have not been investigated. In addition to C4ST-1 and C4ST-2, several other sulfotransferases that belong to the same HNK-1 sulfotransferase (HNK-1-1ST) family have also been cloned, although they catalyze different regioslective sulfations of other types of glycan chains: HNK-1ST, which facilitates 3-O-sulfation of a GlcUA residue in HNK-1 antigen precursor oligosaccharide chains of glycoproteins and/or glycolipids (33, 34), and N-acetylgalactosamine 4-O-sulfotransferases-1 and -2 (GalNAc4ST-1 and GalNAc4ST-2), which catalyze 4-O-sulfation at the nonreducing terminal GalNAc residues in the GalNAcβ1-4GlcNAcβ1-containing sequences in both N- and O-glycans (35–38).

In an attempt to identify additional sulfotransferase(s) by public database searches, we found and identified another human GalNAc 4-O-sulfotransferase, which acted primarily on DS. During characterization of this enzyme, the cDNA and the catalytic activity of the identical gene product were reported, and the enzyme was designated as D4ST-1 (39). The acceptor specificity was also characterized to some extent using dermatan, nearly exhaustively desulfated DS, as an acceptor. In this study, we found that partially desulfated DS also serves as an excellent acceptor, which allowed us to investigate more detailed acceptor specificity toward the recognition sequences of D4ST-1 compared with those of C4ST-1 and -2.

EXPERIMENTAL PROCEDURES

Materials—35S-Labeled 3’-phosphoadenosine 5’-phosphosulfate (PAPS) (1.69 mCi/mmol) was purchased from PerkinElmer Life Sciences. Unlabeled PAPS and GalNAc 4-O-sulfate were obtained from Sigma. The following sugars and enzymes were purchased from Seikagaku Corp. (Tokyo, Japan): chondroitin (a chemically desulfated derivative of whale cartilage CS-A); various CS preparations including CS-A, CS-B (porine skin DS), CS-C, CS-D, and CS-E; six unmodified standard disaccharides derived from CS (i.e. Δ1,4-DS, Δ1,4-4S, Δ1,6-6S, Δ1,4-diDS, Δ1,4-diDS4, and Δ1,3-triS); conventional and highly purified (prooase-free) chondroitinase ABC (EC 4.2.2.4) from Proteus vulgaris; chondroitinase AC-I (EC 4.2.2.5) from Flavobacterium heparinum; and chondroitinase B (EC 4.2.2) from F. heparinum. A Superdex™peptide column was obtained from Amersham Pharmacia Biotech Inc. (Alto, CA, USA) by two-round PCR using specific primer sets corresponding to the 5’- and 3’-noncoding regions, based on the human genomic sequence. The first PCR was performed with a forward primer, 5’-CAA CTA CCC GCG GTC CCA GA-3’, and a reverse primer, 5’-CAT CCA GGA TCC TGC GAA GC-3’, followed by nested PCR with nested primers: a forward primer, 5’-ACC CCT TGA GCA CCA TGT CTC-3’, and a reverse primer, 5’-ACC TGG CTT GTA AAC CAC TC-3’. Each PCR was carried out with ROD-Plus (TOYOBO, Tokyo) in the presence of 5% (v/v) dimethyl sulfoxide by 30 cycles at 94 °C for 30 s, 55 °C for 42 s, and 68 °C for 2.5 min. The amplified cDNA fragment of an expected size (~1.1 kb) was subcloned into a pGEM®-T Easy vector (Promega, Tokyo) and sequenced in a 377 DNA sequencer (PerkinElmer Life Sciences), which identified the clone as the recently reported D4ST-1 (39). The additional 5’-noncoding sequence was also amplified using a human placenta cDNA library as a template by PCR with a gene-specific reverse primer and a gt10 insert screen amplifier (Clontech).

Construction of Expression Vectors Encoding Soluble Forms of Sulfotransferases—The cDNA encoding a truncated form of D4ST-1 lacking the first NH2-terminal 62 amino acid residues was amplified by PCR with the pGEM®-T Easy vector containing the full coding sequence of the protein using a 5’ primer containing an in-frame BamHI site (5’-GCG GAT CCG GCA TCC TGG AGA TG-3’) and a 3’ primer containing a BamHI site located 27 bp downstream from the stop codon (5’-CGG GAT CCG GCG TTT AAA CCA GTC CC-3’). PCR was carried out with Pfu polymerase (Promega) at 30 cycles at 95 °C for 42 s, 65 °C for 42 s, and 72 °C for 3.5 min. The PCR products of the expected size were digested with BamHI, cloned into the BamHI site of an expression vector, pEF-BOS/IP (42), and sequenced. The resultant vector contained the cDNA encoding a fusion protein that had an NH2-terminal cleavable insulin leader peptide and a protein A IgG-binding domain followed by a truncated form of D4ST-1.

Likewise, other expression constructs of the following sulfotransferases were designed to exclude the membrane-spanning segment and to include the stem region of the longest possible size. In the case of C4ST-1 (31, 32), the cDNA fragment encoding a truncated form of the protein, lacking the NH2-terminal first 57 amino acid residues, which contained a putative propeptolytic cleavage site in the middle of the protein, was amplified by PCR using a human placenta cDNA library (Clontech) as the template with a 5’ primer containing an in-frame BamHI site (5’-CGG GAT CCC TCC AGG AGG TTC ACA AC-3’) and a 3’ primer corresponding to 16 bp downstream from the stop codon (5’-CGG GAT CCT AAA AAG AGT GAT TCT CT-3’).

In the case of human C4ST-2 (31), the cDNA encoding a truncated form of the protein, lacking the NH2-terminal first 76 amino acid residues, was amplified by PCR using the EST clone (GenBank™ accession number AA182540, IMAGE Consortium cDNA clone, ID number 613430) obtained from Genome System, Inc. as the template with a 5’ primer containing an in-frame BamHI site (5’-CGG GAT CCG GCG TGG TGG AAA CCA GTC CC-3’) and a 3’ primer corresponding to 21 bp downstream from the stop codon (5’-CGG GAT CCT AAA AAG AGT GAT TCT CT-3’).

Expression of the Soluble Forms of the Recombinant Sulfotransferases—Each expression plasmid (6.7 μg) was transfected into COS-1 cells in 100-mm plates using FuGENE™6 (Roche Applied Science) according to the manufacturer’s instructions. Two days after transfection, half of the medium of the cell culture was incubated with 10 μl of IgG-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. The enzyme-bound beads were washed with and resuspended in each assay buffer described below and used as enzyme sources for sulfotransferase assays.

Sulfotransferase activities toward CS/DS variants were assayed by the method described previously (43) with slight modifications. Briefly, the standard reaction mixture (60 μl) contained 10 μl of the resuspended beads, 50 mM imidazole-HCl, pH 6.8, 2 mM dithiothreitol, 10 μM [35S]PAPS (~1 or 3 × 105 dpm), and an acceptor polysaccharide preparation (10 nmol as GlcUA). The reaction mixtures were incubated at
37 °C for 1 h and subjected to gel filtration using a syringe column packed with Sephadex G-25 (superfine) (44). [35S]Sulfate incorporation into polysaccharides was quantified by determination of the radioactivity in the flow-through fractions by liquid scintillation counting.

Identification of the Transferase Reaction Products—The [35S]-labeled chondroitin and partially desulfated DS were isolated by gel filtration as described above, dried, and subjected to exhaustive digestion with chondroitinase ABC, AC-I, or B (45). The digest was analyzed by anion exchange HPLC on an anion-bound silica PA63 column (46) or by gel filtration on a Superdex™ Peptide column (Amersham Biosciences) equilibrated with 0.2 M NH₄HCO₃ (47) as described previously. Chondro-4-O-sulfatase digestion was conducted as previously reported (48). A large scale D4ST-1 reaction product, required for a detailed analysis of the sulfation sites, was prepared using partially desulfated DS (8 μmol as GlcUA) as an acceptor. The reaction sample was exhaustively digested with chondroitinase AC-I, and the digest was fractionated by gel filtration on a Superdex™ Peptide column as described above. Radioactive fractions corresponding to tetra- and hexasaccharides were pooled and further fractionated by anion exchange HPLC on an anion-bound silica column as described above. Eluates were monitored by absorption at 232 nm. Each radioactive tetra- or hexasaccharide fraction was pooled and desalted through a column (0.8 × 57 cm) of Seph- adex G-25 (fine) (Amersham Biosciences). The disaccharide composition of each isolated fraction was determined by HPLC analysis of the chondroitinase ABC digest. Digestion of the isolated hexasaccharide fractions with a highly purified chondroitinase ABC preparation was carried out as described previously (49).

Delayed Extraction Matrix-assisted Laser Desorption Ionization Time-of-flight (DE MALDI-TOF) Mass Spectrometry—DE MALDI-TOF mass spectra in the positive ion mode of the isolated tetra- and hexa- saccharide fractions were recorded in the linear mode in a Voyager DE-PRO (PerSeptive Biosystems, Framingham, MA) (50). Each oli- gosaccharide fraction (30 pmol) was mixed with a small volume of an aqueous solution (10 mg/ml) of a matrix, 2,5-dihydroxybenzoic acid. An aliquot (1 μl) of this sample-matrix mixture was placed on the sample plate well, dried under an air stream, and analyzed.

500-MHz 1H NMR—The isolated oligosaccharide fractions for NMR analysis were repeatedly exchanged in H₂O with intermit- tent lyophilization. The 500-MHz 1H NMR spectra of the oligosaccharide fractions 4-III and 6-II were recorded in a Varian VXR-500 spectrometer at a probe temperature of 26 °C as reported previously (45, 51, 52). Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly relative to acetone (δ 2.225) in D₂O (53).

RESULTS

Evolutionary Relations of Chondroitin/Dermatan GalNAc 4-O-Sulfotransferases—A TBLASTN search, using the catalytic domain of a previously cloned human C4ST-1 (31, 32), showed two human cDNAs encoding putative sulfotransferases with PAPS binding motifs (54) and a type II transmembrane topology. The deduced amino acid sequence of one of the two cDNAs (GenBank™ accession number AB066595) was identical to D4ST-1, which catalyzes preferentially 4-O-sulfation of GalNAc residues in dermatan rather than chondroitin (39). It showed a significant homology to the HNK-1ST family members of hu- man origin (C4ST-1 (31, 32), C4ST-2 (31), GalNAc4ST-1 (35, 36, 38), GalNAc4ST-2 (37, 38), and HNK-1ST (34)) and is the fifth family member. The other cDNA also shared significant sequence identities and structural similarities with the same family members and was identified as chondroitin 4-O-sulfo- transferase-3 (C4ST-3) recently reported as the seventh member (55). As shown in Fig. 1, the evolutionary relations of these gene products showed that D4ST-1 was the closest to C4ST-2, and C4ST-3 was most similar to C4ST-1, suggesting that the sulfotransferases responsible for 4-O-sulfation of CS/DS should be categorized as a “CS/DS 4-O-sulfotransferase subfamily.”

Differential Preferences of D4ST-1, C4ST-1, and C4ST-2 to- ward Chondroitin and Partially Desulfated DS—To facilitate the functional analysis of D4ST-1, a soluble form of the protein was generated by replacing the first 62 amino acids with a cleavable insulin signal sequence and a protein A IgG-binding domain as described under “Experimental Procedures,” and then the soluble chimeric sulfotransferase was transiently expressed in COS-1 cells. The recombinant enzyme secreted into the culture medium was adsorbed to IgG-Sepharose beads for elimination of endogenous sulfotransferases, and then the enzyme-bound beads were used for enzyme assays.

In view of its evolutionary relation to the CS/DS 4-O- sulfotransferase subfamily members, the purified fusion protein was examined using various CS/DS acceptor substrates. As shown in Table I, the enzyme preferentially transferred [35S]sulfate from [35S]PAPS to partially desulfated DS (2.9 pmol), respectively, was detected. Under the same conditions, negligible activities were detected toward natural CS variants A-E. d Porcine skin DS.

| Acceptor | D4ST-1 | C4ST-1 | C4ST-2 | C4ST-3 |
|----------|--------|--------|--------|--------|
| Partially desulfated DS | 748 | 208 | 84 | <1 |
| CS-A | <1 | 81 | 6 | <1 |
| CS-B | 17 | 12 | 2 | <1 |
| CS-C | <1 | 54 | 8 | <1 |
| CS-D | <1 | 81 | 18 | <1 |
| CS-E | <1 | <1 | <1 | <1 |

a The values represent the averages of two independent experiments.

b No detectable sulfotransferase activities were detected towards CS/DS variants tested when the vector-control was used as an enzyme source.

c When sulfotransferase activity of C4ST-3 was assayed at 28 °C as reported previously (55) for 12 h, the appreciable incorporation of sul- fate into chondroitin (4.7 pmol) and partially desulfated DS (2.9 pmol), respectively, was detected. Under the same conditions, negligible activities were detected toward natural CS variants A-E.

d Porcine skin DS.

The apparent evolutionary relations of seven HNK-1ST family members. The predicted amino acid sequences of seven HNK-1ST family members are compared using the GENETYX-MAC (version 10) software. The length of each horizontal line is proportional to the degree of the amino acid sequence divergence.

FIG. 1. Comparison of acceptor specificity of D4ST-1, C4ST-1, and C4ST-2

The recombinant D4ST-1, C4ST-1, C4ST-2, and C4ST-3 were assayed using each of various GAG polysaccharides as the acceptor (10 nmol as GlcUA) under the reaction conditions described under “Experimental Procedures.” The reaction products were separated from [35S]PAPS by gel filtration on a syringe column packed with Sephadex G-25 (super- fine). The radioactivity was measured by liquid scintillation counting.

![Figure 1](http://www.jbc.org/Downloaded from http://www.jbc.org/ on July 25, 2018)
containing 4-O-sulfated units at 31%. No detectable sulfotransferase activity was observed when a control sample prepared from the pEF-BOS/IP expression vector-transfected cells was used as an enzyme source.

We also examined sulfotransferase activities of C4ST-1 and C4ST-2 toward a series of CS/DS substrates using truncated forms of recombinant C4ST-1 and C4ST-2 under the same assay conditions as those for D4ST-1 for comparison. As shown in Table I, a soluble form of the recombinant C4ST-1 efficiently transferred sulfate to both chondroitin and partially desulfated DS with ~2-fold greater incorporation into the former than into the latter with far less activity toward other CS variants. Although a soluble form of C4ST-2 exhibited lower activity compared with D4ST-1 and C4ST-1, it also exhibited appreciable activity toward CS variants in addition to partially desulfated DS as in the case of C4ST-1. A soluble form of C4ST-3 showed negligible activity toward all the tested acceptors when compared with the other enzymes in our assay system (Table I).

FIG. 2. Gel filtration chromatographic analysis of 35S-labeled sulfotransferase reaction products prepared using three different sulfotransferases as enzyme sources and partially desulfated DS as the sulfate acceptor. An aliquot of 35S-labeled sulfotransferase reaction products, prepared using D4ST-1 (A and B), C4ST-1 (C and D), or C4ST-2 (E and F) with partially desulfated DS as the sulfate acceptor, was digested with chondroitinase AC-1 (A, C, and E) or chondroitinase B (B, D, and F). One-half of each digest was analyzed by gel filtration on a Superdex™ Peptide column as described under “Experimental Procedures.” The broken line in A indicates the elution profile of the intact 35S-labeled sulfotransferase reaction products obtained with D4ST-1 and partially desulfated DS. The vertical lines at the top of each panel indicate the elution positions of standard even-numbered oligosaccharides derived from porcine skin DS or GalNAc 4-O-sulfate: 1, octasaccharides; 2, hexasaccharides; 3, tetrasaccharides; 4, disaccharides; 5, GalNAc 4-O-sulfate.

FIG. 3. Anion exchange HPLC analysis of 35S-labeled sulfotransferase reaction products prepared using three different sulfotransferases as enzyme sources and partially desulfated DS as the sulfate acceptor. An aliquot of 35S-labeled sulfotransferase reaction products, prepared using D4ST-1 (A and B), C4ST-1 (C and D), or C4ST-2 (E and F) with partially desulfated DS as the sulfate acceptor, was digested with chondroitinase AC-1 (A, C, and E) or chondroitinase B (B, D, and F). One-half of each digest was analyzed by anion exchange HPLC on an amine-bound silica PA03 column using a linear NaH2PO4 gradient from 16 to 530 mM over a 60-min period. The vertical lines indicate the elution positions of authentic unsaturated disaccharides: 1, ΔDi-0S; 2, ΔDi-6S; 3, ΔDi-4S; 4, ΔDi-diS; 5, ΔDi-diSe; 6, ΔDi-triS.

FIG. 4. Comparison of sulfation sites within partially desulfated DS chains by D4ST-1, C4ST-1, and C4ST-2. The trisaccharide sequences, -GlcUA/ΔH9252 1–3GalNAc/ΔH9252 1–4GlcUA- (open bar) and -IdoUA/ΔH9251 1–3GalNAc/ΔH9252 1–4IdoUA- (solid bar), which served as 4-O-sulfation sites within partially desulfated DS chains upon incubation with D4ST-1, C4ST-1, or C4ST-2, were individually quantified by digestions using chondroitinase AC-1 or B, respectively, followed by HPLC determination of 35S-labeled ΔDi-4S as shown in Figs. 2 and 3.
4-O-Sulfation Sites within DS—To further distinguish the acceptor specificity of D4ST-1 from those of C4ST-1 and C4ST-2, an aliquot of 35S-labeled reaction products, which were obtained by incubation of a truncated form of D4ST-1, C4ST-1, or C4ST-2 with partially desulfated DS, was structurally characterized. The 35S-labeled products, obtained from each trans-ferase reaction, were digested exhaustively with either chondroitinase AC-I or chondroitinase B, which cleaves GalNAcβ1-4GlcUA linkages or GalNAcβ1-4IdoUA linkages, respectively. It should be noted that the sulfation of GalNAc residues flanking a target IdoUA residue is essential for the action of chondroitinase B (45, 56), although chondroitinase AC-I reactions are not much influenced by the sulfation pattern of neighboring sugar residues (58). One-half of each digest was analyzed by gel filtration (Fig. 2) or anion exchange HPLC (Fig. 3).

The gel filtration chromatograms revealed that the depolymerized 35S-labeled products eluted at positions corresponding to disaccharides, tetrasaccharides, hexasaccharides, and larger oligosaccharides (Fig. 2). The integrated radioactivity of each disaccharide peak (Fig. 2, A–F) observed on gel filtration was in good agreement with that of ΔDi-4S detected by anion exchange HPLC using the other half of each lyase digest (Fig. 3, A–F), demonstrating that 35S-labeled ΔDi-4S was released from -GlcUA-GalNAc(4S)-GlcUA- or -IdoUA-GalNAc(4S)-IdoUA- sequence by chondroitinase AC-I or chondroitinase B digestion, respectively (4S represents 4-O-sulfate).

In the analysis of radiolabeled products from the D4ST-1 reaction, only a small proportion (8%) of the total radioactivity was detected at the position of ΔDi-4S for the chondroitinase AC-I digest, whereas as much as 32% of the total radioactivity was identified as ΔDi-4S for the chondroitinase B digest (Fig. 2, A and B). Interestingly, in the case of the C4ST-2 reaction products, comparable proportions of radioactivity (nearly 20–25% of the total) were recovered as ΔDi-4S after digestion with chondroitinase AC-I or B (Fig. 2, E and F). These results are summarized in Fig. 4, which revealed marked differences among the three sulfotransferases in terms of the preference for the isomeric structures of the uronic acids flanking the target GalNAc residue. C4ST-1 showed a marked preference for GalNAc residues in the GlcUA-rich regions typical of CS, which are also dispersed in DS chains, being consistent with previous findings (30–32). In contrast, D4ST-1 was found to utilize mainly GalNAc residues in the IdoUA-rich repeating region. C4ST-2, whose substrate preference was not fully characterized previously (31), catalyzes the sulfation of GalNAc residues in the GlcUA-rich regions in addition to those in the GlcUA-rich regions.

Structural Determination of the Tetra- and Hexasaccharides Generated from D4ST-1 Reaction Products—Gel filtration analysis of the exhaustive chondroitinase AC-I digest of D4ST-1, C4ST-1, or C4ST-2 sulfotransferase reaction products showed several radioactive peaks including tetrasaccharides, hexasaccharides, octasaccharides, and higher oligosaccharides in addition to disaccharides (Fig. 2, A, C, and E). These oligosaccharides were generated most likely from the oligosaccharide sequences containing two, three, four, or more consecutive

![Image](http://www.jbc.org/)

**Fig. 5.** Subfractionation of the tetra- and hexasaccharide fractions by anion exchange HPLC. The 35S-labeled sulfotransferase reaction products, which were obtained on a large scale using D4ST-1 and partially desulfated DS as the acceptor, were digested with chondroitinase AC-I and fractionated by gel filtration as described in the legend to Fig. 2. The tetra- (A) and hexasaccharide (B) fractions obtained by size fractionation were individually chromatographed on an amine-bound silica PA-03 column using a linear salt gradient, as indicated by the dashed line. Vertical lines indicate the elution positions of the authentic unsaturated CS disaccharides as described in the legend to Fig. 3.

![Image](http://www.jbc.org/)

**Fig. 6.** DE MALDI-TOF mass spectra of fractions 4-III and 6-II. Representative DE MALDI-TOF mass spectra of fractions 4-III (A) and 6-II (B) were recorded in a positive ion mode with 2,5-dihydroxybenzoic acid as the matrix. Major molecular ion signals were assigned as summarized in Table II.
TABLE II
MALDI-TOF/MS analysis of the tetra- and hexasaccharide fractions isolated from D4ST-1 reaction products

| Fraction | Yield (nmol) | Compositional assignment |
|----------|--------------|--------------------------|
| 4-III    | 73           | HexUA1HexUA2HexNAc2(OSO3H) |
| 4-V      | 56           | HexUA1HexUA2HexNAc2(OSO3H) |
| 6-I      | 28           | HexUA1HexUA2HexNAc3(OSO3H) |
| 6-II     | 32           | HexUA1HexUA2HexNAc3(OSO3H) |
| 6-III    | 16           | HexUA1HexUA2HexNAc3(OSO3H) |
| 6-IV     | 47           | HexUA1HexUA2HexNAc3(OSO3H) |

*Expressed as nanomoles of each oligosaccharide prepared from D4ST-1 reaction products, which were obtained with partially desulfated DS (8 mol% as uronic acid) as the acceptor substrate.}

repeating disaccharide units, -IdoUAα1–3GalNAc-, flanked by two -GlcUAβ1–3GalNAc- sequences on both sides.

To locate the sulfation sites within the oligosaccharides, the chondroitinase AC-I digest from large scale D4ST-1 reaction products was fractionated by gel filtration. The tetra- and hexasaccharide fractions were further subfractionated by anion exchange HPLC. Five (4-I to 4-V) and four (6-1 to 6-IV) major UV-absorbing peaks were isolated from the tetra- and hexasaccharide fraction, respectively (Fig. 5). Among the isolated fractions, major radioactive fractions 4-III, 4-V, 6-I, 6-II, 6-III, and 6-IV were subjected to structural analysis as described below. Fractions 4-I and 4-II were not radiolabeled and therefore were not analyzed.

DE MALDI-TOF mass spectrometry analysis of the isolated oligosaccharide samples in positive ion mode defined their molecular masses, from which the composition and number of sulfate groups present in each fraction were inferred. In the positive ion mode, DE MALDI-TOF mass spectrometry analysis of sulfated oligosaccharides, monoisotopic masses of an \([M + x + 1Na\] \text{H} \] \type{type} type (where M represents the fully protonated form of an oligosaccharide) were preferentially observed. Representative spectra of fractions 4-III and 6-II are shown in Fig. 6. The molecule-related ion signal clusters at \(m/z\) 884, 906, and 928 afforded by fraction 4-III (Fig. 6A) corresponded, respectively, to mono-, di-, and trisodiated molecular ions of \([M + Na]^{+}\), \([M + 2Na-H]^{+}\), and \([M + 3Na-2H]^{+}\) as \(\Delta\text{HexUA}_{x}\text{HexUA}_{y}\text{HexNAc}_{z}\) with an O-sulfate group, where \(\text{HexUA}, \Delta\text{HexUA}, \text{and HexNAc}\) represent hexuronic acid, unsaturated hexuronic acid, and N-acetylgalactosamine, respectively. Fraction 6-II (Fig. 6B) showed the molecule-related ion signal clusters at \(m/z\) 1365, 1387, 1409, and 1431, corresponding, respectively, to tri-, tetra-, penta-, and hexasodiated \(\Delta\text{HexUA}_{x}\text{HexUA}_{y}\text{HexNAc}_{z}\) with two O-sulfate groups. The assignments of the molecule-related ion signals of fractions 4-III and 6-II in addition to other tetra- and hexasaccharide fractions (4-V, 6-I, 6-II, 6-III, and 6-IV) are summarized in Table II.

The disaccharide composition of each isolated tetra- and hexasaccharide fraction was determined by digestion with chondroitinase ABC in conjunction with HPLC (46), and the results are summarized in Table III. The digestion of each oligosaccharide fraction gave only two kinds of unsaturated disaccharide units, \(\Delta\text{Di}-0S\) and/or \(\Delta\text{Di}-4S\), suggesting that the sulfation positions were restricted to C-4 positions of GalNAc residues. Since the oligosaccharide fractions were isolated from the radiolabeled partially desulfated DS after exhaustive digestion with chondroitinase AC-I, the internal uronic acid residues in the major component(s) in each fraction were IdoUA residues, as expected.

TABLE III
Disaccharide composition of the isolated oligosaccharides derived from the D4ST-1 reaction products

| Fraction | Composition | \(\Delta\text{Di}-0S\) | \(\Delta\text{Di}-4S\) |
|----------|-------------|----------------------|----------------------|
| 4-III    | Di-4S       | 102                  | 102                  |
| 4-V      | ND\(^{a}\)  | 204                  | 204                  |
| 6-I      | 269         | 135                  |                      |
| 6-II     | 133         | 276                  |                      |
| 6-III    | 123         | 282                  |                      |
| 6-IV     | ND          | 415                  |                      |

\(^{a}\) ND, not detected.

\(^{b}\) Recoveries of the disaccharides relative to each parent oligosaccharide were calculated based on absorption at 232 nm.
The digestion of fractions 4-V and 6-IV with chondroitinase ABC gave a single peak at the elution position of /H9004 Di-4S (Table III). Based on these and the results from the DE MALDI-TOF mass spectrometry analysis (Table II), the following structures are proposed as the major compounds in fractions 4-V and 6-IV, respectively: fraction 4-V, /H9004 HexUA /H9251 1–3GalNAc(4S) /H9252 1–4IdoUA /H9251 1–3GalNAc(4S); fraction 6-IV, /H9004 HexUA /H9251 1–3GalNAc(4S) /H9252 1–4IdoUA /H9251 1–3GalNAc(4S).

The digestion of fraction 4-III or 6-II with chondroitinase ABC gave two major products, /H9004 Di-0S and /H9004 Di-4S, in a molar ratio of 1.0:1.0 or 1.0:2.1, respectively, as determined by HPLC (Table III). Hence, fractions 4-III and 6-II contained monosulfated tetrasaccharide and disulfated hexasaccharide, respectively, as major components, being consistent with the results from the DE MALDI-TOF analysis. The oligosaccharide fractions 4-III and 6-II were then analyzed using 500-MHz 1H NMR spectroscopy. The one-dimensional and two-dimensional correlation spectroscopy (COSY) spectra of these fractions (4-III and 6-II) are depicted in Figs. 7 and 8, respectively. The proton chemical shifts were assigned by the analysis of the two-dimensional COSY spectra. These NMR findings are summarized in Table IV.

The chemical shifts of most of the protons of the tetrasaccharide in fraction 4-III and the hexasaccharide in fraction 6-II were identical with those reported for the corresponding protons of analogous tetra- and hexasaccharides (52). All of the internal uronic acid residue(s) of the oligosaccharides in fractions 4-III and 6-II were confirmed as IdoUA residues based upon the chemical shifts (δ 4.926 and 4.881 for fraction 4-III; δ 4.911, 4.867, and 4.881 for fraction 6-II) and the coupling constants J1,2 (2.5 and 2.0 Hz for fraction 4-I; 3.5, 3.5, and 3.5 Hz for fraction 6-II) of the anomeric proton signals. The coupling constants J1,2 of /H9251 IdoUA and /H9252 GlcUA of CS/DS have been reported to be 3.0 and 8.0 Hz, respectively (59, 60). Compared with the proton chemical shifts reported for the reference compound, /H9004 HexUA /H9251 1–3GalNAc(4S) /H9252 1–4IdoUA /H9251 1–3GalNAc(4S) (52), a large upfield shift of H-4 of GalNAc-3 (0.534 ppm) was observed on NMR of fraction 4-I, suggesting that the GalNAc-1 was 4-O-sulfated, whereas the C-4 position of the GalNAc-3 residue was not. Likewise, compared with the proton chemical shifts reported for the reference compound, /H9004 HexUAα1–
suggested that fraction 6-I and 6-III contained mono- and disulfated hexasaccharides, respectively, as major components. Further enzymatic characterization of these fractions was carried out using a highly purified preparation of chondroitinase ABC, which exerts an exolytic action when acting on CS hexasaccharide unlike a conventional preparation of chondroitinase from the same bacterial source; it does not degrade tetrasaccharides, but it degrades hexasaccharides into disaccharides and tetrasaccharides, which are generated from the nonreducing and reducing terminus of the parent hexasaccharide, respectively (49). The digestion of fraction 6-I (1 nmol as hexasaccharide) with the enzyme preparation gave 1.04 nmol of ΔDi-4S and two unsaturated tetrasaccharides (0.48 and 0.61 nmol) (Fig. 9A), which eluted at the same positions as fractions 4-III and 4-IV, respectively. The disaccharide composition of fraction 4-IV was identical to that of fraction 4-III (data not shown), suggesting that fraction 4-IV had the monosulfated tetrasaccharide structure of ΔHexUAα1–3GalNAcβ1–4IdoUAα1–3GalNAcβ1–4IdoUAα1–3GalNAc. These results suggest that fraction 6-I contains the following two hexasaccharide structures (designated fractions 6-Ia and 6-Ib) at a molar ratio of 1.0:1.3: fraction 6-Ia, ΔHexUAα1–3GalNAcβ1–4IdoUAα1–3GalNAcβ1–4IdoUAα1–3GalNAc(4S); fraction 6-Ib, ΔHexUAα1–3GalNAcβ1–4IdoUAα1–3GalNAcβ1–4IdoUAα1–3GalNAc(4S). Likewise, fraction 6-III (1 nmol) yielded 1.02 nmol ΔDi-4S and two tetrasaccharide species (0.62 and 0.48 nmol), which eluted at the same positions as fraction 4-III and the aforementioned fraction 4-IV after digestion with highly purified chondroitinase ABC (Fig. 9B). Thus, the following two hexasaccharide structures are proposed as the major compounds (designated fraction 6-IIda and 6-IIdb) in fraction 6-III at a molar ratio of 1.3:1.0: fraction 6-IIda, ΔHexUAα1–3GalNAcβ1–4IdoUAα1–3GalNAcβ1–4IdoUAα1–3GalNAcβ1–4IdoUAα1–3GalNAcβ1–4IdoUAα1–3GalNAc. Determination of the Sulfation Sites Catalyzed by D4ST-1 within the Tetra- and Hexasaccharide Sequences—Table V summarizes the structures of the major tetra- and hexasaccharide components, which were isolated in this study from the radiolabeled partially desulfated DS after digestion with chondroitinase AC-I. Therefore, the ΔHexUA at the nonreducing ends in individual oligosaccharide structures are derived from GlcUA residues, and the reducing GalNAc residues are derived from GalNAc-GlcUA linkages. Since the partially desulfated DS used as an acceptor substrate was 4-O-sulfated on 31% of the component GalNAc residues, the newly formed 35S-labeled sulfation sites by the action of D4ST-1 within several structurally defined oligosaccharides were determined as follows.

The structure of 35S-labeled fraction 6-I suggests that D4ST-1 transferred sulfate to the GalNAc residue in the disaccharide unit on the reducing side and/or to that in the internal disaccharide unit of the following sequence, -GlcUAβ1–3GalNAcβ1–4IdoUAα1–3GalNAcβ1–4IdoUAα1–3GalNAcβ1–4GlcUA-. The structure of 35S-labeled fraction 4-III provides direct evidence that the D4ST-1 transferred sulfate to the GalNAc residue on the reducing side of the nonsulfated sequence -GlcUAβ1–3GalNAcβ1–4IdoUAα1–3GalNAcβ1–4GlcUA-. Hence, D4ST-1 can catalyze 4-O-sulfation of GalNAc residues not only in the -IdoUAα1–3GalNAcβ1–4IdoUAα1- but also in the -IdoUAα1–3GalNAcβ1–4GlcUA- sequence.

Fractions 4-V and 6-III contained disulfated structures with 4-O-sulfated GalNAc residues on the reducing and nonreducing side of IdoUA residue(s). Interestingly, the specific radioactivity of fraction 4-V was ~6.8-fold higher than that of fraction 4-III (Table V), suggesting that the GalNAc residues on the
The nonreducing side of IdoUA residues (i.e. in -GlcUA/H9252 1–3Gal-NAc/H9252 1–4IdoUA- sequences) may be sulfated by D4ST-1, although the opposite possibility also exists. To clarify this point, the trisulfated hexasaccharide (6-IV) was digested with highly purified chondroitinase ABC, taking advantage of its exolytic action on a hexasaccharide to produce a disaccharide and a tetrasaccharide from the nonreducing and reducing sides of the parent hexasaccharide, respectively. The enzyme yielded two radioactive peaks corresponding to the elution positions of Di-4S and disulfated tetrasaccharide \[\text{HexUA}/H9004 1–3\text{GalNAc(4S)}\] with 33 and 67% of the total radioactivity of the parent oligosaccharide, respectively (Fig. 10), suggesting that D4ST-1 at least catalyzed to a considerable degree the 4-O-sulfation of the GalNAc residue located at the nonreducing side of the hexasaccharide sequence GlcUAa1–3GalNAc(4S)β1–4IdoUAa1–3GalNAc(4S)β1–4IdoUAa1–3GalNAc(4S) that had been embedded in the parent DS polysaccharide. Namely, D4ST-1 was involved in 4-O-sulfation of a GalNAc residue in the sequence -GlcUA/H9252 1–3GalNAc/H9252 1–4IdoUA-.

In the case of disulfated oligosaccharide fractions (6-II and 6-III), the positions of radioactive sulfate group(s) in their sequences could not be determined in this study due to the low incorporated radioactivity. Although fraction 4-V had a high specific radioactivity, the location of the radiolabeled sulfate was not analyzed due to the difficulty in discriminating the two sulfate groups.

In strong contrast to the radioactive peaks corresponding to the sulfated tetra- and hexasaccharides with retention times between 24 and 55 min, which were generated by chondroitinase AC-I (Fig. 3A) or B (Fig. 3B) digestion of the D4ST-1 transferase reaction products, the C4ST-1 reaction products, prepared with partially desulfated DS as an acceptor, yielded radiolabeled \[\text{HexUA}/H9004\] as a predominant peak along with much smaller proportions of radioactive tetra- and hexasaccharide peaks upon digestion with chondroitinase AC-I (Fig. 3C), and the chondroitinase B digest gave no significant radioactive peaks (Fig. 3D). These results suggested that C4ST-1 utilized predominantly the sequences containing consecutive GlcUA residues but not those adjacent to IdoUA. Chromatographic patterns of the chondroitinase AC-I and B digests of the

### TABLE IV

| Sugar and proton | Fraction 4-III | Fraction 6-II |
|-----------------|---------------|---------------|
|                 | α             | β             | α             | β             |
| GalNAc-1        |               |               |               |
| H-1             | 5.210 (2.5)   | ND            | 5.210 (3.5)   | ND            |
| H-2             | 4.356         | ND            | 4.351         | ND            |
| H-3             | 4.169         | ND            | 4.169         | ND            |
| H-4             | 4.73          | ND            | 4.73          | ND            |
| H-5             | 4.188         | ND            | 4.09          | ND            |
| H-6             | ND            | ND            | ND            | ND            |
| H-6'            | ND            | ND            | ND            | ND            |
| NAc             | 2.053         |               | 2.051         |               |
| IdoUA-2         |               |               |               |
| H-1             | 4.926 (2.5)   | 4.881 (2.0)   | 4.911 (3.5)   | 4.867 (3.5)   |
| H-2             | 3.533         | 3.548         |               |               |
| GalNAc-3        |               |               |               |
| H-1             | 4.613 (8.5)   |               | 4.66 (ND)     |               |
| H-2             | 3.986         |               | 4.05          |               |
| H-3             | 3.910         |               | 4.05          |               |
| H-4             | 4.090         |               | 4.67          |               |
| H-5             | 3.737         |               | ND            |               |
| H-6             | ND            |               | ND            |               |
| H-6'            | ND            |               | ND            |               |
| NAc             | 2.078         |               | 2.077         |               |
| IdoUA-4         |               |               |               |
| H-1             | —             |               | 4.881 (3.5)   |               |
| H-2             | —             |               | 3.521         |               |
| H-3             | —             |               | 3.900         |               |
| H-4             | —             |               | 4.095         |               |
| H-5             | —             |               | 4.727         |               |
| GalNAc-5        |               |               |               |
| H-1             | —             |               | 4.611 (8.0)   |               |
| H-2             | —             |               | 3.981         |               |
| H-3             | —             |               | 4.09          |               |
| H-4             | —             |               | 3.92          |               |
| H-5             | —             |               | 3.52          |               |
| H-6             | —             |               | ND            |               |
| H-6'            | —             |               | ND            |               |
| NAc             | —             |               | 2.077         |               |
| ΔHexUA-6"       |               |               |               |
| H-1             | 5.189 (3.5)   |               | 5.185 (3.5)   |               |
| H-2             | 3.794         |               | 3.798         |               |
| H-3             | 4.094         |               | 4.090         |               |
| H-4             | 5.895         |               | 5.896         |               |

a Values for fraction 4-III represent those of ΔHexUA-4.

Chondroitin/Dermatan GalNAc 4-O-Sulfotransferases

Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but actually measured indirectly in reference to acetone (δ 2.225 ppm) in 2H2O at 26 °C. The estimated error for the values to two decimal places was only ±0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ±0.002 ppm. Coupling constants $J_{1,2}$ (in Hz) of GalNAc, IdoUA, and ΔHexUA residues are given in parentheses. ND, not determined; —, not occurring.
Chondroitin/Dermatan GalNAc 4-O-Sulfotransferases

C4ST-2 reaction products were very similar to those obtained from the D4ST-1 reaction products, except for the disaccharide peak (Figs. 2, A, B, E, F, and 3, A, B, E, and F). These results suggest that C4ST-2 may possess both properties of D4ST-1 and C4ST-1. Hence, C4ST-2 can be considered as C4ST-2/D4ST-2.

DISCUSSION

The evolutionary relations shown in Fig. 1 suggest that D4ST-1 and C4ST-1, -2, and -3 constitute a CS/DS 4-O-sulfotransferase subfamily in the HNK-1ST family as proposed in this study. The close evolutionary relation between D4ST-1 and C4ST-2/D4ST-2 is consistent with their substrate specificities as discussed below. D4ST-1, C4ST-1, C4ST-2, and C4ST-3, cloned from mouse and human (30–32, 39, 55), exhibit GalNAc 4-O-sulfotransferase activity toward dermatan and chondroitin. However, their specificities, especially toward DS linkage, have not been fully clarified. In this study, we investigated the acceptor specificities of these enzymes except for C4ST-3, which was phylogenetically closest to C4ST-1 but showed negligible catalytic activity toward the above mentioned substrates. Although the low activity was reportedly due to its thermal instability (55), specific substrates other than chondroitin may exist for C4ST-3 in view of its restricted expression profile in tissues such as liver and kidney (55).

The acceptor specificity of D4ST-1 toward CS/DS variants was clearly distinct from those of C4ST-1 and -2 (Table 1). Among natural CS/DS variants, only CS-B (DS) was utilized by D4ST-1, whereas C4ST-1 and -2 showed broader specificity, exhibiting significant activities toward various natural CS/DS variants. With chondroitin and partially desulfated DS as an acceptor, significantly higher activities were detected for all three enzymes compared with those obtained with natural CS/DS as acceptors. D4ST-1 exhibited a stronger preference to partially desulfated DS. In contrast, C4ST-1 showed a greater preference for chondroitin, and C4ST-2 exhibited comparable preferences for both acceptors, although C4ST-2 activity especially toward partially desulfated DS was significantly lower than those of D4ST-1 and C4ST-1. Whereas these results are basically consistent with those reported recently by Evers et al. (39), the novel acceptor specificity of D4ST-1 was also revealed in this study, as described below, when the reaction products were analyzed in detail.

Analysis of the transferase reaction products clearly showed that the observed differential specificities of the three enzymes represent differences in their preferences for the GalNAc residues flanked by either IdoUA or GlcUA residue(s) in the polymers, as evidenced by the relative abundance of 34S-labeled disaccharide (ΔDi-4S) in the chondroitinase AC-I or B digest of the 34S-labeled DS product (Fig. 4). D4ST-1 predominantly utilized GalNAc residues in the -IdoUA1–3GalNAcβ1–4IdoUA sequence, whereas C4ST-1 efficiently worked on GalNAc residues in -GlcUAβ1–3GalNAcβ1–4GlcUA- and -GlcUAβ1–3GalNAcβ1–4IdoUA-. C4ST-2 used GalNAc residues in both sequences to comparable extents. Chondroitinase AC-I digestion of the D4ST-1 reaction products, obtained with partially desulfated DS as acceptor, yielded multiple radioactive tetra- and larger oligosaccharides containing internal IdoUA residue(s) in addition to disaccharides. The structural determination of the isolated tetra- and hexasaccharides provided detailed information about the 4-O-sulfation sites within the sugar chain. It was clearly demonstrated for the first time that D4ST-1 could catalyze 4-O-sulfation of GalNAc residues not only in the sequence -IdoUA1–3GalNAcβ1–4IdoUA- but also in -IdoUA1–3GalNAcβ1–4GlcUA- and -GlcUAβ1–3GalNAcβ1–4IdoUA-. The demonstrated sequence preferences of D4ST-1, C4ST-1, and C4ST-2 are illustrated in Fig. 11.

During the course of this study, Evers et al. (39) reported the acceptor specificity of the same enzyme, which acted preferentially on nearly exhaustively desulfated dermatan prepared from porcine intestine. After digestion of the transferase reaction products with chondroitinas AC-I and B, a tetrasaccharide ΔHexUA-GalNAc-IdoUA-GalNAc(4S) and a hexasaccharide ΔHexUA-GalNAc(4S)-GlcUA-GalNAc-GlcUA-GalNAc were isolated, respectively, as major fragments, suggesting that they were derived from a parent sequence, -GlcUA-GalNAc-IdoUA-GalNAc(4S)-GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc-IdoUA-GalNAc-. Thus, they concluded that D4ST-1 shows a strong preference for IdoUA-GalNAc flanked by GlcUA-GalNAc but not for IdoUA-GalNAc flanked by IdoUA-GalNAc, IdoUA-rich repeating regions containing -IdoUA1–3GalNAcβ1–4IdoUA- sequences that were scarcely utilized as sulfation sites. The contradictory findings between their and our studies are probably due to the structural differences of the acceptors. In contrast to the nonsulfated dermatan used in their study, our partially desulfated DS containing 4-O-sulfated GalNAc at 31% also served as an excellent acceptor for D4ST-1, and the GalNAc residues located on both reducing and nonreducing sides of internal IdoUA residues were efficiently sulfated by the enzyme, despite its lower frequency of free GalNAc residues in the polymer compared with the exhaustively desulfated form. Although the reactions observed with nonsulfated dermatan probably represent initial sulfation reactions, they probably do not display an overall picture of the DS synthetic processes involving D4ST-1 to form a mature highly sulfated chain. In contrast, the reactions observed with partially desulfated DS appear to include multiple distinct sulfation reactions of D4ST-1 in terms of the sulfate acceptor sequence, which take place at various stages of the biosynthetic process required for the maturation of DS chains as discussed below.
Comparison of the specific radioactivity of the isolated tetra- and hexasaccharide fractions revealed the effects of preexisting sulfate groups on the subsequent sulfation reactions. The specific radioactivity of fraction 4-V was 6.8-fold higher than that of fraction 4-III, and that of 6-IV was 5.2-fold higher than that of fraction 6-III (Table V). These results suggest that preexisting sulfate groups promoted the formation of the authentic unsaturated CS disaccharides as described in the legend to Fig. 3. The peak marked by an asterisk is derived from the enzyme reaction buffer.

The results of the experiment described under “Experimental Procedures” show that the eluates of chondroitinase ABC and analyzed by HPLC on an amine-bound silica column were digested with a highly purified preparation of chondroitinase ABC. The elution positions of the authentic unsaturated CS disaccharides are as described in the legend to Fig. 3. The peak marked by an asterisk is derived from the enzyme reaction buffer.

**Table V**

Structure of the radioactive tetra- and hexasaccharides isolated from D4ST-1 reaction products, which were prepared using partially desulfated DS as the acceptor substrate.

| Fraction no. | Specific 35S radioactivity | Structures |
|--------------|---------------------------|------------|
| 4-III        | 70                        | \( \Delta \text{HexUA}1–3\text{GalNAc}β1–4\text{IdoUA}α1–3\text{GalNAc}(4S) \) |
| 4-V          | 477                       | \( \Delta \text{HexUA}1–3\text{GalNAc}β1–4\text{IdoUA}α1–3\text{GalNAc}(4S) \) |
| 6-Ia         | 82                        | \( \Delta \text{HexUA}1–3\text{GalNAc}β1–4\text{IdoUA}α1–3\text{GalNAc}(4S) \) |
| 6-Ib         | 482                       | \( \Delta \text{HexUA}1–3\text{GalNAc}β1–4\text{IdoUA}α1–3\text{GalNAc}(4S) \) |
| 6-II         | 192                       | \( \Delta \text{HexUA}1–3\text{GalNAc}(4S)β1–4\text{IdoUA}α1–3\text{GalNAcβ1–4\IdoUAα1–3\text{GalNAc}(4S) \) |
| 6-IIIa       | 998                       | \( \Delta \text{HexUA}1–3\text{GalNAc}(4S)β1–4\text{IdoUA}α1–3\text{GalNAcβ1–4\IdoUAα1–3\text{GalNAc}(4S) \) |
| 6-IV         |                           | \( \Delta \text{HexUA}1–3\text{GalNAc}(4S)β1–4\text{IdoUA}α1–3\text{GalNAcβ1–4\IdoUAα1–3\text{GalNAc}(4S) \) |

* 4S represents the 4-O-sulfate group.

Values represent the specific radioactivities of hexasaccharide subfractions 6-1 and 6-III.

**Fig. 10.** HPLC analysis of an enzyme digest of fraction 6-IV using a highly purified preparation of chondroitinase ABC. Fraction 6-IV (3 nmol) was digested with a highly purified preparation of chondroitinase ABC and analyzed by HPLC on an amine-bound silica column as described under “Experimental Procedures.” The eluates were monitored by absorption at 232 nm (A) and by 35S radioactivity (B). The arrow in B indicates the elution position of the \( \Delta \text{HexUA}1–3\text{GalNAc}(4S) \) disaccharide fraction. The vertical lines at the top of A indicate the elution positions of the authentic unsaturated CS disaccharides as described in the legend to Fig. 3. The peak marked by an asterisk is derived from the enzyme reaction buffer.

**Fig. 11.** Schematic representation of the acceptor sequence specificities of D4ST-1, C4ST-1, and C4ST-2/D4ST-2 toward a CS/DS polysaccharide backbone. The arrows indicate the potential sulfation sites in the schematic repeating disaccharide sequence of a CS/DS hybrid structure catalyzed by D4ST-1, C4ST-1, and C4ST-2/D4ST-2. The thickness of each arrow represents the relative preference of each enzyme for \( \text{IdoUA-GalNAc} \)-\( \text{IdoUA} \)-, \( \text{IdoUA-GalNAc} \)-\( \text{GlcUA} \)-, and \( \text{GlcUA-GalNAc-IdeoUA} \)-rich sequences by D4ST-1. The activity of C4ST-1 toward the \( \text{GlcUA-GalNAc-IdeoUA} \)-sequence has been reported in Ref. 57.

In the absence of PAPS, the epimerization reaction in the nonsulfated chondroitin is reversible and reaches a particular equilibrium state, where the \( \text{GalNAc} \)-\( \text{IdoUA} \)-configuration dominates over the \( \text{IdoUA} \)-configuration (61). The degree of interconversion to \( \text{IdoUA} \) is much lower than that of actual \( \text{IdoUA} \) content observed in native DS (62). A marked 4-O-sulfation modification of dermatan has been observed using human skin fibroblast microsomes as an enzyme source (29). Since the microsome preparation utilizes dermatan more efficiently than chondroitin, it has been proposed that \( \text{IdoUA} \) residues may enhance the sulfation of their neighboring GalNAc residues (29). Furthermore, based on differential enzyme activities of C5-epimerase reflecting different IdoUA/GlcUA ratios in contrast to high 4-O-sulfotransferase activities in the used tissues, it was recently postulated that C5-epimerase is the rate-limiting factor for DS synthesis (63). D4ST-1, which exhibits a striking preference for DS lineage, probably contributes significantly to DS synthesis (63).

Although the D4ST-1 message is ubiquitously expressed in adult tissues (39), the \( \text{IdoUA} \)-containing disaccharide cluster shows a tissue-specific nature in abundance and distribution in the polysaccharide backbone (23, 63) (see Ref. 64 and references therein). Native DS is composed of a periodic and wave-like arrangement of \( \text{IdoUA-GalNAc}(4S) \)-containing disaccharide repeats and \( \text{GlcUA-GalNAc}(4S) \)-containing disaccharide repeats in a tissue-specific manner (23). Although the specific activity of D4ST-1 reported by Evers et al. (39) suitably explains the
initial reactions for the formation of isolated IdoUA-GalNAc(4S)-rich regions (30–32) but also in IdoUA-containing regions of DS. Furthermore, the present study demonstrated involvement of C4ST-1 in the synthesis of the IdoUA-containing region of DS. Taken together, the collaborative or competitive contributions of these three sulfotransferases in different ratios in distinct tissues probably takes part in tissue-specific structural expression of DS, although the expression level of C5-epimerase is probably the rate-limiting factor for the DS formation. Molecular cloning of C5-epimerase(s) will make it possible to investigate possible concerted actions between the C5-epimerization and 4-O-sulfation reactions, which will allow us to depict an overall picture of the modification reactions required for the synthesis of mature DS chains.

Acknowledgments—We thank Dr. K. Tsuchiya for preparation of the expression vector pEF-BOS/IP and Dr. S. Nadanaka for preparation of partially desulfated DS. We also thank M. Hattori for technical assistance.

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J. Biol. Chem. 2003, 278:36115-36127.
doi: 10.1074/jbc.M306044200 originally published online July 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306044200

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