Recently, it has become evident that chondroitin sulfate (CS) glycosyltransferases, which transfer glucuronic acid and/or N-acetylglactosamine residues from each UDP-sugar to the nonreducing terminus of the CS chain, form a gene family. We report here a novel human gene (GenBank™ accession number AB088602) that possesses a sequence homologous with the human chondroitin sulfate synthase-1 (CSS1) gene, formerly known as chondroitin synthase. The full-length open reading frame consists of 882 amino acids and encodes a typical type II membrane protein. This enzyme contains a β3-glycosyltransferase motif and a β4-glycosyltransferase motif similar to that found in CSS1. Both the enzymes were expressed in COS-7 cells as soluble proteins, and their enzymatic natures were characterized. Both glucuronyltransferase and N-acetylgalactosaminyltransferase activities were observed when chondroitin, CS polymer, and their corresponding oligosaccharides were used as the acceptor substrates, but no polymerization reaction was observed as in the case of CSS1. The new enzyme was thus designated chondroitin sulfate synthase-3 (CSS3). However, the specific activity of CSS3 was much lower than that of CSS1. The reaction products were shown to have a GlcUAβ1-3GalNAc linkage and a GalNAcβ1-4GlcUA linkage in the nonreducing terminus of chondroitin resulting from glucuronyltransferase activity and N-acetylglactosaminyltransferase activity, respectively. Quantitative real time PCR analysis revealed that the transcript level of CSS3 was much lower than that of CSS1, although it was ubiquitously expressed in various human tissues. These results indicate that CSS3 is a glycosyltransferase having both glucuronyltransferase and N-acetylgalactosaminyltransferase activities. It may make a contribution to CS biosynthesis that differs from that of CSS1.

Chondroitin sulfate (CS) proteoglycans are located in the extracellular matrix and on cell surfaces in various kinds of human tissues. Some CS proteoglycans provide high osmotic pressure and water retention, and others may modulate cell adhesion to the extracellular matrix, proliferation, and morphogenesis (1, 2). The biosynthetic assembly of chondroitin sulfate proteoglycans is characterized by the following sequential processes: (i) synthesis of the core protein; (ii) xylosylation of specific Ser moieties of the core protein; (iii) addition of two Gal residues to the Xyl; (iv) completion of common tetrasaccharide linkage region by the addition of a glucuronic acid (GlcUA) residue; (v) addition of an GalNAc residue to initiate the chondroitin/dermatan sulfate biosynthesis; (vi) repeated addition of GlcUA residues alternating with GalNAc residues to grow the large heteropolymer glycosaminoglycan chains; and (vii) modification of these growing glycosaminoglycan chains by variable O-sulfation and by variable epimerization of GlcUA to IdoUA.

The assembly of the linkage region on the core protein followed by glycosaminoglycan polymerization and modification occurs in the intracellular membrane system, which is composed of the endoplasmic reticulum and Golgi apparatus (3, 4). With the exception of the polysaccharide chain-initiating Xyl transferase, which is found in the endoplasmic reticulum (5), all of the enzymes are firmly attached to the Golgi membranes and may work in an orchestrated manner. Some of these biosynthetic enzymes are found in serum or in the culture medium of the cells (4, 6). The enzymes responsible for the synthesis of the linkage regions in proteoglycans, Xyl transferase (6), Gal transferase I (7, 8), Gal transferase II (9), as well as GlcUA transferase I (10, 11), which act sequentially to transfer Xyl, Gal, GlcUA from their respective sugar nucleotide precursors to the acceptor core protein, have been cloned. We have been interested in characterizing the modification reactions, especially sulfations, because specific regional structures created by these modifications allow chondroitin sulfate to interact with other molecules, including cytokines. The modifications also allow regulation of the assembly and activities of other proteins in extracellular and pericellular matrices (12–

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**MOLECULAR CLONING AND CHARACTERIZATION**

Chondroitin Sulfate Synthase-3

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The abbreviations used are: CS, chondroitin sulfate; CSS, chondroitin sulfate synthase; HS, heparan sulfate; GlcUA, glucuronic acid; (β3)GlcUA-T, (β1,3-)glucuronyltransferase; (β3 or 4)Gal-T, (β1,3 or 1,4-)galactosyltransferase; (β4)GalNAc-T, (β1,4-N-acetylgalactosaminyltransferase; (α4)Gn-T, (α1,4-N-acetylgalactosaminyltransferase; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MES, 2-(N-morpholino)ethanesulfonic acid.

1 The abbreviations used are: CS, chondroitin sulfate; CSS, chondroitin sulfate synthase; HS, heparan sulfate; GlcUA, glucuronic acid; (β3)GlcUA-T, (β1,3-)glucuronyltransferase; (β3 or 4)Gal-T, (β1,3 or 1,4-)galactosyltransferase; (β4)GalNAc-T, (β1,4-N-acetylgalactosaminyltransferase; (α4)Gn-T, (α1,4-N-acetylgalactosaminyltransferase; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MES, 2-(N-morpholino)ethanesulfonic acid.
Recent progress with the human genome project and the expansion of other data bases, such as expressed sequence tags (ESTs) and full-length cDNAs, have enabled a wider search for novel genes that are homologous to known genes. Kitagawa et al. (22) identified a human chondroitin synthase from the novel genes that are homologous to known genes. This enzyme has the dual ability to sulfotransferase (19), uronyl-2-O-sulfotransferase (20), and N-acetylgalactosamine-4-sulfate-6-O-sulfotransferase (21). The sulfation of chondroitin sulfate ordinarily proceeds along with polymerization at the Golgi apparatus. Thus, in order to address control mechanisms of the sulfation reaction, the enzymes involved in the chain synthesis should also be studied, especially the chondroitin sulfate elongation enzymes.

By a similar search of the database for homologues, five enzymes are likely responsible for chondroitin/dermatan sulfate synthase-2, the fifth chondroitin sulfate glycosyltransferase cloned, has only GlcUAT-II activity, and the others were named CSGalNAcT-1, CSGalNAcT-3Gal, and the others were named CSGalNAcT-1, CSGalNAcT-3Gal, and CSGalNAcT-4Gal. The second and fourth chondroitin sulfate glycosyltransferases, respectively. UDP-[3H]GalNAc (7.0 Ci/mmol) and UDP-[3H]Gal (20 Ci/mmol) were purchased from ICN Biomedicals (Irvine, CA) and ARC (St. Louis, MO), respectively. UDP-[3H]GalNAc (7.0 Ci/mmol) and UDP-[3H]Gal (20 Ci/mmol) were from PerkinElmer Life Sciences.

Chondroitin (a chemically desulfated derivative of whale cartilage chondroitin sulfate A), chondroitin sulfate A (whale cartilage), dermatan sulfate (pig skin), chondroitin sulfate C (shark cartilage), chondroitin sulfate D (shark cartilage), chondroitin sulfate E (seabird cartilage), hyaluronic (rooster comb), heparan sulfate (pig aorta), D-Acetylchondroitin 4-sulfotransferase (EC 2.3.1.49 from Acremonium sp.), and chondroitinase ACII (EC 4.2.2.5 from Arthrobacter aurescens) were from Seikagaku Corp. (Tokyo, Japan). Testicular hyaluronidase (EC 3.2.1.35, H6254, type V from sheep testes), β-glucuronidase (EC 3.2.1.31, G0501, type B-10, from bovine liver), heparin (bovine intestine), Galβ1–3Galβ1–4GlcNAc, α-GlcUA-O-nitrophenyl, anti-FLAG BioM2 antibody, anti-FLAG M2-agarose gel, and pFLAG-CMV1 were from Sigma. The pcDNA3.1 plasmid was from Invitrogen. The Superdex™ Peptide HR10/30 column, HiLoad 16/60 Superdex 30-pg column, Fast Desalting column HR10/10, and PD10 desalting column were purchased from Amersham Biosciences. N-Acetylheparan, GlcNAc-O-benzyl, GlcNAc, Galβ1–3Galβ1–4GlcNAc, Galα1–3Galβ1–4GlcNAc, Galα1–3Galβ1–4GlcNAc, Galβ1–3Galβ1–4GlcNAc, and Galβ1–3Galβ1–4GlcNAc were kindly provided by Seikagaku Corp.

**Cloning of CSS3 and Construction of CSS3 and CSS1 Expression Vectors**—A BLAST search of the EST databases was performed using the amino acid sequences of the cloned human chondroitin sulfate glycosyltransferases, CSS1 (GenBank™ accession number AB023207), CSS2 (GenBank™ accession number AB086063), CSSGlUT (GenBank™ accession number AB037823), CSSGalNAcT-1 (GenBank™ accession number AB081516), and CSSGalNAcT-2 (GenBank™ accession number AB079252) as a query, and a novel EST clone was found (GenBank™ accession number AC092419). As the sequence was complete, a GENSCAN search of human genomic databases was performed. The predicted sequence was confirmed with the following two sets of primers: set 1, 5′-ATGCGCTTGGCCTGTCGCCGCCGT-3′ and 5′-CGTCCCCGCTGCTTGGCTACT-3′; set 2, 5′-AGTGCGCCACACGCGGCGGAGC-3′ and 5′-TCAGGAGAGAGTCGTTGAC-3′. The full-length cDNA was amplified by PCR with the following primers: 5′-AGCCACAACGGCGCGCCGAGC-3′ and 5′-GCTTACATATGCTGTCGCCGTCGTCGCCGAC-3′ and 5′-GCTTACATATGCTGTCGCCGTCGTCGCCGAC-3′. The amplified fragment was inserted into the HindIII and XbaI sites of pFLAG-CMV1. The putative catalytic domain of CSS1 (amino acids 130–883) was expressed as a secreted protein fused with a FLAG peptide in COS-7 cells. An ~2.3-kb DNA fragment was amplified by PCR using the Marathon-Ready™ cDNA derived from human brain (Clontech) as a template, with two primers, 5′-CCGAACTTTCGGCGGGGAGGCGGCG-3′ and 5′-AGACTGTCCAGGCGGCGGATTCGTTACT-3′. The amplified fragment was inserted into the HindIII and XbaI sites of pFLAG-CMV1. The putative catalytic domain of CSS1 (amino acids 47–802) was expressed as a secreted protein fused with a FLAG peptide in COS-7 cells. An ~2.3-kb DNA fragment was amplified by PCR using a DNA clone, Kauza DNA Research Institute number, RIAA0990, with 5′-AAGCGCTTTCGCGGAGGCGGCGGATTCGTTACT-3′ and 5′-GCTTACATATGCTGTCGCCGTCGTCGCCGAC-3′. The amplified fragment was inserted into the NotI and XbaI sites of pFLAG-CMV1.

**Purification of FLAG-tagged Recombinant Enzymes from Culture Supernatant**—COS-7 cells (ATCC CRL-1651) were co-transfected with the expression plasmid and pcDNA3.1 by using TransFast™ (Promega, Madison, WI) according to the manufacturer’s instructions. Transfected cells were selected using 600 μg/ml G418 in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum (HyClone Laboratories, Logan, UT), 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin G and cloned by limiting dilution. Cloned cell lines were tested for synthesis and secretion of the recombinant protein by immunoprecipitation and Western blotting of supernatants using an anti-FLAG BioM2 antibody (Sigma). The secreted enzyme was purified by affinity chromatography using an anti-FLAG M2-agarose gel (Sigma). The conditioned medium and gel were mixed overnight at 4 °C and centrifuged for 5 min, and the supernatant was aspirated. The gel was washed five times with 10 ml of 20% (v/v) glycerol in 50 mM Tris-HCl, pH 7.4, and resuspended in the same buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) to produce a 50% slurry. The immobilized enzyme was stable at 4 °C for at least 4 weeks. The amount of recombinant protein recovered was estimated by immunoblotting. It was separated by SDS-PAGE (10% gel), transferred onto polyvinylidene difluoride membrane (Immobilon, Millipore), and probed with FLAG peptide antibody BioM2 (Sigma). FLAG-tagged bacterial alkaline phosphatase (FLAG-BAP, molecular mass 49 kDa) was used as a standard to estimate the relative amount, as described previously (27). Blotting and probing were performed according to the manufacturer’s instructions, followed by horseradish peroxidase-conjugated streptavidin. Immunoblot bands using positive buffer controls were visualized with a chemiluminescence detection system (Amersham Biosciences) with 10-s exposures. The CSS3, CSS1, and BAP protein bands were quantified by densitometric scanning of the digitized image using NIH image (version 1.61) software. The standard curve for each substrate was generated by increasing the

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-[14C]GlcUA (313 mCi/mmol) and UDP-[3H]Gal (20 Ci/mmol) were purchased from ICN Biomedicals (Irvine, CA) and ABC (St. Louis, MO), respectively. UDP-[3H]GalNAc (7.0 Ci/mmol) and UDP-[14C]GlcNAc (200 mCi/mmol) were from PerkinElmer Life Sciences. Chondroitin (a chemically desulfated derivative of whale cartilage chondroitin sulfate A), chondroitin sulfate A (whale cartilage), dermatan sulfate (pig skin), chondroitin sulfate C (shark cartilage), chondroitin sulfate D (shark cartilage), chondroitin sulfate E (seabird cartilage), hyaluronic (rooster comb), heparan sulfate (pig aorta), D-Acetylchondroitin 4-sulfotransferase (EC 2.3.1.49 from Acremonium sp.), and chondroitinase ACII (EC 4.2.2.5 from Arthrobacter aurescens) were from Seikagaku Corp. (Tokyo, Japan). Testicular hyaluronidase (EC 3.2.1.35, H6254, type V from sheep testes), β-glucuronidase (EC 3.2.1.31, G0501, type B-10, from bovine liver), heparin (bovine intestine), Galβ1–3Galβ1–4GlcNAc, α-GlcUA-O-nitrophenyl, anti-FLAG BioM2 antibody, anti-FLAG M2-agarose gel, and pFLAG-CMV1 were from Sigma. The pcDNA3.1 plasmid was from Invitrogen. The Superdex™ Peptide HR10/30 column, HiLoad 16/60 Superdex 30-pg column, Fast Desalting column HR10/10, and PD10 desalting column were purchased from Amersham Biosciences. N-Acetylheparan, GlcNAc-O-benzyl, GlcNAc, Galβ1–3Galβ1–4GlcNAc, Galα1–3Galβ1–4GlcNAc, Galα1–3Galβ1–4GlcNAc, Galβ1–3Galβ1–4GlcNAc, and Galβ1–3Galβ1–4GlcNAc were kindly provided by Seikagaku Corp.
Fig. 1. Amino acid sequence alignment and genomic structure of CSS3 and CSS1. Alignment of the two enzymes sequence was performed by GENETYX (A). Introduced gaps are shown with hyphens. The putative transmembrane domains are underlined. DXD motifs, the β3-glycosyltransferase motif, and the β4-glycosyltransferase motif are boxed. Identical amino acids are indicated by asterisks. The conserved or nonconserved possible N-glycosylation sites are indicated by closed or open arrowheads, respectively. B, the genome structure of CSS3 (upper) and CSS1 (lower) genes was constructed by comparison of their genomic DNA sequences (GenBank™ accession numbers NT_029975.3 and NT_035326.1, respectively) and their cDNA sequences. Exon regions are denoted by boxes. The translation initiation (ATG) and termination (TGA or TAA) codons are also shown. Black horizontal bars denote the introns.
FIG. 2. Estimation of the amount of FLAG epitope-tagged CSS3 and CSS1 proteins. A and B, Western blot analyses of FLAG-tagged BAP protein and FLAG-tagged CSS3 and CSS1. The proteins were isolated from serial dilutions of the culture medium of COS-7 transfectants that were stably expressing FLAG-tagged CSS3 and CSS1 protein, respectively. The intensity of the 49-kDa band (BAP protein) and the 90-kDa bands (CSS3 and CSS1 proteins) increased with increasing concentrations of FLAG-tagged BAP protein and the volume of the medium, respectively. C and D, depiction of the relationship between the content of BAP protein and the band density; linear correlations were noted ($R^2 = 0.991$ and 0.967 for CSS3 and CSS1, respectively). E and F, depiction of the relationship between the volume of the medium and the concentrations of CSS3 and CSS1 proteins, respectively, as derived from the BAP standard curve; linear correlations were again observed ($R^2 = 0.992$ and 0.972 for CSS3 and CSS1, respectively). The amounts of recombinant soluble CSS3 and CSS1 proteins are expressed in arbitrary units of intensity, with 1 unit equivalent to 10 ng of FLAG-tagged BAP protein.
amount of FLAG-tagged BAP protein on the same blotting membrane as the CSS samples. The band intensity and the concentration of the recombinant CSS proteins (90 kDa) in the medium exhibited a linear correlation. The amounts of recombinant CSS proteins could therefore be estimated accurately from the standard curve, which was generated using known amounts of FLAG-tagged BAP protein (49 kDa). The amount of recombinant enzyme protein is expressed in arbitrary units, with each unit of intensity equivalent to 10 ng of FLAG-BAP protein (27).

Preparation of Acceptor Substrate—Glycosaminoglycan polymers were purchased from Seikagaku Corp. For the GlcAT-II assay, chondroitin sulfate A–E, chondroitin, hyaluronan, heparan sulfate, and N-acetyl heparosan were digested with β-glucuronidase prior to the assay (27). Even- and odd-numbered oligosaccharides of chondroitin sulfate isoforms, chondroitin, heparan sulfate, heparin (100 μg each), oligosaccharides of chondroitin, chondroitin sulfate isoforms, and hyaluronan (1 nmol each). The standard reaction mixture for GalNAcT-II contained 10 μl of the resuspended gel and acceptor substrate, 0.307 nmol of UDP-[14C]GlcUA (2.22 × 10^5 dpm), 50 mM MES, pH 5.8 or 6.2, and 20 mM MnCl₂ in a total volume of 30 μl. The reaction mixture for GlcAT-II contained 10 μl of the resuspended gel and acceptor substrate, 0.307 nmol of UDP-[14C]GlcUA (6.66 × 10^5 dpm), 50 mM MES, pH 6.2, and 10 mM MnCl₂ in a total volume of 30 μl. The reaction mixtures were incubated at 37 °C for 2 h with mixing. The reaction mixture for CSS3 and CSS1 polymerization reactions contained 10 μl of the resuspended gel and 1 nmol of CS-C10, 10 nmol of UDP-[3H]GalNAc (2.77 × 10^5 dpm).

Fig. 3. Effects of buffer and pH on the GlcAT-II and GalNAcT-II activities of CSS3 and CSS1. The effects of pH on the CSS3- (A and C) and CSS1-catalyzed (B and D) transfers of GlcUA (A and B) and GalNAc (C and D) to CS-C11 and CS-C10, respectively, were determined under standard assay conditions with different buffers at a final concentration of 50 mM. The buffers are sodium acetate (open circles), MES-NaOH (closed circles), imidazole-HCl (open triangles), and Tris-HCl (closed triangles). Data represent the average of two independent experiments.

Glycosyltransferase Assays—The glycosyltransferase activities were investigated using radioactive forms of UDP-GlcUA, UDP-GalNAc, UDP-GlcNAc, UDP-Gal, and various acceptor saccharide substrates, including chondroitin polymer, various chondroitin sulfate isoforms, hyaluronan, heparan sulfate, heparin (100 μg each), oligosaccharides of chondroitin, chondroitin sulfate isoforms, and hyaluronan (1 nmol each). The standard reaction mixture for GalNAcT-II contained 10 μl of the resuspended beads and acceptor substrate, 0.32 nmol of UDP-[3H]GalNAc (6.66 × 10^5 dpm), 50 mM MES, pH 6.2, and 10 mM MnCl₂ in a total volume of 30 μl. The reaction mixture for GlcAT-II contained 10 μl of the resuspended gel and the acceptor substrate, 0.307 nmol of UDP-[3H]GlcUA (2.22 × 10^5 dpm), 50 mM MES, pH 5.8 or 6.2, and 20 mM MnCl₂ in a total volume of 30 μl. The reaction mixtures were incubated at 37 °C for 2 h with mixing. The reaction mixture for CSS3 and CSS1 polymerization reactions contained 10 μl of the resuspended gel and 1 nmol of CS-C10, 10 nmol of UDP-[3H]GalNAc (2.77 × 10^5 dpm).
dpm), 10 nmol of UDP-GlcUA, 50 mM MES, pH 6.2, and 10 mM MnCl₂ in a total volume of 30 μl. The reaction mixture for polymerization reactions carried out by Escherichia coli K4 strain chondroitin polymerase contained 1 nmol of CS-C₁₀, 50 mM Tris-HCl, pH 7.2, 20 mM MnCl₂, 0.1 M (NH₄)₂SO₄, 1 M ethylene glycol, 10 nmol of UDP-[³H]GalNAc (2.77 × 10⁴ dpm), 10 nmol of UDP-GlcUA, and 1 g of the enzyme preparation in a total volume of 30 μl. The reaction mixtures were incubated at 37 °C (for CSS3 and CSS1) or at 30 °C (for E. coli K4 strain chondroitin polymerase) overnight. The reaction was then stopped by boiling for 5 min, and radiolabeled products were separated from free UDP-[³H]GalNAc or UDP-[¹⁴C]GlcUA by gel filtration by using the Superdex™ Peptide HR10/30 column (10 × 300 mm) with 0.2 M NaCl as an eluant or by using the HiLoad 16/60 Superdex 30-pg column (16 × 600 mm) with 0.2 M NH₄HCO₃ as an eluant (27). The labeled products recovered were quantified by liquid scintillation counting. For the acceptor oligosaccharide substrates with an aromatic residue (methoxy-phenyl-, benzyl-, or 4-nitrophenyl-) at the reducing terminus, the reaction products were diluted with 1 ml of 0.5 M NaCl and applied to a Sep-Pak C₁₈ cartridge (100 mg; Waters, Milford, MA) (27). The cartridge was washed with 3 ml of 0.5 M NaCl and then 3 ml of water, the product was eluted with 50% methanol, and the radioactivity of all fractions was measured by liquid scintillation counting.

Identification of the Enzyme Reaction Products—Each product of the GlcUAT-II reaction using chondroitin or CS₁₁ and of the GalNAcT-II reaction using chondroitin was isolated by gel filtration column chromatography using the Superdex™ Peptide HR10/30 column. The radioactive peak containing the product was pooled and desalted with the Fast Desalting column HR10/10 using distilled water as an eluant, and was lyophilized. In order to identify the linkage structure, the dried sample (about 20 pmol of radiolabeled material) from GlcUAT-II reaction was incubated with one of the following: 1) 100 milliunits of chondroitinase ACII in a total volume of 100 μl of 100 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 100 mM CaCl₂. The reaction was stopped by boiling for 5 min, and the products were separated by gel filtration using the Superdex™ Peptide HR10/30 column (10 × 300 mm) with 0.2 M NaCl as an eluant. The products eluted were quantified by liquid scintillation counting. For the acceptor oligosaccharide substrates with an aromatic residue (methoxy-phenyl-, benzyl-, or 4-nitrophenyl-) at the reducing terminus, the reaction products were diluted with 1 ml of 0.5 M NaCl and applied to a Sep-Pak C₁₈ cartridge (100 mg; Waters, Milford, MA) (27). The cartridge was washed with 3 ml of 0.5 M NaCl and then 3 ml of water, the product was eluted with 50% methanol, and the radioactivity of all fractions was measured by liquid scintillation counting.
The enzyme digests were analyzed using the same Superdex™ Peptide column and desalted with the Fast Desalting column HR10/10 using stop the reaction. The radioactive peak containing the product was collected. The solution was then boiled for 5 min to remove denaturants.

Buffer, pH 4.5, at 37 °C

The radioactive peak containing the product was collected. The solution was then boiled for 5 min to remove denaturants.

Table I

| Acceptor substrate         | Specific activity of radiolabeled sugar incorporationa |
|----------------------------|-----------------------------------------------------|
|                            | GlcUA-T II   | GalNAc-T II   | GlcUA-T II   | GalNAc-T II   |
| Glycosaminoglycan polymerb |            |              | 143          | 153          |
| Chondroitin                | 7.7         | 15           | 7.3          | 5.6          |
| Chondroitin sulfate A      | 0.4         | 0.4          | 8.1          | 7.2          |
| Dermatan sulfate           | ND          | 0.6          | 8.7          | 8.2          |
| Chondroitin sulfate C      | 0.2         | 0.6          | 5.9          | 7.3          |
| Chondroitin sulfate D      | 0.2         | 0.6          | 3.6          | 4.1          |
| Chondroitin sulfate E      | 0.1         | 0.6          | 11           | 5.2          |
| Heparan sulfate            | 0.2         | 0.6          | ND           | ND           |
| N-Acetyllnephranose        | ND          | ND           | ND           | ND           |
| Heparin                    | ND          | ND           | ND           | ND           |

b Glycosaminoglycan oligosaccharide

Undecasaccharide for GlcUA-T II

CH11                      | 13          | ND           | 99           | ND           |
CS-A11                    | 21          | ND           | 200          | ND           |
CS-C11                    | 23          | ND           | 240          | ND           |
HA10                      | ND          | ND           | ND           | ND           |

Decasaccharide for GalNAc-T II

CH10                      | ND          | 4.4          | ND           | 176          |
CS-A10                    | ND          | 8.2          | ND           | 289          |
CS-C10                    | ND          | 9.5          | ND           | 300          |
HA10                      | ND          | 0.3          | ND           | ND           |

Linkage region oligosaccharides

Galβ1-3Galβ1-4Xy1β1-OMP   | ND          | ND           | ND           | ND           |
GlcUAβ1-3Galβ1-3Galβ1-4Xy1β1-OMP | ND          | ND           | ND           | ND           |

a The values represent the averages of two independent experiments.
b Poly saccharide substrates for GlcUA-T II were used after β-glucuronidase treatment.
c ND, not detected.

d 7.4, containing 30 mM sodium acetate at 37 °C overnight; or 2) 1 unit of β-glucuronidase in a total volume of 100 μl of 100 mM sodium acetate buffer, pH 5.0, at 37 °C overnight. To confirm the linkage structure, assays were carried out to determine whether the product could serve as an acceptor for E. coli K4 strain chondroitin polymerase, which synthesizes chondroitin, and if the resultant products could be digested completely with chondroitinase ACII (29) Briefly, 20 pmol of the radiolabeled material was lyophilized and served as a substrate for E. coli K4 strain chondroitin polymerase. The reaction was performed at 30 °C overnight in a 50-μl solution containing 50 mM Tris-HCl, pH 7.2, 20 mM MnCl2, 0.1 mM (NH4)2SO4, 1 M ethylene glycol, 20 pmol of the radiolabeled material was digested completely with chondroitinase ACII (29) Briefly, 20 pmol of the radiolabeled material was lyophilized and served as a substrate for E. coli K4 strain chondroitin polymerase.

RESULTS

Molecular Cloning of CSS3 and Determination of Its Nucleotide and Amino Acid Sequences—A BLAST search of the EST database was performed using the amino acid sequences of the cloned human chondroitin sulfate glycosyltransferases as a query, and a novel EST was found (GenBank™ accession number AC04219). As the sequence was incomplete, a GENSCAN search of human genomic data bases was performed. The predicted sequence was confirmed by PCR with two sets of primers as follows: set 1, 5′-ATGGCTGTGCTCTGACAGATAGTCA-3′ and the reverse primer for CSS1, 5′-ATGGCTGTGCTCTGACAGATAGTCA-3′, and the probe for CSS1, 5′-ATGGCTGTGCTCTGACAGATAGTCA-3′, which contains a minor groove binder. PCR products were measured continuously with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The relative amounts of the transcripts were normalized to the amount of GAPDH transcript in the same cDNA sample.

Quantitative Analysis of the CSS3 Transcript in Human Tissues by Real Time PCR.—For quantification of CSS3 transcripts, the real time PCR method was employed, as described in detail previously (30). Marathon Ready cDNA derived from various human tissues was purchased from Clontech. Standard curves for the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, were generated by serial dilution of pCR2.1 (Invitrogen) DNA containing the GAPDH gene. The primer set and probe for CSS3 are as follows: the forward primer, 5′-CCCCAGAAAAAGTCTCTATCTCAGGA-3′, and the reverse primer, 5′-AACCTCTTCAATATTGTGCACCTTTATGACGTTGTTGACT-3′, and the probe, 5′-ATGAGTGGTTCATGCGGCG-3′, which contains a minor groove binder. The primer sets and probes for CSS1 are as follows: the forward primer for CSS1, 5′-AGTGTGCTGTGCTCTGACAGATAGTCA-3′, and the reverse primer for CSS1, 5′-AGTGTGCTGTGCTCTGACAGATAGTCA-3′, and the probe for CSS1, 5′-AGTGTGCTGTGCTCTGACAGATAGTCA-3′, which contains a minor groove binder. PCR products were measured continuously with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The relative amounts of the transcripts were normalized to the amount of GAPDH transcript in the same cDNA sample.

Comparison of Amino Acid Sequences between CSS3 and CSS1—The deduced amino acid sequence of the clone exhibited high homology (62%) with CSS1, as shown in Fig. 1A. Hydropathy plots of the amino acid sequence revealed one hydrophobic stretch, located at position 9–29, as in CSS1 (Fig. 1A, underlined). Two DXXD motifs, which are conserved in many glycosyltransferases and function as key sequences for divalent cation binding, as well as other motifs that are conserved in...
1,3-glycosyltransferases (β3GTs) and 1,4-glycosyltransferases (β4GTs), were found, with one in the N-terminal and one in the C-terminal region, respectively (Fig. 1A, boxed). A comparison of the cysteine residue locations in the predicted proteins encoded by CSS3 (11 cysteines) and by CSS1 (11 cysteines) showed good conservation of 10 of these cysteines (Fig. 1A, boldface). Two of the three potential N-glycosylation sites in CSS3 appeared to be conserved in both enzymes (Fig. 1A, closed arrowheads). The third potential N-glycosylation site was located close to the C terminus of both enzymes (Fig. 1A, open arrowheads). A remarkable difference between the two amino acid sequences was seen in the lengths of their N-terminal stem regions. The proline-rich stem region of CSS3 was about 90 amino acids in length and was somewhat longer than the corresponding region in CSS1.

Genome Organization and Chromosome Localization—A comparison of the cDNA sequence with the genomic sequence on chromosome 5 revealed that the CSS3 gene spans over 287 kb, because of its long second intron (~280 kb), and that it consists of at least three exons (Fig. 1B, uppercase). In contrast, the CSS1 gene spans over 75 kb. Its genomic organization, including the exon-intron boundaries, is similar to that of the CSS3 gene (Fig. 1B, lowercase) and consists of three discrete exons in the coding region (27). The CSS3 and CSS1 genes were located on human chromosome 5q31 and 15q26.3, respectively.
Estimation of the Amount of FLAG Epitope-tagged CSS3 and CSS1 Proteins—To facilitate functional analysis of the putative glycosyltransferase, a soluble form of the protein was generated by replacing the first 129 and 46 amino acids of CSS3 and CSS1, respectively, with the preprotrypsin signal sequence and a FLAG tag, as described under “Experimental Procedures.” The soluble putative glycosyltransferases were expressed in COS-7 cells as recombinant enzymes fused with the FLAG tags. The fused enzymes expressed in the medium were adsorbed onto anti-FLAG M2 antibody-conjugated agarose gels to eliminate endogenous glycosyltransferases, and the enzyme-bound gels were then used for the various reactions. The amounts of FLAG-tagged CSS3 and CSS1 were estimated using FLAG-BAP as a standard (Fig. 2, A and B), as described previously (27). The amount of FLAG-BAP was correlated with the densitometric units obtained by measurement of each band intensity ($R^2 = 0.991$ and 0.967 for CSS3 and CSS1, respectively), as shown in standard curves in Fig. 2, C and D. The amounts of recombinant CSS3 and CSS1 proteins were determined by defining 10 ng of the FLAG-BAP as 1 unit. One unit of CSS3 and of CSS1 was obtained from 19.9 (Fig. 2E) and 134.9 ml (Fig. 2F) of the pooled medium, respectively.

Acceptor Substrate Specificities of CSS1 and CSS3—The acceptor specificities of the truncated CSS3 and CSS1 proteins recovered from COS-7 transfectant cells were determined by testing a variety of glycosaminoglycans and their oligosaccharides as acceptor substrates. As expected from the homologies noted above, preliminary experiments showed that the CSS3 glycosyltransferase had dual enzymatic activities, a GlcUAT-II activity for CS-C11 and a GalNAcT-II activity for CS-C10. Therefore, the effects of buffers and pH on the enzymatic activities of both recombinant glycosyltransferases were examined and compared because those for CSS1 have not been determined (22). As shown in Fig. 3, CSS3 exhibited optimum activity at pH 5.8 and 6.2 in MES buffer with GlcUAT-II and GalNAcT-II, respectively (Fig. 3, A and C). CSS1 exhibited optimum activities at pH 5.8 in MES buffer with both GlcUAT-II and GalNAcT-II (Fig. 3, B and D). The reactions catalyzed by each of these enzymes were carried out with the appropriate buffer conditions.

Divalent cations were essential for the activity of both enzymes, and 10 mM EDTA completely abolished all enzymatic activities (Fig. 4). For CSS3, Co$^{2+}$ evoked the highest level of activity under standard assay conditions, and Mn$^{2+}$ was 31 and 64% as effective as Co$^{2+}$ for GlcUAT-II and GalNAcT-II activity, respectively. Cd$^{2+}$ was also effective, as was Mn$^{2+}$ (Fig. 4A). In the case of CSS1, Co$^{2+}$ evoked the highest level of activity under standard assay conditions with the substrate GlcUAT-II, whereas Cd$^{2+}$ and Mn$^{2+}$ were 85 and 70% as effective as Co$^{2+}$, respectively. Mn$^{2+}$ exhibited the highest activity for GalNAcT-II under standard assay conditions, and Co$^{2+}$ and Cd$^{2+}$ were 70 and 53% as effective as Mn$^{2+}$, respectively (Fig. 4B). The optimal concentrations for both enzymes were 20 mM Mn$^{2+}$ for the GlcUAT-II substrate and about 10 mM Mn$^{2+}$ for the GalNAcT-II substrate (Fig. 5, A and B). Under the established standard incubation conditions described under “Experimental Procedures,” GlcUA incorporation into CS-C11 and GalNAc incorporation into CS-C10 were proportional to the incubation time for up to 4 h, for each of the enzymes (data not shown). The specificity of the recombinant CSS3 and CSS1 toward each UDP-sugar donor substrate was analyzed using a series of radiolabeled molecules UDP-GlcUA, UDP-Gal, UDP-GalNAc, and UDP-GlcNAc under optimized conditions. Both CSS3 and

![Graph](image)

**Fig. 7.** Polymerization reactions carried out by CSS3 (A), CSS1 (B), and E. coli K4 strain chondroitin polymerase (C) using chondroitin sulfate oligosaccharide as an acceptor. $^3$H-Labeled oligosaccharide chains obtained from the incubation with the enzymes, CS-C10 oligosaccharide, and $^3$HUDP-GalNAc alone (open circles) or with the enzymes, CS-C10 oligosaccharide, $^3$HUDP-GalNAc, and UDP-GlcUA (closed circles), were chromatographed on a Superdex Peptide column. Effluent fractions were monitored by $^3$H radioactivity. Numbered arrowheads 10–12 indicate the elution positions of chondroitin sulfate-derived authentic decasaccharide to dodecasaccharide, respectively. The total volume was at fraction number 45 (data not shown).
CSS1 were able to catalyze efficiently the transfer of GlcUA from UDP-GlcUA to the acceptor CS-C11 and of GalNAc from UDP-GalNAc to the acceptor CS-C10. In contrast, the other radiolabeled nucleotide sugars tested (UDP-Gal, UDP-GalNAc, and UDP-GlcNAc for CS-C11; UDP-GlcUA, UDP-Gal, and UDP-GlcNAc for CS-C10) were not substrates of the recombinant CSS3 and CSS1 (data not shown). Furthermore, various monosaccharides including GlcUAβ-O-4-nitropheryl, GlcNAcα-O-benzyl, GlcNAcβ-O-benzyl, Galα-O-benzyl, Galβ-O-benzyl, and GalNAcα-O-benzyl and GalNAcβ-O-benzyl were not efficient acceptor substrates for any of the UDP-sugars tested as donors (data not shown).

**Acceptor Activity of Polymer Chondroitin and Various Chondroitin Sulfate Isoforms**—To characterize the substrate specificity of the purified recombinant CSS3 and CSS1, chondroitin, chondroitin sulfate isoforms, and other glycosaminoglycans were tested as acceptor substrates. As shown in Table I, chondroitin was the best substrate and the other polymer chondroitin sulfate isoforms were poor acceptors for enzymatic reactions catalyzed by both CSS3 and CSS1. The specific activity of CSS1 for chondroitin was about 18- and 10-fold more than the activity of CSS3 for GlcUA-T-II and GalNAcT-II, respectively.

**Acceptor Activity of Chondroitin and Chondroitin Sulfate Oligosaccharides**—As shown in Table I, both enzymes apparently showed GlcUA-T-II activity toward undecasaccharides having GalNAc in their nonreducing termini. These substrates were prepared from the CS isoforms and chondroitin. The activities of CSS3 for the CS-A and CS-C undecasaccharides were 1.6- and 1.7-fold higher than its activity for the chondroitin undecasaccharide, and for CSS1 these activities were 2.0- and 2.4-fold higher. The activity of each enzyme toward the hyaluronic undecasaccharide was negative. On the other hand, both enzymes apparently showed GalNAcT-II activity toward decasaccharides having GlcUA at their nonreducing termini, which were prepared from CS isoforms and chondroitin. The activities of CSS3 for the CS-A and CS-C decasaccharides were 1.9- and 2.1-fold higher than that for the chondroitin decasaccharide, and these activities of CSS1 were 1.6- and 1.7-fold higher, whereas the activity for the hyaluronan decasaccharide was negative for each enzyme. The specific activity of CSS1 for chondroitin sulfate oligosaccharide was about 10- and 32-fold higher than those of CSS3 for transferring GlcUA-T-II to CS-C11 and for transferring GalNAcT-II to CS-C10, respectively. The apparent $K_m$ values for UDP-GlcUA for GlcUA-

analyzed for radioactivity. Arrows indicate the elution positions of the authentic saturated disaccharide (closed arrowhead, GlcUAβ1–3GalNAc) or free GlcUA (open arrowhead). B, the GlcUA-T-II reaction products from CS11 were recovered from a Superdex™ Peptide column and subjected to chondroitin polymerization with E. coli K4 strain chondroitin polymerase or following chondroitinase AC-II digestion of the resultant polymer, as described under “Experimental Procedures.” The [14C]GlcUA-labeled CS-C11 oligosaccharide transferred by CSS3 (open circles), the sample polymerized by E. coli K4 strain chondroitin polymerase (closed circles), or the chondroitinase AC-II digest of the E. coli K4 strain chondroitin polymerase products (closed triangles) was applied to a column of Superdex™ Peptide, and the respective fractions (0.5 ml each) were analyzed for radioactivity. Arrows indicate the elution positions of the authentic saturated disaccharide (closed arrowhead, GlcUAβ1–3GalNAc). C, the GalNAcT-II reaction products with polymer chondroitin recovered from a Superdex™ Peptide column were then subjected to digestion with chondroitinase AC-II or α-N-acetylgalactosaminidase as described under “Experimental Procedures.” The undigested sample (open circles), the chondroitinase AC-II digest (closed circles), or the α-N-acetylgalactosaminidase digest (closed triangles) was applied to a column of Superdex™ Peptide, and the respective fractions (0.5 ml each) were analyzed for radioactivity. Arrows indicate the elution positions of the authentic free GalNAc (open arrowhead).
TII activities of CSS3 and CSS1 to the acceptor, CS-C11, were 360 μM (R² = 0.993) and 21 μM (R² = 0.997), respectively. The apparent Kₘ values for UDP-GalNAc for GalNAc-TII activities of CSS3 and CSS1 to the acceptor, CS-C10, were 221 μM (R² = 0.997) and 167 μM (R² = 0.983), respectively.

Effects of the length of oligosaccharides on CSS3 and CSS1 activities were determined using CS-C and chondroitin oligosaccharides as acceptors (Fig. 6). For both the GlcUA-TII and the GalNAc-TII activity of CSS3, the CS-C oligosaccharides were better acceptor substrates than the chondroitin oligosaccharide (Fig. 6, A and C). In the case of chondroitin oligosaccharides, the longer oligosaccharides served as better acceptors for CSS3 than did the shorter oligosaccharides (Fig. 6, A and C, closed circles). On the other hand, in CSS3 activity toward CS-C oligosaccharides, CS-C9 was the most efficient acceptor for GlcUA-TII, and CS-C8 was the most efficient acceptor for GalNAc-TII (Fig. 6, A and C, open circles). A similar tendency was also observed in CSS1 activity toward CS-C oligosaccharides, although CS-C11 is the most efficient acceptor for GlcUA-TII, and CS-C12 is the most efficient acceptor for GalNAc-TII (Fig. 6, B and D, open circles). To examine whether the CSS3 enzyme has other glycosyltransferase activities, several substrates were tested as acceptors, Galβ1–3Galβ1–4Xylβ1–O-methoxyprenyl and GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1–O-methoxyprenyl (with GlcUA-T-I and GalNAc-T-I for the glycosaminoglycan linkage region, respectively) (Table I), Galβ1–3GalNAcO–O-benzyl (human natural killer cell-1 epitope synthase), and Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (lactosamine tetrasaccharide), but no activity was detected (data not shown). These results again suggested that CSS3 is responsible for the chondroitin sulfate elongation but not for the linkage tetrasaccharide or activity toward other substrates, as described previously for CSS1 (22). Both CSS3 and CSS1 have dual glycosyltransferase activities of GlcUA-T-II and GalNAc-T-II, which are responsible for synthesizing the repeating disaccharide units of chondroitin sulfate. However, incubations of the recombinant soluble enzymes with CS-C10 in the presence of UDP-[3H]GlcUA and UDP-GlcUA in vitro, despite the individual transferase activities observed for CSS3 and CSS1, did not result in polymerization (Fig. 7, A and B, closed circles). In contrast, E. coli K4 strain chondroitin polymerase yielded radiolabeled polymer chondroitin chains when the incubation was carried out with UDP-[3H]GlcUA and UDP-GlcUA in vitro (Fig. 7C, closed circles). All of the enzymes demonstrated [3H]GlcNAc monosaccharide transfer to CS-C10 in the presence of UDP-[3H]GlcNAc alone (Fig. 7, open circles). These results suggest that CSS3 and CSS1 cannot produce chondroitin polymer upon incubation with UDP-sugars and oligosaccharide acceptor substrates in vitro, whereas the E. coli K4 strain chondroitin polymerase can carry out this reaction.

**Analysis of CSS3 Reaction Products**—To identify the GlcUA-T-II reaction products, chondroitin polymer was labeled with [14C]UDP-GlcUA by CSS3 under optimized conditions, and the products were isolated and then subjected to gel filtration analysis after treatment with chondroitinase AC-II or β-glucuronidase. As shown in Fig. 8A, the labeled products were completely digested by chondroitinase AC-II or β-glucuronidase, quantitatively yielding two 14C-labeled peaks at the positions of [14C]GlcUAβ1–3GalNAc and of free [14C]GlcUA, respectively. These findings indicate that a GlcUA residue was transferred to the nonreducing terminal GalNAc residue of the chondroitin polymer through a β-linkage. Furthermore, 14C-labeled chondroitin sulfate dodecasaccharide was used as an acceptor for a chondroitin polymerase from an E. coli K4 strain (Fig. 8B) (29). This reaction was performed in the presence of the enzyme and two donor substrates, UDP-GalNAc and UDP-GlcUA. The reaction products showed a molecular mass of ∼3000 Da that was speculated to be the product resulting from transfer of approximately five sugar residues. These products were digested by chondroitinase AC-II, yielding unsaturated GlcUAβ1–3GalNAc disaccharides. These results indicated the
**FIG. 10.** A, schematic structural comparison of the six cloned members of CS glycosyltransferases family. B, diagram shows the catalytic activities of the glycosyltransferases involved in the synthesis of CS. C, phylogenetic trees. A, all of the members carry out glycosyltransferase activities that are possibly involved in CS biosynthesis. The putative trans-membrane domains (TM) are indicated by slashed boxes. Conserved
In this report, we have described the cloning and characterization of a novel human chondroitin sulfate synthase-3 (CSS3), which is homologous with the human chondroitin sulfate synthase-1, formerly known as chondroitin synthase (22). We have designated these enzymes, which exhibit both GlcUAT-II activity and GalNAcT-II activity for chondroitin chain elongation in vitro, CSS (chondroitin sulfate synthase). The CSS3 enzyme is the third CSS and the sixth chondroitin sulfate glycosyltransferase identified.

The high amino acid sequence similarity, which includes many characteristic motifs shared between CSS3 and CSS1, suggests that the enzymes have related functions and evolutionary origins. A remarkable difference in two amino acid sequences, however, was seen in the lengths of their N-terminal stem regions. The stem region in CSS3 was about 90 amino acids longer than that in CSS1. This extra region consists of a proline-rich sequence that has no sequence similarity to any other proteins found in various data bases. The longer stem region presumably not only separates the catalytic domain from the membrane proximal segment by an appropriate distance, but it also may have a function specific to CSS3. For example, it may determine the localization of CSS3 in the Golgi membrane or may allow it to participate in interactions with other molecules (31). Alternatively, the proline chain may be cleaved in this region (32). Chromosomal assignments of the six human chondroitin glycosyltransferases, CSS1, CSS3, CSS2, CSGlcUAT, CSGalNAcT-1, and CSGalNAcT-2, indicate that these genes are localized on different chromosomes, 15q26.3, 5q31, 2p36.1, 7q36, 8q21.3, and 10q11.22, respectively, despite the significant similarities of the nucleotide and predicted amino acid sequences among the six genes (22–26). Homologues of human CSS1 and CSS3 have been identified in Caenorhabditis elegans and in Drosophila, suggesting the duplication might have occurred after the evolutionary branch point leading to Drosophila. Interestingly, a homologue of human CSGalNAcT-1 and -2 is absent from C. elegans but is present in Drosophila, and a homologue of human CSGlcUAT or CSGalNAcT is absent from both C. elegans and Drosophila. Differences in amino acid sequences were used to measure the evolutionary relationships between human CSS3 and other members of the CS glycosyltransferase family (Fig. 10C). The resulting dendrogram indicated that human CS glycosyltransferase genes may be classified into three groups (CSS1/CSS3, CSS2/CSGlcUAT, and CSGalNAcT-1/CSGalNAcT-2), with all groups in the family roughly equidistant from each other (Fig. 10C). This observation suggests that the CS glycosyltransferase family emerged early in evolution and has undergone recent duplication.

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specific molecular chaperone (Cosmc: core 1 molecular chaperone) in assisting the folding/stability of GlcNAc and GlcUA to an oligosaccharide acceptor transferase activity in mammalian cells (36), and both GlcNAc and GlcUA transferase activity than EXT2 when expressed independently, but both subunits are essential for activity in vivo. Experimentally, EXT1 was seen to have a more robust activity than EXT2 when expressed independently, but both subunits apparently can catalyze individual reactions. In the case of CS biosynthesis, CSS3 may work with other chondroitin sulfate glycosyltransferases as a co-polymerase in vivo. In fact, none of the purified, soluble versions of CSS1, CSS2, and CSS3 can produce chondroitin polymer upon incubation with UDP-sugars and oligosaccharide acceptor substrates in vitro, whereas the E. coli K4 strain chondroitin polymerase can carry out this reaction (Fig. 7) (29).

In conclusion, at least six CSS glycosyltransferases, CSS1, CSS3, CSS2, CSGlcUA-T, CSGalNAcT-1, and CSGalNAcT-2, form a gene family and can be classified into three groups based on their structures and enzymatic specificities (Fig. 1A). They may play roles at different stages of CS synthesis in a co-operative or orchestrated manner (Fig. 10B). The recent cloning, expression, and characterization of many glycosyltransferases have led to great progress in understanding chondroitin sulfate biosynthesis. However, none of these enzymes alone can form an entire glycosaminoglycan chain, and the coordination of complex cellular machinery may be required for CS elaboration. To understand the nature of the complexes formed by these molecules, further information is required on interactions between the various membrane-bound enzymes including CS sulfotransferases, their appropriate substrates, and membrane-bound nascent proteoglycans. Such information will be critical to determine the specific structural basis underlying the efficiency of the reactions carried out during formation of the CS chain.

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