Characterization of the d-Glucuronyl C5-epimerase Involved in the Biosynthesis of Heparin and Heparan Sulfate*

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The murine gene for the glucuronyl C5-epimerase involved in heparan sulfate biosynthesis was cloned, using a previously isolated bovine lung cDNA fragment (Li, J.-P., Hagner-McWhirter, Å., Kjellén, L., Palgi, J., Jalkanen, M., and Lindahl, U. (1997) J. Biol. Chem. 272, 28158–28163) as probe. The ~11-kilobase pair mouse gene contains 3 exons from the first ATG to stop codon and is localized to chromosome 9. Southern analysis of the genomic DNA and chromosome mapping suggested the occurrence of a single epimerase gene. Based on the genomic sequence, a mouse liver cDNA was isolated that encodes a 618-amino acid residue protein, thus extending by 174 N-terminal residues the sequence deduced from the (incomplete) bovine cDNA. Comparison of murine, bovine, and human epimerase cDNA structures indicated 96–99% identity at the amino acid level. A cDNA identical to the mouse liver species was demonstrated in mouse mast cells committed to heparin biosynthesis. These findings suggest that the iduronic acid residues in heparin and heparan sulfate, despite different structural contexts, are generated by the same C5-epimerase enzyme. The catalytic activity of the recombinant full-length mouse liver epimerase, expressed in insect cells, was found to be ~2 orders of magnitude higher than that of the previously cloned, smaller bovine recombinant protein. The ~52-kDa, similarly highly active, enzyme originally purified from bovine liver (Campbell, P., Hannessson, H. H., Sandback, D., Rodén, L., Lindahl, U., and Li, J.-P. (1994) J. Biol. Chem. 269, 26953–26958) was found to be associated with an ~22-kDa peptide generated by a single proteolytic cleavage of the full-sized protein.

Heparin and heparan sulfate (HS)† are both linear, sulfated glycosaminoglycans based on a common carbohydrate backbone of alternating d-glucosamine (GlcN) and hexuronic acid (d-glucuronic (GlcUA) or l-iduronic acid (IdoUA)) units. Both polymer types are synthesized as proteoglycans. Heparin occurs in connective tissue-type mast cells, whereas HS has a ubiquitous distribution and is produced by almost all mammalian cells. The biosynthesis of heparin and HS follows a common pathway that involves: (i) formation of a GlcUA-Gal-Gal-Xyl-Ser carbohydrate-protein linkage region; (ii) assembly of repeating -GlcUA-GlcNAc- disaccharide units; and (iii) modification of the [GlcUA-GlcNAc]n polymers (1, 2). The latter process includes N-deacetylation and N-sulfation of GlcNAc units, C5-epimerization of GlcUA to IdoUA units, and O-sulfation at various sites of the sugar residues. Heparin is highly modified, heavily N- and O-sulfated and with more IdoUA than GlcUA units. Generally, a predominant proportion of heparin chains consists of trisulfated -IdoUA(2-OSO3)-GlcNSO3(6-OSO3)- units. By comparison, HS has a more variable and heterogeneous structure, with highly modified, less modified, and unmodified sequences arranged in domain-type fashion (3, 4). The IdoUA units occur in essentially two types of domains, composed of contiguous N-sulfated, and of alternating N-sulfated and N-acetylated disaccharide units, respectively (5). The sequence variability of the chains is believed to reflect the functional role of HS glycosaminoglycans (GAGs) in specific interactions with different proteins (1–4, 6). IdoUA residues generally appear to promote protein binding due to their conformational flexibility and have been identified as invariant constituents of protein-binding HS domains (4). The reaction catalyzed by the C5-epimerase therefore is crucial for many biological functions of heparin and HS.

The mechanisms in control of the structural diversity of heparin and, in particular, of HS are only partly understood but clearly rely on the substrate specificities of the enzymes involved (2, 4). The enzymes required to synthesize a HS chain have all been cloned. Notably, several of these proteins, including species committed to polymer modification, occur in multiple forms that are encoded by different genes. Some of these species have been shown to differ with regard to kinetic properties and/or substrate specificity from the respective homologous forms (7–10).

The IdoUA units of heparin and HS chains occur in widely different structural contexts, which range from the minimally sulfated -GlcNSO3-IdoUA-GlcNAc- sequence found in HS molecules to the extensively sulfated -GlcNSO3(6-OSO3)-IdoUA(2-OSO3)-GlcNSO3(6-OSO3)- structure typical of heparin (4). These findings raise the question as to whether the C5-epimerase, similar to other enzymes in the same biosynthetic
electrophoresis; kb, kilobase pair; FCS, fetal calf serum; bp, base pair; ORF, open reading frame; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; NDST, N-deacetylation/N-sulfotransferase.

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‡ The abbreviations used are: HS, heparan sulfate; GlcUA, D-glucuronic acid; IdoUA, L-iduronic acid; GAG, glycosaminoglycan; FISH, fluorescence in situ hybridization; DAPI, 4',6-diamidino-2-phenylindole; αManR, 2,5-anhydromannose (formed by reduction of terminal 2,5-anhydromannose residues with NaBH4); PAGE, polyacrylamide gel
process, also occurs in genetically distinct forms. More specifically, is the same epimerase committed to the formation of heparin, in the mast cell, and to HS, in other cells? Following the isolation of the GlcUA C5-epimerase from bovine liver, the corresponding cDNA was cloned from bovine lung, and a re- 
combinant protein with significant catalytic activity was ex-
pressed in insect cells (11). In the present study we have addressed the question of genetic polymorphism by cloning the murine C5-epimerase gene. Only one form of the gene was found. Moreover, we have cloned the C5-epimerase cDNA from mouse liver that generates HS, and from mouse mastcymo-
toma cells that produce heparin. The results strongly suggest that the same enzyme protein is implicated in both biosynthetic processes. Finally, analysis of the epimerase gene structure indicated that the bovine cDNA previously cloned (11) was incomplete, as ~28% of the 5’-terminal coding region was miss-
ing. This conclusion was verified by cloning and expression of the full-length protein, with the catalytic activity much higher than that of the previously expressed truncated form.

EXPERIMENTAL PROCEDURES

Isolation and DNA Sequence Analysis of Mouse Genomic Clones—A mouse genomic library (Lambda FX-II from Stratagene) was screened with a 1407-bp DNA probe from the bovine epimerase coding sequence, labeled with [32P]dCTP (PerkinElmer Life Sciences). Approximately 2 × 106 phages were plated at a density of 250,000 plaques per 20 × 
20-cm plate, and duplicate nylon filters were prepared from each plate. Hybridization was performed at 60 °C in 5’-Denhardt’s hybridization solution, containing 100 μg of salmon sperm DNA/ml. The final washes were in 0.1× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS. Plaques that produced positive signals on the filters were selected for second and third round screening. Five positive clones were found. Two of the clones were digested, clone 5a with EcoRI and clone 64 with SacI, and the resultant fragments were cloned into pUC119 and BlueScript vectors, respectively. The insert-containing plasmids were purified using the Qiagen plasmid kit and subsequently sequenced. Nucleotide sequencing was performed using the BigDye termino-

**Fig. 1.** Diagrammatic representation of the mouse C5-epimerase gene. A, alignment of the five genomic clones studied. The restriction sites in clones 5a (EcoRI) and 64 (SacI) are indicated by vertical lines. B, organization of the gene. The coding sequences of the exons are shown as black boxes, and the non-coding sequences as shadowed boxes. Introns are represented by horizontal lines. The in-
tron fragment marked by *, generated by digestion of clone 5a with EcoRI, was used as a probe in Southern analysis (Fig. 2).

**Table I**

| Exon no. | 5’-Splice acceptor and 3’-end sequence | Exon size | 3’-End sequence and 3'-splice donor | Intron size |
|---------|---------------------------------------|-----------|------------------------------------|-----------|
| 1       | >586 GGTGAAGgggtgtaggt               | >1500     |                                    |           |
| 2       | ttagagGTGTGgCCA                      | 246       | TGTCAGCaGgtgtaggt                 | >7000      |
| 3       | cccagcAAACACgATG                    | >1025     |                                    |           |

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**Exon 1** **Exon 2** **Exon 3**

| Exon size | Intron-exon boundary sequences of the mouse C5-epimerase gene |
|-----------|---------------------------------------------------------------|
|            | Exon no. | 5’-Splice acceptor and 3’-end sequence | Exon size | 3’-End sequence and 3’-splice donor | Intron size |
| bp        | bp       |---------------------------------------|-----------|------------------------------------|-----------|
| 1         | >586     | GGTGAAGgggtgtaggt                     | >1500     |                                    |           |
| 2         | ttagag   | GTGTGgCCA                             | 246       | TGTCAGCaGgtgtaggt                 | >7000      |
| 3         | cccagc   | AAACACgATG                            | >1025     |                                    |           |
corresponding to bp 287–321 of the bovine cDNA reported earlier (11). The sense primer is 5’-ATGCGTTGTTTGGCAGCTCGGGTCAA, corresponding to bp 1–26 of the open reading frame of mouse cDNA. The antisense primer is 5’-GCAGCCCTTGGGCCACAGTCCAGTCATTGGGCTTGC corresponding to bp 287–321 of the bovine cDNA reported earlier (11).

For cloning of the 5’-terminal portion of bovine C5-epimerase cDNA, PCR was performed using a bovine lung gt10 cDNA library (CLONTECH) as template under the conditions described above. The sense primer is 5’-GCAGCCCTTGGGCCACAGTCCAGTCATTGGGCTTGC corresponding to bp 287–321 of the bovine cDNA reported earlier (11).

All PCR products were directly cloned into a TOPO™-TA Cloning vector (Invitrogen) according to the protocol provided by the manufacturer and were subsequently sequenced. The sequencing was carried out as described for the genomic DNA.

Expression of the Marine C5-epimerase Recombinant Protein—Based on the cDNA sequence of the mouse C5-epimerase, new primers were designed and used for generation of a fragment without transmembrane domain for recombinant expression. PCR was performed by using the full-length mouse cDNA as template under the same conditions. The RT-PCR reaction mixture was used for amplification of C5-epimerase cDNA using the primer pair described above under the same conditions. The resulting fragment was inserted into a pIZ/V5 expression vector (Invitrogen) and subsequently sequenced. The expression construct was introduced into Sf9 insect cells, and the cells were cultured according to the manufacturer’s instructions. The medium was collected and analyzed for epimerase activity.

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Enzyme Purification and Characterization—Medium containing recombinant epimerase (100 ml) was applied to a 20-ml column of O-desulfated heparin immobilized on Sepharose (16), equilibrated in 50 mM HEPES buffer, pH 7.4, 100 mM KCl, 15 mM EDTA. After extensive washing with the same buffer, the bound material was eluted with the same HEPES buffer containing 300 mM KCl, 15 mM EDTA. The eluted material was concentrated by centrifugation in an Ultraclear-MC 10,000 filter unit (Millipore).

Bovine liver C5-epimerase was extracted and purified to homogeneity as described (16). Peptide sequences were determined as described (11). Purified enzyme preparations were analyzed by SDS-PAGE (17). After electrophoresis, gels were stained with silver and documented.

Determination of Epimerase Activity—Epimerase assay was based on the release of \(^4\text{H}\) (recovered as \(^3\text{H}_2\text{O}\)) from a C5-\(^4\text{H}\)-labeled polysaccharide substrate, as described (11). For determination of enzyme activity, tissues were freshly dissected from a (A/Sn × Leaden) F1 mouse that had been inoculated previously with Furth mastocytoma cells in a hind leg (18). The tissues were immediately homogenized in 10 volumes of 50 mM HEPES, pH 7.4, containing 100 mM KCl, 15 mM EDTA, 1% Triton X-100, and protease inhibitors (16). Lysates were shaken at 4 °C for 30 min and centrifuged. The supernatants were collected and assayed for enzyme activity (11) and for total protein (19). Controls were performed to ascertain that all determinations of epimerase activity in tissue extracts fell within the linear range of the assay.

Enzymatic conversion of GlcUA to IdoUA units was directly demonstrated using purified recombinant mouse C5-epimerase (50 ng of protein in 100 μl of incubation mixture) and a metabolically \(1\text{-}\(^4\text{H}\)\)glucose-labeled [4GlcUA\(\beta\)1–4GlcNSO3\(\alpha\)1–1\(\alpha\)] polysaccharide substrate, as described (11). Briefly, the partially epimerized polysaccharide was subjected to deaminative cleavage with nitrous acid (pH 1.5 reaction) followed by reduction, and the resultant labeled GlcUA-αMan\(\alpha\) (glucuronyl-2,5-anhydromannitol) and IdoUA-αMan\(\alpha\) (iduronyl-2,5-anhydromannitol) disaccharides were separated by paper chromatography and quantified by acitllination counting.

Labeling and Preparation of Gliosaminoglycans from Mast Cells—Mastocytoma cells established from the Furth tumor (15) were cultured in RPMI 1640 medium (Life Technologies, Inc.) containing 50 μg/ml penicillin, 50 μg/ml streptomycin, and 10% FCS. To label the cells, 50
mCi of Na\textsuperscript{35}SO\textsubscript{4} (Amersham Pharmacia Biotech) was added per ml of culture medium. After incubation for 24 h, the medium was collected, and the cells were lysed in 50 mM HEPES, pH 7.4, containing 0.1 M KCl and 1% Triton X-100. The cell lysate was kept on ice for 30 min and centrifuged. The supernatant and medium were pooled and treated with 0.5M NaOH at 4 °C overnight and subsequently neutralized. The sample, containing released GAG chains, was diluted and applied to a DEAE-Sephacel column (4 ml) equilibrated in 0.05M NaAc, pH 4.0, containing 0.05 M NaCl. Labeled GAGs were eluted with a gradient of 0.05–1.5 M NaCl in the same buffer. The fractions containing labeled GAGs were pooled and concentrated. Analytical chromatography on DEAE-Sephacel was performed using the same buffer and salt gradient but in a high pressure liquid chromatography system. Treatments of GAGs with chondroitinase ABC (20) or with nitrous acid at pH 1.5 (21) were carried out as described. Unlabeled heparin and chondroitin sulfate standards were detected by the carbazole reaction for hexuronic acid (22).

**RESULTS**

**Organization of the Mouse Epimerase Gene**—Screening a genomic library from mouse liver with the previously described \textsuperscript{32}P-labeled bovine epimerase cDNA (11) as a probe yielded five positive clones that were purified and further characterized. The two largest clones, 5a and 64, were 16–18 kb in size, whereas the remaining three ranged 8–12 kb. All clones showed sequence overlap with at least three of the other clones (Fig. 1). Digestion of clone 5a with \textit{Eco}RI released seven fragments that were subsequently cloned into pUC119. Clone 64 was cleaved with \textit{Sac}I, and the resultant 3 major fragments were cloned into BlueScript. Analysis of exon-intron organization revealed that the C5-epimerase is encoded by only 3 exons, of which the largest one (exon 3) encodes more than 50% of the protein (Fig. 1B). The genomic sequence defining the exon/intron boundaries (splice sites) follows the gt/ag consensus rule (Table I). The precise match between the open reading frame (ORF) sequence in the exons and the cDNA (data not shown) suggests that the identified genomic clone represents the functional gene for the C5-epimerase.

**Southern Analysis and Chromosome Localization**—In order to...
to address whether the C5-epimerase is encoded by a single gene in the mouse genome, we performed Southern blot hybridization of genomic DNA that had been digested with restriction enzymes as indicated in Fig. 2. The probe used was an intron fragment (indicated by the asterisk in Fig. 1A) generated from clone 5a by digestion with EcoRI. This 800-bp probe detected a single band of the same size in the sample cleaved with EcoRI and single bands of the expected sizes (estimated based on restriction enzyme mapping) upon cleavage with HindIII (3.5 kb), NcoI (5 kb), SacI (6.5 kb), and PstI (8 kb).

By using the full-length mouse epimerase cDNA as probe, a single chromosomal locus was detected by FISH mapping in both mouse and human chromosomes. The FISH detection efficiency with this probe was 85% on mouse chromosomes and 63% on human chromosomes. The mouse epimerase is localized to chromosome number 9 (Fig. 3A) and the human epimerase to chromosome number 15 (Fig. 3B), in accordance with the homology relationship between human and mouse genomes. The loci were further defined based on the combined information from 10 photos each. The epimerase probe thus was mapped to mouse chromosome 9, region C and D (Fig. 3C), and to human chromosome 15, region q23-q24 (Fig. 3D). These findings along with the homology between human and mouse genomes indicated that the C5-epimerase is encoded by a single gene.

![Alignment of C5-epimerase amino acid sequences](image-url)

**FIG. 6.** Alignment of C5-epimerase amino acid sequences. Deduced polypeptide structures based on cDNAs from mouse liver, mouse mast cells (MC), human brain, and bovine lung are aligned. The numbers indicate the amino acid residues of the mouse proteins. Identical residues are boxed and shaded. The dashes indicate missing amino acids.
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C5-epimerase revealed a single transcript of cDNA probe corresponding to the C-terminal region of bovine tissue Northern mRNA blot by hybridization with a32P-labeled Northern analysis of a mouse multiple encoded by a single gene. The protein molecular weight by SDS-PAGE and stained with silver. About 100 ng of each preparation was analyzed nant mouse enzyme expressed in insect cells was prepared as described (16). About 100 ng of each preparation was analyzed fied as described (16). About 100 ng of each preparation was analyzed

**Expression of the C5-epimerase Transcript and Enzyme Activity in Mouse Tissues**—Northern analysis of a mouse multiple tissue Northern mRNA blot by hybridization with a32P-labeled cDNA probe corresponding to the C-terminal region of bovine C5-epimerase revealed a single transcript of ~5 kb in all tissues examined. The highest expression level was seen in liver, whereas small amounts of transcript were seen in spleen (Fig. 4A). Yet epimerase activity in spleen was similar to that in kidney or lung (Fig. 4B). Although we have no explanation to this discrepancy, we note that two other enzymes involved in the biosynthesis of HS, N-deacetylace/N-sulfotransferase 1 and 2-O-sulfotransferase, showed similar low levels of mRNA expression in spleen (23, 24). Mouse mastocytoma showed high epimerase activity (Fig. 4B) and contained the same ~5-kb epimerase transcript (11) as the other murine tissues.

**Assessment of Epimerase Structure and Activity**—Previous studies in our laboratory led to the isolation of a highly active ~52-kDa epimerase from bovine liver (16) and to the subsequent cloning of a bovine lung cDNA (11). This cDNA encoded a protein consisting of 444 amino acid residues. N-terminal analysis of the purified liver enzyme showed that it lacked 73 amino acid residues predicted from the cDNA and thus represented a truncated form. Yet the recombinant protein, expressed in insect cells, had a catalytic activity much lower than that of the purified liver enzyme. Although this discrepancy could have several reasons, we noted that also the cloned protein could be incomplete, since the 5'-end of the cDNA open reading frame was not defined with certainty. With the gene structure for the epimerase at hand it became possible to reassess this question, by renewed cDNA isolation based on the genomic sequence information. Appropriate oligonucleotide primers were therefore designed based on the genomic sequences, as described under “Experimental Procedures,” and used for PCR amplification using mouse liver cDNA as template. Agarose gel electrophoresis of the PCR product revealed one strong band of ~2 kb in size, and this product was directly inserted into a TOPTM-TA cloning vector. Double-strand sequencing of six selected clones revealed an epimerase cDNA encompassing the entire ORF in exon sequences of the corresponding genomic DNA. The ORF of the 1854-bp sequence encodes a protein of 618 amino acid residues, with a strongly hydrophobic domain close to the N terminus of the deduced polypeptide (indicated in Fig. 5).

Mouse mast cell C5-epimerase cDNA was derived through the same procedure as was used to clone the liver cDNA. The two sequences are identical (Fig. 6). Low stringency PCR (annealing at lower temperatures with degenerate primers) using the mast cell cDNA as template failed to reveal any additional, related cDNA (data not shown). BLAST search yielded a cDNA sequence from human brain (AB020643 in GenBankTM) with 93% identity to the mouse C5-epimerase at the nucleotide level and 96% identity at the amino acid level. The previously described incomplete bovine lung epimerase cDNA structure was extended by cloning of the missing 5'-terminal portion, revealing a total sequence highly similar to those of the murine and human structures (90% identity to the mouse sequence at the nucleotide level and 96% identity at the amino acid level). The deduced amino acid sequences of epimerase from mouse liver, mouse mast cells, bovine lung, and human brain are aligned in Fig. 6.

Following expression in insect cells, purification of the recombinant mouse protein on a column of immobilized O-desulfated heparin yielded a product that migrated as a single ~70-kDa protein on SDS-PAGE (Fig. 7), thus in agreement with the molecular mass calculated from the amino acid sequence (70,096 Da). By contrast, the purified bovine liver epimerase gave two bands under the same reducing conditions, corresponding to ~52- and ~22-kDa polypeptides (Fig. 7). Tryptic digestion of the latter component yielded a peptide (not shown) with an N-terminal sequence identical to residues 119–131, common to all cloned epimerase species (Fig. 6). We conclude that the ~22-kDa polypeptide represents a cleavage product of the original enzyme that remained associated with the ~52-kDa polypeptide throughout the purification procedure.

The recombinant full-length mouse epimerase, expressed in insect cells, showed a catalytic activity of ~1 × 10^9 cpn 3H/h/mg protein, thus almost as high as that of the purified bovine liver enzyme (9.6 × 10^9 cpn 3H/h/mg protein) (16), and much higher than that of the recombinant (truncated) bovine lung enzyme (~1 × 10^8 3H cpn H/h/mg protein) previously reported (11).

The ability of the recombinant mouse epimerase to actually convert GlcUA to IdoUA units was ascertained by incubation with a chemically N-sulfated, metabolically radiolabeled K5 polysaccharide substrate. The product was depolymerized by treatment with HNO2, and the resultant disaccharides were reduced, purified, and analyzed by paper chromatography. About one-third of the GlcUA residues had been converted to IdoUA (Fig. 8D), indicating equilibrium of C5-epimerization (25), whereas GlcUA only was found in control incubations (Fig. 8A).

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**FIG. 7.** SDS-PAGE of purified epimerase samples. The recombinant mouse enzyme expressed in insect cells was prepared as described under “Experimental Procedures,” and bovine liver enzyme was purified as described (16). About 100 ng of each preparation was analyzed by SDS-PAGE and stained with silver. *Lane 1,* purified bovine enzyme; *lane 2,* recombinant mouse enzyme. The protein molecular weight standards are indicated.

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^2^ The ~22-kDa fragment was observed in the initial purification of the bovine liver enzyme but was not identified due to contamination with peptides from a leaky concanavalin A column (11).
Heparin Production in Murine Mast Cells—The murine mast cells shown to express the epimerase gene were analyzed with regard to GAG biosynthesis. Following metabolic labeling with \[^{35}S\]sulfate, polysaccharides were isolated and subjected to anion-exchange chromatography (Fig. 9A). Part of the highly retarded, labeled material was eliminated by digestion with chondroitinase ABC (Fig. 9B), in accord with previous findings of “oversulfated” chondroitin sulfate in similar cells (26). The remaining, major polysaccharide component emerged at the same elution position as commercial heparin and was susceptible to degradation by nitrous acid (Fig. 9C), thus demonstrating that the mast cells had indeed synthesized heparin. No significant amounts of nitrous acid-sensitive material appeared at the elution position expected for HS. These findings indicate that the GlcUA C5-epimerase committed to heparin biosynthesis is the same as that involved in the formation of HS in other cells.

**DISCUSSION**

The murine GlcUA C5-epimerase gene consists of 3 exons and spans 11 kb from the first ATG to the stop codon. The five genomic clones obtained all relate to the same gene, as indicated by restriction enzyme mapping (not shown in detail; results outlined in Fig. 1). Southern analysis of mouse genomic DNA by hybridization with a genomic DNA probe detected a single band for the products of each restriction enzyme applied (Fig. 2). Further evidence for a single gene encoding the epimerase was obtained by FISH mapping of murine and human chromosomes (Fig. 3). The human gene, located at chromosome 15, also contains 3 exons (AC026992 in GenBank™), which are similar in size to those of the murine gene. Alignment of polypeptide sequences deduced from the epimerase cDNAs isolated from bovine and murine tissues and from the reported human brain cDNA (AB020643 in GenBank™) points to highly conserved structures (Fig. 6), with more than 90% similarity between any two different species at the nucleotide level and 96–99% similarity at the amino acid level.

Heparin is synthesized exclusively by mast cells, whereas HS is produced by a large variety of cells. Whereas the two biosynthetic pathways involve basically similar reactions, the heparin chain is more extensively modified. Recent findings indicate that several of the polymer-modifying enzymes occur in various genetically distinct forms. The \(N\)-deacetylase/\(N\)-sulfotransferase (NDST) that converts \(N\)-acetylated to \(N\)-sulfated glucosamine units thus has been found in four variant forms, encoded by different genes (7, 10, 27–29). Similarly, there are at least five distinct glucosamine 3-\(O\)-sulfotransferases (8) and three 6-\(O\)-sulfotransferases (9). By contrast, only one hexuronyl 2-\(O\)-sulfotransferase has been reported so far, and this enzyme has been demonstrated to catalyze the 2-\(O\)-sulfation of both GlcUA and IdoUA residues (30, 31) (notably, an analogous bifunctional 2-\(O\)-sulfotransferase has been implicated in dermatan sulfate biosynthesis (32)). Results obtained after targeted gene disruption of NDST-1 and NDST-2 suggest that these isoforms may be preferentially committed to the biosyn-
the C5-epimerase reaction occur in different structural contexts in heparin and HS. In heparin the vast majority of such units are located between two adjacent N-sulfated GlcN residues, whereas in HS about half of the IdoUA units occur in domains composed of alternating N-sulfated and N-acetylated GlcN residues (5). We therefore anticipated also that the epimerase might present in different forms, committed to the formation of IdoUA units in different types of sequences. On the contrary, the results obtained in the present study point to the involvement of the same epimerase enzyme in heparin and HS biosynthesis. A single gene was implicated, as discussed above. A single ~5-kb transcript was detected in all mouse tissues analyzed, including mastocytoma (11). Moreover, identical cDNA structures were expressed in a murine mast cell, found to produce heparin but no significant amounts of HS, and in liver, known to synthesize HS (37). In accord with these findings, epimerase preparations derived from mouse mastocytoma and mouse liver showed highly similar kinetic properties (38).

Cloning and expression of mouse liver cDNA, based on the epimerase gene structure, yielded a protein composed of 618 amino acid residues, thus considerably larger than either the recombinant bovine lung enzyme (444 residues (11)) or the original preparation purified from bovine liver (371 residues (16)). Given the structure of the full-length protein reported here, these discrepant results can all be explained in terms of the same epimerase gene. As shown in Fig. 10, the bovine lung cDNA thus was incomplete, lacking a 5'-terminal sequence corresponding to 173 amino acid residues, whereas the purified bovine liver enzyme was truncated due to proteolytic cleavage. Interestingly, both the full-length recombinant (Fig. 10C) and the truncated liver (Fig. 10A) enzymes showed essentially similar, high catalytic activities, more than 2 orders of magnitude higher than that of the recombinant, intermediate-sized, bovine lung enzyme (Fig. 10B). This intriguing observation relates to the fact that the bovine liver enzyme apparently remained associated with the truncated N-terminal ~22-kDa peptide throughout the isolation procedure. The purified bovine liver epimerase and the recombinant mouse liver enzyme thus are similar, except for a proteolytic cleavage in the polypeptide, between residues Ser-246 and Lys-247 of the bovine lung enzyme, the N-terminal domain of the native enzyme molecule would seem to be essential for the generation of catalytic activity. This domain contains the transmembrane region and, presumably, a luminal stem portion but may conceivably contribute directly to the catalytic mechanism, irrespective of whether the 618-residue polypeptide is intact or cleaved. Alternatively, the N-terminal portion may be essential for proper folding of the protein but not for the catalytic activity per se. So far we cannot discriminate between these possibilities, since we have been unable to separate the ~52- and ~22-kDa components of the extracted bovine liver enzyme, except by SDS-PAGE. The enzyme activity was not affected by

Fig. 9. Anion-exchange chromatography of mast cell polysaccharides. Cultured mast cells were metabolically labeled with [35S]sulfate and commercial heparin and analyzed by chromatography on DEAE-Sephacel (start of gradient 0.05–1.5M NaCl, indicated by arrow below C). Effluent fractions were analyzed for radioactivity (filled circles) or for hexuronic acid (shown in A only) by the carbazole reaction (open circles). The peak elution positions of standard chondroitin sulfate (CS; ~1 sulfate/disaccharide unit, thus not "oversulfated") and heparin (Hep) are indicated by arrowheads in A. Samples were untreated (A); digested with chondroitinase ABC (B); or reacted with nitrous acid (C).

Fig. 10. Schematic representation of the three C5-epimerase preparations studied. A, the purified bovine liver enzyme (two peptides); B, the previously cloned bovine epimerase (11); C, the recombinant mouse full-length enzyme.

thesis of HS and heparin, respectively. An NDST-2 knockout mouse thus was unable to synthesize heparin and showed abnormal mast cells but seemed otherwise healthy and produced apparently normal HS (33, 34), whereas elimination of NDST-1 resulted in a severely compromised HS biosynthesis and a lethal phenotype (35, 36). The IdoUA units generated in
inclusion of reducing agents such as diithiothreitol in the assay medium. Furthermore, a non-reduced sample of purified bovine epimerase showed the same two ~52- and ~22-kDa components on SDS-PAGE as were seen with reduced samples (data not shown). These findings suggest that the two fragments of the protein are tightly associated by non-covalent linkage. Attempts to renature epimerase activity from single or combined fractions following SDS-PAGE have been unsuccessful. The cleaved ~52-kDa component was consistently seen in several different enzyme preparations. Moreover, it was noted that >90% of the epimerase activity solubilized in buffer containing 1% Triton X-100 (and protease inhibitors) could be released from freshly homogenized bovine liver in the absence of any added detergent.3 These observations raise the possibility that the proteolytic cleavage had occurred in vivo and not during purification of the epimerase. Similar degradation was not observed in the insect cells used to express the recombinant mouse liver enzyme (Fig. 7).

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3 J.-P. Li, unpublished information.