Formation of HNK-1 Determinants and the Glycosaminoglycan Tetrasaccharide Linkage Region by UDP-GlcUA:Galactose β1,3-Glucuronosyltransferases*

(Received for publication, October 8, 1998, and in revised form, December 21, 1998)

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While expression-cloning enzymes involved in heparan sulfate biosynthesis, we isolated a cDNA that encodes a protein 65% identical to the UDP-GlcUA:glycoprotein β1,3-glucuronosyltransferase (GlcUAT-P) involved in forming HNK-1 carbohydrate epitopes (3OSO 3GlcUA 3Gal-) on glycoproteins. The cDNA contains an open reading frame coding for a protein of 335 amino acids with a predicted type II transmembrane protein orientation. Cotransfection of the cDNA with HNK-1 3-O-sulfotransferase produced HNK-1 carbohydrate epitopes in Chinese hamster ovary (CHO) cells and COS-7 cells. In vitro, a soluble recombinant form of the enzyme transferred GlcUA in β-linkage to Gal β1,3/4GlcNAcβ1,3Galβ-O-naphthalenemethanol, which resembles the core oligosaccharide on which the HNK-1 epitope is assembled. However, the enzyme greatly preferred Gal β1,3Galβ-O-naphthalenemethanol, a disaccharide component found in the linkage region tetrasaccharide in chondroitin sulfate and heparan sulfate. During the course of this study, a human cDNA clone was described that was thought to encode UDP-GlcUA:Galβ1,3Galβ-R glucuronosyltransferase (GlcUAT-I), involved in the formation of the linkage region of glycosaminoglycans (Kitagawa, H., Tone, Y., Tamura, J., Neumann, K. W., Ogawa, T., Oka, S., Kawasaki, T., and Sugahara, K. (1998) J. Biol. Chem. 273, 6615–6618). The deduced amino acid sequences of the CHO and human cDNAs are 95% identical, suggesting that they are in fact homologues of the same gene. Transfection of a CHO cell mutant defective in GlcUAT-I with the hamster cDNA restored glycosaminoglycan assembly in vivo, confirming its identity. Interestingly, transfection of the mutant with GlcUAT-P also restored glycosaminoglycan synthesis. Thus, both GlcUAT-P and GlcUAT-I have overlapping substrate specificities. However, the expression of the two genes was entirely different, with GlcUAT-I expressed in all tissues tested and GlcUAT-P expressed only in brain. These findings suggest that, in neural tissues, GlcUAT-P may participate in both HNK-1 and glycosaminoglycan production.

* This work was supported by Grants GM33063 and CA46462 from the National Institutes of Health (to J. D. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Glucuronic acid has been found in several types of complex carbohydrates expressed by vertebrate and invertebrate cells, including the sulfated glycosaminoglycan chains of proteoglycans and HNK-1 carbohydrate epitopes (3OSO 3GlcUA 3Gal-R). HNK-1 (human natural killer cell carbohydrate antigen-1) was originally described on human natural killer cells (1), but later studies showed that it was present in greatest abundance in the nervous system on subsets of glycolipids (2, 3), glycoproteins (4), and proteoglycans (5). In contrast, glycosaminoglycans are ubiquitously distributed among tissues, usually in covalent linkage to proteoglycan core proteins (6). Both HNK-1 and glycosaminoglycans can bind a variety of proteins that participate in cell-cell, cell-extracellular matrix, and cell-signal signaling during development (4, 6). The presence of GlcUA in both types of glycans and the partial overlap in their ligand binding properties suggest the possibility that their synthesis may be coordinated as well.

Much information is available about the enzymes involved in the addition of GlcUA to these glycans. Assembly of HNK-1 occurs by the transfer of GlcUA from the high energy donor UDP-GlcUA to a terminal galactose residue linked β1,4 to GlcNAc, followed by sulfation of the GlcUA residue at C-3. The glucuronosyltransferase associated with HNK-1 was first demonstrated in embryonic chick brain extracts using neolactotetraosylceramide as acceptor (7). The same activity was found later in rat brain using both neolactotetraosylceramide and asialoorosomucoid as substrates (7–9). By partially purifying the enzymes and noting differences in phospholipid activation and pH dependence, Oka et al. (9) concluded that the glucuronosyltransferase involved in the synthesis of HNK-1 epitopes on glycoproteins (GlcUAT-P) differs from the one that acts on glycolipids (GlcUAT-L). This hypothesis was confirmed recently in studies of recombinant GlcUAT-P, which selectively adds GlcUA to glycoprotein substrates (10). The gene and cDNA encoding GlcUAT-L have not yet been identified.

Glycosaminoglycan biosynthesis begins by the formation of the tetrasaccharide linkage intermediate -GlcUAβ1,3Galβ1,3Galβ1,4Xylβ-O-Ser. This intermediate serves as the primer for heparan sulfate and chondroitin sulfate assembly, which arises from the alternating addition of β-GlcNAc and β-GlcUA or β-GalNAc and β-GlcUA residues, respectively, to the linkage tetrasaccharide. Three GlcUA-transferases are thought to catalyze the addition of GlcUA: one involved in the formation of the linkage region tetrasaccharide (GlcUAT-I) (11, 12) and two...
that polymerize the different chains (13). The latter activities may be part of bifunctional enzymes in which the same protein catalyzes the alternating addition of a HexNAc residue and GlcUA (14, 15). GlcUA-T-I, in contrast, is much like GlcUA-T-P in that it transfers GlcUA from UDP-GlcUA to a β-linked Gal residue. The enzyme was first described in embryonic chick cartilage (12) and partially purified from embryonic chick brain (11) and a mouse mastocytoma (16). Interestingly, these early studies showed that crude enzyme preparations transferred GlcUA not only to substrates derived from the linkage region, such as Galβ1,3Gal and Galβ1,3Galβ1,4Xyl, but also to lactose (Galβ1,4Glc) and N-acetyllactosamine (Galβ1,4GlcNAc), the precursor of HNK-1.

These findings raised the question of whether formation of the linkage region and HNK-1 determinants is catalyzed by the same enzyme. Curenton et al. (17) provided evidence that GlcUA-T-I is distinct from the enzyme involved in HNK-1 formation based on partial separation of the activities and substrate competition studies. The cloning of a cDNA for GlcUA-T-P confirmed that at least two enzymes exist, but detailed analysis of substrate specificity was not done. In the present report, we isolated a cDNA encoding a hamster glucuronosyltransferase that is 65% identical to GlcUA-T-P (10) and 95% identical to human GlcUA-T-I (18), which was cloned while these experiments were under way. Analysis of the recombinant enzymes showed significant overlap in substrate specificity, and transfection experiments revealed that both enzymes will produce HNK-1 carbohydrate epitopes and facilitate glycosaminoglycan biosynthesis.

**EXPERIMENTAL PROCEDURES**

Cell Cultures—Chinese hamster ovary (CHO-K1, ATCC CCL-61), COS-7 (ATCC CRL-11651), and Lec2 (ATCC CRL-1736) cells were obtained from the American Type Culture Collection (Manassas, VA). Mutants pgsG-110, -114, and -224 were isolated by direct selection of glycosaminoglycan-deficient CHO-K1 cells and will be described in greater detail elsewhere.1 Lec2-GlcUA-T-P is a subclone of Lec2 stably expressing HNK-1 GlcUA-T-P and was kindly provided by E. Ong and M. Fukuda (Burnham Institute, La Jolla, CA). All of the cell lines were grown under an atmosphere of 5% CO₂ in air and 100% relative humidity. CHO cells and the various transfectants were maintained in Ham’s F-12 growth medium (Hyclone Laboratories) supplemented with 7.5% (v/v) fetal bovine serum (Hyclone Laboratories), 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin G. Sulfate-free medium was prepared from individual components (19), substituting chloride salts against phosphate-buffered saline (20). COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. Lec2 cells were maintained in α-minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics. Lec2-GlcUA-T-P cells were maintained in complete medium with 0.2 mg/ml (active) G418 (Life Technologies, Inc.).

Cloning of a Novel Glucuronosyltransferase from Chinese Hamster Ovary Cells—pgsD-H661, a CHO mutant defective in heparin sulfate biosynthesis (14), was stably transfected with a CHO-K1 cDNA library in pcDNA1 (Invitrogen) and screened for formation of heparin sulfate biosynthesis.2 A PCR fragment was prepared using the genomic DNA from the correctant as a template and the SP6 and T7 flanking sequences of the integrated vector as primers. PCRs were carried out with Taq DNA polymerase (Life Technologies, Inc.) in a Perkin-Elmer Model 2400 thermal cycler (35 cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min, with a final incubation at 72 °C for 10 min). The PCR fragment was cloned into pGEM-T (Promega), and the sequence was determined on both strands by the dyeodeoxy chain termination method (25) with [³²P]dCTP by random oligonucleotide priming (Prime-IT II labeling kit, Stratagene) and purified with an Elute-tip (Schleicher & Schüll). Hybridization was carried out for 16 h at 42 °C in the same buffer containing 1 × 10⁶ cpm/ml [³²P]-labeled probe, and positive clones were detected by conventional autoradiography. One of the plasmids obtained had the full-length sequence and was named pcDNA1-GlcUA-T-X in initial experiments. This was later shown to encode a section of GlcUA-T-I and therefore was renamed GenBank™/EBI accession number AF113703.

Mouse multiple-tissue poly(A)⁺ RNA (Clontech) was hybridized using gel-purified full-length cDNA for GlcUA-T-I as a probe essentially according to the manufacturer’s recommendation. Briefly, the solution was prewarmed to 68 °C, and the blot was prehybridized for 30 min. The Expresshyb solution was replaced with fresh solution containing 1 × 10⁶ cpm/ml [³²P]-labeled probe and hybridized at 68 °C for 1 h. The blot was rinsed and washed for 30–40 min at room temperature in 2× SSC containing 0.5% SDS with several changes of buffer and then for 40 min at 50 °C in 0.1× SSC containing 0.1% SDS, with one change of wash. Hybridization was detected with a PhosphorImager (Storm 860, Molecular Dynamics, Inc.).

Expression of the Protein A-GlcUA-T-I and Protein A-GlcUA-T-P Fusion Proteins—The cDNA fragment encoding amino acids 39–335 of GlcUA-T-I (the putative stem region and catalytic domain) was prepared by PCR using pcDNA1-GlcUA-T-I as a template. The fragment was fused in frame to the C terminus of protein A in pRK5-F10-PROTA (21). The 5′-primer for PCR was GCCGAAATTCGAGTTCACAGTGGCCTCC- TCC, and the 3′-primer was GGCGGAATTCGTGATCCAGAAGTGTG- GCC (the EcoRI site is shown in boldface letters, and the coding sequence of GlcUA-T-I is underlined). PCR was carried out with Pfu polymerase (Clontech; 25 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, followed by a final incubation at 72 °C for 7 min). The PCR products were cloned into pCR-Script Amp SK (+) (Stratagene), and the sequences were determined. The clone with the correct sequence was digested by EcoRI and ligated into the EcoRI site of pRK5-F10-PROTA to yield pPROTA-GlcUA-T-I.

The cDNA fragment encoding amino acids 39–347 of rat GlcUA-T-P (GenBank™/EBI accession number D88305) (10) was prepared by PCR using pcDNA3-GlcUA-T-P as a template (kindly provided by M. Faulsfeld, University of York). The 5′-primer was GCCCGAATTCGAGTTCACAGTGGCCTCC- TCC, and the 3′-primer was GCCCGAATTCGAGTTCACAGTGGCCTCC- TCC (the EcoRI site and XhoI sites are shown in boldface letters). Expression of soluble recombinant enzyme was measured after transfection of COS-7 cells using Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s instructions. The supernatant was centrifuged for 5 min at top speed in an IEC clinical centrifuge at 4 °C to sediment cell debris. The supernatant and the supernatant was removed and incubated with rabbit IgG against agarose beads (10 μl of beads/ml of sample; Sigma) with end-over-end mixing at 4 °C for 24–48 h. The samples were centrifuged for 5 min, and the supernatant was aspirated. The beads were washed twice with 10 ml of 20% (v/v) glycerol and 50 μl Trits-Cl, pH 7.4, and resuspended in the same buffer containing protease inhibitors (10 μl/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) to achieve an ~50% (v/v) slurry. The immobilized enzyme was stable at 4 °C for at least 4 months.

Isolation of Cell Lines Stably Expressing GlcUA-T-I and GlcUA-T-P—The cDNA insert of pcDNA1-GlcUA-T-I was digested by HindIII and XhoI and cloned into pcDNA3 (Invitrogen), yielding pcDNA3-GlcUA-T-I. Lec2, wild-type CHO-K1, and pgsG mutant cells were transfected with pcDNA3-GlcUA-T-I or pcDNA3-GlcUA-T-P using Lipofectamine, and stable transfectants were selected using 0.4 mg/ml (active) G418. Individual colonies were screened for HNK-1 expression by glycosaminoglycan production, and positive ones were isolated with glass cloning rings and expanded.

**Immunofluorescence Staining of Cells with Anti-HNK-1 Antibody**—Cells (1 × 10⁶) were transfected with pcDNA1-HNK-1 or pcDNA1-HNK-1-30ST (where 30ST is 3-O-sulfotransferase; M. Fukuda). Two days later, the monolayers were washed twice with cold PBS and fixed at 4 °C for 15 min with 4% (v/v) paraformaldehyde. Cells were washed twice with PBS, blocked at room temperature for 10 min with 2% (w/v) bovine serum albumin in PBS, and incubated at room temperature for 30–40 min with mouse monoclonal anti-HNK-1 antibody (Becton Dickinson Advanced Cellular Biology) diluted 1:100 in buffer. Primary anti-

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1. Two of these mutants lack endogenous GlcUA-transferase I activity (X. Bai, G. Wei, A. Sinha, and J. D. Esko, unpublished results).
2. G. Wei, X. Bai, and J. D. Esko, unpublished results.
tibody was removed, and the cells were incubated at room temperature for 20–30 min with fluorescein isothiocyanate-conjugated goat anti-mouse IgM antibody (Sigma) diluted 1:100 with buffer. Cells were washed again with PBS, mounted with antifade reagent (Molecular Probes, Inc.), and examined by fluorescence microscopy using a Zeiss epifluorescence microscope equipped with a fluorescein filter set and a 153 mm/sodium taurodeoxycholate, 0.1 mM sodium acetate, pH 5.0, and 2.5 units/μl β-glucuronidase from limpets or Helix pomatia (Sigma), and the mixture was incubated overnight at 37 °C. The sample was diluted with 1 ml of 0.5 mM NaCl and separated on a Sep-Pak C18 cartridge as described above. The flow-through fraction, wash, and eluant were collected, and radioactivity was measured by liquid scintillation spectrometry.

**Purification of Glycosaminoglycan Chains**—Cells were labeled for 24 h with 10 μCi/ml H2[35]SO4 (1600 Ci/mmol; NEN Life Science Products) in sulfate-free medium. [35S]-Labeled glycosaminoglycan chains were isolated by anion-exchange chromatography as described previously (28) and analyzed by anion-exchange HPLC using a 7.5 mm inner diameter × 7.5-cm column of DEAE-3SW (TosoHaas, Montgomeryville, PA). The column was equilibrated in 10 mM KH2PO4 buffer, pH 6.0, containing 0.2% (w/v) Tween 20 and 0.2 mM NaCl. The glycosaminoglycans were eluted with a linear gradient of NaCl (0.2–1 M) in the same buffer using a flow rate of 1 ml/min and by increasing the NaCl concentration by 10 mM/min. The effluent from the column was monitored for radioactivity with an in-line radioactivity detector (Radium Plc Onebeta, Packard Instrument Co.) with sampling rates every 6 s. The data were averaged over 1-min intervals.

**RESULTS**

Isolation of a cDNA Clone for a Glucuronosyltransferase from Chinese Hamster Ovary Cells—During a series of experiments to identify genes involved in glycosaminoglycan synthesis (see “Experimental Procedures”), we found a cDNA clone that showed high homology to the recently cloned rat glycoprotein glucuronosyltransferase involved in the assembly of HNK-1 epitopes (GlcUT-P) (10). This clone, originally designated GlcUT-X, contained sequence homologous to the C-terminal ectodomain of GlcUT-P, but lacked an ATG start codon. Using the insert as a probe, we screened a commercial CHO-K1 cDNA library and isolated six different clones. Two of the longest cDNAs were potentially full-length based on the presence of putative start and stop codons. DNA sequencing revealed a single open reading frame with a potential Kozak consensus sequence for ribosome recognition just upstream from the ATG start codon and a polyadenylation signal located close to a poly(A) run (Fig. 1). The open reading frame encoded a 335-amino acid protein. Kyte-Doolittle hydrophathy analysis (38) indicated one potential transmembrane domain consisting of 18 hydrophobic amino acid residues located 7 amino acids from the initiating Met residue. A relatively proline-rich segment followed the hydrophobic section, and one potential N-glycosylation site in the putative ectodomain was present at Asn-299 (NCT, marked by an asterisk). These characteristics are common to type II transmembrane proteins and to many known Golgi glycosyltransferases (27). Overall, the sequence exhibited 65% identity to rat GlcUT-P, and therefore, we tentatively characterized the cDNA as a homologue of this enzyme.

GlcUT-X was expressed in various adult mouse tissues as measured by Northern blot analysis using the full-length cDNA as a probe. As shown in Fig. 2, a single transcript of ~1.8 kilobase pairs was obvious in adult liver, brain, and heart; was moderately expressed in lung, skeletal muscle, kidney, and testis; and was barely present in spleen. This distribution differs significantly from the expression of GlcUT-P, which has two transcripts, one at 4.0 kilobase pairs and a minor one at 1.9 kilobase pairs, and is more strongly expressed in brain and liver. Furthermore, most non-neural tissues do not normally express HNK-1 epitopes, suggesting that GlcUT-X might not participate in HNK-1 formation. As these studies were under way, Kitagawa et al. (18) cloned a GlcUA-transferase cDNA from a human placenta cDNA library using PCR and degenerate primers based on the sequence of GlcUT-P. Comparing its sequence with GlcUT-I and GlcUT-P activities were assayed under optimized conditions using Galβ3Glcβ3-O NM as a substrate.4 HNK-1 3-O-sulfotransferase activity was measured essentially according to the method of Ong et al. (25), but using 2 μl GlcUAβ3Glcβ3-O NM as an acceptor.

**β-Glucuronidase Digestion—**Radioactive product was dried and dissolved in 60 μl of solution containing 1.53 μg/ml sodium taurodeoxycholate, 0.1 mM sodium acetate, pH 5.0, and 2.5 units/μl β-glucuronidase from limpets or Helix pomatia (Sigma). The mixture was incubated overnight at 37 °C. The sample was diluted with 1 ml of 0.5 mM NaCl and separated on a Sep-Pak C18 cartridge as described above. The flow-through fraction, wash, and eluant were collected, and radioactivity was measured by liquid scintillation spectrometry.
The putative transmembrane domain is underlined, and the asterisk indicates a potential N-glycosylation site. The polyadenylation consensus sequence and the putative Kozak sequence are double-underlined.

FIG. 1. Nucleotide and deduced amino acid sequences of the hamster GlcUA-T-I cDNA. The putative transmembrane domain is underlined, and the asterisk indicates a potential N-glycosylation site. The polyadenylation consensus sequence and the putative Kozak sequence are double-underlined.

FIG. 2. Northern analysis of GlcUA-T-I in mouse adult tissues. Each lane contained 2 μg of poly(A)1 RNA. A shows the results using the 32P-labeled full-length cDNA probe. B shows hybridization with the human β-actin probe provided by the manufacturer (CLONTECH). Kb, kilobase pairs; GlcAT-I, GlcUA-T-I.

(GenBankTM/EBI accession number AB009598) with that of GlcUA-T-X showed that they were 95% identical at the amino acid level (Fig. 3), suggesting that they most likely represent the same enzyme from different species. Based on the ability of the transferase to attach GlcUA to Galβ1,3Galβ1,4Xylβ-O-Ser and its lack of activity with glycoprotein substrates, Kitagawa et al. concluded that the enzyme was involved in forming the linkage tetrasaccharide present in glycosaminoglycans, such as heparan sulfate and chondroitin sulfate. This enzyme is known as GlcUA-transferase I (GlcUA-T-I) to distinguish it from other GlcUA-transferases involved in polymerization of glycosaminoglycans (13). The high homology of GlcUA-T-I (which is designated GlcUA-T-I below) and GlcUA-T-P raised the question of whether these transferases might participate in both HNK-1 synthesis as well as glycosaminoglycan formation.

GlcUA-T-I Can Generate HNK-1 Determinants in COS-7 and Lec2 Cells—To test if GlcUA-T-I can produce HNK-1 epitopes, we cotransfected the cDNA encoding CHO GlcUA-T-I and the human HNK-1 3'-O-sulfotransferase (25) into COS-7 and Lec2 cells. Lec2 cells lack the Golgi CMP-sialic acid transporter, and therefore, the cells accumulate oligosaccharides that terminate with Gal residues that can serve as acceptors for GlcUA addition (10, 28). Like wild-type CHO cells, Lec2 also does not express HNK-1 3'-O-sulfotransferase. Therefore, control Lec2 cells and those transfected with only GlcUA-T-I or HNK-1 3'-O-sulfotransferase did not stain with anti-HNK-1 antibody (Fig. 4, A, C, and D, respectively). However, when Lec2 cells were cotransfected with both GlcUA-T-I and HNK-1 3'-O-sulfotransferase, the cells stained with anti-HNK-1 antibody, suggesting that GlcUA-T-I induces HNK-1 expression on cell-surface glycoconjugates (Fig. 4B). GlcUA-T-I was also introduced into COS-7 cells, which have endogenous HNK-1 3'-O-sulfotransferase (25). Therefore, transfection with GlcUA-T-I with or without sulfotransferase resulted in HNK-1 expression (Fig. 4, F and H). In this regard, GlcUA-T-I behaved exactly the same as GlcUA-T-P (25), but the staining of cells transfected with GlcUA-T-I was somewhat weaker (data not shown). Nevertheless, these findings showed that GlcUA-T-I can produce HNK-1 epitopes in vivo in different cell lines.

Conceivably, GlcUA-T-I and GlcUA-T-P may form HNK-1 determinants on different glycoproteins or glycolipids. To test this idea, we analyzed HNK-1-reactive glycoconjugates in Lec2 cells which have endogenous HNK-1 3'-O-sulfotransferase (25). Therefore, transfection with GlcUA-T-I with or without sulfotransferase resulted in HNK-1 expression (Fig. 4, F and H). In this regard, GlcUA-T-I behaved exactly the same as GlcUA-T-P (25), but the staining of cells transfected with GlcUA-T-I was somewhat weaker (data not shown). Nevertheless, these findings showed that GlcUA-T-I can produce HNK-1 epitopes in vivo in different cell lines.
total cell extracts (Fig. 5). Several prominent bands ranging from 65 to 100 kDa were present in cells stably transfected with GlcUAT-P, whereas only a minor band at ~110 kDa was seen in cells containing GlcUAT-I. The difference in reactivity was not due to variation in the level of expression of the enzymes since in vitro assays indicated that they did not vary significantly (7.2 ± 0.2 pmol/min/mg for GlcUAT-P, 7.9 ± 2.2 pmol/min/mg for GlcUAT-I, and 0.42 ± 0.01 pmol/min/mg for 3-O-sulfotransferase activity). All of the bands were peptide N-glycosidase F-sensitive, indicating that the epitope was assembled on N-linked oligosaccharides found on glycoproteins. Further characterization of the reactive bands has not yet been done.

Comparison of Substrate Specificity of GlcUAT-I and GlcUAT-P—To further analyze the substrate specificity of these GlcUA-transferases, the ectodomains of GlcUAT-I and GlcUAT-P were fused to the IgG-binding domain of protein A, and the chimeras were expressed in COS-7 cells. The secreted enzymes were absorbed to IgG-agarose, washed, and assayed with various acceptors and UDP-[3H]GlcUA. As shown in Table I, both glucuronosyltransferases can act on a variety of synthetic substrates containing terminal \( \beta \)-linked galactose residues. In general, disaccharides were better substrates than monosaccharides, but the individual enzymes showed strong differences in substrate utilization. As expected, GlcUAT-P could transfer GlcUA to asialofetuin and asialomucin containing terminal Gal residues, whereas GlcUAT-I had no detectable activity toward asialoglycoproteins and negligible activity with asialoglycosphingolipid substrates with or without added phospholipids (8). GlcUAT-I had the highest activity with Galβ1,3Galβ-O-R (where \( R = \) naphthalenemethanol or benzyl alco-
Western blot analysis of HNK-1-containing glycoproteins. Cell lines stably expressing GlcUAT-I (GlcAT-I) or GlcUAT-P (GlcAT-P) and transiently transfected with pcDNA3-HNK-1 3OST were solubilized, and an aliquot of cell protein was analyzed by SDS-PAGE. The gel was blotted onto nitrocellulose, and HNK-1-containing bands solubilized, and an aliquot of cell protein was analyzed by SDS-PAGE.

**Table I**

| Acceptor                      | Enzyme activity (GlcAT-I) | GlcAT-P |
|-------------------------------|--------------------------|---------|
| Monosaccharides (1 mM)        |                          |         |
| Galb1,4GlcNAcb-O-naphthenemethanolate | <0.1                    | <0.1    |
| Galb1,4GlcNAcb-O-naphthenol    | <0.1                    | 0.1     |
| Galb1,4GlcNAcb-O-1-naphthenol  | <0.1                    | 1.0     |
| Galb1,4GlcNAcb-O-2-naphthenol  | 0.3                     | 40      |
| Galb1,4GlcNAcb-O-p-naphthenol  | 0.1                     | <0.1    |
| Disaccharides (1 mM)          |                          |         |
| Galb1,4GlcNAcb-O-NM           | 0.9                     | 46      |
| Galb1,4GlcNAcb-O-NM           | 0.9                     | 56      |
| Galb1,4GlcNAcb-O-NM           | 1.2                     | 13      |
| Galb1,4GlcNAcb-O-NM           | 2.8                     | 41      |
| Galb1,4GlcNAcb-O-benzyl        | 1.4                     | 33      |
| Galb1,4GlcNAcb-O-NM           | 116                    | 54      |
| Galb1,4GlcNAcb-O-benzyl        | 78                     | 49      |

Kinetic analysis of GlcUAT-I activity showed that the recombinant enzyme formed products in proportion to time for up to 5 h with all of the tested substrates (Fig. 6A). The apparent $K_m$ values for Galb1,4GlcNAcb-O-NM, Galb1,4GlcNAcb-O-NM, and Galb1,4GlcNAcb-O-NM were 1.8, 2.9, and 3.2 mM, respectively, whereas the $K_m$ for Galb1,4GlcNAcb-O-NM was significantly lower (0.67 mM) (Fig. 6B). Furthermore, the apparent $V_{max}$ was much greater for Galb1,4GlcNAcb-O-NM (>10-fold) than for the other substrates, suggesting that GlcUAT-I is much less reactive with substrates related to glycoproteins and is more reactive with those involved in glycosaminoglycan assembly. In contrast, GlcUAT-P was much more promiscuous, reacting with many substrates.

**Figure 6. Dependence of GlcUAT-I activity on time and acceptor concentration.** Four synthetic substrates, Galb1,3Galb-O-NM ( ), Galb1,3GalNAcb-O-NM ( ), Galb1,3GlcNAcb-O-NM ( ), and Galb1,4GlcNAcb-O-NM ( ), were tested as substrates with recombinant GlcUAT-I (see Experimental Procedures). Data are single point determinations. A, dependence of enzyme activity on time; B, dependence of enzyme activity on acceptor concentration.

Restoration of Glycosaminoglycan Synthesis by Both Glucuronosyltransferases in a Glycosaminoglycan-deficient Mutant—

The high reactivity of GlcUAT-I with Galb1,3Gal containing disaccharides supported the idea that this enzyme is involved in glycosaminoglycan biosynthesis (18). To test this hypothesis directly, we transfected a pair of glycosaminoglycan-deficient mutants of CHO cells defective in GlcUAT-I. Introduction of the cDNA for GlcUAT-I resulted in restoration of glycosaminoglycan biosynthesis as measured by the incorporation of HPLC confirmed that it consisted of a mixture of heparan sulfate and chondroitin sulfate chains (Fig. 7). Interestingly, transfection by GlcUAT-P also corrected the deficiency in the mutant (Table II). The relative level of the transfected GlcUA-transferases varied from 0.3 to 70 times of the endogenous value for GlcUAT-I in the wild-type, but glycosami-
noglycan synthesis was restored under all conditions. These results suggested that both enzymes could facilitate formation of glycosaminoglycan chains.

DISCUSSION

In this report, we have described the isolation and characterization of a cDNA clone encoding a novel hamster glucuronosyltransferase (GlcUAT-I). Our initial characterization of the enzyme suggested that it might be a homologue of GlcUAT-P based on the high degree of homology (65% identity) (Fig. 3) and its ability to produce HNK-1-reactive material in the presence of HNK-1 3-O-sulfotransferase (Fig. 4). However, Northern blot analysis showed that the enzyme had a markedly different tissue distribution from HNK-1, with expression in virtually all tissues tested (Fig. 2). In contrast, HNK-1 (and GlcUAT-P) is restricted to brain and neurons (10). Furthermore, much less material contained HNK-1 determinants in cells transfected with hamster GlcUAT-I compared with GlcUAT-P (Fig. 5). These findings suggested that hamster GlcUAT-I might be involved in the formation of another type of GlcUA-containing glycoconjugate. To test this hypothesis, we examined a number of synthetic and modified natural substrates as acceptors using recombinant forms of the enzymes. Hamster GlcUAT-I transfected GlcUA efficiently to compounds containing Galβ1,3Gal, which resembles the linkage tetrasaccharide found in heparan sulfate and chondroitin sulfate (Table I). As these studies were under way, Kitagawa et al. (18) reported a cDNA encoding an enzyme thought to be GlcUAT-I, which is involved in glycosaminoglycan biosynthesis. Hamster GlcUAT-I shows 95% identity to human GlcUAT-I (Fig. 3), suggesting that it most likely represents the same gene, but from a different species. Correction of a mutant defective in GlcUAT-I by transfection with hamster GlcUAT-I supported this idea (Fig. 7 and Table II). Interestingly, GlcUAT-P also corrected the mutant, suggesting that it might work equally well in forming HNK-1 determinants as well as the linkage region of glycosaminoglycans.

The substrate specificity of GlcUAT-I has been debated ever since the enzyme activity was first described in cartilage and brain extracts (11, 12). In these early studies, relatively crude enzyme preparations were found to catalyze the transfer of GlcUA not only to glycosaminoglycan linkage region fragments (Galβ1,3Gal), but also to related compounds terminating in galactose, such as lactose (Galβ1,4Glc) and Galβ1,4GlcNAc, the precursor of HNK-1. HNK-1 had not yet been described when these studies were done (1), and therefore, it was not appreciated that the apparent reactivity with Galβ1,4GlcNAc was most likely due to another GlcUA-transferase in the crude tissue extracts (GlcUAT-P). Several years ago, Curenton et al. (17) studied whether the glucuronosyltransferases for making HNK-1 epitopes and the glycosaminoglycan linkage region were the same enzyme using embryonic chick brain as an enzyme source. They found that the activity related to glycosaminoglycan assembly was firmly membrane-associated, whereas the activity related to HNK-1 formation was readily solubilized, suggesting that they were separate entities. Furthermore, no activity toward Galβ1,4GlcNAc acceptors was detected in embryonic chick cartilage extract, which is a rich source of GlcUAT-I, but not GlcUAT-P. Based on these results, they concluded that two different enzymes catalyze the formation of linkage region fragments and HNK-1 determinants. More recent molecular cloning experiments support the idea that multiple enzymes exist, but the data presented here using recombinant forms of the enzymes suggest that they may be more promiscuous with respect to substrate utilization than previously appreciated.

Given the apparent overlap in behavior of the enzymes, especially GlcUAT-P, what can we say about their relative contribution to glycosaminoglycan and HNK-1 synthesis? Under normal conditions, GlcUAT-I probably does not give rise to HNK-1 determinants since the enzyme reacts relatively poorly with precursor glycoproteins, glycolipids, and synthetic disaccharides related to HNK-1 (Table I). Furthermore, transfection of wild-type CHO or COS-7 cells with only HNK-1 3-O-sulfotransferase does not result in expression of glycoproteins reactive with HNK-1 antibodies, yet these cells express endogenous GlcUAT-I activity. The inability of GlcUAT-I to form HNK-1 under these conditions might be due to differences in subcellu-
lar location of the enzyme and macromolecular precursors of HNK-1. However, Sugumaran et al. (29, 30) have suggested that GlcUAT-I may be located in medial- and trans-Golgi fractions based on sucrose density gradient fractionation of chondrocytes. HNK-1 precursors are also likely to arise in these compartments of the Golgi since the terminal Gal residue on glycoprotein substrates is attached by β1,4-galactosyltransferase (lactose synthase), which has been immunocytochemically located in the medial- and trans-aspects of the Golgi (31). Thus, it is more likely that the poor reactivity of GlcUAT-I with precursors of HNK-1 explains why the endogenous enzyme does not give rise to HNK-1 determinants under normal conditions.

How do we explain the expression of HNK-1 epitopes after introduction of GlcUAT-I into cells (Figs. 4 and 5)? Expression of HNK-1 determinants in CHO and COS-7 cells under these conditions may merely reflect the high level of enzyme activity achieved by plasmid amplification and strong expression from the cytomegalovirus promoter, which overpowers the weak activity of the enzyme with glycoprotein substrates. Analysis of the enzyme activity in extracts prepared from stable transfectants indicates that the enzyme is enhanced, but not in all cases (Table II). Curiously, SDS-PAGE analysis of modified proteins indicates that those bearing HNK-1 determinants differ in cells transfected with GlcUAT-I and GlcUAT-P. Perhaps this reflects differences in protein substrate recognition or subcellular localization of the transfected enzyme and substrates. Identification of the reactive glycoproteins and more precise localization studies of the transferases may shed light on this issue.

The fact that GlcUAT-P can bypass a mutation in glycosaminoglycan biosynthesis (Table II) presumably reflects variation in the acceptor substrates (Table I). These findings suggest that GlcUAT-P and GlcUAT-I do not show any homology to the colipids (7, 8); and possibly other enzymes inferred from enzymes involved in elongation of heparan sulfate and chondroitin sulfate (15, 30); hyaluronan synthases (32); GlcUAT-L, -M, and -I (33, 34). Overall, GlcUAT-P and GlcUAT-I do not show any homology to the hyaluronan synthases and the putative heparan sulfate copolymerase recently reported by Lind et al. (35).

The difference in substrate selectivity of GlcUAT-I and GlcUAT-P presumably reflects variation in the acceptor substrate-binding sites of the proteins. Unfortunately, >40% of the residues differ between the two enzymes, making it difficult to pinpoint specific residues that impart selectivity by merely comparing sequences. However, it should be possible to study the enzyme structure by swapping contiguous blocks of residues. This approach helped define sites in lysosomal enzymes that are recognized by the phosphotransferase that adds GlcNAc-P to terminal mannose residues only on lysosomal enzymes (36, 37). A similar strategy applied to GlcUAT-I and GlcUAT-P might provide insight into active-site residues as well as features of the proteins that confer substrate specificity.

Acknowledgments—We thank H. Freeze (Burnham Institute, La Jolla, CA) for many helpful conversations, M. Fukuda for providing several reagents, and K. Sugahara for sharing data while our experiments were under way.

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