Anticoagulant Heparan Sulfate Precursor Structures in F9 Embryonal Carcinoma Cells

(Received for publication, October 20, 1998, and in revised form, November 18, 1998)

Lijuan Zhang‡§, Keichi Yoshida¶, Jian Liu‡, and Robert D. Rosenberg‡**

From the ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, the ¶Department of Medicine, Harvard Medical School, Beth Israel Hospital, Boston, Massachusetts 02215, and ¶¶Tokyo Research Institute of Sekagaku Corp., Higashi-yamato-cho, Tokyo 207, Japan

To understand the mechanisms that control anticoagulant heparan sulfate (HSact) biosynthesis, we previously showed that HSact production in the F9 system is determined by the abundance of 3-O-sulfotransferase-1 as well as the size of the HSact precursor pool. In this study, HSact precursor structures have been studied by characterizing [6-3H]GlcN metabolically labeled F9 HS tagged with 3-O-sulfates in vitro by 3'-phosphoadenosine 5'-phospho-35S and purified 3-O-sulfotransferase-1. This later in vitro labeling allows the regions of HS destined to become the antithrombin (AT)-binding sites to be tagged for subsequent structural studies. It was shown that six 3-O-sulfation sites exist per HSact precursor chain. At least five out of six 3-O-sulfate-tagged oligosaccharides in HSact precursors bind AT, whereas none of 3-O-sulfate-tagged oligosaccharides from HSinact precursors bind AT. When treated with low pH nitrous or heparitinase, 3-O-sulfate-tagged HSact and HSinact precursors exhibit clearly different structural features. 3-O-Sulfate-tagged HSact hexasaccharides were AT affinity purified and sequenced by chemical and enzymatic degradations. The 3-O-sulfate-tagged HSact hexasaccharides exhibited the following structures, ΔUA-[6-3H]GlcNAc6S-GlcUA-[6-3H]GlcNS4S±6S-IdceA2S-[6-3H]GlcNS6S. The underlined 6- and 3-O-sulfates constitute the most critical groups for AT binding in view of the fact that the precursor hexasaccharides possess all the elements for AT binding except for the 3-O-sulfate moiety. The presence of five potential AT-binding precursor hexasaccharides in all HSact precursor chains demonstrates for the first time the processive assembly of specific sequence in HS. The difference in structures around potential 3-O-sulfate acceptor sites in HSact and HSinact precursors suggests that these precursors might be generated by different concerted assembly mechanisms in the same cell. This study permits us to understand better the nature of the HS biosynthetic pathway that leads to the generation of specific saccharide sequences.

Different heparin/heparan sulfate (HS)1 sequences bind to a large number of growth factors and cytokines (1–6), enzymes (7), protease inhibitors (8–12), virus proteins (13), and selectins (14). Such sequences are usually synthesized in the right place (15) and at the right time (16, 17). These HS species are involved in development, angiogenesis, lipid metabolism, coagulation, virus infection, and inflammation (18–22). To synthesize HS oligosaccharide sequences that bind to these specific protein ligands probably requires the synthesis of specific HS precursor structures. HS precursor structures are then acted upon by a unique sulfotransferase, which is positioned at the end of the biosynthetic pathways and whose levels control the concentration of the specific HS component that is the product of the biosynthetic pathway.

The pentasaccharide sequence, GlcNAc/NS6S-GlcUA-GlcUA-GlcNS3S-GlcUA, represents the minimum sequence for antithrombin (AT) binding, where the boldface 3S and 6S constitute the most critical elements involved in the interaction (8, 9). The first sugar residue is either N-acetylated or N-sulfated. The third sugar residue is either with or without a 6-O-sulfate, depending on the source of heparin from which these sequences were originally characterized (23). The AT-binding sequence also exists in HS, but such sequences have never been fully characterized due to limited materials. HSact produced by endothelial cells (1% total HS) is responsible in part for the nonthrombogenic properties of blood vessels (24). Even though it constitutes a small portion of HStotal, the relative abundance of the AT-binding sequence is at least 10-fold greater than would be predicted by completely random assembly of disaccharide constituents (25). These observations suggest that production of the AT-binding sequence requires the coordinated action of several biosynthetic enzymes, i.e. the enzymes that catalyze chain polymerization, GlcNAc N-deacetylation and N-sulfation, GlcUA epimerization, 2-O-sulfation of uronic acid residues, and 3-O- and 6-O-sulfation of glucosaminyl residues. Multiple forms of N-sulfotransferases (26–30) and 3-O-sulfotransferases (31)2 have been reported recently. Due to the substrate specificity of the enzymes involved, the initial distribution of N-sulfate groups strongly influences the subsequent epimerization and O-sulfations (32, 33). The downstream O-sulfation may also be able to influence N-sulfation as well (34). However, the mechanisms that control the

* This work was supported in part by National Institutes of Health Grants 5-P01-HL41484 and HL66385. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of National Research Service Award Postdoctoral Fellowship.

** To whom correspondence and reprint requests should be addressed: Massachusetts Institute of Technology, Bldg. 68-480, 77 Massachusetts Ave., Cambridge, MA 02139. Tel.: 617-253-8804; Fax: 617-258-6553; E-mail: rdrosen@MIT.EDU.

† The abbreviations used are: HS, heparan sulfate; HSact, anticoagu-

lantly active heparan sulfate; HSinact, anticoagulantly inactive heparan sulfate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane-
sulfonic acid; DMEM, Dulbecco's modified Eagle's medium; GAG, gly-
cosaminoglycan; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; AT, antithrombin; 3-O-ST-1, glucosaminyl 3-O-sulfotransferase-1; 3-O-ST, N-deacetylase/N-sulfotransferase; 3-O-ST, iduronic/glucuronic acid 3-O-
sulfotransferase; 6-O-ST, glucosaminyl 6-O-sulfotransferase; GlcUA, glucuronic acid; IdceA, iduronic acid; HPLC, high pressure liquid chromato-

matography; MES, 4-morpholinooethylsulfonic acid.

2 Liu, J., Shworak, N. W., Sinay, P., Schwartz, J. J., Zhang, L., Fritze, L. M. S., and Rosenberg, R. D. (1999) J. Biol. Chem. 274, in press.
Anticoagulant Heparan Sulfate Biosynthesis

coordinated action of these enzymes to generate AT-binding sequences or other sequences in HS and heparin are unknown.

To delineate the biosynthetic pathway that regulates HS act synthesis, our laboratory has purified as well as molecularly cloned 3-O-ST-1 (EC 2.8.2.23). It was demonstrated that 3-O-ST-1, existing in limited amount, acts upon HS act precursor to produce HS act and HS inact precursor to produce 3-O-sulfated HS act (31, 35). When 3-O-ST-1 is no longer limiting in the F9 cell system, the capacity for HS act generation is determined by the abundance of HS act precursors (36). We have reported that overall HS act and HS inact structures are different at the disaccharide level in the F9 cell system. In vitro 3-O-sulfation with purified 3-O-ST-1 can tag the regions of the HS act precursors destined to become AT-binding sites and allow the HS act precursors to be captured. The tagged regions can then be structurally examined (36). Based on the above observations, we have studied the HS act and HS inact precursor structures by characterizing [6-3H]Glcn metabolically labeled F9 HS tagged with 3-O-sulfates in vitro by PAP35S and purified 3-O-ST-1. We have found that HS act and HS inact precursors in F9 cells have different epimerization and O-sulfation patterns around the 3-O-sulfate acceptor sites in the intact glycosaminoglycan (GAG) chain. We have sequenced the 3-O-sulfate-tagged HS act precursor hexa-O-saccharides that represent the 3-O-sulfate acceptor sites in the HS act precursors, which are destined to become AT-binding sites. This region contains five hexa-O-saccharides, i.e. (△UA-[6-3H]GlcNAc6S-GlcUA-[6-3H]GlcNS8S=6S-IdceA2S-[6-3H]GlcNS6S), which possess the correct positioning of all critical groups except for the absence of the 3-O-sulfate residue required for AT binding in all HS act precursor chains. This finding demonstrates for the first time the overall structure of the HS act precursors. This information permits us to speculate about the nature of the HS biosynthetic pathway that leads to the generation of specific oligosaccharide sequences.

EXPERIMENTAL PROCEDURES

Cell Culture—F9 cells were grown on gelatin (Sigma) coated (0.1%) tissue culture dishes in Dulbecco’s modified (DMEM) containing 10% heat-inactivated calf serum (Irvine Scientific), penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml) under an atmosphere of 5% CO2, 95% air and 100% relative humidity. All tissue culture media and reagents were purchased from Life Technologies, Inc., unless otherwise indicated.

HS Preparation—The methods for HS preparation have been described previously (30). In brief, cell monolayers were labeled with 100–1000 μCi/ml sodium [35S]sulfate (carrier-free, ICN) overnight by incubation in sulfate-deficient DMEM, penicillin G (100 units/ml), and 10% (v/v) fetal bovine serum that had been exhaustively diazylated against phosphate-buffered saline and supplemented with 200 μM Na2SO4. [6-3H]Glucosamine labeling were conducted in 100 μCi/ml (30 μg/ml) sodium [35S]sulfate (carrier-free, ICN) overnight in 1 mg/ml glucose DMEM with 10% (v/v) diazylated fetal bovine serum. GAGs were isolated from both monolayer and media. Purified GAGs were resuspended in 0.5 M NaBH4 at 0.4 M NaOH and incubated at 4 °C for 24 h to reduce the chains from the core protein. β-Elimination was stopped by adding 5-μl aliquots of 5 M acetic acid until bubble formation ceased. The released GAG chains were purified by DEAE-Sepharose (Sigma) chromatography followed by ethanol precipitation and then resuspended in water. GAGs were digested with 20 μl of chondroitin ABC (Seikagaku) in 50 ml Tris-HCl and 50 mM sodium acetate buffer (pH 8.0). Complete digestion of chondroitin sulfate by chondroitin ABC was ensured by monitoring the extent of conversion of the carrier to disaccharides (100 μg = 1.14 absorbance units at 232 nm). HS was purified from chondroitin-degraded products by phenol/chloroform extraction, ethanol precipitation, and diethyl ether precipitation. After washing the pellets with 0.5 ml of 75% ethanol, HS was suspended in H2O for further analysis.

The quality of HS chains prepared was evaluated by anion exchange HPLC (TSK DEAESw, 8 cm × 7.5 mm inner diameter, Tosohas Inc.). Samples were eluted with a linear gradient of 0.2 to 1 M NaCl in 10 mM KH2PO4, pH 6.0, containing 0.2% CHAPS at a flow rate of 1 ml/min, and radioactivity in the effluent was determined by on-line liquid scintillation spectrometry (Packard).

The absolute amounts of the unlabeled HS were determined by hydrolyzing HS for 3 h with 6 N HCl and 0.1% phenol (v/v) at 100 °C and determining the levels of glucosamine on an Applied Biosystems model 420 Amino Acid Derivatizer with on-line model 130A PTC amino acid analyzer.

HS act and HS inact Precursors were Tagged by 3-O-Sulfation with Purified 3-O-ST-1 and PAPS—The standard 50-μl reaction contains 400 units of purified 3-O-sulfotransferase-1 (3-O-ST-1) (EC 2.8.2.23), 60 μg of bovine serum albumin, metabolically labeled [3H]HS, [35S]HS, or cold HS chains prepared from F9 cells suspended in 1 mM CaCl2, 5 mM MgCl2, 5 mM MnCl2, 0.375 mg/ml protamine, 0.4 mg/ml chondroitin sulfate, 1% Triton X-100, 50 mM MES (pH 7.0), and 1 mM PAPS or 25 μM (1 × 105 cm) PAP35S. The reactions were incubated at 37 °C overnight and then terminated by boiling for 1 min. After adding 100 μg of chondroitin sulfate as cold carrier, the radiolabeled HS was purified by phenol/chloroform extraction, DEAE-Sepharose chromatography, and ethanol precipitation. After washing the pellets with 0.5 ml of 75% ethanol, 3-O-sulfate-tagged HS act and HS inact precursors were separated by AT affinity assay as described below.

Separation of 3-O-Sulfate-tagged HS act and HS inact Precursors by AT Affinity Assay—AT affinity assay was used as described previously (36). In brief, AT complexes were created by mixing 3-O-sulfate-tagged HS act in 500 μl of HB (150 mM NaCl, 10 mM Tris-Cl (pH 7.4)) with 2.5 mM AT/CaCl2, 10 mM sodium [35S]sulfate, 0.002% Triton X-100, 100 μg/ml chondroitin sulfate, 0.5 mM CaCl2, 0.5 mM MgCl2, and 60 μl of HB containing ~50% concanavalin A-Sepharose 4B was then added. AT complexes were bound to concanavalin A by mixing the reaction mixtures for 1 h at room temperature. Beads were pelleted by a brief centrifugation at 10,000 × g. The supernatant was collected, and the beads were washed three times with 1.25 ml of HB containing 0.0004% Triton X-100. The supernatant and washing solutions were combined as 3-O-sulfate-tagged HS act or HS inact precursors. 3-O-Sulfate-tagged HS act precursors were eluted with three successive 200-μl washes of HB containing 1.0 mM NaCl, 0.0004% Triton X-100 and pooled. After adding 100 μg of chondroitin sulfate as cold carrier to 3-O-sulfate-tagged HS act precursors, the pooled 3-O-sulfate-tagged HS act and HS inact precursors were cleaned by phenol/chloroform extraction followed by DEAE-Sepharose chromatography and ethanol precipitation. After washing the pellets with 0.5 ml of 75% ethanol and dried briefly by Speed-Vac, 3-O-sulfate-tagged HS act and HS inact precursors were resuspended in H2O and used for chemical and enzymatic structural studies.

Low pH Nitrous Acid Degradation—3-O-Sulfate-tagged HS act and HS inact precursors or 3-O-sulfate-tagged tetra- or hexa-O-saccharides were equilibrated with 100 mM ammonium bicarbonate at a flow rate of 4 ml/h. 200 μl of radiolabeled sample mixed with dextran blue (5 μg) and phenol red (5 μg) was loaded on the column. 0.4 ml per fraction was collected. A 10-μl sample from each fraction was counted for 3H and/or 35S radioactivity unless otherwise indicated. The desired fractions were pooled and dried by Speed-Vac to remove ammonium bicarbonate and used for further analysis. A small amount of free sulfate generated during the experimental procedures was quantitated by re-running the collected disaccharide fractions on a polyamine HPLC. The free sulfate is subtracted from disaccharides to get the accurate di-, tetra-, and oligosaccharide quantitations indicated under “Results.”

Digestion of 3-O-Sulfate-tagged HS act and HS inact Precursors with Heparitinase I, Heparitinase II, Heparitinase, and Heparitinase IV—Heparitinase I (EC 2.4.2.8.2), heparitinase II (no EC number), heparitinase (EC 4.2.2.7) were obtained from Seikagaku; heparitinase IV was from Dr. Yoshida, Seikagaku Corp., Tokyo, Japan. Heparitinase I recognizes the following sequences: GlcNAcNS6S=6S(R)-[GlcUA-GlcNAc/NS8S=6S-IdceA2S-GlcNS6S] (35). Each reaction contains 0.5 mg of the unlabeled HS and 1000 units of heparitinase I, II, III, or IV as described above. The reaction products and references can be found in the Seikagaku’s catalog.

The digestion of 3-O-sulfate-tagged HS act and HS inact precursors was carried out in 100 μl of 40 mM ammonium acetate (pH 7.0) containing 1 mM CaCl2, with 2 milliunits of enzyme or 2 milliunits of each heparitinase I, heparitinase II, and heparitinase. The digestion was incubated at
Six known heparan sulfate disaccharide standards (Seikagaku) were carried out by HPLC using an amine-bonded silan PA01 column (0.46 × 25 cm) (PAMM-HPLC) (YMC). 100-μl aliquots containing 2 nmol of each of the six heparan sulfate disaccharide standards (Seikagaku) were included with all analytic runs. The PAMM-HPLC was eluted with H₂O for 5 min followed by 0–100% KH₂PO₄ linear gradient for 100 min at a flow rate of 1 ml/min. The elution of cold disaccharide standards was monitored by on-line UV monitoring, and radiolabeled disaccharides were monitored by on-line liquid scintillation spectrometry (Packard Instrument Co.). Each fraction represents a 0.5-ml elution volume.

Sequential Enzymatic Degradation of Δ^4,5-Tetrasaccharides—Δ^4-Glycuronidase (no EC number) and Δ^4-glycuronate 2-0-sulfatase (no EC number) were from Dr. Yoshida, Seikagaku Corp., Tokyo, Japan. α-N-Acetylgalcosamine 6-O-sulfate sulfatase (6-O-sulfatase) (no EC number) and α-N-acetylgalcosaminidase (no EC number) were purified from bovine kidney in our laboratory. No other enzymatic activity was detected in these enzyme preparations. GlcUA-[3H]anManR3S and GlcUA-[3H]anManR3S6S standards were prepared as described previously (31). GlcUA-anManR3S and GlcUA-anManR3S6S standards were prepared from 3-0-S-sulfated cold F9 HS. In brief, after hydrazinolysis, 3-0-S-sulfate-tagged F9 HS was treated with high pH nitrous acid (pH 4.0) and low pH nitrous acid (pH 1.5). The resulting disaccharides were reduced with NaBH₄ and desalted by Bio-Gel P2 chromatography. 3-0-S-Sulfated-tagged disaccharides were collected by ion pairing reverse phase HPLC as previously reported (37). The identity of GlcUA-anManR3S and GlcUA-anManR3S6S was further confirmed by co-chromatography on ion pairing reverse phase HPLC with GlcUA-[3H]anManR3S and GlcUA-[3H]anManR3S6S standards. GlcUA-anManR3S and GlcUA-anManR3S6S are the only disaccharides generated by 3-ST-1 and PAPS-tagging of cold F9 HS.

3-0-S-sulfate-tagged tetrasaccharides were digested with 10% of Δ^4-glycuronate 2-0-sulfatase or Δ^4-glycuronidase in a total volume of 100 μl of 50 mM imidazole HCl buffer (pH 6.5) at 37°C overnight. 5% (5 μl) of the reaction mixture was combined with six HS disaccharide UV standards and GlcUA-[3H]anManR3S6S standards (2000 rpm) in a total volume of 100 μl and analyzed by polyamine HPLC. Completion of the digestion was confirmed by polyamine HPLC. 40 μl of 0.5 M sodium acetate (pH 5.5), 55 μl of H₂O, and 10 μl 6-O-sulfatase were added to 85 μl of reaction mixture. The 6-O-sulfatase digestion was completed in 2 days at 37°C as monitored by polyamine HPLC as described above. 100 μl of 6-O-sulfatase-treated materials was diluted with 300 μl of H₂O, 2 μl of 16 μM acetic acid and then 5 μl of α-N-acetylgalcosaminidase (3.5 μg) were added (pH 4.2). α-N-Acetylgalcosaminidase digestion was completed overnight at 37°C as monitored by polyamine HPLC.

RESULTS

The HS[^ac] Precursors Contain Six Potential AT-binding Sites Whose Structure Is Completed by in Vitro 3-O-Sulfation—We have previously reported that F9 cells make 1% HS[^ac] and 29% HS[^inac] precursors, and the metabolically labeled GlcUA-anMan[^35S] (6%) and GlcUA-anMan[^35S]6S[^35S] (12%) accounted for about 15% of O-sulfated disaccharides in 1% F9 HS[^ac] (36). The high percentage of 3-O-sulfated disaccharides in the biosynthesized HS[^ac] chain suggests that there are multiple potential 3-O-sulfation sites in the F9 HS[^rec] precursor chain. To estimate this parameter, [6-3H]GlcN metabolically labeled HS[^rec] precursor was isolated by exhaustive conversion to HS[^ac] by exposure in vitro to pure 3-0-ST-1 as well as PAPS and subsequent captured by AT affinity chromatography. The 3-O-sulfate-tagged HS[^rec] precursors were subjected to a mixture of heparitinase I, heparitinase II, and heparinase digestion to confirm the identity of the F9 HS[^ac] precursor chain. Then, the HS[^ac] precursors were digested with 3-ST-1 and PAPS.

The HS[^ac] peaks have the structure GlcUA-Gal-Gal-Xyl- in the linkage region. The HS[^ac] peaks were identified by comparison to standards (“Experimental Procedures”). The two HS[^ac] peaks from Fig. 2 were further analyzed to estimate the number of 3-O-sulfation sites per HS[^ac] precursor chain. We first assessed the molecular weight for F9 HS by SDS-polyacrylamide gel electrophoresis as described previously (39), where M_r = 2.112 × 10^5. The HS[^ac] exhibits an average molecular weight (M_r) of 42,000 (data not shown). Based on this and the average disaccharide molecular mass of 500 Da, there are about 84 disaccharides per chain, i.e., 82 disaccharides in the repetitive -(UA-GlcNAc)₃₉ region and one tetrasaccharide GlcUA-Gal-Gal-Xyl- in the linkage region. Assuming the average HS consists of 82 disaccharides in the repetitive -(UA-GlcNAc)₃₉ region, the number of di- and tetrasaccharides per 3-O-sulfated HS[^ac] precursor chain are summarized in Table I. There are 4 × ΔUA-GlcNAc6S-GlcUA-Gal-Gal-Xyl- in the linkage region.
GlcNS\textsubscript{S3S} and 2 × ΔUA-GlcNAc\textsubscript{6S}-GlcUA-GlcNS\textsubscript{3S6S} per chain, therefore a total of six 3-O-sulfate sites per HS\textsuperscript{act} precursor chain.

The sum of N-sulfates, 2-O-sulfate, 6-O-sulfates, and 3-O-sulfates for each sugar residues is provided in Table I and equals 100% of the total sulfate groups per 3-O-sulfate-tagged chain, which corresponds to a total of 71 sulfate groups. Of these, 37 sulfate groups or 52% are O-sulfates. Furthermore, since 3-O-sulfates comprise 14% of all metabolically labeled O-sulfates (36). We can calculate that each endogenous HS\textsuperscript{act} chain contains 5.2 3-O-sulfation sites (37 × 14%).

By treating cold F9 HS with PAP\textsuperscript{35S} and 3-O-ST-1, we found that 6 pmol of \([35S]\)sulfates from PAP\textsuperscript{35S} are transferred to 3-O-sulfate positions in 1 pmol of F9 HS\textsuperscript{act} precursors (data not shown). Thus, from the evidence presented above, we concluded that there are six 3-O-sulfate acceptor sites per HS\textsuperscript{act} precursor chain, which can be captured as 3-O-sulfated tetrasaccharides as indicated above.

We then estimated the number of 3-O-sulfate acceptor sites per HS\textsuperscript{inact} precursor chain. We previously reported that metabolically labeled GlcUA-anMan\textsubscript{p35S} (5%) and GlcUA-anMan\textsubscript{p235S6S35S} (5%) accounted for 10% of O-sulfated disaccharides in retinoic acid and dibutylryl cAMP plus theophilline F9 HS\textsuperscript{inact}. However, endogenous retinoic acid and dibutylryl cAMP plus theophilline F9 HS\textsuperscript{inact} are composed of 3-O-sulfated HS\textsuperscript{inact} as well as HS\textsuperscript{act} and HS\textsuperscript{inact} precursors that have never been 3-O-sulfated (36). To obtain a more accurate estimate, cold F9 HS was exhaustively converted with PAPS/PAP\textsuperscript{35S} and 3-O-ST-1. After AT affinity fractionation to remove all 3-O-sulfate-tagged HS\textsuperscript{act} precursors, we found that 6 pmol of \([35S]\)sulfates from PAP\textsuperscript{35S} are transferred to 3-O-sulfate positions in 1 pmol of F9 HS\textsuperscript{inact} precursors (data not shown). Therefore, we conclude that there are six 3-O-sulfate acceptor sites in HS\textsuperscript{inact} precursors as well.

3-O-Sulfate-tagged Tetrasaccharide Structures—To characterize the two tetrasaccharide structures in Fig. 2B, we repeated the experiment to obtain the tetrasaccharides using different F9 HS preparations. To this end, either cold F9 HS was tagged with radiolabeled 3-O-sulfates or \textsuperscript{35}SO\textsubscript{4} metabolically labeled HS was tagged with cold 3-O-sulfates \textit{in vitro} by PAPS/PAP\textsuperscript{35S} and purified 3-O-ST-1. Affinity purified 3-O-sulfate-tagged HS\textsuperscript{act} precursors were subjected to heparitinase I, heparinase II, and heparinase digestion. The radiolabeled tetrasaccharides were collected from Bio-Gel P2 chromatography. The identical two tetrasaccharide peaks with the same 2 to 1 ratio of tetrasaccharide I to tetrasaccharide II were found after polyamine HPLC chromatography among the three 3-O-sulfate-tagged tetrasaccharide sources (data not shown). This result suggests that we can characterize the tetrasaccharides from any 3-O-sulfate-tagged F9 HS\textsuperscript{act} precursors.

The 3-O-S\textsubscript{35S} tagged tetrasaccharide I and tetrasaccharide II from cold F9 HS\textsuperscript{act} were separated by polyamine HPLC chromatography and collected for the tetrasaccharide sequencing analysis. A fraction of each tetrasaccharide collected was rerun on polyamine HPLC (Fig. 3, A and B). Tetrasaccharide I and tetrasaccharide II were first treated with low pH nitrous acid, which cleaves the GlcNS±6S-GlcUA/IdceA±2S linkage but not the GlcNAc±6S-GlcUA/IdceA±2S linkage in the tetrasaccharide. However, after low pH nitrous acid treatment and NaBH\textsubscript{4} reduction, both tetrasaccharide peaks remained as tetrasaccharides as judged by the Bio-Gel P2 profile (data not shown), but the elution time on polyamine HPLC was altered (Fig. 3, C and D). The alteration in this parameter corresponds to the lost of one N-sulfate and ring contraction from the reducing end GlcN residue of both tetrasaccharides.

The tetrasaccharides collected were then treated with Δ\textsuperscript{4,5}glycuronate 2-O-sulfatase. The retention time of tetrasaccharide I and tetrasaccharide II remained the same, whereas the 2-O-sulfate in the internal Δ\textsuperscript{4,5}UA2S-GlcNS UV standard was completely removed as judged by the shift of UV absorb-
TABLE I

Di- and tetrascarharide compositions of 3-O-sulfate-tagged HS\textsuperscript{act} precursors

| Name | 3-O-Sulfate-tagged [\(^3\)H]HS\textsuperscript{act} precursors |
|------|----------------------------------------------------------|
|      | No. of sugars/chain                                      |
| 1    | \(\Delta U4\)-[\(^3\)H]GlcNAc                           |
|      | 37                                                      |
| 2    | \(\Delta U4\)-[\(^3\)H]GlcNAc6S                         |
|      | 5                                                       |
| 3    | \(\Delta U4\)-GlcNS                                     |
|      | 16                                                      |
| 4    | \(\Delta U4\)-GlcNS6S                                  |
|      | 3                                                       |
| 5    | \(\Delta U4S\)-[\(^3\)H]GlcNS                          |
|      | 3                                                       |
| 6    | \(\Delta U4S\)-GlcNS6S                                 |
|      | 6                                                       |
| 7    | \(\Delta U4A\)-[\(^3\)H]GlcNAc6S-GlcUA-\[\(^3\)H]GlcNS6S |
|      | 4                                                       |
| 8    | \(\Delta U2A\)-[\(^3\)H]GlcNAc6S-GlcUA-\[\(^3\)H]GlcNS6S |
|      | 2                                                       |
|      | Number of disaccharides per chain                        |
|      | 82                                                      |
|      | Number of NS per chain                                  |
|      | 34                                                      |
|      | Number of 2S per chain                                  |
|      | 9                                                       |
|      | Number of 3S per chain                                  |
|      | 6                                                       |
|      | Number of 6S per chain                                  |
|      | 22                                                      |
|      | Number of total sulfates per chain                      |
|      | 71                                                      |


In vitro 3-O-sulfated [\(^3\)H]HS\textsuperscript{act} precursors were depolymerized with a mixture of heparitinase I, heparitinase II, and heparinase I. The resulting unsaturated di- and tetrascarharides were originally separated on a Bio-Gel P2 column (Fig. 1) and were then further resolved by polyamine HPLC (Fig. 2). The number of each di- and tetrascarharide per chain was determined in two steps. Initially, we determined the radioactivity per di- or tetrascarharide. To this end, the sum of radioactivity in all di- and tetrascarharide peaks was divided by 82 to determine the average radioactivity per disaccharide and 41 to determine the average radioactivity per tetrascarharide. Subsequently, the total radioactivity in each of various di- or tetrascarharide peaks was divided by the average radioactivity per di- or tetrascarharide to determine the number of a particular di- or tetrascarharide in a specific peak. The numbers represent the average of two separate experiments. Total N-sulfates, 2-O-sulfate, 6-O-sulfates, and 3-O-sulfates per chain were added according to the number of these sulfates in each di- and tetrascarharide species. It should be noted that these data are consistent with previously published data from our laboratory, which indicates that 3-O-sulfated di-scarharides comprise 18% of all molecular labeled O-sulfatedascarharides in F9 HS\textsuperscript{act} (GlcUA-anMan\(_3\)\(^3\)SM, 6%, and GlcUA-anMan\(_3\)\(^3\)S\(^6\)SM, 12%) (36). In addition, direct comparison of the number of 3-O-sulfate incorporations per HS\textsuperscript{act} precursor chain (6 pmol 3\(^3\)SM/pmol F9 HS) are also consistent with the values provided.

From the above results, we concluded that the first tetrascarharide peak was divided by the average radioactivity per di- or tetrascarharide to determine the number of a particular di- or tetrascarharide in a specific peak. The numbers represent the average of two separate experiments. Total N-sulfates, 2-O-sulfate, 6-O-sulfates, and 3-O-sulfates per chain were added according to the number of these sulfates in each di- and tetrascarharide species. It should be noted that these data are consistent with previously published data from our laboratory, which indicates that 3-O-sulfated di-scarharides comprise 18% of all molecular labeled O-sulfatedascarharides in F9 HS\textsuperscript{act} (GlcUA-anMan\(_3\)\(^3\)SM, 6%, and GlcUA-anMan\(_3\)\(^3\)S\(^6\)SM, 12%) (36). In addition, direct comparison of the number of 3-O-sulfate incorporations per HS\textsuperscript{act} precursor chain (6 pmol 3\(^3\)SM/pmol F9 HS) are also consistent with the values provided.

To the number of these sulfates in each di- and tetrascarharide species. It should be noted that these data are consistent with previously published data from our laboratory, which indicates that 3-O-sulfated di-scarharides comprise 18% of all molecular labeled O-sulfatedascarharides in F9 HS\textsuperscript{act} (GlcUA-anMan\(_3\)\(^3\)SM, 6%, and GlcUA-anMan\(_3\)\(^3\)S\(^6\)SM, 12%) (36). In addition, direct comparison of the number of 3-O-sulfate incorporations per HS\textsuperscript{act} precursor chain (6 pmol 3\(^3\)SM/pmol F9 HS) are also consistent with the values provided.

The elution position of peak I on polyamine HPLC suggests that this tetrascarharide may have only one sulfate group. After \(\Delta 4\)-glycosaminidase treatment, the resulting products eluted at the same position as GlcNAc-GlcUA-anMan\(_3\)\(^3\)S in Fig. 3G. After \(\alpha\)-N-acetylglycosaminidase treatment, it coeluted with GlcUA-[\(^3\)H]Man\(_3\)\(^3\)S standard. From the above evidence, we concluded that peak I has the structure of \(\Delta 4\)-UA-GlcNAc-GlcUA-anMan\(_3\)\(^3\)S. The critical 6-O-sulfate group at residue 2 is missing in this tetrascarharide. This observation explains the fact that a population of 3-O-sulfate-tagged HS\textsuperscript{act} precursors within the GlcNAc-GlcUA-GlcNS\(^6\)S context failed to bind to AT. However, we cannot quantitate how many 6-O-sulfates are missing in the 3-O-sulfate-tagged HS\textsuperscript{act} precursors within the GlcNS-GlcUA-GlcNS\(^3\)S context because heparitinase treatment does not preserve all the 3-O-sulfate acceptor sites of these precursors. Our inability to capture all of the 3-O-sulfate-tagged tetrascarharides from the HS\textsuperscript{act} precursors is probably due to the heterogeneity in the structure of these precursors. This heterogeneity results from the fact that we have not, as yet, identified the ligands for 3-O-sulfated HS\textsuperscript{act} materials and hence cannot purify the 3-O-sulfated HS\textsuperscript{act} precursors.

Five of Six 3-O-Sulfate-tagged Oligosaccharides in HS\textsuperscript{act} Precursors Bind to AT—Both HS\textsuperscript{act} and HS\textsuperscript{inact} precursors contain about six 3-O-sulfation sites per chain. 3-O-Sulfate-tagged HS\textsuperscript{act} precursors binds to AT, and 3-O-sulfate-tagged HS\textsuperscript{inact} precursors do not. The next question we asked is how many of the 3-O-sulfate acceptor sites (potential AT-binding sites) in 3-O-sulfate-tagged HS\textsuperscript{act} precursors actually bind to AT. We reasoned that heparitinase I digestion should leave AT-binding sites intact since all the 3-O-sulfate-tagged HS\textsuperscript{act} precursors remained as 3-O-sulfate-tagged tetrascarharide after heparitinase I, heparitinase II, and heparinase digestion (Fig. 1). If HS has the same AT-binding sequence as in heparin, i.e. GlcNAc\(^6\)S-GlcUA-GlcNS\(^6\)S-IdceA2S-GlcNS6S, the reducing end IdceA2S-GlcNS6S cannot be cleaved by heparitinase I ("Experimental Procedures" (38)). To this end, 3-O-ST-1 and PAP\(^3\)S-labeled, AT affinity purified HS\textsuperscript{act} and HS\textsuperscript{inact} were digested with 2 milligrams of heparitinase I overnight. A fraction of the digested materials from the 3-O-sulfate-tagged HS\textsuperscript{act} and HS\textsuperscript{inact} precursors were used in the AT affinity assay. We found that, for the digested HS\textsuperscript{act}, the extent of AT binding was approximately equal to the undigested HS\textsuperscript{act} control. To check the heparitinase I digestion reaction, the rest of the digested 3-O-sulfate-tagged HS\textsuperscript{act} and HS\textsuperscript{inact} materials were
analyzed by Bio-Gel P-6 chromatography. The digestion patterns were reproducibly obtained, and sample profiles are provided (Fig. 5). For 3-O-sulfate-tagged HS act precursors, the data show that 85% remained larger than hexasaccharides, 14% were tetrasaccharides, and 0.5% were disaccharides (Fig. 5A). For 3-O-sulfate-tagged HS inact precursors, only 14% remained larger than hexasaccharides, 52% were tetrasaccharides, 33% were disaccharides (Fig. 5B).

Each fraction of oligosaccharides equal to or larger than hexasaccharides in 3-O-sulfate-tagged HS act and HS inact precursors were collected and lyophilized for the AT affinity assay. All the oligosaccharides from 3-O-sulfate-tagged HS act precursors bound to AT, but the oligosaccharides from 3-O-sulfate-tagged HS inact precursors did not. Since 85% of the 3-O-sulfate-tagged HS act oligosaccharides bind to AT and there are six 3-O-sulfation sites per chain, 6 × 85% = 5.1. It implies that at least five out of six 3-O-sulfate acceptor sites possess the correct positioning of all critical groups except for the 3-O-sulfate for AT binding in all HS act precursor chains. This experiment also suggests that HS act and HS inact precursors have distinct sulfation patterns around the potential 3-O-sulfate acceptor sites.

The majority of 3-O-sulfate-tagged HS inact precursors should have GlcUA at their reducing end, i.e. -GlcUA-GlcNS₆S-²GlcUA, since GlcUA residues at this position are apparently cleaved by heparitinase I in order to generate 85% of 3-O-sulfated di- (33%) and tetrasaccharides (52%). In contrast, GlcUA-GlcNS₃S-²GlcA₆S-² should be the main 3-O-sulfate-tagged HS act sequence since it is uncleavable by heparitinase I. It is interesting to note that 33% of 3-O-sulfate-tagged HS inact precursors can be degraded into disaccharides and 0.5% of 3-O-sulfate-tagged HS act precursors can be degraded into disaccharides. This result implies that 3-O-sulfation is not the only factor that determines whether 3-O-sulfated sequences are preserved as tetrasaccharides following the digestion with heparitinase.

**Different Sulfation Distribution around 3-O-Sulfate Acceptor Sites in HS act and HS inact Precursors**—To compare further the sulfation patterns in HS act and HS inact precursors, [6-³H]GlcN metabolically labeled HS was treated with 3-O-ST-1 and
PAP35S. The 3H and 35S double-labeled HS was AT affinity fractionated into 3-O-sulfate-tagged HSact and HSinact precursors. The low pH nitrous-treated and NaBH4 reduced products were analyzed by Bio-Gel P-6 chromatography (Fig. 6). These conditions depolymerize HS between GlcNS and GlcUA/IdceA residues, liberating oligosaccharides whose length reflects the spacing between N-sulfated GlcN units. The 3H radioactivity profiles are similar; therefore, the distribution of GlcNS residues are similar in HSact and HSinact precursors outside 3-O-sulfate acceptor sites. However, 3-O-sulfate acceptor sites in HSact and HSinact precursors are different because their 35S radioactivity profiles are distinct. The 3-O-sulfate acceptor sites in HSact precursors should be located in GlcUA/IdceA-GlcNAc6S-GlcUA-GlcNS35S6S sequences since 88% of 3-O-sulfated materials remained as tetrasaccharides (Fig. 6A). In contrast, 3-O-sulfate acceptor sites in HSinact precursors should...
be located mainly in GlcUA/IdeA-GlcNS6S-GlcUA-GlcNS5S6S±6S sequences since 60% of 3-O-sulfated materials was cleaved into disaccharides (Fig. 6B).

3-O-Sulfate-tagged HS\textsuperscript{act} Hexasaccharide Structures—The tetrasaccharide analysis, heparitinase I, and low pH nitrous acid treatment data imply that the dominant 3-O-sulfate acceptor sites in HS\textsuperscript{act} precursor are GlcUA/IdeA-GlcNAc6S-GlcUA-GlcNS5S6S±6S-IdeA±2S, whereas the dominant 3-O-sulfate acceptor sites in HS\textsuperscript{inact} precursor are GlcUA/IdeA-GlcNS/Ac±6S-GlcUA-GlcNS5S6S±6S-GlcUA. These results further clarify why 3-O-sulfate-tagged HS\textsuperscript{act} precursors bind AT and 3-O-sulfate-tagged HS\textsuperscript{inact} precursors do not. To obtain the minimum AT-binding sequence in 3-O-sulfate-tagged HS\textsuperscript{act} precursors, the \textsuperscript{3}H and \textsuperscript{35}S double-labeled HS\textsuperscript{act} and HS\textsuperscript{inact} precursors were subjected to limited digestion with heparitinase I and heparitinase II. According to recently published work on the exolytic and processive mechanism of depolymerization of HS by heparitinase II (41), this digestion condition should preserve some of the AT-binding hexasaccharide structures. The digested products were analyzed by Bio-Gel P2 chromatography (Fig. 7). The major hexasaccharide peak from 3-O-sulfate-tagged HS\textsuperscript{act} precursors was collected and lyophilized. AT affinity purified, \textsuperscript{3}H and \textsuperscript{35}S double-labeled hexasaccharides were loaded on a polyamine HPLC column (Fig. 8). Two peaks (hexasaccharide I and hexasaccharide II) were found and collected for further analysis.

Hexasaccharide I and hexasaccharide II were digested with heparitinase IV. All the hexasaccharides were degraded into di- and tetrasaccharides, which were separated and collected after Bio-Gel P2 chromatography (data not shown). The \textsuperscript{3}H-labeled disaccharides from both hexasaccharide I and heparitinase II coeluted at the same position as hexasaccharide II and hexasaccharide I were treated with low pHi nitrous acid and NaBH\textsubscript{4} reduced. The products were fractionated into disaccharide and tetrasaccharide by Bio-Gel P2 chromatography and collected for further analysis. The \textsuperscript{3}H-labeled disaccharides from both heparitinase I and heparitinase II coeluted at the same position as hexasaccharide II and hexasaccharide I eluted at the same position as \textsuperscript{3}H-labeled disaccharides from both heparitinase I and heparitinase II. According to recently published work on the exolytic and processive mechanism of depolymerization of HS by heparitinase II (41), this digestion condition should preserve some of the AT-binding hexasaccharide structures. The digested products were analyzed by Bio-Gel P2 chromatography (Fig. 7). The major hexasaccharide peak from 3-O-sulfate-tagged HS\textsuperscript{act} precursors was collected and lyophilized. AT affinity purified, \textsuperscript{3}H and \textsuperscript{35}S double-labeled hexasaccharides were loaded on a polyamine HPLC column (Fig. 8). Two peaks (hexasaccharide I and hexasaccharide II) were found and collected for further analysis.

Hexasaccharide I and hexasaccharide II were digested with heparitinase IV. All the hexasaccharides were degraded into di- and tetrasaccharides, which were separated and collected after Bio-Gel P2 chromatography (data not shown). The \textsuperscript{3}H-labeled disaccharides from both hexasaccharide I and heparitinase II both co-eluted with ΔUA2S-GlcNS6S UV standard on polyamine HPLC (Fig. 9, A and B). The \textsuperscript{3}H and \textsuperscript{35}S double-labeled tetrasaccharide from heparitinase I has the structure ΔUA-\textsuperscript{3}H[GlcNAc6S-GlcUA-\textsuperscript{3}H[GlcNS3S6S]. The tetrasaccharide from heparitinase II has the structure ΔUA-\textsuperscript{3}H[GlcNAc6S-GlcUA-\textsuperscript{3}H[GlcNS3S6S] because they have the same retention time on polyamine HPLC as tetrasaccharide I in Fig. 3A and tetrasaccharide II in Fig. 3B. This experiment suggests that the AT-binding hexasaccharide I has the structure [6-\textsuperscript{3}H[GlcNAc6S-GlcUA-\textsuperscript{6-3H}[GlcNS3S6S-UA2S-6-3H[GlcNS6S] and the hexasaccharide II has the structure [6-\textsuperscript{3}H[GlcNAc6S-GlcUA-\textsuperscript{6-3H}[GlcNS3S6S-UA2S-6-3H[GlcNS6S].

To determine the identity of the UA2S in both hexasaccharides, hexasaccharide I and hexasaccharide II were treated with low pH nitrous acid and NaBH\textsubscript{4} reduced. The products were fractionated into disaccharide and tetrasaccharide by Bio-Gel P2 chromatography and collected for further analysis. The \textsuperscript{3}H-labeled disaccharides from both heparitinase I and heparitinase II coeluted at the same position as ΔUA-GlcNAc6S-GlcUA-\textsuperscript{3}H[GlcNS3S6S-UA2S-6-3H[GlcNS6S] and the hexasaccharide II has the structure ΔUA-GlcNAc6S-GlcUA-GlcNS3S6S-IdeA2S-6-3H[GlcNS6S].

Based on the data presented in this paper, the differences between 3-O-sulfate acceptor sites in HS\textsuperscript{act} and HS\textsuperscript{inact} precursors are summarized in Fig. 10. It is important to note that the heterogeneity in the HS\textsuperscript{inact} precursors results in a generation of a large number of potential 3-O-sulfate acceptor sites. Those structures for which we have evidence are provided in the figure. In this regard, the second sugar residue in HS\textsuperscript{inact} precursors is either a GlcNS (60%) or GlcNAc (40%), and the fifth sugar residue in HS\textsuperscript{inact} precursors is either a GlcUA (85%) or IdeA (15%). Furthermore, the 6-O-sulfate groups on residue 2 and 6 of the 3-O-sulfate acceptor site on the HS\textsuperscript{inact} precursors can be present or absent. These alterations distinguish the potential 3-O-sulfate acceptor sites of the HS\textsuperscript{inact} precursors from that of the HS\textsuperscript{act} precursors whose sequence is uniquely defined. The many possible combinations of alterations in the HS\textsuperscript{inact} precursor acceptor sites reflect the underlying heterogeneity of the HS\textsuperscript{inact} precursors. For this reason, we are unable to preserve the major 3-O-sulfate acceptor hexasaccharide sequence in HS\textsuperscript{inact} precursors and cannot quantitatively provide the actual sequence of the 3-O-sulfate acceptor sites in HS\textsuperscript{inact} precursors. However, it should be emphasized that the critical 6-O-sulfate is always present in HS\textsuperscript{act} precursors, whereas it can be missing in HS\textsuperscript{inact} precursors.

**DISCUSSION**

This article presents an analysis of AT-binding HS\textsuperscript{act} precursor sequences in F9 embryonal carcinoma cells. At least five out of six 3-O-sulfate acceptor oligosaccharides possess the correct positioning of all critical AT-binding groups except 3-O-sulfate in all HS\textsuperscript{act} precursor chains. It is interesting to note that four AT-binding oligosaccharides in each LTA cell HS\textsuperscript{act} chain are present as shown by an AT-protected heparin lyase assay in our laboratory. To possess multiple AT-binding sites in each HS\textsuperscript{act} chain suggests that HS\textsuperscript{act} biosynthesis is not a random process but a highly repetitive, highly organized operation. Based on the data presented in this paper, the schematic model for the structure of HS\textsuperscript{act} precursors in F9 cells is advanced in [3] J. Liu and R. D. Rosenberg, unpublished results.
In this model, there are six potential AT-binding hexasaccharides that exhibited all groups necessary for AT binding except for the 3-O-sulfate moieties. Six potential AT-binding hexasaccharides are equal to 22% of the chain length of HS act precursors (18 of 82 disaccharides per chain) and contain 26 of 37 O-sulfates in 3-O-sulfate-tagged HS act precursors. Beyond the six potential AT-binding hexasaccharides, there are 5 DUA-GlcNAc6S, 3 DUA2S-GlcNS, 3 DUA-GlcNS6S, 16 DUA-GlcNS, and 37 DUA-GlcNAc disaccharides to complete the structure of the HS act precursor (Table I). 5 DUA-GlcNAc6S, 3 DUA2S-GlcNS, and 3 DUA-GlcNS6S are the 11 O-sulfated residues outside the AT-binding domain that need to be placed to complete the primary structure of HS act precursor (Fig. 11).

The reason we proposed that there are six instead of five potential AT-binding hexasaccharides in F9 HS act precursors is that we could place five out of six DUA2S-GlcNS6S residues in potential AT-binding domains in F9 HS act precursors. We suspect that heparitinase I may contain trace heparitinase II contaminants that generate 15% of the tetrasaccharides in 3-O-sulfate-tagged HS act precursors (Fig. 5). More likely, endo-D-glucuronidases may cleave mature HS chains limitedly in cultured F9 cells before HS chains were prepared for our structural studies (see “Experimental Procedures”). The general presence of endo-D-glucuronidases in variety of tissue and cells has been reported (41–48). Endo-D-glucuronidases recognize the sequences GlcUA/IdceA-GlcNAc/NS$_6$-GlcUA$_2$GlcNAc/NS$_6$3S$_6$. The arrow indicates the cleavage site (49, 50). The cleavage eliminates both AT- and potential AT-binding sites (50). The endo-D-glucuronidases extensively cleave the newly synthesized heparin ($M_r$ 60,000–100,000) to generate fragments that are stored in cytoplasmic granules of mast cells ($M_r$ 5,000–25,000). This may explain why commercial heparin ($M_r$ 5,000–25,000) contains limited numbers of AT-binding sites per chain (38). In contrast, endo-D-glucuronidases may cleave in a limited fashion the endogenous HS chains (48, 49). This limited cleavage may explain why we can place five out of six DUA2S-GlcNS6S residues in potential AT-binding domains in F9 HS act precursors.

It is apparent from our model that there is a template for HS act precursor formation that requires correct N-sulfation, epimerization, 2-O-, and 6-O-sulfation at all six sites along the chain during the biosynthesis. What remains to be explained is how this is accomplished. F9 HS act chain contains almost all the detectable 3-O-sulfated disaccharides (GlcUA-anMan$_R$3S and GlcUA-anMan$_R$3S6S) in HS total. The 3-O-sulfated disaccharides accounted for about 18% of O-sulfated disaccharides in the HS act chain (36). Our current study showed that there are six 3-O-sulfate acceptor sites per HS act chain. Therefore, 3-O-sulfates are added in an all or none manner to the six 3-O-sulfate acceptor sites in HS act precursor in a concerted fashion to generate the AT-binding sites during biosynthesis. Based on this observation, our current model is that after chain polymerization, all other chain modification reactions, i.e. N-sulfation, epimerization, 2-O-, 6-O-sulfation, occur at all six regions in the same fashion as 3-O-sulfation of HS act precursors. To explain this concerted mechanism, we propose that N-STs recognize and sulfate a three-dimensional feature of the
hs^act precursors - Six 3-o-sulfation sites / chain

\[
\begin{align*}
\text{UA} - \text{GlcNAc} - \text{GlcA} - \text{GlcNS} - \text{IdA} - \text{GlcNS} \\
\pm \text{6-OSO}_3 & \quad \pm \text{2-OSO}_3 \\
3 \text{-OSO}_3 & \quad 6 \text{-OSO}_3
\end{align*}
\]

hs^inact precursors - Six 3-o-sulfation sites / chain

\[
\begin{align*}
\text{UA} - \text{GlcNAc} - \text{SO}_3 & \quad \text{UA} - \text{GlcNAc} - \text{GlcA} - \text{GlcNS} - \text{IdA} - \text{GlcNS} - \text{IdA} - \text{GlcNAC} \\
\pm \text{6-OSO}_3 & \quad \pm \text{6-OSO}_3 \\
\text{GlcA} 85\% & \quad \text{IdA} 15\%
\end{align*}
\]

26 0-sulfates in AT-binding hexasaccharides

\[
\begin{align*}
6S & \quad 3S & \quad 6S \\
\text{UA} - \text{GlcNAc} - \text{GlcA} - \text{GlcNS} - \text{IdA} - \text{GlcNS} & \quad \pm \text{6S} & \quad \pm \text{2S}
\end{align*}
\]

11 0-sulfates outside AT-binding hexasaccharides

This study shows that F9 cells produce different HS structures. The previous HS^act-deficient mutants and 3-O-ST-1 studies in our laboratory suggested that the same cells make both HS^act and HS^inact precursors (35, 53), and the same HS proteoglycan core proteins carry both HS^act and HS^inact chains (54). A Chinese hamster ovary cell mutant defective in N-ST makes both fully sulfated and undersulfated HS chains (55). A human colon carcinoma cell makes both fully sulfated and undersulfated HS chains (56). All these observations suggest that different biosynthesis schemes that generate different HS structures occur in the same cells. In addition to the increasing numbers of specific HS domain structural studies in different tissues and cells (57–61), we suggest that different HS biosynthetic pathways exist, which generate HS with different structures and biologic functions. Evaluation of the different pathways will eventually delineate how the HS biosynthesis is regulated.

Currently we do not know where HS^act and HS^inact precursor biosynthesis pathways diverge. The constant presence of the critical 6-O-sulfate groups only in HS^act precursors indicates that the pathway is either set up by or diverges before the critical 6-O-sulfation. It is possible that specific isoforms of 6-O-ST are involved in HS^act precursor pathways, and the existing structures around the 3-O-sulfate acceptor site favor the action of a 6-O-ST isofrom. Furthermore, the existence of auxiliary proteins that modulate the action of N-ST, epimerase, 2-O-ST, and 6-O-ST for HS^act precursor formation have not been excluded.

In conclusion, the decision to synthesize HS^act or HS^inact depends on the presence of specific HS precursor intermediates, specific modification enzymes, and perhaps auxiliary factors in the Golgi biosynthesis machinery. Currently, we have obtained a series of Chinese hamster ovary mutants defective in HS^act precursor formation. Complementation of these mutations will provide us with molecular details about the required elements for HS^act precursor biosynthesis. This information should allow us to formulate a more definitive model of the HS^act biosynthetic pathway. This model will be evaluated by reconstituting the sequential biosynthetic apparatus using different recombinant modification enzymes/proteins.

Acknowledgments—We thank Peter Blaiklock for careful reading of this manuscript and members of the Rosenberg laboratory for their insightful comments.

REFERENCES
1. Ashikari, S., Habuchi, H., and Kimata, K. (1995) J. Biol. Chem. 270, 29586–29593
2. Feyzi, E., Lustig, F., Fager, G., Spillmann, D., Lindahl, U., and Salmivirta, M. (1997) J. Biol. Chem. 272, 5518–5524
3. Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) J. Biol. Chem. 268, 23906–23914
4. Lyon, M., Daekin, J. A., Mizuno, K., Nakamura, T., and Gallagher, J. T. (1994)

FIG. 10. 3-O-Sulfate-tagged hexasaccharide structures in F9 HS^act and HS^inact precursors. The structures are based on the following data: 1) Six 3-O-sulfate acceptor sites per HS^act and HS^inact precursors; 2) Low pH nitrous acid, heparitinase I, and heparitinase II degradations of 3-O-sulfate-tagged HS^act and HS^inact precursors; 3) tetrasaccharide structures in 3-O-sulfate-tagged HS^act and HS^inact precursors; 4) hexasaccharide structures in 3-O-sulfate-tagged [3H]HS^act precursors. The boxes in 3-O-sulfate-tagged HS^act precursors correspond to the sulfate residues existing in 3-O-sulfate-tagged HS^act precursors. The 3-O-sulfate-tagged HS^act and HS^inact precursor sequences are different at all levels. IdA indicates IdceA; GlcA indicates GlcUA.
