Structural Analysis of Glycosaminoglycans in Drosophila and Caenorhabditis elegans and Demonstration That tout-velu, a Drosophila Gene Related to EXT Tumor Suppressors, Affects Heparan Sulfate in Vivo*

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We have devised a sensitive method for the isolation and structural analysis of glycosaminoglycans from two genetically tractable model organisms, the fruit fly, Drosophila melanogaster, and the nematode, Caenorhabditis elegans. We detected chondroitin/chondroitin sulfate- and heparan sulfate-derived disaccharides in both organisms. Chondroitinase digestion of glycosaminoglycans from adult Drosophila produced both nonsulfated and 4-O-sulfated unsaturated disaccharides, whereas only unsulfated forms were detected in C. elegans. Heparin lyses released disaccharides bearing N-, 2-O-, and 6-O-sulfated species, including mono-, di-, and trisulfated forms. We observed tissue- and stage-specific differences in both chondroitin sulfate and heparan sulfate composition in Drosophila. We have also applied these methods toward the analysis of tout-velu, an EXT-related gene in Drosophila that controls the tissue distribution of the growth factor Hedgehog. The proteins encoded by the vertebrate tumor suppressor genes EXT1 and 2, show heparan sulfate co-polymerase activity, and it has been proposed that tout-velu affects Hedgehog activity via its role in heparan sulfate biosynthesis. Analysis of total glycosaminoglycans from tout-velu mutant larvae show marked reductions in heparan sulfate but not chondroitin sulfate, consistent with its proposed function as a heparan sulfate co-polymerase.

Proteoglycans consisting of core proteins with glycosaminoglycan chains are abundant molecules, found both in the extracellular matrix and on the cell surface. These diverse molecules serve a wide range of functions, from affecting the compressive properties of cartilage to growth factor reception. Until recently, proteoglycans were studied principally in vertebrate systems. However, genetic experiments in the fruit fly, Drosophila, have established that proteoglycans, and their associated glycosaminoglycans, are required for normal development of this invertebrate model organism (reviewed in Ref. 1). A Drosophila member of the glypican family, division abnormally delayed (daily)1 (2, 3), affects signaling mediated by two conserved growth factors, Wingless, a member of the Wnt family, and Decapentaplegic, a transforming growth factor-ß/bone morphogenetic protein-related protein (3, 4). Wnts and transforming growth factor-ß/bone morphogenetic proteins are important patterning molecules in vertebrate and invertebrate species, and studies of Drosophila and Caenorhabditis elegans have identified many of the evolutionarily conserved components of these signaling systems (5, 6).

Mutations affecting genes encoding proteins related to known glycosaminoglycan biosynthetic enzymes have also been described in Drosophila. sugarless shows striking homology to UDP-glucose dehydrogenase (7–9) and affects signaling mediated by multiple growth factors, including Wingless, Decapentaplegic, and the fibroblast growth factor receptor-related proteins Heartless and Breathless (10). sulfateless encodes a protein similar to N-deacetylase/N-sulfotransferase and is also required for Wingless-mediated and fibroblast growth factor receptor signaling (10, 11). Both sugarless and sulfateless mutations disrupt glycosaminoglycan-modification of Dally in vivo, supporting their assignment as glycosaminoglycan biosynthetic enzymes (3, 11). pipe, a gene required for establishing the embryonic dorsal-ventral axis, encodes a protein with significant homology to heparan sulfate 2-O-sulfotransferase genes in vertebrates (12). Finally, tout-velu (ttv), a gene related to the tumor suppressor genes, (EXT1 and 2) has been shown to affect events directed by Hedgehog, a Drosophila homolog of Sonic Hedgehog and Indian Hedgehog (13). It is not known, however, whether tout-velu, like the vertebrate EXT1 and 2 genes, encodes an enzyme with heparan sulfate co-polymerase activity (14).

Biochemical studies of proteoglycans from both Drosophila and C. elegans have been reported. In addition to the glypican, Dally, a Drosophila syndecan has been identified and shown to be heparan sulfate-modified (15). Heparan sulfate and chondroitin sulfate polymers have been detected in extracellular matrix preparations of Drosophila, material that could be radioabeled with 35SO4 and degraded with either chondroitinase ABC or nitrous acid (16). Other proteoglycan or proteoglycan-like molecules have been described in Drosophila, including DROP-1 (17), Papilin (18), and macrophage-derived proteoglycan-1 (a hemocyte/macroage-derived protein of the extracellular matrix) (19). A gene encoding a protein related to perlecan, unc-52, has been studied in some detail in C. elegans and shown to affect muscle attachment and sarcomere organization (20, 21).

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1 The abbreviations used are: dally, division abnormally delayed; ttv, tout-velu; HPLC, high performance liquid chromatography; EXT, exostin.
Although these findings collectively show that proteoglycans exist in Drosophila and C. elegans, performing critical functions during development, very little is known about the different glycosaminoglycan structures found in these organisms. A detailed understanding of proteoglycan and glycosaminoglycan functions in these systems will require structural information that can be used in conjunction with genetic and molecular data. We therefore devised a method for structural analysis of glycosaminoglycan-derived disaccharides suitable for the relatively small samples that can be easily obtained from these animals. We have applied these methods toward identifying tissue-specific and developmental stage-specific distributions of glycosaminoglycans, as well as to characterize glycosaminoglycans in animals bearing mutations in ttk, a gene proposed to affect heparan sulfate biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following standard unsaturated disaccharides from heparan sulfate were purchased from Sigma: 2-acetamido-2-deoxy-4-O-(4-deoxy-a-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA-GlcNAc), 2-deoxy-2-sulfamido-4-O-(4-deoxy-a-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA-GlcNAcS), 2-acetamido-2-deoxy-4-O-(4-deoxy-a-D-threo-hex-4-enopyranosyluronic acid)-6-O-sulfamido-D-glucose (ΔUA2S-GlcNAc), 2-deoxy-2-sulfamido-4-O-(4-deoxy-2-O-sulfamido-a-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA2S-GlcNAcS), 2-deoxy-2-sulfamido-4-O-(4-deoxy-2-O-sulfamido-a-D-threo-hex-4-enopyranosyluronic acid)-6-O-sulfamido-D-glucose (ΔUA2S-GlcNaCS), 2-deoxy-2-sulfamido-4-O-(4-deoxy-2-O-sulfamido-a-D-threo-hex-4-enopyranosyluronic acid)-6-O-sulfamido-D-glucose (ΔUA2S-GlcNaCS), 2-acetamido-2-sulfamido-4-O-(4-deoxy-a-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA-GlcNAc), and heparan sulfate from bovine kidney.

The following standard unsaturated disaccharides from chondroitin sulfate were obtained from Seikagaku America (Falmouth, MA): 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-D-glucose (ΔDI-HA), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-D-galactose (ΔDI-DS), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-6-sulfamido-D-galactose (ΔDI-DS), 2-acetamido-2-deoxy-3-O-(2-O-sulfamido-β-D-gluco-4-enopyranosyluronic acid)-D-glucose (ΔDI2S-GlcNAc), and 2-acetamido-2-deoxy-3-O-(2-O-sulfamido-β-D-gluco-4-enopyranosyluronic acid)-4,6-di-O-sulfamido-D-galactose (ΔDI2S-GlcNAc).

**Preparation and Enzymatic Digestion of Chondroitin Sulfate**—Adult whole animals or tissues were obtained by collection from agar plates, washed, and separated from E. coli on a 0.2% agarose gel gradient as described (24). For the analysis of both C. elegans and Drosophila, whole animals or tissues were first lyophilized to dryness. Approximately 20 mg of lyophilized sample was then homogenized with 1.0 ml of acetone. The homogenate was washed with acetone and dried. The pellet was extracted in 1.0 ml of 0.5% SDS, 0.1 M NaOH, 0.8% NaBH₄, for 16 h at room temperature with constant stirring. Two hundred μl of 1.0 M sodium acetate and 300 μl of 1 M HCl were then added, the solution was filtered, and 200 μl of 1 M HCl was added to the filtrate. Insoluble material was removed by centrifugation at 2500 × g for 10 min at 4 °C. Seven ml of ethanol was added to the supernatant and chilled for 2 h at 0 °C, and the crude glycosaminoglycan fraction collected by centrifugation at 2500 × g for 10 min at 4 °C. The resulting precipitate was dissolved in 250 μl of water, and the crude glycosaminoglycan solution was diluted to 100 μl with water and used for the determination of chondroitin sulfate. For chondroitinase digestion, 5 μl of 0.2 M Tris-acetate buffer (pH 8.0) and 10 μl of an aqueous solution containing chondroitinase ABC (50 mIU) and chondroitinase ACII (50 mIU) were added to a 20-μl portion of the sample solution and incubated at 37 °C for 3 h. An 8-μl portion of this mixture was loaded onto the high performance liquid chromatograph. For the analysis of Drosophila heparan sulfate, 5 μl of 0.1 M acetic acid buffer (pH 7.0) with 10 mM calcium acetate and 15 μl of an aqueous solution containing heparin lyase mixture (Seikagaku America), heparin lyase I (1 mIU), heparin lyase II (1 mIU), and heparin lyase III (1 mIU) were added to a 5-μl portion of sample. The mixture was incubated at 37 °C for 16 h, and an 8-μl aliquot was loaded onto the high performance liquid chromatograph. For the analysis of heparan sulfate from C. elegans, chondroitin was removed from the crude glycosaminoglycan solution prior to heparin lyase digestions by chondroitinase treatment followed by separation with Ultrafree-MC DEAE membrane. Enzymatic digestion with a heparin lyase mixture was then carried out as described above, with the exception that Sigma enzymes were used.

**Genetic Analysis of tout-velue**—We used tout-velue (tm-020082) mutants for our analysis, the only allele for which genetic studies have been described (13). Dpy[rj008] was obtained by collection from agar plates, washed, and separated from E. coli on a 0.2% agarose gel gradient as described (24). The following standard unsaturated disaccharides from heparan sulfate were purchased from Sigma: 2-acetamido-2-deoxy-4-O-(4-deoxy-a-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA-GlcNAc), 2-deoxy-2-sulfamido-4-O-(4-deoxy-a-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA-GlcNAcS), 2-acetamido-2-deoxy-4-O-(4-deoxy-a-D-threo-hex-4-enopyranosyluronic acid)-6-O-sulfamido-D-glucose (ΔUA2S-GlcNAc), 2-deoxy-2-sulfamido-4-O-(4-deoxy-2-O-sulfamido-a-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA2S-GlcNAcS), 2-deoxy-2-sulfamido-4-O-(4-deoxy-2-O-sulfamido-a-D-threo-hex-4-enopyranosyluronic acid)-6-O-sulfamido-D-glucose (ΔUA2S-GlcNaCS), 2-deoxy-2-sulfamido-4-O-(4-deoxy-2-O-sulfamido-a-D-threo-hex-4-enopyranosyluronic acid)-6-O-sulfamido-D-glucose (ΔUA2S-GlcNaCS), 2-acetamido-2-sulfamido-4-O-(4-deoxy-a-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA-GlcNAc), and heparan sulfate from bovine kidney.

The following standard unsaturated disaccharides from chondroitin sulfate were obtained from Seikagaku America (Falmouth, MA): 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-D-glucose (ΔDI-HA), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-D-galactose (ΔDI-DS), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enopyranosyluronic acid)-6-sulfamido-D-galactose (ΔDI-DS), 2-acetamido-2-deoxy-3-O-(2-O-sulfamido-β-D-gluco-4-enopyranosyluronic acid)-D-glucose (ΔDI2S-GlcNAc), and 2-acetamido-2-deoxy-3-O-(2-O-sulfamido-β-D-gluco-4-enopyranosyluronic acid)-4,6-di-O-sulfamido-D-galactose (ΔDI2S-GlcNAc).

**Preparation and Enzymatic Digestion of Heparan Sulfate**—To 230 μl of crude glycosaminoglycan sample, 50 μl of 0.3 M sodium phosphate buffer (pH 6.0) was added, and the solution was applied on an Ultrafree-MC DEAE membrane, which had been equilibrated with sodium phosphate buffer (pH 6.0) containing 0.15 M NaCl. The fractions eluted with 0.5 M NaCl in the same buffer were collected, desalted with Biomax-5, evaporated, and resuspended in 12 μl of water in preparation of heparin lyase digestion. For the analysis of Drosophila heparan sulfate, 5 μl of 0.1 M acetic acid buffer (pH 7.0) with 10 mM calcium acetate and 15 μl of an aqueous solution containing heparin lyase mixture (Seikagaku America), heparin lyase I (1 mIU), heparin lyase II (1 mIU), and heparin lyase III (1 mIU) were added to a 5-μl portion of sample. The mixture was incubated at 37 °C for 16 h, and an 8-μl aliquot was loaded onto the high performance liquid chromatograph. For the analysis of heparan sulfate from C. elegans, chondroitin was removed from the crude glycosaminoglycan solution prior to heparin lyase digestions by chondroitinase treatment followed by separation with Ultrafree-MC DEAE membrane. Enzymatic digestion with a heparin lyase mixture was then carried out as described above, with the exception that Sigma enzymes were used.

**RESULTS**

**Sample Preparation and HPLC for the Determination of Glycosaminoglycans**—We established a protocol for highly reproducible and sensitive HPLC analysis of unsaturated disaccharides from chondroitin sulfate and heparan sulfate in C. elegans and Drosophila. The coefficient of variation for each unsaturated disaccharide was less than 5% (adult Drosophila, n = 5). The lower determination limits of the HPLC for chondroitin sulfate and heparan sulfate were approximately 0.5 and 1.5 ng, respectively. All results described below were reproduced in at least duplicate experiments.

**Analysis of Glycosaminoglycans in C. elegans**—The complete digestion of material from C. elegans with both chondroitinase ABC and ACII released disaccharides that co-chromatographed with ΔDI-OS using reverse phase ion-pair chromatography (Fig. 4).
The identity of Di-0S was established using graphitized carbon chromatography, which can resolve Di-0S and Di-HA, showing that chondroitin is found in this invertebrate organism (data not shown) (25). Digestion with chondroitinase ACII alone generated the same chromatographic profile obtained with ACII plus ABC, indicating that the majority of chondroitin contains glucuronic acid and not iduronic acid (data not shown). We did not detect Di-4S, Di-6S, and other over-sulfated disaccharides found in vertebrate chondroitin sulfate in these unfractionated glycosaminoglycan preparations from C. elegans, nor were we able to detect Di-HA in our preparations. The compositions of unsaturated disaccharides produced from chondroitin in worms is listed in Table I.

The disaccharide profile of worm heparan sulfate we observed is relatively simple, with UA-GlcNS, UA2S-GlcNS, and UA2S-GlcNS6S species in nearly equal amounts and nonsulfated UA-GlcNAc representing about 50% of the total. The composition of disaccharides produced from heparan sulfates in worms, compared with that derived from bovine kidney, is provided in Table II. The identities of all disaccharides have been confirmed by comparison with standards using two HPLC separation methods, reversed phase ion-pair (23) and graphitized carbon chromatography, which can resolve Di-0S and Di-HA, showing that chondroitin is found in this invertebrate organism (data not shown) (25). Digestion with chondroitinase ACII alone generated the same chromatographic profile obtained with ACII plus ABC, indicating that the majority of chondroitin contains glucuronic acid and not iduronic acid (data not shown). We did not detect Di-4S, Di-6S, and other over-sulfated disaccharides found in vertebrate chondroitin sulfate in these unfractionated glycosaminoglycan preparations from C. elegans, nor were we able to detect Di-HA in our preparations. The compositions of unsaturated disaccharides produced from chondroitin in worms is listed in Table I.

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Analysis of Glycosaminoglycans from Drosophila—As for the analysis of glycosaminoglycan from *C. elegans*, the identities of *Drosophila* disaccharides were established by comparison with standards using two distinct separation methods, reverse phase ion-pair (23) and graphitized carbon chromatography (25). Digestion of unfractionated glycosaminoglycans from adult flies with chondroitinases yields disaccharides that co-chromatograph with ΔDi-0S and ΔDi-4S (Fig. 1C). As we observed for material from *C. elegans*, the equivalent release of disaccharides with chondroitinase ACII compared with digestion with both ACII and ABC indicates that the majority of polymers are glucuronate containing and hence derived form chondroitin sulfate, not dermatan sulfate (data not shown). The identity of the 4S species was confirmed by its conversion to ΔDi-0S with chondro-4-sulfatase. Chondroitin sulfate from adult flies has a relatively low degree of sulfation, and as in the human serum protein bikunin, only 4-O-sulfated disaccharides are represented (26). ΔDi-HA from *Drosophila* was not detectable using these methodologies. The disaccharide composition of adult *Drosophila* chondroitin sulfate is listed in Table I.

Heparan sulfate disaccharides are also found in glycosaminoglycan preparations from adult flies (Fig. 2C). Fly heparan sulfate shows a greater degree of complexity than samples from *C. elegans*, with ΔUA-GlcNAc, ΔUA-GlcNS, ΔUA-GlcNac6S, ΔUA-GlcNS6S, ΔUA2S-GlcNS, and ΔUA2S-GlcNS6S species generated by digestion with a mixture of heparin lyases I, II, and III. To determine whether the material we detected in *Drosophila* is a typical heparan sulfate polymer, we identified the disaccharides released by treatment with heparin lyase I or III. Heparin lyase I generated ΔUA-GlcNS6S, ΔUA2S-GlcNS6S disulfated disaccharides, and ΔUA2S-GlcNS6S trisulfated disaccharide (data not shown). Heparin lyase III generated ΔUA-GlcNac, ΔUA-GlcNS, ΔUA-GlcNac6S, and ΔUA-GlcNS disaccharides (data not shown). These findings demonstrate that material from *Drosophila* has heparin lyase disaccharide profiles typical of heparan sulfate and is not the unusual glycosaminoglycan found in the snail *Achatina fulica* that is resistant to heparin lyase I and III digestion (27). Overall, the proportion of sulfated disaccharides is high, representing about 69% of total compared with 47% in bovine kidney. The composition of unsaturated disaccharides produced from adult *Drosophila* heparan sulfates is given in Table II.

Analysis of Drosophila Tissues and Developmental Stages—In vertebrates, structural variants of glycosaminoglycans show tissue-specific distributions (28, 29). To determine whether *Drosophila* tissues also show reproducible differences in glycosaminoglycans we examined different tissues and developmental stages in parallel samples from whole adult bodies, ovaries, embryos, and third instar larvae. We detected reproducible differences in the disaccharide profiles of heparan sulfates isolated from *Drosophila* (Fig. 3 and Table III). For example, third instar larvae show a reduced percentage of ΔUA-GlcNS, compared with embryos and adults. Embryos show higher relative amounts of ΔUA-GlcNS6S compared with larvae, adults, or ovaries. Ovaries show a significantly higher proportion of ΔUA2S-GlcNS. The ratios of heparan sulfate to chondroitin sulfate also vary widely in *Drosophila*. The ovary showed the greatest proportion of heparan sulfate (heparan sulfate:chondroitin sulfate, 0.74), with larvae showing the lowest (0.06). The degree of 4-O-sulfation of chondroitin sulfate also differed among tissues, ranging from 36% in the ovary to 11% in whole adult flies (Table I).

Analysis of tout-velu, a Gene Related to the Vertebrate Heparan Sulfate Co-polymerases EXT1 and EXT2—One of the utilities of structural analyses of glycosaminoglycans in *Drosophila* and *C. elegans* is to examine the effects of removing specific gene functions on the sugar polymers synthesized in an intact animal. We have used the analytical methods we developed to examine glycosaminoglycans in animals bearing mutations in *ttv*, a gene with 56% amino acid identity to the vertebrate tumor suppressor gene, *EXT1* (13). Both *EXT1* and 2 have been shown to encode enzymes with heparan sulfate co-polymerase activity, suggesting that *ttv* may also affect the synthesis of heparan sulfate (14). We examined glycosaminoglycans in third instar larvae homozygous for a null allele of *ttv*. *ttv* mutant larvae show a markedly reduced amount of heparan sulfate with the same disaccharide composition as wild type. Chondroitin sulfate, however, is unaffected by *ttv*, consistent with *ttv* encoding a heparan sulfate-specific co-polymerase.

**DISCUSSION**

*Drosophila* and *C. elegans* as Model Organisms for Studying Proteoglycan and Glycosaminoglycan Functions—Many studies in *Drosophila* have documented the important role of proteoglycans in developmental patterning. Yet at the structural level, little is known about glycosaminoglycans in *Drosophila* or *C. elegans*, another model organism that offers a powerful array of genetic and molecular tools. We describe here a sensitive method for analysis of disaccharides derived from chondroitin and heparan sulfates in these model organisms.

Analysis of Chondroitin Sulfate—Chondroitin polymers are found in adult *Drosophila* and *C. elegans*.*Chondroitin*-derived disaccharides in *C. elegans* were not sulfated, and only ΔDi-4S was detected in *Drosophila*. This is in contrast to chondroitin sulfate found in cartilage from a wide range of animals, including the squid, in which the vast majority of disaccharides released by chondroitinase treatment are sulfated at either the 4 or 6-O position (30). Equivalent release of disaccharides with chondroitinase ABC and ACII, or ACII digestion alone, suggests that dermatan sulfate is either not found, or represented at very modest levels in *Drosophila* and *C. elegans*.

It is interesting that the profiles of disaccharides generated by chondroitinase treatment of *C. elegans* and *Drosophila* ma-
glycosaminoglycans in Drosophila and C. elegans

Fig. 3. A comparison of Drosophila heparan sulfate from different tissues and developmental stages. A, chromatograms of a, ovaries; b, embryos; c, larvae and d, adults. Other conditions were as described for Fig. 2. B, percentages of unsaturated disaccharides from Drosophila heparan sulfates: different tissues and developmental stages. Row a, ovaries; row b, embryos; row c, larvae; row d, adults. Open boxes, \(\Delta U-A\) GlcNAc; dotted boxes, \(\Delta U-A\) GlcNS; light striped boxes, \(\Delta U-A\) GlcNAc6S; medium striped boxes, \(\Delta U-A\) GlcNS6S; heavy striped boxes, \(\Delta U-A\) 2S-GlcNS; filled boxes, \(\Delta U-A\) 2S-GlcNS6S.

Material resemble those generated from treatment of human bikunin (26), an abundant serum protein component of the inter-\(\alpha\)-trypsin inhibitor family of protease inhibitors (reviewed in Ref. 31). This suggests that the chondroitin-modified proteins in these invertebrates may include protease regulators. In fact, a gene with striking homology to mouse bikunin is found in C. elegans, showing greater than 40% amino acid identity over a stretch of 100 amino acids (GenBank\textsuperscript{TM} accession number U64857). The C. elegans gene encodes a protein most similar to tissue factor pathway inhibitor, a member of the bovine pancreatic trypsin inhibitor/Kunitz family of protease inhibitors.

Analysis of Heparan Sulfate—Heparin lyase treatment of glycosaminoglycans from Drosophila and C. elegans releases disaccharides found in vertebrates. Our analysis provides the first direct evidence for disaccharides bearing \(N\)-, 2-O-, and 6-O-sulfations in these organisms. We did not detect \(\Delta U\) 2S-GlcNAc or \(\Delta U\) 2S-GlcNAc6S in either of these organisms using the methods that we developed for microdetermination of glycosaminoglycans, but it remains possible that these forms exist, albeit at levels below our current detection limits. Our methods, using heparin lyase digestion of small quantities of crude glycosaminoglycans, is not suitable for detection of 3-O-S sequences. We plan further characterization of heparan sulfate from Drosophila and C. elegans using larger scale preparation of purified material and NMR spectroscopy.

Our findings show that enzymes required for biosynthesis and modification of heparan sulfate must exist in Drosophila and C. elegans. Indeed, genes encoding proteins with significant homology to EXT1 (13), \(N\)-deacetylasen-sulfotransferase (10), C5 glucuronyl epimerase (GenBank\textsuperscript{TM} accession number P46555), and heparan-sulfate sulfotransferase (12, 32) enzymes from vertebrates are represented in Drosophila and C. elegans. Our analysis of \(ttv\) (see below) indicates that like its vertebrate homolog, EXT1, Ttv affects heparan sulfate biosynthesis. Structural studies of glycosaminoglycans from animals bearing mutations in these genes will show which genes are required for the generation of specific glycosaminoglycan forms in vivo.

One of the most striking features of glycosaminoglycans is their structural diversity, with discrete structural variants found in different tissues (28, 33). Specific forms of heparan sulfate are also associated with different disease states and ages (29, 34). These findings suggest that different forms of heparan sulfate are important for influencing the biological function of the associated proteoglycan. To determine whether Drosophila could provide a model system for exploring the function of different heparan sulfate structural variants, we examined glycosaminoglycans from different developmental stages and tissues. Indeed, Drosophila does show tissue- and stage-specific modifications of both heparan and chondroitin sulfate. For example, levels of \(\Delta U\) GlcNS6S are relatively higher in embryos compared with larvae and adults. Given the importance of GlcN 6-O-sulfate groups for binding several growth factors (reviewed in Ref. 33) the levels of these disaccharides are potentially important in regulating growth factor signaling throughout development (36, 37).

The differences in 2-O-sulfated disaccharides in the ovary compared with other tissues and stages is worthy of note. Recently it has been shown that pipe, a gene required for establishing dorsal-ventral polarity in the embryo, encodes a protein related to heparan sulfate 2-O-sulfotransferase (12). pipe is expressed only in the ventral follicle cells of the ovary that surround the developing oocyte, and is required for the proteolytic activation of the protein ligand, Spätzle. Spätzle in turn activates Toll, a Drosophila homologue of the vertebrate interleukin 1 receptor that specifies ventral cell fates in the future embryo (reviewed in Ref. 38). We demonstrate here that 2-O-sulfated heparan sulfate disaccharides are found in the ovary, in a proportion distinct from that found in embryos, whole adults, or larvae. These findings show that a heparan sulfate 2-O-sulfotransferase must exist in Drosophila, and the homology of pipe to vertebrate proteins with this activity
TABLE III

Compositions of unsaturated disaccharides produced from heparan sulfates in Drosophila

|                | ΔUA\(\text{GlcNAc}\) | ΔUA\(\text{GlcNS}\) | ΔUA\(\text{GlcNAc6S}\) | ΔUA\(\text{GlcNS6S}\) | ΔUA\(\text{2S-GlcNS}\) | ΔUA\(\text{2S-GlcNS6S}\) | Total amount | In dry tissue | In body |
|----------------|---------------------|---------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------|-------------|---------|
| Ovary          | 29.8                | 28.2                | 1.1                     | 14.9                    | 20.8                    | 5.2                     | 41.6        | 1.7         |
| Embryo         | 30.8                | 27.4                | 2.0                     | 23.3                    | 12.0                    | 4.5                     | 26.0        | 0.054       |
| Larva (3rd)    | 38.6                | 20.6                | 1.6                     | 19.2                    | 15.3                    | 5.6                     | 11.9        | 4.4         |
| Wild type      | 30.8                | 26.8                | 3.1                     | 18.3                    | 17.4                    | 3.6                     | 27.3        | 5.5         |
| 
| Wild type      | ND                  | ND                  | ND                      | ND                      | ND                      | ND                      | ND          | ND          |
| ttt/ttv         | ND                  | ND                  | ND                      | ND                      | ND                      | ND                      | ND          | ND          |
| Adult          | 30.8                | 26.8                | 3.1                     | 18.3                    | 17.4                    | 3.6                     | 27.3        | 5.5         |

* ND, not detected.

TABLE IV

Ratios of heparan sulfate to chondroitin sulfate in Drosophila

|                | Total amount (HS) | Total amount (CS) | HS/CS |
|----------------|-------------------|-------------------|-------|
|                | In dry tissue     | In body           |       |
|                | ng/mg             | ng/mg             |       |
| Ovary          | 41.6              | 1.7               | 0.741 |
| Embryo         | 26.0              | 0.054             | 0.274 |
| Larva (3rd)    | 11.9              | 4.4               | 0.057 |
| Wild type      | 10.6              | 3.1               | 0.644 |
| ttt/CyO        | ND                | ND                | ND    |
| ttt/ttv        | ND                | ND                | ND    |
| Adult          | 27.3              | 5.5               | 0.101 |

* ND, not detected.

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Fig. 4. Analysis of heparan sulfate from Drosophila bearing mutations in ttt. A, wild type; B, ttt/CyO; C, ttt/ttv. Other conditions were as described for Fig. 2.
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