Heparan sulfate, one of the most abundant components of the cell surface and the extracellular matrix, is involved in a variety of biological processes such as growth factor signaling, cell adhesion, and enzymatic catalysis. The heparan sulfate chains have markedly heterogeneous structures in which distinct sequences of sulfate groups determine specific binding properties. Sulfation at each different position of heparan sulfate is catalyzed by distinct enzymes, sulfotransferases. In this study, we identified and characterized Drosophila heparan sulfate 6-O-sulfotransferase (dHS6ST). The deduced primary structure of dHS6ST exhibited several common features found in those of mammalian HS6STs. We confirmed that, when the protein encoded by the cDNA was expressed in COS-7 cells, it showed HS6ST activity. Whole mount in situ hybridization revealed highly specific expression of dHS6ST mRNA in embryonic tracheal cells. The spatial and temporal pattern of dHS6ST expression in these cells clearly resembles that of the Drosophila fibroblast growth factor (FGF) receptor, breathless (btl). RNA interference experiments demonstrated that reduced dHS6ST activity caused embryonic lethality and disruption of the primary branching of the tracheal system. These phenotypes were reminiscent of the defects observed in mutants of FGF signaling components. We also show that FGF-dependent mitogen-activated protein kinase activation is significantly reduced in dHS6ST double-stranded RNA-injected embryos. These findings indicate that dHS6ST is required for tracheal development in Drosophila and suggest the evolutionarily conserved roles of 6-O-sulfated heparan sulfate in FGF signaling.

Heparan sulfate proteoglycans are ubiquitously present on the cell surface and in the extracellular matrix and are known to be involved in a variety of biological phenomena, including cell proliferation, differentiation, cell adhesion, angiogenesis, blood coagulation, lipid metabolism, and viral and bacterial infections. These diverse mechanisms of action are achieved by interactions between the specific structures of heparan sulfate and the binding proteins. The backbone of heparan sulfate is a polysaccharide chain composed of alternating D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine units, and some GlcA residues are converted into L-iduronic acids. The microheterogeneity of the heparan sulfate structures is mainly produced by the nonrandom introduction of N-, 2-O-, 6-O-, and 3-O-sulfate groups (1, 2). Thus, the biological functions of heparan sulfate proteoglycans are controlled by biosynthetic events, which define the fine structures of heparan sulfate.

For many years, functional studies of structurally complex heparan sulfate have focused on vertebrate tissues and cultured cells. Recent studies, however, have shown that heparan sulfate is also found in simple model organisms such as Drosophila melanogaster and Caenorhabditis elegans (3). The structural features of heparan sulfate in Drosophila are similar to those found in vertebrates. In addition, the analyses of heparan sulfate in Drosophila mutants have shown that the biosynthesis apparatus is also conserved in this organism (4). Therefore, on the basis of their homology to vertebrate heparan sulfate-modifying genes, it is now possible to analyze the function of modified heparan sulfate in vivo using these genetically tractable organisms.

Recently, genetic studies on mutants for heparan sulfate sulfotransferases indicated that the regulated synthesis of heparan sulfate affects morphogenesis during development. Bullock et al. (5) reported that heparan sulfate 2-O-sulfotransferase (HS2ST)† knock-out mice showed renal agenesis as well

† The abbreviations used are: HS2ST, heparan sulfate 2-O-sulfotransferase; HSPG, heparan sulfate proteoglycan; HS6ST, heparan sulfate 6-O-sulfotransferase; FGF, fibroblast growth factor; dARNT, Drosophila aryl hydrocarbon receptor nuclear translocator; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; CDSNS-heparin, completely desulfated and N-sulfated heparin; NS-heparosan, deacetylated and N-sulfated heparosan; CDNSNa-ceramic, completely desulfated N-acetylated heparan; ΔUA-GlcNAc, 2-acetamido-2-deoxy-α-D-4-(4-deoxy-a-L-threo-hex-epi-hexopyranosyluronic acid)-D-glucose; ΔUA-GlcNS, 2-deoxy-2-sulfamido-4-O-(4-deoxy-a-L-threo-hex-epi-hexopyranosyluronic acid)-D-glucose; ΔUA-GlcNAc6S, 2-acetamide-2-deoxy-α-D-4-(4-deoxy-a-L-threo-hex-epi-hexopyranosyluronic acid)-D-glucose; ΔUA-GlcNS6S, 2-deoxy-2-sulfamido-4-O-(4-deoxy-a-L-threo-hex-epi-hexopyranosyluronic acid)-D-glucose; ΔUA2S-GlcNS6S, 2-deoxy-2-sulfamido-4-O-(4-deoxy-a-L-threo-hex-epi-hexopyranosyluronic acid)-D-glucose; HPLC, high performance liquid chromatography; ENG, Engelbreth-Holm-Swarm; PCR, polymerase chain reaction; BDGP, Berkeley Drosophila Genome Project; DPE, downstream promoter element; RNAi, RNA interference; FGF, fibroblast growth factor receptor; bFGF, basic fibroblast growth factor; EST, expressed sequence tag; CNS, central nervous system; OPC, outer proliferative center; dMAPK,
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as defects in eyes and skeleton. In Drosophila, pipe encodes a protein that is structurally similar to HS2ST, and the ventral expression of pipe in the somatic cells of ovary is required for the formation of embryonic dorsoventral polarity (6). Mutations in sulfatless (sfl), which encodes a heparan sulfate N-deacetylase/N-sulfotransferase, affect Wingless, FGF, and Hedgehog signaling pathways during development (7–9). These findings have shown that differentially modified heparan sulfate can regulate growth factor signaling.

It is well established that heparan sulfate binds to bFGF and is required for the activation of the signal transduction pathway. However, the mechanism by which heparan sulfate promotes bFGF action is not clearly understood. It has been variously proposed that heparan sulfate induces a conformational change in bFGF (10), serves as a bridge between bFGF and FGFR (11), or promotes dimerization of FGFRs (12). It has been reported that specific fine structures of heparan sulfate have a critical role for binding to bFGF. Biochemical studies have shown that N- and O-sulfation is required for binding of bFGF to heparan sulfate (13). On the other hand, studies using biological assays have shown that, in addition to 2-O-sulfation, 6-O-sulfation is required for the activation of bFGF signaling (14, 15). This may reflect the fact that heparan sulfate interacts with not only FGF's but also their receptors. Recent crystallographic analysis also supported the idea that 6-O-sulfation promotes the dimerization of FGFRs (16).

In Drosophila, one FGF ligand, Branchless (Bnl), and two FGFRs, Heartless (Htl) and Breathless (Btl) have been identified and characterized (17–19). btl and bnl are required for the normal development of the Drosophila respiratory system, branching trachea that deliver air to all tissues (19, 20). The Drosophila tracheal system shares many features with the vertebrate vascular system and is therefore regarded as a simple model to study tubulogenesis (21). Branching morphogenesis in both Drosophila and mammals is regulated by growth factors FGF and vascular endothelial growth factor (22, 23), cadherins (24, 25), and hypoxia-inducible factor-1-like basic helix-loop-helix transcription factors (26–28). Heparan sulfate is also critical for both Drosophila tracheogenesis and vertebrate angiogenesis (7, 29).

In this study, we examined the structure and function of Drosophila 6-O-sulfotransferase (dHS6ST). We present here the cDNA and deduced amino acid sequences of dHS6ST and the characterization of its enzymatic activity. In situ hybridization of dHS6ST mRNA revealed that dHS6ST is expressed in specific patterns in several tissues. During embryogenesis, dHS6ST mRNA was detected in developing tracheal precursor cells that also express a Drosophila FGF receptor, btl. Blocking the dHS6ST activity by double-stranded RNA interference caused embryonic lethality and defects in the migration of the tracheal cells. In addition, Btl-dependent activation of MAPK, which is seen normally in tracheal cells, is significantly decreased in these embryos. These results suggested the possible involvement of 6-O-sulfated heparan sulfate in the modulation of FGF signaling during tracheogenesis in Drosophila.

EXPERIMENTAL PROCEDURES

Fly Strains and Chemicals—Oregon-R was used as a wild type of Drosophila melanogaster. 1-ve-1 is an enhancer trap line at the tracheal-less (trh) locus (30). A Drosophila cDNA library was obtained from CLONTECH. A rabbit anti-p-galactosidase was purchased from Cappel. A mouse anti-dpMAPK was purchased from Sigma. [35S]SIPAPS was prepared as described previously (31). Unlabeled PAPS was from Sigma, diphosphorylated mitogen-activated protein kinase; MAPK, mitogen-activated protein kinase; CME, central midline element; dsRNA, double-stranded RNA.
halocarbon oil. Early embryos of 1-eve-1, the tracheless (trh)-lacZ line, were collected within 2 h of egg laying and injected with dHS6ST double-stranded RNA (dsRNA) or a buffer only. Injected embryos were allowed to develop at 25 °C for 11 h to observe tracheal phenotypes or for 7 h to detect MAPK activation, and subjected to the staining procedure.

**Immunohistochemistry**—β-Galactosidase expressed in 1-eve-1 enhancer trap embryos was detected using rabbit polyclonal antiserum (Cappel) at a 1:500 dilution in PBT (5% goat serum, 0.3% Triton X-100) and DPE are indicated by underline and double underline, respectively. Consensus sequences of the initiator and DPE are shown below the corresponding regions. The asterisk represents the position of 5′ end of the obtained dHS6ST cDNA.

**RESULTS**

Cloning of dHS6ST cDNA and Genomic Organization of the dHS6ST—A genomic sequence, AE003728, which has been mapped to 92C on the third chromosome (BDGP), was identified as a clone bearing sequences with significant homology to mouse heparan sulfate 6-O-sulfotransferase-1 (mHS6ST-1) (35). We named this gene presumptive Drosophila heparan sulfate 6-O-sulfotransferase (dHS6ST). To isolate a dHS6ST cDNA, a Drosophila larval cDNA library was screened with the PCR-amplified genomic fragment as a probe. From ~2 × 10^5 plaques, four independent positive clones were obtained and sequenced.

Comparison of the dHS6ST cDNA and the AE003728 genomic sequences revealed that dHS6ST spans 79.0 kilobase pairs of the genome and contains six exons separated by five introns, with the second intron being more than 67 kilobase pairs in size (Fig. 1A). Around the genomic region corresponding to the 5′ end of the cloned dHS6ST cDNA, we identified the sequence TCAGTT, which matches the Drosophila consensus sequence for an initiator “TCA(G/T)T(T/C)” (+1 site is underlined, Fig. 1B) (41–43). Although a typical TATA box was not found in this region, the sequence GGACGT is highly similar to the downstream promoter element (DPE), which is contained in many TATA-less promoters in Drosophila, at a typical position from the initiator sequence (+28 to +34 in Fig. 1B) (44). The occurrence of these sequences predicted the transcription start site as the A residue shown by +1 in Fig. 1B. The cDNA clone that we have sequenced therefore appears to be nearly full-length. Northern blot analysis showed that dHS6ST encodes a single major transcript of ~3.1 kilobase pairs (data not shown).

BDGP suggested the existence of several genes around the dHS6ST locus (Fig. 1A). miranda, which encodes a cytoskeletal structural protein affecting the asymmetric division of neuroblasts, was contained within in the fifth intron of dHS6ST (45, 46). An EST clone, GH26202, derived from an adult head cDNA library, was mapped to the second intron of the dHS6ST gene. Furthermore, the 5′ sequence of an EST clone, LD07688, was found to start at 499 base pairs upstream of the 5′ end of dHS6ST in a reverse direction.

**Primary Structure of dHS6ST**—The complete cDNA and predicted amino acid sequences for dHS6ST are presented in Fig. 2A. The amino-terminal sequence contains three in-frame ATG codons. Out of three ATGs, the third one appears to be the most likely initiation codon, judging from the Drosophila consensus translation initiation sequence (47). The pattern of the hydrophathy plot for the amino acid sequence started from the third ATG also matches those for mammalian homologues of dHS6ST (Fig. 2B) (35, 48). The predicted protein was composed of 432 amino acid residues with two putative N-linked glycosylation sites. A hydrophathy plot of dHS6ST showed the type II transmembrane structure of a Golgi resident protein with one prominent hydrophobic segment in the amino-terminal region that extends from amino acid residue 9 to 20.

A comparison of the primary structures of mammalian and Drosophila HS6STs revealed that the amino acid sequence of dHS6ST is 53%, 54%, 46%, and 53% identical to the sequences of human HS6ST (hHS6ST), mouse HS6ST-1, -2, and -3 (mHS6STs), respectively. Although relatively low levels of similarity were observed in both the amino- and carboxyl-terminal regions among HS6STs, the amino acid sequences of the central region are highly conserved (Fig. 3). Putative PAPS binding sites were present in these regions of all these proteins (49, 50). Furthermore, all HS6STs bear two N-glycosylation sites at conserved positions. The predicted amino acid sequence of dHS6ST therefore shows all the structural features of HS6STs reported previously.

**Characterization of Heparan Sulfate 6-O-Sulfotransferase Activity of dHS6ST**—To determine whether the isolated dHS6ST cDNA encodes a protein with HS6ST activity, we examined the activity of dHS6ST expressed in tissue culture cells. dHS6ST cDNA was inserted in a mammalian expression vector, pFLAG-CMV2, and COS-7 cells were transfected with this construct or with pFLAG-CMV2 alone as a control. HS6ST activity in the cells transfected with dHS6ST was approximately twice as high as that with the control vector, whereas the dHS6ST cDNA insert did not increase the HS2ST activity (Table I). These results confirmed that recombinant dHS6ST produces HS6ST activity.

To further analyze this enzyme, we purified the epitope-
tagged dHS6ST protein and examined the sulfotransferase activity using several heparin derivatives, heparan sulfates, and chondroitin as substrates (Table II). The dHS6ST was able to transfer sulfate to CDSNS-heparin, NS-heparosan, heparin, and heparan sulfate but was marginally active using CDSNAc-heparin and chondroitin as substrates. The ratio of the activity of dHS6ST toward NS-heparosan to that toward CDSNS-heparin was 0.36. Since NS-heparosan contains only glucuronic acid and not its epimer iduronic acid, this result suggests a preference of dHS6ST for iduronic acid adjacent to the targeted N-sulfoglucosamine.

We analyzed the structures of the 35S-labeled products obtained by incubation with purified dHS6ST and CDSNS-heparin or heparan sulfate from a mouse EHS tumor (Fig. 4). In both products, most of the radioactivity in the disaccharide fractions was at the position of DUA-GlcNS6S. We also found a small peak at the position of DUA2S-GlcNS6S from heparan sulfate. These results showed that dHS6ST transfer sulfate preferentially to position 6 of GlcNSO3 residues. Since it has been reported that the substrate specificity of mHS6ST-2 is affected by the concentration of the substrates (35), we analyzed the dependence of the substrate preference of dHS6ST on substrate concentration. The ratio of the sulfotransferase activity toward NS-heparosan to that toward CDSNS-heparin was low at all concentrations tested (data not shown), suggesting the substrate specificity of dHS6ST is insensitive to the concentrations of acceptor substrate. Taken together, our findings indicated that dHS6ST shows HS6ST activity with preference for specific substrates.

Expression Patterns of dHS6ST mRNA in Embryos and Imaginal Discs—The expression patterns of dHS6ST mRNA were determined by in situ hybridization of whole-mount embryos and imaginal discs (Fig. 5). We observed high levels of accumulation of dHS6ST mRNA in syncytial blastoderm stage embryos indicating that dHS6ST is a maternally supplied product (Fig. 5A). During early gastrulation, zygotic expression of dHS6ST was detected in many tissues, including the invaginating mesoderm (Fig. 5B). In the stage 10 embryos, we detected highly specific expression of dHS6ST mRNA in tracheal precursor cells (Fig. 5C). dHS6ST expression in the invaginating tracheal precursor cells is maintained through stage 12 (Fig. 5, D and E). This spatial and temporal expression pattern of dHS6ST in tracheal precursor cells was strikingly similar to that of breathless (btl), a Drosophila FGF receptor (18). By stage 16, however, dHS6ST became uniformly expressed in various tissues (Fig. 5F). The restricted expression pattern of dHS6ST may reflect the requirements of the dHS6ST function in tracheal development.

In Drosophila, another FGF receptor homologue is encoded by heartless (htl) (17). htl is expressed in the invaginating
Fig. 3. Comparison of the amino acid sequences of human, mouse, and Drosophila HS6STs. Drosophila and mammalian HS6STs are aligned using the program ClustalW (57). Indicated regions (5'PSB and 3'PSB) are highly conserved among virtually all HS6STs that are predicted to serve in 3'-phosphoadenosine 5'-phosphosulfate (PAPS) binding (49, 50). Arrowheads indicate conserved potential N-glycosylation signals. Consensus residues (*) are indicated for each position where five HS6STs exhibit identical amino acids.

TABLE I

| Plasmids          | HS6ST | HS2ST |
|-------------------|-------|-------|
|                   | pmol/min/mg protein |            |
| pFLAG-CMV2        | 1.8 ± 0.2 | 4.6 ± 0.5 |
| pFLAG-CMV2-dHS6ST | 3.6 ± 0.8 | 3.8 ± 0.1 |

TABLE II

| Substrates       | Relative sulfotransferase activity of dHS6ST
|------------------|-----------------------------------|
|                  | %                                |
| CDSN-heparin     | 100                               |
| CDSNac-heparin   | 2.2                               |
| NS-heparan       | 39.5                              |
| Heparan sulfate (mouse EHS tumor) | 28.0                           |
| Heparan sulfate (pig norta) | 12.8                           |
| Chondroitin      | 1.5                               |

mesoderm during embryogenesis, in the morphogenetic furrow of the eye disc and surrounding the outer proliferative center (OPC) of the larval CNS (51). The expression pattern of dHS6ST partially overlapped with that of hitl in these tissues (Fig. 5, B, H, and I). In third instar larvae, dHS6ST mRNA was detected in actively dividing neuroblasts, including cells anterior to the morphogenetic furrow of the eye disc (Fig. 5H) and OPC of the CNS (Fig. 5I). This observation suggests the possible involvement of dHS6ST in hitl-mediated neuronal development. We also observed high levels of expression of dHS6ST mRNA throughout the wing disc (Fig. 5G) and in the lobula of the CNS (Fig. 5I). The similarity of expression patterns between dHS6ST and Drosophila FGF receptors raises the possibility that 6-O-sulfated heparan sulfate is required for all FGF signaling during Drosophila development.

dHS6ST Double-stranded RNA Inhibits FGF Signaling during Tracheal Formation—To elucidate the function of dHS6ST, we performed RNA interference (RNAi), a gene silencing strategy mediated by dsRNA (39). Approximately 75% of the control embryos, which had been injected with buffer only, hatched normally, while the remaining control embryos died owing to mechanical trauma (Table III). Most of the dHS6ST dsRNA-injected embryos (94%) died prior to the point immediately before hatching, although they survived to stage 17, the final stage of embryogenesis (Table III). This observation suggested that dHS6ST is essential for viability at late embryonic stages. Since whole mount in situ hybridization revealed expression of dHS6ST in the tracheal precursor cells, we tested whether disruption of dHS6ST activity affects tracheogenesis. The tracheal system of the Drosophila embryo form by a sequential series of branching steps that can be visualized by following the expression of 1-eve-1, an enhancer trap line for the tracheless (trh) gene (26, 52). We injected dsRNA into the 1-eve-1 embryos at the syncytial blastoderm stage and collected embryos at stage 13, when primary branches continue to grow toward their targets. In 58% of dHS6ST dsRNA-injected embryos, branch formation was disordered while the levels of 1-eve-1 expression seemed to be comparable to the control embryos (Table III and Fig. 6). Twenty-five percent of these embryos exhibited stalled branches and disconnected dorsal trunks in all segments. The remaining 33% of dHS6ST dsRNA-injected embryos showed migration defects of the dorsal branch or lateral trunk in one to several segments. In buffer-injected embryos, 21% showed minor tracheal defects, and no control embryos showed severe tracheal disruption as described for dHS6ST dsRNA-injected animals (Table III). These results indicated that blocking dHS6ST activity disrupted the branching of tracheal precursor cells without affecting tracheal cell differentiation.

Expression patterns of dHS6ST mRNA and the tracheal
tracheal precursor cells at stage 10 (A) and stage 11 (C) dHS6ST stages (D). During early gastrulation, zygotic expression of accumulation of products derived from CDSNS-heparin (A) and heparan sulfate (B). The arrows indicate the elution positions of ΔUA-GlcNAc6S (1), ΔUA-GlcNS (2), ΔUA-GlcNS6S (3), ΔUA2S-GlcNS (4), and ΔUA2S-GlcNS6S (5).

**Fig. 6.** Disruption of dHS6ST function by RNA interference caused the branching defects of the tracheal system. I-eve-1 embryos, which bear an enhancer trap P element insertion in the trh gene, were injected with buffer (A) or dHS6ST dsRNA (B). Tracheal phenotypes were observed at stage 13 by staining with anti-β-galactosidase antibody. Arrows in B point to sites of misguided or stalled branching. C and D, the effect of injection of dHS6ST dsRNA on the activity of MAPK at the tips of the migrating tracheal precursor cells of stage 11 embryos was examined. When buffer was injected as a control, most embryos showed normal diphospho-MAPK signals in tracheal precursor cells around tracheal pit (C). Injection of dHS6ST dsRNA disrupted activation of MAPK (D). Arrowheads in C and D indicate tracheal pits. Anterior of the embryos are oriented to the left, and embryos are shown in lateral views.

| Injected materials | Phenoypes | Lethality (%) | Tracheal abnormality | Reduction of MAPK activation |
|--------------------|-----------|---------------|---------------------|-----------------------------|
| Buffer             | 25        | 21            | 26                  |                             |
| dHS6ST dsRNA       | 96        | 58            | 58                  |                             |

phenotypes of dHS6ST dsRNA-injected embryos suggested that dHS6ST is involved in btl-mediated signaling. To test this possibility, we examined the effects of injection of dHS6ST dsRNA on the activity of MAPK, a downstream transducer of FGF signaling. Activated form of MAPK can be visualized by staining with anti-dpMAPK antibody (53, 54). When buffer was injected as a control, most of the embryos showed normal MAPK activation in tracheal precursor cells, while a modest reduction of MAPK activation was observed in 26% of embryos (Table III and Fig. 6C). In contrast, in 58% of dHS6ST dsRNA-injected embryos, anti-diphospho-MAPK immunoreactivity around tracheal pit was below the level of detection (Fig. 6D).

Our findings strongly suggest that 6-O-sulfated heparan sul-
Drosophila Heparan Sulfate 6-O-Sulfotransferase

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Tracheal development.

We have shown here that dHS6ST is expressed in embryos and imaginal tissues. The expression of dHS6ST mRNA during development coincides with FGF-responsive cells known to synthesize Drosophila FGFRs, breathless (btl) and heartless (htl). Particularly in embryonic stages, the pattern of dHS6ST expression in the tracheal cells is analogous to those of btl. Activation of several genes required for tracheal tubular formation, including btl, is regulated by trachealless (trh), which encodes a transcription factor belonging to the basic helix-loop-helix-PAS (Per-ARNT-sim) protein family (26, 55). The heterodimers of Trachealless and Drosophila aryl hydrocarbon receptor nuclear translocator (dARNR) bind to the specific sequence, TACGTT, in the btl regulatory region and regulate its expression in tracheal cells as well as midline precursor cells (55). We identified a Trachealless/dARNR binding site in the first intron of dHS6ST, suggesting that the expression of dHS6ST might be regulated in the same fashion as that of btl by these transcription factors (Fig. 7).

RNAi experiments showed that dHS6ST is required for normal tracheal formation. The expressivity of the phenotypes observed in dHS6ST dsRNA-injected embryos was variable, from stalled primary branches in all segments to migration of 6-desulfated heparin, which binds to bFGF but

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