The formation of heparan sulfate (HS) chains is catalyzed by glycosyltransferases encoded by EXT (hereditary multiple exostosis gene) family members. Genetic screening for mutations affecting morphogen signaling pathways in Drosophila has identified three genes, tout-velu (ttv), sister of tout-velu (sotv), and brother of tout-velu (botv), which encode homologues of human EXT1, EXT2, and EXT3, respectively. So far, in vitro glycosyltransferase activities have been demonstrated only for BOTV/DEXT3, which harbors both N-acetylgalactosaminyltransferase-I (GlcNAcT-I) and N-acetylgalactosaminyltransferase-II (GlcNAcT-II) activities responsible for the chain initiation and elongation of HS, and no glucuronyltrans- ferase-II (GlcAT-II) activity. Here we demonstrated that TTV/ DEXT1 and SOTV/DEXT2 had GlcNAcT-II and GlcAT-II activities required for the biosynthesis of repeating disaccharide units of the HS backbone, and the coexpression of TTV with SOTV markedly augmented both glycosyltransferase activities when compared with the expression of TTV or SOTV alone. Moreover, the polymerization of HS was demonstrated on a linkage region analogue as an acceptor substrate by BOTV and an enzyme complex composed of TTV and SOTV (TTV-SOTV). In contrast to human, TTV-SOTV accepts an acceptor substrate by BOTV and an enzyme complex composed of TTV and SOTV (TTV-SOTV). In contrast to human, TTV-SOTV accepts an acceptor substrate by BOTV and an enzyme complex composed of TTV and SOTV (TTV-SOTV).

In HS biosynthesis, the tetrasaccharide sequence (GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1-) is first assembled on a specific serine residue of a core protein. HS polymerization then occurs with GlcNAc and GlcUA transferred by the action of an enzyme complex consisting of EXT1 (6) and EXT2 (7) of the EXT (exostosin) gene family (8, 9). These genes are involved in hereditary multiple exostosis, which is an autosomal dominant disorder characterized by the formation of a cartilage-capped tumor, caused by mutations in either EXT1 or EXT2 (10). The family of EXT genes has been extended to include three EXT-Like genes, EXT1L1, EXT1L2, and EXT3L3 (10–14). Among the three EXT proteins, we demonstrated that EXT1L1 possessed only GlcNAc transferase II (GlcNAcT-II) activity, which is responsible for HS chain elongation (15), whereas EXT2L2 shows GlcNAc transferase I (GlcNAcT-I) activity, which is involved in HS chain initiation (16–18). Most interestingly, it has been revealed that EXT3L3 possesses both GlcNAcT-I and GlcNAcT-II activities, which are involved in HS chain initiation and elongation (15). Although EXT-Like genes are thought to be involved in the synthesis of HS on the basis of their enzymatic activities in vitro, none have been linked to hereditary multiple exostosis. Therefore, the functions of EXT-Like genes in vivo are still unknown.

EXT genes have been well conserved between humans and Drosophila. In Drosophila, three EXT family genes named tout-velu (ttv), sister of tout-velu (sotv), and brother of tout-velu (botv), which encode homologues of human EXT1, EXT2, and EXT3, respectively, have been identified (19–23). Mutations in ttk, sotv, and botv impaired Hh, Wg, and Dpp signaling activities, as well as the distribution of morphogens as a result of defective biosynthesis of HS in vivo (21). Han et al. (22) also demonstrated that botv null embryos exhibited stronger segment polarity phenotypes than sotv null embryos. Moreover, biochemical and immunohistochemical studies in Drosophila have revealed that HS levels are dramatically reduced in the absence of TTV, SOTV, or BOTV (21–23). Nonetheless, in vitro glycosyltransferase activities have been demonstrated only for BOTV/DEXT3, which harbors both GlcNAcT-I and GlcNAcT-II activities involved in the initiation and elongation of HS, and no GlcAT-II activity (24). Recently, Han et al. (22) demonstrated that TTV and SOTV form a complex and colocalize in vivo, which are properties similar to those of vertebrate EXT1 and EXT2. Here we investigated HS polymerization by using recombinant soluble forms of ttk, sotv, and botv in combination to elucidate the mechanism behind the biosynthesis of HS in Drosophila, and we demonstrated that the polymerization was performed by a complex of TTV and SOTV (TTV-SOTV) as an enzyme source. These results indicated that the mechanism of polymerization by TTV-SOTV was analogous to that recently shown for human EXT1 and EXT2. Moreover, we showed that BOTV/DEXT3 had a critical role as a trigger of initiation of HS synthesis on the tetrasaccharide linkage region. These findings led us to conclude that TTV, SOTV, and BOTV play essential roles in the biosynthesis of full-length HS in Drosophila.

**EXPERIMENTAL PROCEDURES**

Materials—UDP-[U-14C]GlcUA (285.2 mCi/mmol) and UDP-[3H]GlcNAc (60 Ci/mmol) were purchased from PerkinElmer Life Sciences. Unlabeled UDP-GlcUA and UDP-GlcNAc were obtained from Sigma. *Flavobacterium heparinum* heparinase I was purchased from "in accordance with 18 U.S.C. Section 1734 solely to indicate this fact."

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank(TM)/EBI Data Bank with accession number(s) AB281331.

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The abbreviations used are: HS, heparan sulfate; EXT, hereditary multiple exostoses gene; Cbz, benzoyloxycarbonyl; GlcNAcT-I and GlcNAcT-II, α-GlcNAc transferase I and II; GlcAT, glucuronyltransferase; MES, 2-(N-morpholino)ethanesulfonic acid; Wt, Wnt; Wingless; Hh, Hedgehog; Dpp, decapentaplegic.
Seikagaku Corp. (Tokyo, Japan). GlcUAβ1–3Galβ1–O-C₆H₄NHCbz was chemically synthesized (25). N-Acetylheparosan oligosaccharides derived from the capsular polysaccharide of Escherichia coli K5 were prepared as described previously (26). A Superdex 75 HR10/30 column was supplied by Amersham Biosciences. pCasper-hsp-ttv, which can rescue ttv-deficient homozygous flies, was from N. Perrimon (Harvard Medical School, Boston) (19).

Construction of Soluble Forms of TTV and SOTV—A cDNA fragment of a truncated form of TTV, lacking the first 41 amino-terminal amino acids, including the putative cytoplasmic and transmembrane domains, was amplified with pCasper-hsp-ttv as a template using a 5′-primer (5′-GAAGATCTGCAAGAGATGGTGGTTC-3′) containing an in-frame BglII site and a 3′-primer (5′-CGGGATCCGCTCTGTGTTT-GGGGGGAGAAG-3′) containing a BglII site located 40 bp downstream from the stop codon. In the case of SOTV, the cDNA fragment encoding a truncated form of SOTV, lacking the first amino-terminal 53 amino acids, including the putative cytoplasmic and transmembrane domains, was amplified with Drosophila EST clone (ID GH02288) as a template using a 5′-primer (5′-CGGGATCCGCTCTGTTGCTTGCTT-CATGG-3′) containing an in-frame BamHI site and a 3′-primer (5′-CGGGATCCGCTCTGTTGCTTGCTTGCGCC-3′) containing a BamHI site located 40 bp downstream of the stop codon. PCR was carried out with KOD polymerase (Toyobo, Osaka, Japan) for 30 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 180 s in 5% (v/v) dimethyl sulfoxide. The PCR fragment was cloned into the BamHI site of pGIR201protA (27), resulting in the fusion of the insulin signal sequence and the protein A sequence present in the vector as described previously (24). The nucleotide sequence of the amplified cDNA was determined in a 377 DNA sequence PE (Applied Biosystems).

Expression of a Soluble Form of TTV, SOTV, or BOTV—The expression plasmid (10.0 μg) was transfected into COS-1 cells on 100-mm plates using SuperFect® (Qiagen) according to the manufacturer’s instructions. For cotransfection experiments, TTV-SOTV, TTV-BOTV, or SOTV-BOTV expression plasmids (5.0 μg each) were cotransfected into COS-1 cells on 100-mm plates using SuperFect medium was collected and incubated with 10 μl of IgG-Sepharose (Amersham Biosciences) for 4 h at 4°C. The beads recovered by centrifugation were washed with phosphate-buffered saline, and then resolved on 7.5% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated for 1 h with mouse IgG antibody. The mouse IgG antibody was diluted 1:1,000 with 25 mM Tris-buffered saline. The bound antibody was detected with mouse IgG conjugated to horseradish peroxidase.

Measurement of Glycosyltransferase and Polymerization Activities—After a 3-day culture at 28°C, 10 ml of the culture medium was collected and incubated with 10 μl of IgG-Sepharose (Amersham Biosciences) for 1 h at 4°C. The beads recovered by centrifugation were washed with phosphate-buffered saline, and then resolved on 7.5% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated for 1 h with mouse IgG antibody. The mouse IgG antibody was diluted 1:1,000 with 25 mM Tris-buffered saline. The bound antibody was detected with mouse IgG conjugated to horseradish peroxidase.

Western Blot Analysis—After 3 days of culture at 28°C, the culture medium was collected and incubated with 10 μl of IgG-Sepharose (Amersham Biosciences) for 1 h at 4°C. The beads recovered by centrifugation were washed with phosphate-buffered saline, and then resolved on 7.5% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated for 1 h with mouse IgG antibody. The mouse IgG antibody was diluted 1:1,000 with 25 mM Tris-buffered saline. The bound antibody was detected with mouse IgG conjugated to horseradish peroxidase.

Measurement of Glycosyltransferase and Polymerization Activities—After a 3-day culture at 28°C, 10 ml of the culture medium was collected and incubated with 10 μl of IgG-Sepharose (Amersham Biosciences) for 1 h at 4°C. The beads recovered by centrifugation were washed with phosphate-buffered saline, and then resuspended in the assay buffer and tested for glycosyltransferase activities as described below. For GlcNAcT activity, the reaction mixture with a total volume of 20 μl contained 10 μl of the resuspended beads, 0.25 mM UDP-[14C]GlcNAc (3.3 × 10⁶ dpm), 100 mM MES buffer, pH 5.8, 10 mM MnCl₂, and either GlcUAβ1–3Galβ1–O-C₆H₄NHCbz (250 nmol) (24) or N-acetylheparosan oligosaccharides with the nonreducing terminal GlcUA, [GlcUA-GlcNAc]ₙ, as acceptors. For GlcAT-II activity, the reaction mixture with a total volume of 20 μl contained 10 μl of the resuspended beads, 0.25 mM UDP-[14C]GlcUA (2.5 × 10⁶ dpm), 100 mM MES buffer, pH 5.8, 10 mM MnCl₂, and N-acetylheparosan oligosaccharides, GlcNAc-[GlcUA-GlcNAc]ₙ, with the nonreducing terminal GlcNAc as an acceptor. Polymerization reactions using N-acetylheparosan oligosaccharides, [GlcUA-GlcNAc]ₙ, with the nonreducing terminal GlcUA as acceptors, were conducted in incubation mixtures containing the following constituents in a total volume of 20 μl: 10 μg of N-acetylheparosan oligosaccharides with the nonreducing terminal GlcUA, 0.25 mM UDP-[14C]GlcUA (5.28 × 10⁵ dpm), 0.25 mM UDP-GlcNAc, 100 mM MES buffer, pH 5.8, and 10 μl of MnCl₂. The mixtures were incubated at 30°C overnight, and the 14-C-labeled products were then separated by gel filtration chromatography on a Superdex 75 column equilibrated and eluted with 0.2 M NH₄HCO₃. Fractions (0.4 ml each) were collected at a rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

Characterization of the Reaction Products—Products of polymerization reactions on N-acetylheparosan oligosaccharides, [GlcUA-GlcNAc]ₙ, with the nonreducing terminal GlcUA were isolated by gel filtration on a Superdex 75 column as described above. The radioactive peak containing the TTV and SOTV reaction product was pooled and evaporated to dry. The [14C]GlcUA-labeled oligosaccharides chains were exhaustively digested with 30 μIU of heparitinase I in a total volume of 50 μl of 20 mM sodium acetate buffer, pH 7.0, containing 2 mM calcium acetate at 37°C overnight. The enzyme digest was analyzed using the Superdex 75 column as described above.

Measurement of Polymerization on a Linkage Region Oligosaccharide Analogue by TTV, SOTV, and BOTV—First, a GlcNAc transfer reaction was conducted using the linkage region oligosaccharide analogue, GlcUAβ1–3Galβ1–O-C₆H₄NHCbz (250 nmol) as an acceptor (24), in an incubation mixture containing the following constituents in a total volume of 20 μl: 10 μl of the soluble form of BOTV-bound beads as enzyme source, 0.25 mM UDP-GlcNAc, 100 mM MES buffer, pH 5.8, 10 mM MnCl₂. The reaction products were then added to the polymerization reaction mixture containing the following constituents in a total volume of 40 μl: 0.25 mM UDP-[14C]GlcUA (5.28 × 10⁵ dpm), 0.25 mM UDP-GlcNAc, 100 mM MES buffer, pH 5.8, 10 mM MnCl₂, and 10 μl of the resuspended beads (TTV-SOTV, TTV-BOTV, or SOTV-BOTV). Each mixture was incubated at 30°C overnight, and the 14C-labeled products were then separated by gel filtration chromatography on a Superdex peptide column equilibrated and eluted with 0.2 M NH₄HCO₃.
NH₄HCO₃. Fractions (0.4 ml each) were collected at a flow rate of 0.4 ml/min, and the measurement of radioactivity was carried out by liquid scintillation counting.

RESULTS

Expression of TTV and SOTV—In previous studies, three EXT family homologues have been identified and named ttv/DEXT1 (CG10117, GenBank™ accession number AB221351), sov/DEXT2 (CG8433, GenBank™ accession number AF145598), and botv/DEXTL3 (CG15110, GenBank™ accession number AB077850). The soluble form of botv was reported to exhibit GlcNAcT-I and -II activities, which are involved in the initiation and elongation of HS, but showed no GlcAT-II activity (24). However, no such glycosyltransferase activities have been detected in addition to the band for BOTV. Thus, TTV and SOTV proteins were unstable at 37 °C, and so the glycosyltransferase activities of TTV and SOTV could not be detected.

Functional Analysis of a Soluble Form of TTV or SOTV—To facilitate the functional analysis of TTV and SOTV, glycosyltransferase activities were measured by using a soluble form of TTV or SOTV as an enzyme source. Each of the protein A-fused proteins expressed in the medium was adsorbed onto IgG-Sepharose beads to eliminate endogenous glycosyltransferases, and the protein-bound beads were then used as an enzyme source. Both bound fusion proteins transferred GlcNAc and GlcUA to N-acetylgalactosamino oligosaccharides [GlcoA-GlcNAc]₃ and GlcNAc-[GlcoA-GlcNAc]₄ respectively, indicating that TTV and SOTV had GlcNAcT-II and GlcAT-II activities, which are involved in the synthesis of the repeating disaccharide units of HS. Moreover, coexpression of the soluble TTV with the soluble SOTV augmented both GlcNAcT-II and GlcAT-II activities when compared with the expression of TTV or SOTV alone (Table 1). These results were analogous to the findings made for human EXT1 and EXT2 (8, 9). However, coexpression of the soluble BOTV with the soluble TTV or SOTV augmented neither GlcNAcT-II nor GlcAT-II activity when compared with the expression of BOTV, TTV, or SOTV alone. These results were consistent with the findings that TTV and SOTV are physiologically associated but do not interact with BOTV (22).

Identification of Polymerization Products—In our previous study, the polymerization of HS in vitro was demonstrated using EXT1 and EXT2.
together as an enzyme source, on an artificial linkage region analogue with a truncated glycosaminoglycan-protein linkage tetrasaccharide, GlcUAβ1–3Galβ1–O-C3H4NHCbz, as an acceptor (9). Hence, it was next investigated whether the coexpression of TTV and SOTV shows HS polymerization activity. Unexpectedly, no polymerization was detected on the incubation of TTV-SOTV with GlcUAβ1–3Galβ1–O-C3H4NHCbz, or GlcUAβ1–3Galβ1–O-C3H4NHCbz as an acceptor in the presence of both UDP-GlcNAc and UDP-[14C]GlcUA. It was predicted that because TTV-SOTV might not have chain-fraction decasaccharides derived from chondroitin, respectively (32). The total volume was at 20-mer in length. The radioactive peaks containing cotransfectant (TTV-SOTV) reaction products were digested with heparitinase I and subjected to gel filtration chromatography as described under “Experimental Procedures.” The radiolabeled products were completely digested by heparitinase I, quantitatively yielding a 14C-labeled peak at the position of [14C]GlcUAα1–4GlcNAc, indicating that the polymerization occurred on N-acetylated when TTV and SOTV were coexpressed (Fig. 2B). Moreover, a few saccharide residues, about 6-mer long, were synthesized on N-acetylated when TTV coexpressed with TTV or SOTV was used as an enzyme source, suggesting that some HS chains were synthesized even in the absence of either TTV or SOTV. The results might account for the residual staining of heparitinase-treated ttv mutant embryos with the 3G10 antibody, which recognizes the heparitinase-generated unsaturated uronic acid epitope (20). To compare the chain length synthesized by all the combinations, the size-defined oligosaccharide, GlcUAβ1–3Galβ1–O-C3H4NHCbz, was used. The linkage region analogue, GlcUAβ1–3Galβ1–O-C3H4NHCbz, was incubated with the recombinant soluble BOTV protein, and then polymerization activity was measured using each of the three combinations, TTV-SOTV, TTV-BOTV, and SOTV-BOTV, as an enzyme source as described under “Experimental Procedures.” As shown in Fig. 3, HS polymerization was demonstrated by all the three combinations, TTV-SOTV, TTV-BOTV, and SOTV-BOTV, as an enzyme source. Moreover, these results altogether indicated that all three Drosophila EXT family members could together achieve polymerization on a linkage region.

**DISCUSSION**

EXT genes have been well conserved between *Drosophila* and humans. So far, these *Drosophila* EXT family genes named tout-velu (ttv, sister of tout-velu (sotv)), and brother of tout-velu (botv), which
encode *Drosophila* homologues of human EXT1, EXT2, and EXT3, respectively, have been identified (19–23). In this study, we demonstrated that the polymerization of HS in *Drosophila* was achieved by an enzyme complex composed of TTV and SOTV (TTV-SOTV), which is analogous to findings made recently for the human EXT1 and EXT2 protein complex (Fig. 4, A and B) (9). In contrast to human EXT1-EXT2, which possess GlcNAcT-I activity for chain initiation, TTV-SOTV exhibited no GlcNAcT-I activity (Table 1), indicating that BOTV/ DEXT3, which is an EXT-Like gene and possesses GlcNAcT-I activity, is indispensable for the biosynthesis of HS chains in *Drosophila*. Thus, all three EXT members in *Drosophila*, TTV, SOTV, and BOTV, appear to be essential for the biosynthesis of HS.

An indispensable role for TTV, SOTV, or BOTV in HS biosynthesis has also been suggested by the observations that the synthesis is diminished or abolished in *Drosophila* bearing a mutation in *ttv*, *sotv*, or *botv* (21–23). In addition, mutations in *ttv*, *sotv*, and *botv* seriously impaired Hh, Wg, and Dpp signaling activities as well as the distribution of these morphogens (21). Thus, these results also indicate that HS is an important regulator of morphogen signaling and the tissue distribution of these morphogens. Notably, Han et al. (22) demonstrated that *botv* null embryos exhibited stronger segment polarity phenotypes than *sotv* null embryos. These results suggested that a mutation in *botv* led to impaired HS biosynthesis because the mutants do not have GlcNAcT-I activity for chain initiation on the linkage region (Fig. 5B). Also, The et al. (20) reported residual staining with a monoclonal antibody 3G10 in *ttv* embryos. These results are consistent with the present finding that HS polymerization occurred when BOTV and TTV or SOTV was used as an enzyme source, although the efficiency of polymerization was low (Fig. 5D). Moreover, Han et al. (22) demonstrated that Wg signaling is defective only in the *botv* mutant or *ttv*-sotv double mutant but not in the *ttv* or *sotv* mutant, whereas the distribution of the Wg morphogen is abnormal in the *ttv*, *sotv*, and *botv* mutants. Despite the existence of a small amount of HS in the *ttv* or *sotv* mutant (Fig. 5D), signaling events performed by Wg were abrogated. These results suggested that there might be a threshold amount of HS for the reception of signaling molecules.

In mammals, the polymerization of HS occurs with GlcNAc and GlcUA transferred alternately by the action of an enzyme complex consisting of EXT1 (6) and EXT2 (7) from the EXT gene family (Fig. 4A) (8, 9). These genes are involved in hereditary multiple exostosis, which is an autosomal dominant disorder characterized by the formation of a cartilage-capped tumor, caused by mutations in either EXT1 or EXT2 (10). EXT1- and EXT2-deficient mice generated by gene targeting failed to undergo gastrulation and died by embryonic day 8.5. Moreover, embryonic stem cells from EXT1- and EXT2-deficient mice showed a complete loss of HS (28, 29). These results indicate that EXT1 and EXT2 are essential for both gastrulation and the biosynthesis of HS early in embryonic development. Recently, it has been reported that EXT1 mutant mice generated by the gene trap method (EXT1<sup>Glu/Glu</sup>) survive to embryonic day 14.5 (30). We revealed that the embryonic fibroblasts from EXT-deficient mice generated by the gene targeting method still produce short HS chains as compared with those from wild-type mice (31). We suggested that because EXT1 gene trap mouse fibroblasts produced small amounts of normal EXT1 transcript, gene trap mice still produce some HS, albeit much less than normal. Thus, the EXT1 gene trap mice survive longer than the EXT1-deficient mice. These results suggested, as in the case of *Drosophila*, that the amount or length of HS chains is the critical determinant of embryonic mouse development, and the function of HS is altered by the amount or chain length.

The family of mammalian EXT genes has been extended by the identification of three EXT-Like genes, EXT1L1, EXT2L2, and EXT3L3 (11–14). Although the EXT-Like genes are predicted to be involved in the synthesis of HS on the basis of their enzymatic activities in *vitro* (15, 17), the functions of these genes *in vivo* have not been demonstrated. In fact, the polymerization of HS on the linkage analogue was achieved by an enzyme complex of EXT1 and EXT2, without the aid of EXTL proteins (8, 9). Most interestingly, the HS chains synthesized on the linkage analogues by the EXT1-EXT2 complex were much longer than those from naturally occurring HS. Thus, it is predicted that these EXTL proteins might regulate the size of HS chains *in vivo*.

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