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Embryonic expression of a *decapentaplegic* gene in the  
oligochaete annelid *Tubifex tubifex*

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Setal sacs

## Abstract

We have cloned and characterized the expression of a *decapentaplegic* homologue (designated *Ttu-dpp*) from the oligochaete annelid *Tubifex tubifex*. RT-PCR analysis and in situ hybridization revealed that *Ttu-dpp* begins to be expressed around the time of the onset of ectodermal germ band (GB) elongation (i.e., the onset of gastrulation). At this time, *Ttu-dpp* expression is detected in the anteriormost part of the GBs. As development proceeds and the GBs elongate, the domain of *Ttu-dpp*-expressing cells extends posteriorly. Then *Ttu-dpp*-expressing cells within the GB are divided into two groups: one group occurs along the ventral midline and coincides with the domain of ventral ganglia; the other is located more dorsally. The latter group of *Ttu-dpp*-expressing cells subsequently undergoes dorsalward expansion, which results in the formation of a lateral stripe of cells in every segment except the first (i.e., segment I). In embryos that undergo body elongation (that is one of the last morphogenetic movements occurring prior to hatchout), *Ttu-dpp* expression in the lateral region is confined to setal sacs, which are arranged in [the same transverse plane](#) around the periphery of each segment (except segment I).

*Keywords:* Annelid; *Tubifex tubifex*; *decapentaplegic* genes; Germ bands; Segments; Setal sacs

## 1. Introduction

*Decapentaplegic* (*dpp*) and *BMP2/4* (vertebrate version of *dpp*) encode signaling molecules (ligands) from the TGF- $\beta$  family. Dpp/BMP2/4 have been known to play a central role in specification of dorsoventral axis during embryonic development in *Drosophila* and *Xenopus* (DeRobertis and Sasai, 1996). Developmental significance of these ligands has also been implicated in cell fate specification in ascidians and axis specification during *Drosophila* imaginal disc development (Lawrence and Struhl, 1996; Miya et al., 1997). In insects (other than fruit fly) and spiders, it has also been suggested that Dpp is involved in leg development, which is accomplished by budding from the embryo proper (Sanchez-Salazar et al., 1996; Niwa et al., 2000; Akiyama-Oda and Oda, 2003; Prpic et al., 2003; Yamamoto et

al., 2004).

Despite its obvious importance, the expression pattern of *dpp* in lophotrochozoans has only been described for two molluscs, *Ilyanassa obsoleta* (Lambert and Nagy, 2002) and *Patella vulgata* (Nederbragt et al., 2002). The former authors reported that *Ilyanassa dpp* mRNA, which associates with centrosomes temporarily, segregate asymmetrically to specific blastomeres during early cleavage stages, while the latter authors confined their observation to the larval stage, and reported that *Patella dpp* is expressed in cells surrounding the shell-forming cells.

In this study, we have isolated a *dpp* homologue from another lophotrochozoan, *Tubifex tubifex* (oligochaete annelid) and examined its expression from 1-cell stage through juveniles.

## 2. Results and discussion

### 2.1. Cloning of *Tubifex* homologue of *decapentaplegic*

Using a set of degenerate primers, we amplified a *decapentaplegic* (*dpp*) homologue from *Tubifex tubifex* cDNA generated from mixed embryonic stages. The amplified fragment was 263 bp long, and the deduced amino acid sequence aligned well with mollusc Dpp and vertebrate BMP2/4 with more than 60% identity (Fig. 1A). An analysis of the phylogenetic relationships of the predicted amino acid sequence indicated that it clusters (though with low bootstrap support) with Dpp, as opposed to other members of BMP family (Fig. 1B). We concluded that this fragment was a portion of the *T. tubifex dpp* orthologue designated *Ttu-dpp*.

We extended the *Ttu-dpp* fragments using gene-specific primers and 3'RACE. The fragments we obtained included the stop codon, 3'untranslated region (73 bp) and a poly-A tail. The initial clone and extended sequences combined gave a 340-bp sequence encoding 88 amino acids (accession number [AB192888](#)). To our knowledge, this is the first *dpp* homologue isolated in an annelid.

### 2.2. Temporal expression profile

To estimate the relative levels of *Ttu-dpp* expression during embryonic development, we performed semi-quantitative RT-PCR with gene-specific primers.

The results are shown in Fig. 2. *Ttu-dpp* transcripts are present at a detectable level in stage 13 embryos (see Fig. 3A), but not in earlier embryos. The transcripts are also present in older embryos up to stage 18; it appears that as development proceeds, the level of *Ttu-dpp* transcripts becomes higher slightly. The results suggest that *Ttu-dpp* begins to be transcribed zygotically around the time of the beginning of gastrulation, i.e., the beginning of germ band elongation.

### 2.3. Spatial expression pattern

The first sign of *Ttu-dpp* expression was detected at the anteriormost part of the ectodermal germ band (GB) in stage 13 embryos (Fig. 4D). In earlier embryos (stages 1-12), there was no indication of *Ttu-dpp* expression (Fig. 4A-C). In embryos processed for *in situ* hybridization with a sense probe, no staining was detected from stage 1 through stage 18 (data not shown).

As the GBs elongate and coalesce with each other along the ventral midline in embryos at stages 13-15 (Fig. 3A-C), the domain of *Ttu-dpp*-expressing cells extended posteriorly along the GB, though these were confined to the anterior half of the GB (Fig. 4E, F). During these stages, there were no regional differences in staining intensity along the dorsoventral axis of the GB.

As embryos enter stage 16 (during which they undergo the first episode of body elongation; Fig. 3D-F), *Ttu-dpp*-expressing cells that comprised the GB became organized in two longitudinal belts on either side of the embryo. One (designated a ventral belt hereafter) was running along the ventral midline and coincided with the domain of ventral ganglia; the other was located more dorsally. These two belts of cells were separated from each other by a zone of *Ttu-dpp*-negative cells (Fig. 4G). The dorsally located belt of *Ttu-dpp*-expressing cells appeared as a chain of cell clusters (as indicated by arrows in Fig. 4G). Interestingly, such a cell cluster was not seen in the first segment (segment I; square bracket in Fig. 4G).

As development proceeds during stage 16, the ectodermal GB (and mesodermal GB) expands dorsally in the anterior part of the embryo, which is accompanied by body elongation therein (Fig. 3D-F). As Fig. 4H shows, *Ttu-dpp*-expressing cells were distributed broadly along the dorsoventral axis in each segment of the anterior part of the embryo. At this stage again, segment I was found to be devoid of such lateral *Ttu-dpp*-expressing cells (Fig. 4H). It seems likely that at

least a part of such lateral cells are derived from previous cell clusters in the dorsal belt.

In stage 17 embryos (Fig. 3G, H), *Ttu-dpp*-expressing cells were organized in two bilateral pairs of dot-like structures in each segment. Furthermore, these structures were arranged in the same transverse plane around the periphery of each segment (Fig. 5A, B). Such *Ttu-dpp* structures were first found in segments II-IV (at early stage 17) and, as development proceeded, they became recognizable in more posterior segments as well (Fig. 5A, B). It should be noted that such dot-like structures were absent in segment I (see Fig. 5A, B). In stage 18 embryos (Fig. 3I) and newly hatched juveniles, such dot-like structures were hardly seen except that particular segments (VII-IX) occasionally exhibited these structures (Fig. 5E).

Close examination showed that the dot-like structures were located in the interior region, but not on the embryo's surface and that they were composed of units of conical shape (see Fig. 5D). If viewed head on, such units individually appeared as a stained circle (Fig. 5C). Dorsally located dot-like structures were comprised of a single stained unit in every segment (Fig. 5D). The ventrally located structures seen in segments II-IX were composed of two stained units (Fig. 5C), while the remaining, posteriorly located dot-like structures were each comprised of a single unit (Fig. 5C). For instance, in embryos at stage 17b (Fig. 3H) which exhibited 21 ventral dot-like structures, the first eight ones (i.e., those in segments II-IX) were found to be composed of two units, while the remaining 13 structures consisted of a single unit. At present, it is unclear whether ventral dot-like structures in posterior segments come to be composed of two units during subsequent development.

Judging from the spatial pattern of their occurrence, it is highly possible that *Ttu-dpp*-positive dot-like structures correspond to setal (chaetal) sacs, which are small groups of cells that produce setae. In fact, similar *Ttu-dpp*-positive structures were detected at the base of setae in some anterior segments of juveniles (a few days after hatchout) (Fig. 5E). In *Tubifex tubifex*, setal sacs occur in every segment of the body except the first and the last (Dixon, 1915), and they are located in the mesodermal region even though they are derived from ectodermal cells originated from the P and Q teloblast lineages (Goto et al., 1999).

### 3. Experimental procedures

### 3.1. Embryos

Embryos of the freshwater oligochaete *Tubifex tubifex* were obtained as described previously (Shimizu, 1982) and cultured at 22°C. For experiments, embryos were freed from cocoons in the culture medium (Shimizu, 1982). Unless otherwise stated, all experiments were carried out at room temperature (20-22°C).

### 3.2. RNA Isolation and cDNA synthesis

Total RNA was isolated from *Tubifex* embryos by using ISOGEN (Nippon Gene) according to the manufacturer's recommendations. Poly (A)<sup>+</sup> RNA was isolated from total RNA using Oligotex-MAG (TaKaRa) according to the manufacturer's instructions. Isolated total RNA and poly (A)<sup>+</sup>RNA were dissolved in RNase-free sterilized H<sub>2</sub>O and stored at -80°C until use. First-strand cDNA was synthesized with a *NotI*-dT18 primer using Time Saver cDNA Synthesis Kit (Amersham Pharmacia Biotech) according to the manufacturer's protocols.

### 3.3. Degenerate PCR

To clone the *decapentaplegic* (*dpp*) gene of *T. tubifex*, we designed degenerate PCR primers based on the amino acid sequences conserved among the *dpp* class genes of insects (*Drosophila melanogaster*, *Schistocerca americana*, *Tribolium castaneum* and *Gryllus bimaculatus*) and *Caenorhabditis elegans*. Forward primers, F1 and F2, corresponded to amino acid sequences LYVDF and W(D/N/Q)DWI(M/I/V)AP, respectively; a reverse primer, R1, corresponded to V(E/L/V)(G/S)CGC. Nucleotide sequences of these primers were 5'-CTNTAYGTNGAYTT-3' (F1), 5'-TGGVAIGAYTGGATVRTNGCN CC-3' (F2), and 5'-CARCAICCRCAISHNWNAC-3' (R1). Primary PCR was performed with primers F1 and R1. Amplification parameters were: 5 min at 95°C, 30 cycles of 60 sec at 95°C, 60 sec at 34°C and 60 sec at 72°C, and final 60-sec extension at 72°C. Nested PCR was performed on 1 µl of this reaction with primers F2 and R1. Amplification parameters for the nested PCR were the same as those for the primary PCR except that the annealing temperature was 53°C.

The PCR products were separated by agarose gel electrophoresis, purified with ultrafree-DA filters (MILLIPORE), and directly cloned into pCR 2.1 TOPO vector

using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The inserted DNA fragments were sequenced using BigDye Terminator v3.1 Cycle Sequencing kit with an ABI Prism 377 DNA Sequencer (Applied Biosystems).

#### 3.4. 3' Rapid amplification of cDNA ends (RACE)

To isolate the 3' portion of *dpp* transcripts, 3' RACE was performed using gene specific primers (GSPs) and an adaptor primer. Nucleotide sequences of these primers were 5'-GCTGGCTATGATGCGTC-3' (GSP1), 5'-CGACCACGCACAGGTTC-3' (GSP2), and 5'-AACTGGAAGAATTTCGCGGCC-3' (adaptor primer). Amplification was carried out with Elongase Enzyme Mix (Invitrogen) and cDNA synthesized with *NotI*-dT18 primer. Primary amplification was performed with GSP1 and adaptor primer. Amplification parameters were: 35 cycles of 94°C for 60 sec, 55°C for 60 sec and 68°C for 4 min. This PCR product was used as a template for nested PCR with GSP2 and adaptor primer; amplification parameters were the same as those for the primary PCR except that the annealing temperature was 63°C. Purification, subcloning and sequencing of amplicons were performed as described above.

#### 3.5. Relative quantification of gene expression by RT-PCR

The mRNA was prepared from *T. tubifex* embryos at selected developmental stages (using 200 embryos for each sample) and reverse transcribed into single-strand cDNA (First-Strand cDNA Synthesis Kit; Amersham Biosciences), which was used as a template for RT-PCR. The amount of cDNA used for each of the following PCR reactions was equivalent to that resulting from 10 embryos. PCR was performed on cDNA with primers GSP1(5'-GCTGGCTATGATGCGTC-3') and GSP3 (5'-CCTCAGTACAACA GTTCC-3'). Amplification parameters were: 30 cycles of 95°C for 30 sec, 52°C for 30 sec and 72°C for 60 sec.  $\beta$ -actin was used as positive control. Primers ( $\beta$ -actinF1 and  $\beta$ -actinR1) for amplification of  $\beta$ -actin were designed from conserved amino acid sequences, WDDMEKI and FEQEMA, respectively. Nucleotide sequences of these primers were 5'-TGGGAYGAYATGGARAARAT-3' ( $\beta$ -actinF1) and 5'-GCCATYTC YTG YTCRAA-3' ( $\beta$ -actinR1). Amplification parameters for  $\beta$ -actin were the same as those for *dpp*, except that the number of cycles was 25. Reactions were agarose-gel electrophoresed, stained with ethidium bromide, and photographed under UV light.

### 3.6. Whole-mount *in situ* hybridization

Digoxigenin (DIG)-labeled RNA probes were prepared according to the protocols described by Ogasawara et al. (2001). [An antisense and sense riboprobes were synthesized with T7 and Sp6 RNA polymerases, respectively.](#) Whole-mount *in situ* hybridization was performed according to the method described previously (Matsuo et al. 2005) with slight modifications. Hybridization (36 h) and subsequent wash were both performed at 55°C.

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Figure legends

Fig. 1. Characterization of *Ttu-dpp*, a *decapentaplegic* homologue from *Tubifex tubifex*. (A) Alignment of the C-terminal domain of Ttu-Dpp with known Dpp/BMP2/4 class proteins. Asterisks represent amino acid identity. Numbers in parentheses indicate the percentage amino acid identity with Ttu-Dpp. (B) Molecular phylogenetic relationship of Ttu-Dpp to other TGF- $\beta$  family proteins. The phylogenetic tree was generated by the neighbor joining method using PAUP\*4.0b10 (Swofford 1998). Numbers are bootstrap values (as percentages of 1000 replications). Lengths of branches are drawn to the scale indicated. Species abbreviations: Ate, *Arachaeearanea tepidariorum* (spider); Cel, *Caenorhabditis elegans* (nematode); Dme, *Drosophila melanogaster* (fruit fly); Hsa, *Homo sapiens* (human); Iob, *Ilyanassa obsoleta* (mollusc); Mmu, *Mus musculus* (mouse); Pvu, *Patella vulgata* (mollusc); Tca, *Tribolium castaneum* (beetle); Ttu, *Tubifex tubifex* (annelid); Xla, *Xenopus laevis* (frog).

Fig. 2. Temporal expression profile of *Ttu-dpp*. RT-PCR analysis showing expression pattern of *Ttu-dpp* transcripts in *Tubifex tubifex* embryos from stage 1 through stage 18. Stage 1, 1-cell; stage 2, 2-cell; stage 5, 8-cell; stage 6, 10-cell; stage 7, 14-cell; stage 8, 24-cell; stages 9-10, formation of M teloblast pair; stage 11, formation of NOPQ proteloblast pair; stage 12, ectodermal teloblastogenesis (for details of developmental stages, see Shimizu, 1982). For stages 13-18, see Fig. 3.  $\beta$ -actin was used as positive control.

Fig. 3. Diagrammatic illustration of developmental stages (13-18) in *Tubifex*. (A)-(C), left side view (upper) plus ventral view (lower); (D)-(I), left side view. Anterior is to the left; dorsal is to the top. Circles at the posterior end of the germ band in (A)-(C) are embryonic stem cells called teloblasts; for brevity of the figure, teloblasts are not included in (D)-(H) though they do exist in these embryos as well (see Goto et al., 1999). Asterisks indicate the position of buccal cavity. pr, prostomium. (A)-(C) Embryos at stages 13-15 undergo gastrulation that consists of ventralward movement of elongating germ bands (shaded) and spreading of micromere-derived epithelial cells (not depicted here) over the endoderm. The germ band is associated, at its anterior end, with an anteriorly located cluster of micromeres (called a micromere cap, mc), and it is initially located at the dorsal side of the embryo (A). Along with their elongation, the germ bands on both sides of the embryo gradually curve round toward the ventral

midline (B) and finally coalesce with each other along the ventral midline (C). (D)-(I) Gastrulation is followed by embryo's body elongation, which begins in the anteriormost region of the embryo (D), continues in an anterior-to-posterior fashion (E-H), and completes in the caudal end at stage 18 (I). Body elongation is accompanied by formation of segmental ectoderm, which is accomplished by dorsalward expansion of germ bands (shaded). (For details of morphogenetic movements, see Shimizu and Nakamoto, 2001)

Fig. 4. Expression of *Ttu-dpp* detected by *in situ* hybridization with an antisense riboprobe. (A)-(C) Expression was not detected in cleavage-stage embryos. (A) 1-cell stage (st. 1), animal pole view. (B) 8-cell stage (st. 5), animal pole view. (C) 24-cell stage (st. 8), left side view. In (B) and (C), some blastomeres are labeled to indicate embryo's orientation. (D)-(F) Embryos undergoing gastrulation. Anterior is to the left; dorsal is to the top. Asterisks indicate the anterior end of germ bands. Dashed lines in (D) and (E) indicate the dorsal and ventral margins of left germ bands. (D) Ventrolateral view of stage 14 embryo (see Fig. 3B). Arrow indicates the position of posteriormost stained cells in the left germ band. (E)-(F) Side view of stage 15 embryo (see Fig. 3C). Double arrowhead and arrow indicate the position of posteriormost stained cells in the left and right germ bands, respectively. Note that the left germ band is out of focus here. (F) shows a cleared specimen. (G), (H) Left side view of stage 16 embryo. Cleared specimens. Anterior half of an embryo (inset) is shown. Thin lines with roman numerals (I-VII) indicate the position of seven consecutive segments. (G) Stage 16a embryo (see Fig. 3D). Stained cells are seen in the prostomium (pr), ventral ganglia, and a longitudinal thin belt of cells that lies at a distance from the ventral ganglia. Three arrows indicate cell clusters in the belt. Note that this belt of stained cells begins at segment II, but not at segment I (square bracket). (H) Stage 16c embryo (see Fig. 3F). Stained cells are seen in the prostomium (pr), ventral ganglia, and the lateral region of the embryo. Lateral stained cells are organized in segmentally iterated stripes that extend along the dorsoventral axis; arrows indicate dorsalmost stained cells in each stripe. Note the absence of such a lateral stripe of cells in segment I. Scale bar in (A): (A-E) 200  $\mu\text{m}$ ; (F) 250  $\mu\text{m}$ ; (G, H) 150  $\mu\text{m}$  (500  $\mu\text{m}$  for insets).

Fig. 5. Expression of *Ttu-dpp* in setal sacs. (A) and (B) Left side view of stage 17 embryos. Dorsal is to the top. Thin lines with roman numerals (I-III) indicate the position of the first three segments. (A) Stage 17a embryo (see Fig. 3G). This embryo exhibited 12 pairs of dot-like structures on the left side. (B) Stage 17b embryo (see Fig. 3H), cleared specimen. This embryo exhibited 18 pairs of dot-like structures on the left side. Note that either in (A) or (B), dot-like structures are absent in segment I. (C), (D) Higher magnification of dot-like structures seen in cleared stage 17b embryos. Anterior is to the left. (C) Head-on view of ventral dot-like structures (arrows) in segments XI-XIII. (D) Dorsal (single arrows) and ventral (double arrows) dot-like structures in segments IV-VI. (E) Side view of a juvenile a few days after hatchout. Cleared specimen. Three segments (VII-IX) are shown. Anterior is to the left and dorsal is to the top. Arrows indicate stained structures located at dorsal setal sacs. Double arrowhead indicates a stained structure in the ventral setal sac. Asterisks indicate dorsal setae. Scale bar in (A): (A) 200  $\mu\text{m}$ ; (B) 250  $\mu\text{m}$ ; (C-E) 100  $\mu\text{m}$ .