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The *Tribolium decapentaplegic* gene is similar in sequence, structure, and expression to the *Drosophila dpp* gene

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Abstract We are characterizing members of the Transforming Growth Factor-β (TGF-β) superfamily in the red flour beetle, *Tribolium castaneum*, in order to examine the evolutionary conservation of the structure and function of TGF-β-like genes during insect development. A *decapentaplegic*-like gene of the TGF-β superfamily was isolated in *Tribolium* (*Tc dpp*) that is similar in sequence, organization, and expression to the *Drosophila melanogaster dpp* gene (*Dm dpp*). Conserved features include a high degree of sequence similarity in both the pro-domain and mature domains of the encoded polypeptide. In addition, the position of an intron within the protein-coding region is conserved in *Tc dpp*, *Dm dpp*, and two bone morphogenetic protein genes of the TGF-β superfamily in humans, BMP2 and BMP4. Consensus binding sites for the *dorsal* transcription factor are found within this intron in *Tc dpp* similar to the intronic location of several *dorsal* binding sites in *Dm dpp*. During embryogenesis, *Tc dpp* is expressed in an anterior cap of serosa cells at the blastoderm stage, in the dorsal ectoderm at the lateral edges of the developing and extended germ band, and in the distal tips of developing embryonic appendages. Several aspects of embryonic expression, similar in both flies and beetles, suggest conserved roles for *dpp* in cellular communication during the development of these distantly related insects.

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Introduction

The Transforming Growth Factor-β (TGF-β) superfamily comprises a diverse group of growth and differentiation factors that are important for cellular communication during invertebrate and vertebrate development (reviewed by Kingsley 1994). Members of this family share a number of structural features. They are secreted polypeptides that are processed to yield a biologically active mature domain derived from approximately 100 amino acids at the carboxy-terminus of the pre-proprotein. All members of the TGF-β family share varying degrees of sequence similarity in the mature domain (reviewed in Burt and Law 1994). Seven precisely spaced cysteine amino acids in the mature domain are conserved in members of the superfamily across phyla. One of these cysteines is used in the formation of intermolecular disulfide bonds between identical members of the superfamily to yield homodimers or between different members of the superfamily to yield heterodimers. These homo- or heterodimers of the mature domain are the bioactive ligands that bind cell surface receptors mediating biological functions (reviewed in Attisiano et al. 1994; Kingsley 1994).

Though members of the TGF-β family were first characterized in vertebrates, three members of this family have been identified in the fruit fly, *Drosophila melanogaster* (Padgett et al. 1987; Wharton et al. 1991; Doctor et al. 1992; Arora et al. 1994). The *Drosophila* genes, *decapentaplegic* (*dpp*), *60A*, and *screw* encode members of the TGF-β superfamily with important functions during development. Genetic and molecular analyses of the *dpp* gene implicate it in a number of functions during *Drosophila* development including embryonic dorsal/ventral axis patterning (Irish and Gelbart 1987; Ferguson and Anderson 1992a,b; Wharton et al. 1993),

induction of specific mesodermal cell fates (Staehling-Hampton et al. 1994; Frasch 1995), embryonic midgut development (Immergluck et al. 1990; Panganiban et al. 1990; Reuter et al. 1990; Staehling-Hampton and Hoffmann 1994), and aspects of axis specification and morphogenesis during imaginal disc development (Spencer et al. 1982; Bryant 1988; Heberlein et al. 1993; Diaz-Benjumea et al. 1994; Nellen et al. 1996). The complex transcriptional patterns of *dpp* expression during *Drosophila* development are controlled by regulatory regions both 5' and 3' of the *dpp* protein-coding region and within an intron that interrupts the protein-coding region; these regulatory regions span over 50 kbp in the *Drosophila* genome (St. Johnston et al. 1990; Masucci et al. 1990; Blackman et al. 1991).

The mature domain of *dpp* gene shares more sequence identity with two vertebrate bone morphogenetic proteins, BMP2 and BMP4 (Wozney et al. 1988), of the TGF-β superfamily than with other members of the superfamily in *Drosophila* (Burt and Law 1994). This sequence similarity underscores a functional relatedness in that disulfide-linked homodimers of the mature domain of *Drosophila dpp c*an induce cartilage and bone formation in a rat subcutaneous bone induction assay (Sampath et al. 1993). Further, BMP4 expression in *Drosophila* can partially rescue *dpp* embryonic mutant phenotypes (Padgett et al. 1993).

The red flour beetle, *Tribolium castaneum*, has emerged as an important system for studies of the molecular analysis of insect development and the degree of conservation of patterning genes and mechanisms among diverse insect species (reviewed in Brown and Denell 1996). Although *Tribolium* has been studied for decades because of its agricultural importance as a major pest of stored grains, recent work has demonstrated the utility of *Tribolium* for genetic analysis and molecular studies of development (Beeman et al. 1989, 1996; Brown et al. 1992; Stuart et al. 1991, 1993; Sulston and Anderson 1996). For example, in a number of recent papers the authors have compared anterior/posterior axis specification in the long germ insect *Drosophila*, where the entire embryo is subdivided into a segmented germ band at the blastoderm stage, and short germ insects such as *Tribolium*, where posterior segments are delineated sequentially as the germ rudiment extends posteriorly (Sommer and Tautz 1993; Brown et al. 1994a,b; Nagy and Carroll 1994; Wolff et al. 1995). The results thus far indicate that the hierarchy of segmentation genes is conserved between fruit flies, flour beetles, and other holometabolous insects (reviewed in Nagy 1994; Tautz and Sommer 1995). Other important aspects of insect embryogenesis have also been investigated in *Tribolium* including the apparent conservation of mesoderm formation through the actions of the *twist* and *snail* genes (Sommer and Tautz 1994). Though the mechanisms of dorsal/ventral axis specification in *Drosophila* have been examined in detail (reviewed in Chasan and Anderson 1993; Morisato and Anderson 1995), little is known about other insects including *Tribolium*. A recent paper on the expression of the *Tribolium zen* gene suggests that conserved mechanisms are likely to function in dorsal/ventral patterning during early embryogenesis (Falciani et al. 1996).

Given the central role of *dpp* during *Drosophila* development in establishing, among other things, cell fates along the dorsal/ventral axis during early embryogenesis and along the proximal/distal axis in developing appendages, we set out to isolate and characterize the *dpp* ortholog in *Tribolium*. In this paper, we report the sequence of the entire protein-coding region of *T. castaneum dpp* (referred to as *Tc dpp*), as well as flanking and intronic regions. Observations of *Tc dpp* expression during embryogenesis suggest similarities in the roles of *dpp* between insects that diverged from a common ancestor approximately 250 million years ago. These initial studies provide the basis for a continuing examination of the roles of TGF-β superfamily members among diverse insect orders.

Materials and methods

cDNA and genomic library screens

A *Tribolium* embryonic cDNA library (2×10^5 pfu) in λgt22 was screened at reduced stringency [37°C for 36–40 h in 30% formamide, 125 mM sodium phosphate pH 7.0, 10% polyethylene glycol 8000, 7% sodium dodecyl sulfate (SDS), 250 mM NaCl]. Membranes were hybridized with a [^{32}P]-radiolabelled 930 bp DNA fragment (from pVGR10; J. Doctor, unpublished) encoding the entire mature domain and a portion of the pro-domain of the *D. melanogaster 60A* gene (Doctor et al. 1992). Following hybridization, the membranes were washed once at 37°C with 2 x SSC (0.3 M sodium chloride, 0.03 M sodium citrate) 1% SDS for 30 min and twice at 37°C with 0.2 x SSC/1% SDS for 30 min each before exposing to X-ray film. The DNA inserts in positive bacteriophage were amplified by PCR (polymerase chain reaction) using primers in the multiple cloning site of λgt22, and the products were subcloned into plasmid pCRII using the TA cloning system (Invitrogen). One subclone, pLOU1, encoded a *Tribolium dpp*-like sequence. A *T. castaneum* genomic DNA library (80,000 pfu) in Lambda Gem-11 (Promega) was screened at high stringency (42°C for 16–20 h in 50% formamide, 4 x SSC, 1% SDS, 5 x Denhardt's solution) with [^{32}P]-radiolabelled pLOU1 as a probe followed by washing once at 65°C with 2 x SSC/1% SDS for 30 min and twice at 65°C with 0.2 x SSC/1% SDS for 30 min each. Genomic DNA restriction fragments were subcloned into pBluescript II KS (Stratagene) and bacteriophage M13 vector, mp19, for DNA sequence determination.

DNA sequencing

Double and single stranded DNA templates were used to determine the sequence of the *Tc dpp* gene using Sequenase Version 2.0 (United States Biochemical Co.). Universal primers and a series of custom synthesized primers (Oligos, etc.) were used to determine the DNA sequence on both strands of approximately 4.9 kbp of *Tribolium* DNA. Nucleotide sequences were compiled and analyzed using MacVector (Version 4.1) sequence analysis software (International Biotechnologies). Sequence alignments were determined using CLUSTAL W (Thompson et al. 1994). Database searches were accomplished using BLAST (Altschul et al. 1990).

Rapid amplification of cDNA ends (RACE)

RACE was carried out using the Marathon cDNA Amplification Kit (CLONTECH Laboratories) as directed. cDNA was synthesized using Moloney Murine Leukemia Virus reverse transcriptase

and 10 μg of total embryonic *Tribolium* RNA as template. Following second strand synthesis, adapters were ligated to the cDNA. For RACE PCR, the Expand Long Template PCR System (Boehringer Mannheim) was used with an adapter primer and a custom synthesized *Tribolium dpp* gene-specific primer (5'–GTGGACT AAGACACGTTGAAAGGG–3'). Amplification products were subcloned into pCRII and sequenced.

In situ hybridization

Digoxigenin-labeled RNA probes were synthesized with the Riboprobe system (Promega) using digoxigenin-labeled uridine triphosphate (UTP; Boehringer-Mannheim). In situ hybridization to fixed embyros was as described by Brown et al. (1994a). Stained embryos were mounted in 70% glycerol/phosphate-buffered saline as whole eggs or were dissected to remove yolk and mounted as a flat preparation. Images were digitally captured; brightness and contrast were adjusted in Adobe Photoshop.

Results

Isolation of a *Tribolium decapentaplegic* gene

We screened a *Tribolium* embryonic cDNA library at reduced stringency with a probe for the *Drosophila 60A* gene and purified six positive bacteriophage clones for analysis. The cDNA inserts were amplified by PCR and directly subcloned into pCRII. One cDNA (pLOU1) was chosen initially for DNA sequence analysis. Though we had used the *Drosophila 60A* gene as a probe, sequence of pLOU1 revealed an encoded polypeptide more similar to *dpp* of *Drosophila* than to *60A*. Subsequent analysis has demonstrated that the other five cDNAs encode portions of the *Tribolium 60A* gene (Pletcher and Doctor, in preparation).

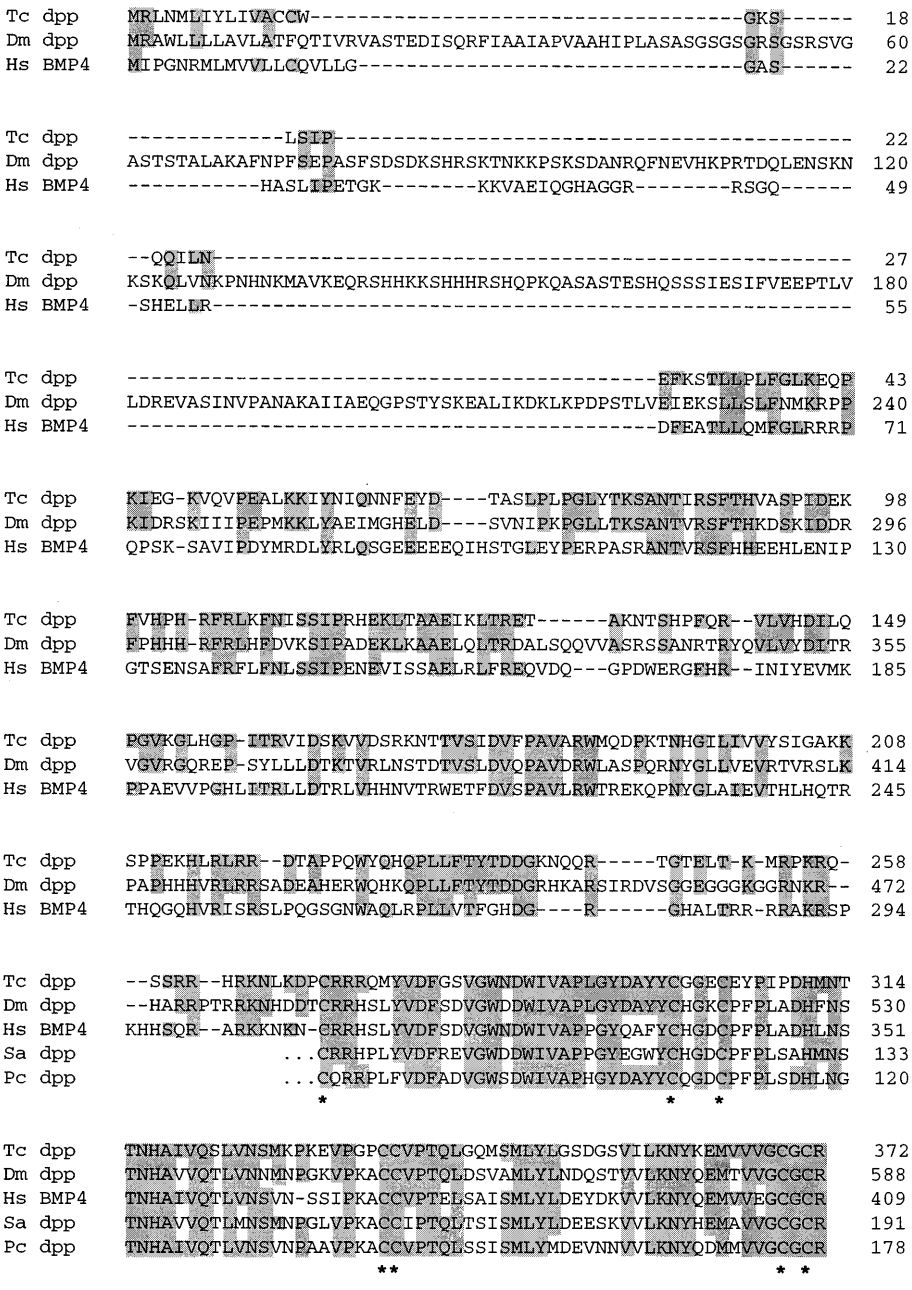


Fig. 1 Comparison of complete amino acid sequences of *Tribolium castaneum decapentaplegic* gene (*Tc dpp*), *Drosophila melanogaster dpp* (*Dm dpp*) and human bone morphogenetic protein gene (BMP4) and the mature domains of *dpp* genes from *Precis coenis* and *Schistocerca americana*. Alignments were done using CLUSTAL W alignment software (Thompson et al. 1994). Amino acid positions are numbered *to the right*. *Shaded boxes* indicate sequence identity between *T. castaneum dpp* (*Tc dpp*; Genbank Accession number U63132) and the other sequences: *D. melanogaster dpp* (*Dm dpp*; Padgett et al. 1987; M30116), *Homo sapiens* BMP4 (*Hs BMP4*; Wozney et al. 1988; M22490), *Precis coenis dpp* (*Pc dpp*; Carroll et al. 1994; L42141), *Schistocerca americana dpp* (*Sa dpp*; Newfeld and Gelbart 1995; V23785). *Asterisks* indicate the seven conserved cysteine residues of Transforming Growth Factor (TGF)-β superfamily members. [An annotated figure of *Tc dpp* DNA and protein sequence can be accessed through the web site of this journal (http://science.springer.de/dge/dge-main.htm)]

Table 1 Comparison of percent amino acid sequence identity of *Tribolium castaneum dpp* and related proteins, see Fig. 1 for alignment; *Pro* pro-domain from first amino acid following putative signal peptidase cleavage site to first cysteine of mature domain., *Mature* mature domain from first cysteine to stop, *Comp* complete polypeptide sequence from initiating methionine to stop, *Tc dpp Tribolium castaneum dpp* (U63132), *Dm dpp Drosophila melanogaster dpp* (Padgett et al. 1987; M30116), *Hs BMP4 Homo sapiens* BMP4 (Wozney *et al.* 1988; M22490), *Pc dpp Precis coenis dpp* (Carroll *et al.* 1994; L42141), *Sa dpp Schistocerca americana dpp* (Newfeld and Gelbart, 1995; V23785)

	Dm dpp			Hs BMP4			Sa dpp	Pc dpp
	Pro	Mature	Comp	Pro	Mature	Comp	Mature	Mature
Tc dpp	49	67	53	34	64	42	63	65
Dm dpp		–		35	76	43	77	73
Hs BMP4					–		76	75

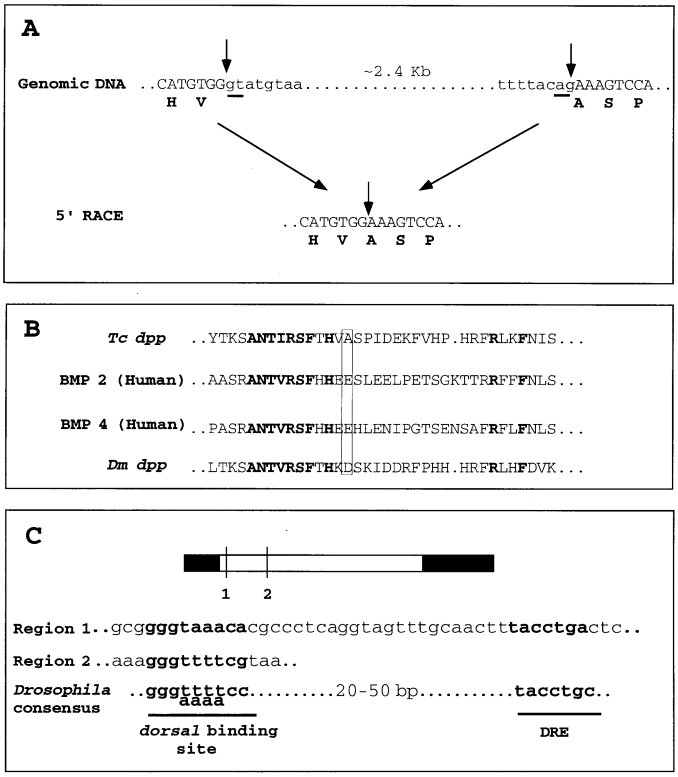


Fig. 2A–C Intron conservation among members of the *dpp* subgroup of the TGF-β superfamily. **A** The nucleotide and deduced amino acid sequence of the *Tribolium dpp* gene near the 5′ and 3′ intron boundaries is shown. Nucleotides within the protein-coding region are *capitalized*. The consensus nucleotide sequences (Padgett et al. 1986) of GT and AG on the 5′ and 3′ boundaries of the intron, respectively, are *underlined*. The nucleotide sequence of a 5′ RACE product shows the position of the intron. **B** Intron splice site conservation between members of the *dpp* subgroup. *Dm dpp*, human BMP2, and BMP4 genes all contain a splice junction after the first base of the codon encoding the amino acid indicated by the *box* (Padgett et al. 1993). The *Tc dpp* gene has a splice site that is exactly conserved (**A**). The codon GAA (encoding alanine) is interrupted by the intron after the G (first base in the codon). **C** Putative *dorsal* binding sites in the intron of the *Tc dpp* gene. A sequence search using the consensus *Drosophila dorsal* protein binding site sequence (G G G A/T A/T A/T A/T C C; Huang et al. 1993) revealed two putative *dorsal* binding sites within the intron of the *Tc dpp* gene. The *schematic diagram* depicts the relative position of these sites (Region *1*, Region *2*) within the intron (*white box*); the protein-coding region is in *black*. A sequence similar to the *Drosophila* consensus dorsal repression element (DRE; T A C C T G C; Huang et al. 1995) is 23 bp downstream from the *dorsal* binding site in Region 1

Sequence analysis of the *dpp*-like cDNA in pLOU1 revealed that it was not full length. We therefore screened a *Tribolium* genomic DNA library at high stringency using pLOU1 as a probe and purified four positive bacteriophage. Regions of *Tribolium* genomic DNA from these bacteriophage were subcloned and the entire protein-coding region as well as genomic DNA flanking the start and stop codons were sequenced on both strands. In addition, we sequenced the entire intron that interrupts the protein-coding region in the pro-domain. We have determined the sequence of approximately 4.9 kbp of *Tribolium* genomic DNA encoding a *dpp*-like member of the TGF-β superfamily (Genbank accession number U63132; see also annotated sequence figure in the website for this journal, http://science.springer.de/dge/dgemain.htm). Southern gel blot analysis indicates that *Tribolium dpp* (*Tc dpp*)is present in a single copy in the red flour beetle genome (not shown).

Sequence and structure of *Tc dpp*

The following results provide evidence that we have isolated the *Tribolium* ortholog of *dpp*. These include extensive sequence similarity to *Dm dpp* in the protein-coding region (Fig. 1) and the precise conservation of the position of an intron in the protein-coding region (Fig. 2).

The *Tc dpp* gene encodes a polypeptide of 372 amino acids with all of the hallmarks of a member of the TGF-β superfamily, including an amino-terminal signal sequence, a pro-domain region, and a mature domain with seven conserved cysteines. The signal sequence, expected in a secreted cellular signaling protein, is typical in having a hydrophobic core and is predicted to be cleaved by signal peptidase after amino acid 20 (Heinje 1986). Three sites for *N*-linked glycosylation are found in the pro-domain and one is located in the mature domain. Three potential dibasic cleavage sites (Barr 1991) that may be used in the proteolytic separation of the pro- and mature domains are found near the first of the seven conserved cysteines in the mature domain. Following the stop codon is a 506 bp untranslated region that is delimited by the site of polyadenlyation in the pLOU1 cDNA. No consensus polyadenylation signal sequence (AATAAA) near the site of polyadenylation at position 4770 is present. Two AATATA sequences, however, are found beginning at positions 4618 and 4691.

Based on the alignment of *Tc dpp*, *Dm dpp* and human BMP4 in Fig. 1, the amino acid sequence of the mature domain (from the first conserved cysteine) of *Tc dpp* is 67% identical to *Dm dpp* and 64% identical to human

BMP4 (Table 1). The percent identity of *Drosophila dpp* and human BMP4 is surprisingly much higher at 76%. A similar degree of sequence identity in the mature domain is also observed when comparing (Fig. 1) butterfly *dpp* (*Precis coenis*, Carroll et al. 1994) and grasshopper *dpp* (*Schistocerca americana*; Newfeld and Gelbart 1995) to human BMP4 (76% and 75%, respectively; Table 1).

Supporting the contention that this *Tc dpp* sequence is the beetle *dpp* ortholog is the high degree of sequence identity in the pro-domain region where there is typically much less sequence conservation among superfamily members. The sequence alignment in Fig. 1 indicates that there is 49% identity in the *dpp* pro-domain region between *Tribolium* and *Drosophila* but only 34% identity between *Tribolium dpp* and human BMP4 and 35% between *Drosophila dpp* and human BMP4 (Table 1). Further, several large blocks of amino acids are precisely conserved between *Tribolium* and *Drosophila dpp* in the pro-domain region. For example, stretches of 16 amino acids (amino acids 75–90 in *Tc dpp*) and 11 amino acids (amino acids 230–240) are conserved. In addition, one of the *N*-linked glycosylation sites (amino acids 110–112) is conserved in the pro-domain between *Tribolium dpp* and human BMP4.

Analysis of Rapid Amplification of cDNA Ends (RACE) products demonstrated that the position of an intron in the pro-domain of the protein-coding region of *Tc dpp* is precisely conserved between the *Tc dpp* and *Dm dpp* genes and the closely related human *BMP2* and *BMP4* genes of the TGF-β superfamily (Fig. 2A and B). Within this conserved intron in the *Dm dpp* gene are several silencer elements that are bound by the *dorsal* transcription factor to repress *Dm dpp* expression during early embryogenesis. Examination of the *Tc dpp* gene sequence revealed two consensus *dorsal* binding sites in the *Tc dpp* intron (Fig. 2C).

Expression of *Tribolium dpp* during embryonic development

Examination of *Tc dpp* transcription by in situ hybridization using an antisense probe reveals a dynamic pattern of *Tribolium dpp* RNA expression during embryogenesis. Expression of *dpp* in *Tribolium* is first detected at the blastoderm stage in the anterior-most region of the serosa (Fig. 3A). This anterior cap of *dpp* expression in the serosa fades as the embryo condenses.

Tc dpp expression is detected after the blastoderm stage as the germ band develops and extends. In an early stage of germ band extension (Fig. 3C), *Tc dpp* is detected along the lateral edges of the embryo parallel to the anterior/posterior axis in ectodermal cells fated to give rise to the dorsalmost cells of the embryo. Region-specific expansions of *Tc dpp* expression within these regions are intriguing and may correspond to the beginnings of limb formation. This lateral expression extends into the gnathal region but apparently not into the more anterior regions of the developing head. At this stage, *Tc dpp* expression is also detected in the developing labrum and in a putative mesodermal region within the posterior growth zone (Fig. 3C).

When the germ band is fully extended and segments are morphologically visible (Fig. 3D), *Tc dpp* expression is detected in the putative dorsal ectoderm, in the labrum, and in the developing antennal, gnathal, and thoracic appendages. In the more posterior segments of this embryo, region-specific expansion of *Tc dpp* expression along the lateral edges is also observed, reminiscent of the staining observed in the more anterior segments at an earlier stage (Fig. 3C).

As appendage development continues, *Tc dpp* RNA is most readily detected in the dorsal aspect of the distal tips of the elongating thoracic appendages (Fig. 3E). At least for the thoracic appendages, *Tc dpp* RNA is also detected in a small region of proximal dorsal cells (i.e. arrows in Fig. 3E). *Tc dpp* expression is still detected in the cells of the putative dorsal ectoderm (Fig. 3F).

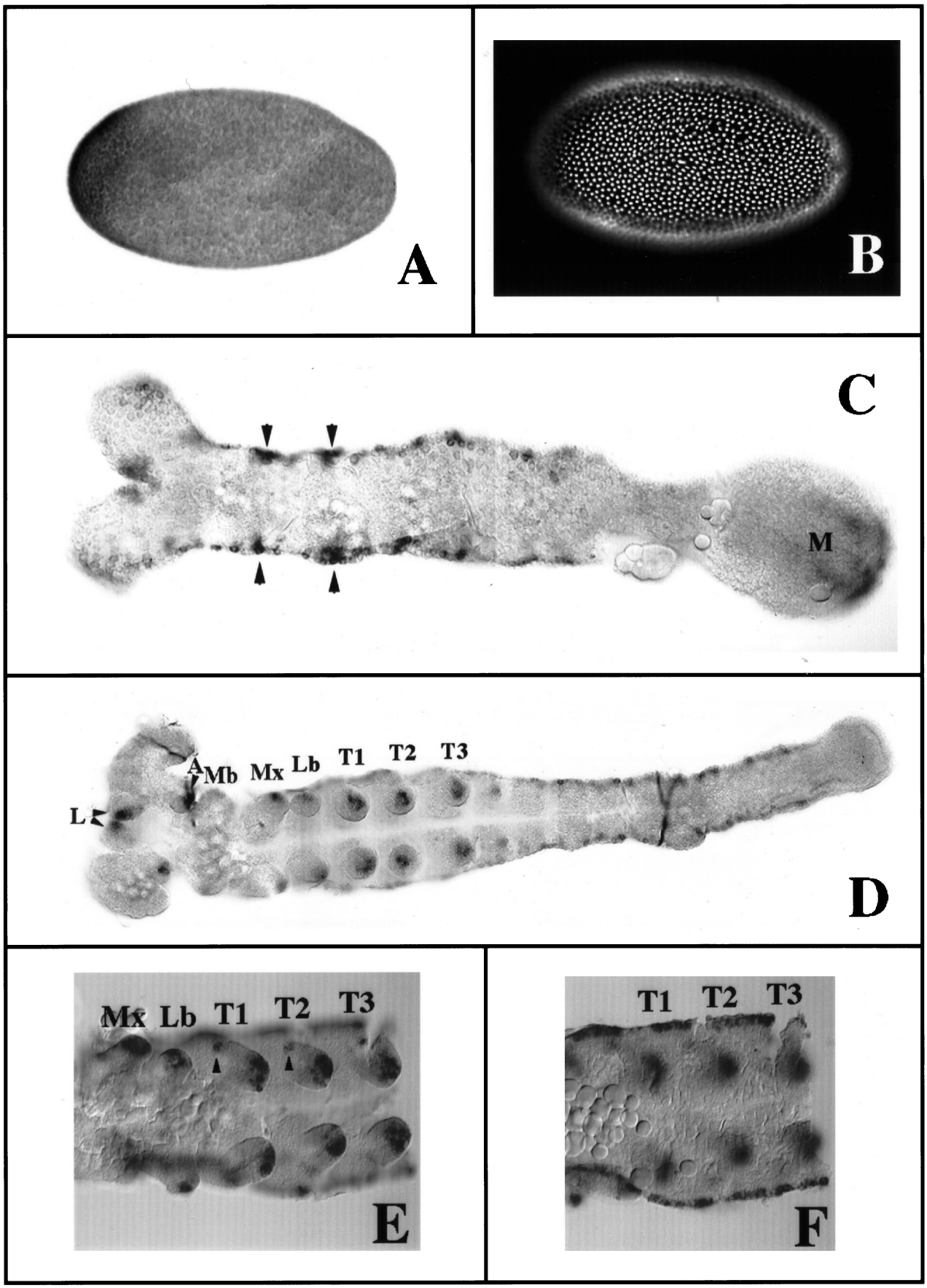
Discussion

The *T. castaneum dpp* ortholog

Three genes, *dpp*, *60A*, and *screw*, encoding members of the TGF-β superfamily have been identified and shown to be important during the development of the dipteran *D. melanogaster*. We set out to isolate and characterize members of the TGF-β superfamily in the red flour beetle, *T. castaneum*, in order to examine the evolutionary conservation of the structure and function of TGF-β-like genes during insect development. In a screen of a cDNA library in search of *Tribolium 60A* orthologs, we serendipitously identified a cDNA encoding a *Tribolium dpp* ortholog, as well as several cDNAs encoding a *Tribolium 60A* ortholog (Pletcher and Doctor, in preparation). The *Tc dpp* cDNA was used to screen a genomic DNA library to facilitate the isolation and sequencing of the entire protein-coding region of *Tc dpp*.

The *Tc dpp* gene encodes a polypeptide of 372 amino acids with a structure typical for members of the TGF-β superfamily, including an amino-terminal signal sequence, a pro-domain region, and a mature domain with seven conserved cysteines. Within the protein-coding region are several consensus sites for *N*-linked glycosylation. Glycosylation of the pro-domain has been documented for TGF-β1 and is essential for secretion of this member of the TGF-β superfamily (Sha et al. 1989). There are three dibasic cleavage sites (Barr 1991) that may be used for proteolytic separation of the pro- and mature domains.

The *Tc dpp* polypeptide has considerable sequence similarity to *Dm dpp* within both the mature and the pro-domain. Both of these insect proteins share sequence similarity to human BMP4, particularly in the mature domain, as indicated in the amino acid alignment of *Tc dpp*, *Dm dpp*, and human BMP4 in Fig. 1. The BMP4 polypeptide is approximately the same length as *Tc dpp*,


A
B
C
M
A
Mb
Mx
Lb
T1
T2
T3
L
D
Mx
Lb
T1
T2
T3
E
T1
T2
T3
F

whereas *Dm dpp* is considerably longer at 588 amino acids. Most of the difference in length is accounted for by a large region in the pro-domain of *Dm dpp* that is not present in the other two polypeptides. It is likely that this sequence information was inserted into *Dm dpp* in the *Drosophila* lineage subsequent to the divergence of the *Tribolium* and *Drosophila* lineages. Examination of *dpp* genes in species more closely related to *D. melanogaster* should help to resolve this question.

Another compelling argument that we indeed have identified the *Tribolium dpp* ortholog is the precise conservation between *Tribolium* and *Drosophila* of the intron in the pro-domain of the protein-coding region (Fig. 2). We confirmed the intron/exon boundaries by Rapid Amplification of cDNA Ends (RACE) using *Tribolium* RNA and a primer designed to a sequence 3' of the intron. The position of this intron is also conserved between *Tc dpp* and *Dm dpp* and the closely related *BMP2* and *BMP4* genes of the TGF-β superfamily. Thus, this aspect of the structure of the *dpp/BMP* genes was present in the ancestral organism that gave rise to the vertebrate and invertebrate lineages and has been maintained over the course of hundreds of millions of years of evolution. The importance of the intron in the protein-coding region for the regulation of *Dm dpp* is well established as the location of a number of enhancer and silencer elements essential for proper *dpp* expression during embryonic development (Huang et al. 1993; Jackson and Hoffmann 1994). As described below, several putative regulatory elements are conserved in the *Tribolium* intron. The role of this intron, if any, in the regulation of the vertebrate BMPs is not known, but its presence should prove useful in identifying *dpp* homologs in other vertebrate and invertebrate species.

The *Dm dpp* gene (St. Johnston 1988) and several mammalian *BMP4* genes (Kurihara et al. 1993; Feng et al. 1995) also have an intron that interrupts the 5′ untranslated region immediately upstream of the start of translation. A consensus 3′ splice site is found in the *Tc dpp* gene 8 bp upstream of the start codon (see the figure accompanying this paper on the web site of this journal; http://science.springer.de/dge/dge-main.htm). We have not yet determined whether this region is a functional splice site during *Tc dpp* gene expression.

Fig. 3A–F Expression of *Tc dpp* RNA during embryonic development detected by hybridization in situ. Hybridization was with a digoxigenin-labeled antisense *Tribolium dpp* RNA probe and immunodetection used the Genius kit of Boehringer-Mannheim. **A** Bright field image showing detection of *Tc dpp* RNA in a blastoderm embryo. Expression is detected in a dorsally-shifted cap at the anterior end of the embryo in the presumptive serosa. **B** Fluorescent counterstaining of the same embryo as in **A** with the nuclear dye Hoechst 33342 demonstrating that the embryo is at the blastoderm stage. **C** Embryo during germ band extension. Ventral view with anterior *to the left*, posterior growth zone *to the right*. RNA is detected in two longitudinal bands in the presumptive dorsal ectoderm. Region-specific expansions of *Tc dpp* expression within these regions are indicated by *arrows*. RNA is also detected in the mesoderm (*M*) in the posterior growth zone. **D** Germ band extended embryo with RNA expression in the three pairs of developing thoracic appendages (*T1, T2, T3*), the gnathal appendages (*Mb* mandibular, *Mx* maxillary, *Lb* labial), the antennae (*A*), and the labrum (*L*). Ventral view with anterior *to the left*, posterior *to the right*. **E** *Tc dpp* RNA expression in thoracic appendages (*T1, T2, T3*); *Tc dpp* RNA is also detected in a small region of proximal dorsal cells (*arrows*). Expression is also detected in the developing gnathal appendages (*Mn* mandibular, *Lb* labial). **F** Thoracic region of same embryo as in **E** with plane of focus along putative dorsal ectoderm showing *Tc dpp* RNA expression

Expression of *Tc dpp* at the blastoderm stage

The expression of *Tc dpp* in serosal cells at the blastoderm stage has similarity to the initial pattern of *Dm dpp* expression in the dorsalmost 40% of the blastoderm prior to nuclear cycle 13. In *Drosophila* this region corresponds to the extraembryonic amnioserosa as well as the dorsal ectoderm (St. Johnston and Gelbart 1987; Ray et al. 1991). That *Tc dpp* is expressed in an anterior cap rather than a dorsal cap is most likely due to topological differences between the two types of embryos. The similarity of *Tc dpp* and *Dm dpp* expression in the blastoderm is paralleled by observations of the expression of the *Tribolium zerknullt* (*Tc zen*) gene (Falciani et al. 1996). The relative patterns of *dpp* and *zen* are similar in beetles and flies. In *Drosophila*, *dpp* and *zen* are initially expressed at the blastoderm stage in the dorsalmost region and in the termini (St. Johnston and Gelbart 1987; Ray et al. 1991). At gastrulation, *dpp* expression is restricted to presumptive dorsal ectoderm whereas *zen* is restricted predominantly to the amnioserosa. In beetles, both *Tc dpp* and *Tc zen* are expressed in an anterior polar cap, and as the embryonic rudiment condenses, *Tc dpp* expression fades and is eventually expressed in the presumptive dorsal ectoderm, and *Tc zen* expression is restricted to the serosa.

The amnioserosa of *Drosophila* is an evolutionary modification of the serosal and amnionic membranes and does not separate into two distinct layers. In the case of the serosa of *Tribolium* and the amnioserosa of *Drosophila*, both of these extra-embryonic membranes cease cell division and become polyploid as they eventually cover the dorsal surface of the embryo during gastrulation (Falciani et al. 1996). The blastoderm stage expression of *Tc dpp* and *Tc zen* in the serosa and *Dm dpp* and *Dm zen* in the amnioserosa supports a relationship between these two tissues. Wolff and colleagues (1995), however, report that the *hunchback* ortholog of *Tribolium* is expressed in an anterior domain overlapping with the expression of *Tc dpp* and *Tc zen*, whereas *hunchback* is not expressed in the amnioserosa of *Drosophila*. The homology relationships between the extra-embryonic membranes of these and other insect species awaits further investigation.

Although the morphogenesis of embryos and extra-embryonic membranes in *Tribolium* and *Drosophila* are quite different, there are striking parallels in the expression of *dpp* and *zen* in related structures. It is likely that regulatory genes, similar to those necessary for the regulation of *dpp* and *zen* in *Drosophila* (Ray et al. 1991),

will be required to specify dorsal/ventral and terminal cell fates in *Tribolium*. Supporting this contention are conserved sequences for binding of the *dorsal* transcription factor that we have identified in the *Tc dpp* gene (discussed below).

Expression of *Tc dpp* during embryonic appendage development

Tc dpp expression is detected later in embryonic development as the germ band extends. In an early stage of germ band extension (Fig. 3C), *Tc dpp* is detected along the lateral edges of the embryo in ectodermal cells that will give rise to the dorsalmost cells of the embryo. The region-specific expansions of *Tc dpp* expression (arrows in Fig. 3C) within these regions may correspond to the beginnings of appendage formation. At this stage, *Tc dpp* RNA expression is also detected in the growth zone as the germ rudiment extends posteriorly. Based on our observations of whole mount embryos (Fig. 3C), we believe that this posterior *Tc dpp* expression is in the mesoderm; sections of fixed material are necessary to confirm this interpretation. The detection of *Tc dpp* in this region is interesting as this expression may indicate a role for *dpp* in short germ insects such as *Tribolium* in maintenance of the growth zone or in the delineation of posterior segment identities.

When the germ band is fully extended and segments are morphologically visible (Fig. 3D), *Tc dpp* expression is detected in the labrum, in the distal tips of the developing antennal, gnathal, and thoracic appendages, and in the putative dorsal ectoderm. In the more posterior segments, region-specific expansion of *Tc dpp* expression along the lateral edges is also observed, but no signal is detected at the posteriormost end of the embryo. As elongation of the thoracic appendages continues, *Tc dpp* RNA is most readily detected in the dorsal aspect of the distal tips of the elongating thoracic appendages (Fig. 3E) and is still detected in the cells of the putative dorsal ectoderm (Fig. 3F).

During imaginal disc development in *Drosophila*, the juxtaposition of expression domains of *Dm dpp* in the dorsal region of the anterior compartment and *wingless* in the ventral region of the anterior compartment is essential for the proper expression of *Distal-less* and the establishment of the proximal/distal axis in developing limbs (Campbell et al. 1993; Diaz-Benjumea et al. 1994). A somewhat similar situation appears during embryonic appendage development in the red flour beetle. The detection of *Tc dpp* in the dorsal aspect of the distal tip of developing thoracic appendages is especially interesting given the expression of the *Tribolium* ortholog of *wingless* in developing appendages from the ventral midline to the distal tip (Nagy and Carroll 1994). Thus, at the distal tip of developing *Tribolium* thoracic appendages, *Tc dpp* is expressed in the dorsal region and *wingless* is expressed in the ventral region. This juxtaposition of *Tc dpp* and *wingless* expression may specify the distalmost region of developing *Tribolium* thoracic appendages. The characterization of the expression of the *Tribolium distal-less* ortholog should prove informative in this regard.

Conserved *dpp* regulatory sequences

The *Dm dpp* gene requires an extensive array of over 50 kbp of regulatory DNA residing 5′ and 3′ of the protein-coding region to ensure proper expression in developing appendages and during other aspects of development (St. Johnston et al. 1990; Masucci et al. 1990; Blackman et al. 1991). It will be interesting to determine whether such large regulatory regions are required for control of *Tc dpp* expression and whether particular sequence elements are conserved among fruit flies, flour beetles, and other insect species. Given the dynamic pattern of *Tc dpp* expression from the initial analysis reported here, it is likely that *Tc dpp* will share a complex regulatory array with *Dm dpp*. We have yet to examine the expression of *Tc dpp* in the developing embryonic gut and during post-embryonic development, such as in the eye during metamorphosis. *Dm dpp* plays important roles during these stages of fruit fly development (i.e. Panganiban et al. 1990; Heberlein et al. 1993).

Similarities in some aspects of the early embryonic expression of *Tc dpp* and *Dm dpp* led us to examine whether any transcriptional regulatory elements are conserved between these species. Early dorsal expression of *Dm dpp* is controlled in part by sequences residing in the intron that interrupts the pro-domain of the protein-coding region (Huang et al. 1993, 1995; Jackson and Hoffmann 1994). In addition to transcriptional enhancers, there are also silencer elements organized into “ventral repressor regions” that contain sequences bound by the *Drosophila dorsal* protein to repress *dpp* expression in ventral nuclei at the blastoderm stage (Huang et al. 1993, 1995). Besides these *dorsal* binding sites, a second type of sequence element, termed a dorsal repression element (DRE), is located within 50 bp 3′ of some *dorsal* binding sites. These DREs are bound by transcription factors similar to NTF-1/Elf-1 (encoded by the *Drosophila grainyhead* gene) and act as co-repressors of *Dm dpp*. We scanned the entire 4.9 kbp of *Tc dpp* sequence for consensus *dorsal* binding sites and found two close matches, both residing within the intron (Fig. 2C). Within 30 bp 3′ of the first of these consensus *Tribolium dorsal* binding sites is a sequence that matches the DRE at six of the seven positions. Thus, we have identified a region in the intron of *Tc dpp* similar in sequence and organization to the ventral repression regions of *Dm dpp*. Whether or not any of these sequences are important for proper *Tc dpp* expression awaits biochemical and transgenic analysis. Nevertheless, the apparent conservation of two components of a ventral repression region in the intron suggests that the early embryonic expression of *Tc dpp* in the serosa may be controlled by similar mechanisms to those employed in the regulation of *Dm dpp*.

In conclusion, we have identified the *dpp* ortholog of the red flour beetle, *T. castaneum*, and have begun to examine its expression during embryonic development. Given the utility of *Tribolium* for combined molecular, genetic, and developmental analysis, we are pursuing further analyses on the embryonic and post-embyonic roles of *Tc dpp* during beetle development.

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