The *tolkin* Gene Is a *tolloid*/BMP-1 Homologue That Is Essential for Drosophila Development

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ABSTRACT

The Drosophila *decapentaplegic (dpp)* gene, a member of the tranforming growth factor β superfamily of growth factors, is critical for specification of the embryonic dorsal–ventral axis, for proper formation of the midgut, and for formation of Drosophila adult structures. The Drosophila *tolloid* gene has been shown to genetically interact with *dpp*. The genetic interaction between *tolloid* and *dpp* suggests a model in which the *tolloid* protein participates in a complex containing the DPP ligand, its protease serving to activate DPP, either directly or indirectly. We report here the identification and cloning of another Drosophila member of the *tolloid*/bone morphogenic protein (BMP) 1 family, *tolkin*, which is located 700 bp 5′ to *tolloid*. Its overall structure is like *tolloid*, with an N-terminal metalloprotease domain, five complement subcomponents C1r/C1s, Uegf, and Bmp1 (CUB) repeats and two epidermal growth factor (EGF) repeats. Its expression pattern overlaps that of *tolloid* and *dpp* in early embryos and diverges in later stages. In larval tissues, both *tolloid* and *tolkin* are expressed uniformly in the imaginal disks. In the brain, both *tolloid* and *tolkin* are expressed in the outer proliferation center, whereas *tolkin* has another stripe of expression near the outer proliferation center. Analysis of lethal mutations in *tolkin* indicate it is vital during larval and pupal stages. Analysis of its mutant phenotypes and expression patterns suggests that its functions may be mostly independent of *tolloid* and *dpp*.

THE transforming growth factor β (TGF-β) superfamily, which consists of the TGF-β, activin, inhibin and *dpp*/BMP (bone morphogenic protein) families (MASSAGUÉ *et al.* 1994), comprises a large family of secreted processed ligands that bind specific cellular receptors to transduce a signal, resulting in the determination of cell fates. First identified by their capacity to induce ectopic bone formation from bone extracts (WOZNEY *et al.* 1988; CELESTE *et al.* 1990), BMPs are involved in the induction and development of bone and other tissues (KINGSLEY *et al.* 1992; STORM *et al.* 1994). The Drosophila *decapentaplegic (dpp)* gene is closely related to the BMPs, exhibiting 75% identity to BMP-2 and BMP-4 in the ligand domain (PADGETT *et al.* 1987; WOZNEY *et al.* 1988). The functional similarity of *dpp* to BMP-4 is demonstrated by the observation that BMP-4 can rescue the lethality of *dpp* null embryos (PADGETT *et al.* 1993), suggesting that many of the components in the pathway are conserved.

Several "accessory" proteins are known to interact with TGF-β-like proteins to modulate their activity. For example, the cleaved precursor domain of TGF-β1, the TGF-β1 binding protein, and the proteoglycan decorin have been shown to form complexes with the bioactive TGF-β ligand to regulate its activity (MIYAZONO *et al.* 1988, 1991; WAKEFIELD *et al.* 1988; GENTRY and NASH 1990; KANZAKI *et al.* 1990; YAMAGUCI *et al.* 1990; BORDER *et al.* 1992). Activin, another TGF-β family member, can be isolated bound to follistatin, an activin binding protein (NAKAMURA *et al.* 1990; HEMMATI-BRIVANLOU *et al.* 1994) that inhibits the normal action of activin.

The best characterized accessory molecules are the members of the *tolloid*/BMP-1 family, a subfamily of the larger protease family, the astacin family. The astacin family of metalloproteases are secreted zymogens that have members in many phyla, and although similar in the protease domain, members have different functions and also contain different regulatory domains in *cis*. The *tolloid*/BMP-1 subfamily members contain one or more epidermal growth factor (EGF) and complement subcomponents C1r/C1s, Uegf, and Bmp1 (CUB) repeats located C-terminal to the protease domain. These two motifs are implicated in protein–protein interactions (VILLIERS *et al.* 1985; WEISS *et al.* 1986; APPELLA *et al.* 1988; BUSBY and INGHAM 1988, 1990; BORK and BECKMANN 1993), which suggests that these metalloproteins form complexes with other proteins. Models for how *tolloid (tld)* might modulate *dpp* activity have been proposed (FINELLI *et al.* 1994; CHILDS and O'CONNOR 1994).

dpp is one of seven zygotic genes involved in dorsal–ventral specification of the Drosophila embryo. Of the other six genes (*tld, screw, shrew, twisted gastrulation, short*

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gastrulation, and *zerknüllt*), *dpp* mutations exhibit the strongest phenotype, which suggests a central role. *dpp* null embryos are ventralized and lack all dorsal epidermal derivatives (IRISH and GELBART 1987). In addition, some alleles of *dpp* are also critical for development of the midgut (SEGAL and GELBART 1985; PANGANIBAN *et al.* 1990; REUTER *et al.* 1990; HURSH *et al.* 1993). The *shortvein* and *disk* regions of *dpp* are required for patterning of adult structures derived from the imaginal disks (SPENCER *et al.* 1982; SEGAL and GELBART 1985). Among the mutant phenotypes of *dpp* are defects in venation and overall morphology of the wing (SPENCER *et al.* 1982; SEGAL and GELBART 1985).

tld was originally identified in saturation screens for patterning mutations as one of seven zygotically expressed genes necessary for proper dorsal–ventral axis determination in Drosophila (WIESCHAUS *et al.* 1984). Later, it was shown that *tld* interacts genetically with the *dpp* gene (FERGUSON and ANDERSON 1992a; FINELLI *et al.* 1994) that is present in the embryo as a dorsal to ventral gradient of activity that is capable of specifying at least three distinct cell fates (FERGUSON and ANDERSON 1992b). The *tld* zymogen has its N-terminal metalloprotease domain followed by two CUB repeats, an EGF repeat, a CUB repeat, another EGF, and two final CUB repeats (SHIMELL *et al.* 1991; FINELLI *et al.* 1994). One model to explain the role of *tld* is that it functions in the embryonic dorsal–ventral DPP pathway by forming a complex containing the DPP ligand via its protein-interacting EGF and CUB domains. The protease activity of *tld* is necessary, either directly or indirectly, for the activation of the DPP complex, which then binds receptor(s) and effects signal transduction. This model is based on the data that antimorphic alleles of *tld* (alleles that enhance the *dpp* embryonic dorsal–ventral phenotype versus a *tld* deficiency) contain nonconservative amino acid changes in the protease domain. The wild-type protease function of *tld* would be abolished, whereas the protein-interacting EGF and CUB repeats remain intact and capable of sequestering a complex containing DPP, rendering it inactive. Most *tld* alleles that do not interact with *dpp* are missense mutations in the C-terminal EGF and CUB repeats or contain termination codons that delete these repeats. These mutant *tld* proteins would not be capable of a protein interaction with the DPP complex, and DPP is free to function. Further support for this model comes from the observation that BMP-1, the human homologue of *tld*, copurifies with several other TGF-β-like BMPs from bone extracts (WOZNEY *et al.* 1988).

It is interesting to note that although the expression patterns of *dpp* and *tld* overlap, they are not identical, raising the possibility that *tld* interacts with other TGF-β-like growth factors, such as *screw* (BRUMMEL *et al.* 1994), *60A* (WHARTON *et al.* 1991; DOCTOR *et al.* 1992), or other unidentified TGF-β-like genes. These models may be complicated by the existence of other *tolloid*/BMP-1 molecules in the fly, which may function with any one or several of these growth factors.

During the cloning of *tld*, we identified another member of the *tolloid*/BMP-1 family in Drosophila, *tolkin (tok)*, which is located 700 bp 5′ to *tld*. *tok* possesses the same general organization as *tld*, with its N-terminal metalloprotease domain followed by two CUB repeats, an EGF repeat, a CUB repeat, another EGF, and two final CUB repeats. *tok* is expressed in the embryo, the larval brain, and also in the imaginal disks. We have generated null alleles of *tok*, and most *tok* mutants die as larvae. Although the mutant phenotype is not fully penetrant, some *tok* larvae have reduced brains and imaginal disks, indicating that *tok* is necessary for proper development of these structures. Homozygous escapers of *tok* null alleles exhibit several wing vein defects, indicating that *tok* is involved in the specification of cell fates in the wing. We characterize the phenotype of *tok* mutants and discuss several possible roles in development for this new member of the *tolloid*/BMP-1 family.

MATERIALS AND METHODS

Cloning and sequencing *tok*: The *tok* gene was identified by low stringency hybridization with a human BMP-1 probe. Lambda phage from a genomic walk of the *tld* region were probed with BMP-1 cDNA sequences under reduced conditions. A phage adjacent to the one containing *tld* was found to hybridize. *Sau*3A fragments from the hybridizing fragment of the lambda phage were cloned into M13 phage and sequenced (MESSING 1983). One fragment showed similarity to the *tolloid*/BMP-1 family, confirming the hybridization data. Genomic fragments of the hybridizing phage were used to screen cDNA libraries (POOLE *et al.* 1985; BROWN and KAFATOS 1988). Several overlapping cDNAs containing *tok* coding sequences were isolated. pTX60.3 contains the 3′ end of the gene and most of the coding region. p535.6 is truncated at the 5′ and 3′ ends and contains part of the 5′ coding sequences. To obtain the remainder of the coding region and the 5′ untranslated sequences, we used anchored PCR, using primers in the cDNA vector and in the coding regions. The SP6 primer, located upstream of the 5′ of cDNAs in pNB40 of N. BROWN's 4–8- and 12–24-hr libraries, was used as the 5′ primer. A primer made to sequences in pTX60.3 was used as the 3′ primer. Areas of highest DNA concentration were cut out from the gel and purified, and a second round of PCR was done using a nested 3′ primer made to pTX60.3 sequences. After another round of nested PCR, a single band was observed and cloned. We sequenced four independent clones and and made a consensus sequence to avoid PCR errors. The *tok* cDNA sequences and parts of the genomic sequences were determined by the dideoxy chain termination technique, as described (FINELLI *et al.* 1994). Deletion clones of the cDNA were constructed according to the protocol described in the Erase-a-Base Kit (United States Biochemical, Cleveland, OH). In addition, primers were made along the cDNA and used to sequence. Computer analysis of sequences was done using the STADEN programs (STADEN 1982, 1984), the GCG programs, FASTA (DEVEREUX *et al.* 1984; PEARSON and LIPMAN 1988) and BLAST (ALTSCHUL *et al.* 1990).

Mutagenesis: Mutations in *tok* were made using both EMS and abortive jumps of a nearby *P*-element construct, P[(w^-,ry^+)G]3 (LEVIS *et al.* 1985). Because *tok* is physically located between this *P* element and *tld*, we could easily map deletions in *tok* (FINELLI *et al.* 1994). P[(w^-,ry^+)G]3 flies were

crossed to a transposase containing strain (ROBERTSON *et al.* 1988) to mobilize the *P* element. Mutant lines that were ry^- and homozygous lethal were collected. We also generated EMS alleles of genes in $tld^{\gamma 1}$, a small deficiency in the *tld/tok* region, using standard EMS procedures (LEWIS and BACHER 1968). The deficiencies generated by the abortive *P*-element jumps that delete *tld* always uncover one other complementation group generated by EMS, suggesting this might correspond to the *tok* gene. DNA from the *tok* gene of this EMS allele was cloned and sequenced and found to contain a lesion in the *tok* gene, tok^1. The other *P*-element deletion lines were then tested against tok^1 for complementation of tok^1 and *tld*. Twenty *P*-element lines failed to complement *tok* but complemented *tld*, which indicates that they delete at least part of *tok*. These *P*-element deletion lines were molecularly mapped. DNA from *tok* deletion mutants was isolated from about 50 adult flies by homogenization with a Kontes grinder in a microfuge tube (FINELLI *et al.* 1994). DNA was digested with either *Eco*RI or *Hin*dIII, and Southern blots were done using the 5′ *P* element, the 3′ *P* element, a 7.5-kb genomic fragment 3′ to the *P* element, and *tok* cDNA sequences as probes. Endpoints of the deletions were determined by comparing changes with the original *P*-element line, P[(w^-, ry^+)G]3, or the balancer chromosome.

Determination of *tok* lethal phase: Egg lays of *trans*-heterozygous alleles of *tok* (tok^1/+ X $tld^{\gamma 1}$/*TM3, Sb, Ser*) indicate that essentially all *tok* null animals hatch. Because *tok* animals survive embryogenesis, we used marked strains to establish the lethal phase at later stages of development. In some cases, the *red* gene, which causes the Malpighian tubules of the larvae to be red, was recombined onto the *tok* chromosome. In other cases, we used *tok/TM6B, Tb* flies, and larvae and pupae that were Tb^+ could be distinguished from those carrying the balancer chromosome. Up to 10% of the animals pupate and up to 3% of *tok* animals eclose. These adult escapers exhibit wing venation defects, primarily affecting the crossveins. Phenotypes of the balancer markers are described in LINDSLEY and ZIMM (1992).

RNA *in situ*: The following constructs were used: pTX60.3, containing a 3.2-kb *tok* cDNA cloned into pBluescript SK+; pF46.2 (FINELLI *et al.* 1994), containing a 3.5-kb *tld* cDNA cloned into pBluescript SK+; and E55 (PADGETT *et al.* 1987), containing a 2.9-kb *dpp* cDNA cloned into pSP65. The purified cDNA inserts were used for RNA *in situ* as described by TAUTZ and PFEIFLE (1989).

Northern analysis: A developmental Northern blot containing Drosophila stages 0–4, 4–8, 8–12, 12–24, L1, L2, L3, early L3, wandering L3, early pupal, late pupal, males and females was probed with the construct pTX60.3.

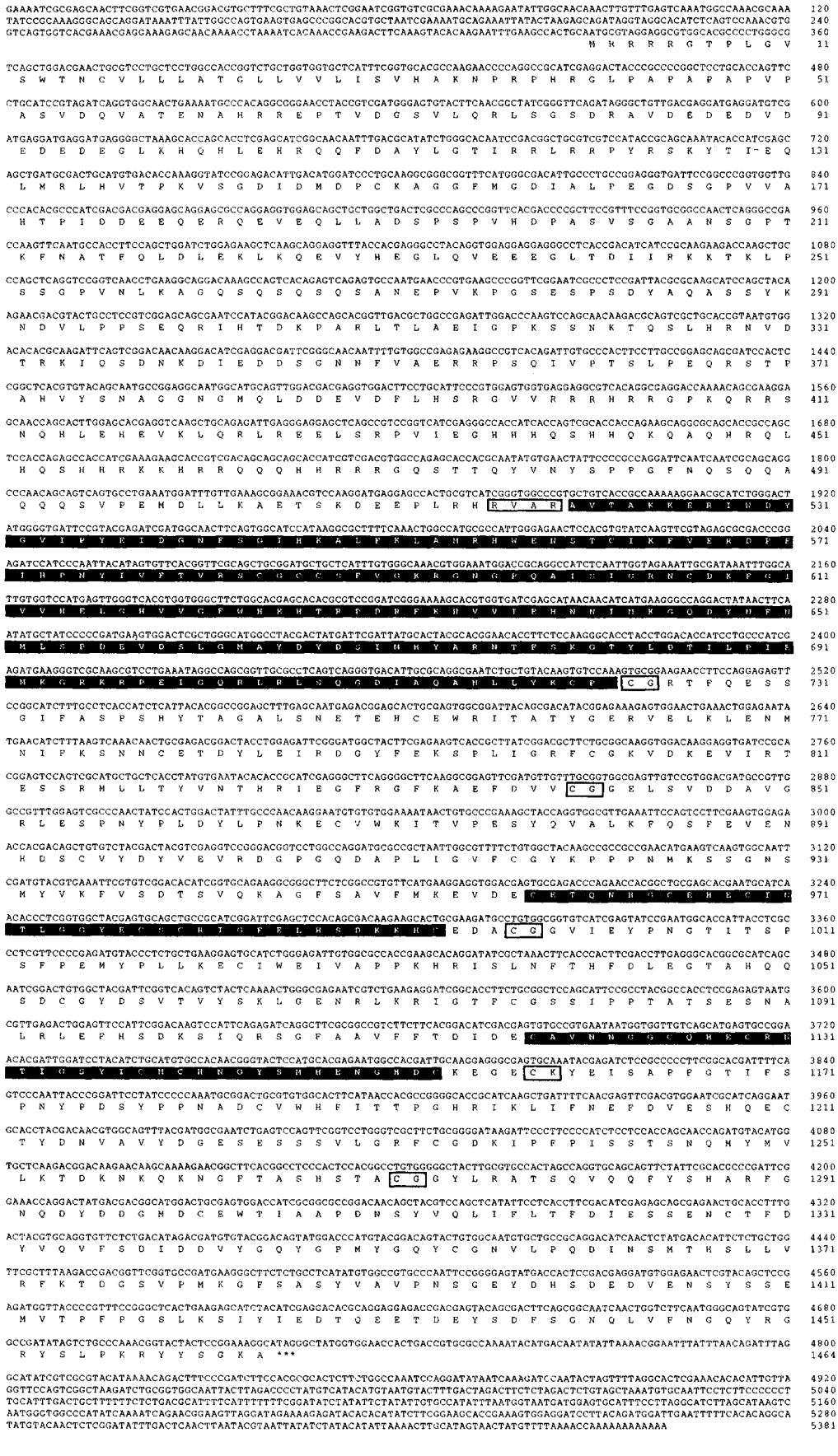
RESULTS

Cloning and sequence analysis of *tok*: To look for potential homologues of the *tolloid*/BMP-1 family in Drosophila, we probed a genomic walk of the *tld* region with the protease domain of human BMP-1. A hybridizing clone was analyzed further by sequence analysis and revealed similarity to *tld* and human BMP-1. We named the gene *tolkin*, because of its relationship to *tld*. The gene has also been previously referred to as *tolloid-related-1 (tlr-1)*; however, the *Toll*-like gene in Drosophila (CHIANG and BEACHY 1994) uses the same abbreviation. To avoid future confusion, we have opted for the name *tolkin*.

Using a *tok* genomic probe, we screened for cDNA clones in Drosophila embryonic and disk libraries. Several cDNAs were isolated, two of which were further characterized and completely sequenced. These cDNAs were truncated at the 5′ end. We therefore cloned the *tok* 5′ sequences using anchored PCR, using primers in the cDNA vector and in the cDNA sequence. This composite sequence is 5.4 kb and encodes a protein of 1464 amino acids (Figure 1).

***tok* is a member of the *tolloid*/BMP-1 family:** The coding region of *tok* was compared with other members of the *tolloid*/BMP-1 family. We note the presence of a potential dibasic cleavage site adjacent to the protease domain, as seen in *tld* and other TGF-β-like molecules (Figure 1). In addition to a zinc-binding metalloprotease domain, it contains two EGF and five CUB repeats, in the same order as those of *tld* and the mouse BMP-1 (Figure 2) (FUKAGAWA *et al.* 1994). For the mouse and human, there is a longer version (two EGFs and five CUB repeats) and a shorter version (one EGF and three CUB repeats) produced by alternative splicing (TAKAHARA *et al.* 1994). Neither *tld* (SHIMELL *et al.* 1991) nor *tok* has the shorter form of these proteins, as shown by developmental northern analysis (data not shown). In the frog, sea urchin BMP-1, and in sea urchin SpAN and BP10, the number and/or arrangement of EGF and CUB repeats is different from that of *tok*, *tld*, and from the long form of human and mouse BMP-1. In addition, the members from these organisms may contain additional repeats, such as a threonine rich region (SpAN and BP10) (REYNOLDS *et al.* 1992) and a thrombospondin repeat (*Caenhorabiditis elegans*) (S. H. CHO and R. W. PADGETT, unpublished data) not found in other members of the family (Figure 2). Consistent with similar structural organization, we note that the dendrogram analysis and sequence alignment of *tolloid*/BMP-1 family members shows that *tok*, *tld*, and BMP-1 are more related to each other in sequence than to the other members of the family (Figure 3).

***tok* has structural differences from *tld*:** *tok* has structural differences from *tld* that are worth noting. The *tok* proregion, the N-terminal region adjacent to the protease domain, is longer than the proregion of *tld* by 393 amino acids. The proregion of *tok*, like that of *tld*, is likely to be proteolytically cleaved near the beginning of the protease domain at dibasic sites (Figure 1) to release the active enzyme. Other members of the astacin family contain about 85–125 amino acids in this region. Structural analysis of the astacin family indicates that the proregion is sequestered in the protease by a salt bridge (BODE *et al.* 1992; YASUMASU *et al.* 1992; GOMIS-RÜTH *et al.* 1993; STÖCKER *et al.* 1993). Because the *tok* proregion is longer, the activity of the *tok* zymogen may be regulated differently than *tld*. Another structural difference of *tok* is in the first CUB domain. *tok* and *tld* both have amino acid inserts at the same position in the first CUB domain, distinguishing them from the other members of the *tolloid*/BMP-1 subfamily and also from conventional CUB repeats (Figure 3). However, the insert in *tld* is 26 amino acids long,



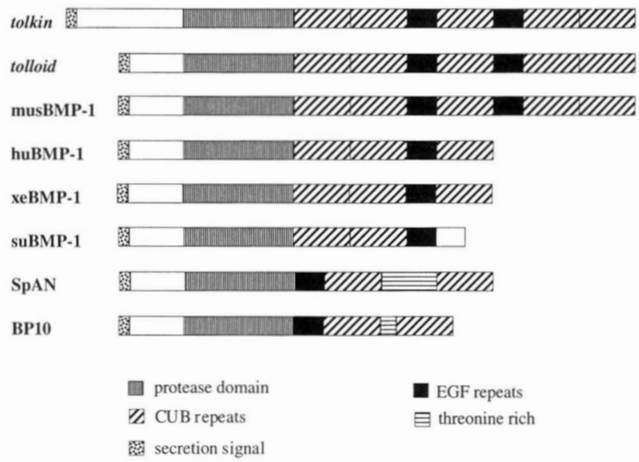


FIGURE 2.—Domain structure and organization of members of the *tolloid*/BMP-1 subfamily. All members contain an N-terminal metalloprotease domain and a proregion, EGF repeats, and CUB repeats. The proregion of *tok* is longer than that of the other members. The threonine region of SpAN consists of short threonine rich repeats. (huBMP-1 is from WOZNEY *et al.* 1988; musBMP-1 is from FUKAGAWA *et al.* 1994; suBMP-1 is from HWANG *et al.* 1994; SpAN is from REYNOLDS *et al.* 1992; BP10 is from LEPAGE *et al.* 1992; Xenopus BMP-1 is from MAÉNO *et al.* 1993)

whereas the insert in *tok* is 7 amino acids long, and this may distinguish their biological activities, specifying interactions with different proteins. We previously noted that some antimorphic mutations of *tld* map to the second CUB repeat (FINELLI *et al.* 1994). This observation, together with the insertion in the first CUB domain, suggests that the first two CUB repeats in these molecules may be important for function, in particular, for specifying interactions with other molecules. It is also interesting to note that there are two alternatively spliced forms of BMP-1 in mouse and humans. The shorter forms do not include the C-terminal EGF and CUB repeats, but both forms always have the first two CUB repeats, further suggesting that these first two repeats are necessary for function (TAKAHARA *et al.* 1994).

***tok* is an essential gene:** Because genetic mutations in *tok* did not exist, we proceeded to induce them to find out if the *tok* gene is necessary for viability. Mutations in the gene were made by EMS and by hybrid dysgenesis of the nearby *P* element, P[(w^-, ry^+)G]3 (LEVIS *et al.* 1985; FINELLI *et al.* 1994) (Figure 4). Twenty-three lines from dysgenic crosses had obtained a lethal hit during the mutagenesis. Three of these lines did not complement *tld*, suggesting that they also delete the *tok* gene, which resides between the *P* element and *tld*. To generate point alleles in *tok*, we generated EMS alleles and tested them for failure to complement $tld^{\gamma 1}$, a small deficiency of the *tld/tok* region (FINELLI *et al.* 1994). Those alleles that complemented *tld* but failed to complement the *P*-element-induced lethal deletions were candidates for mutations in the *tok* gene. One such candidate allele was identified and sequenced. This allele, tok^1, was found to contain a stop codon in the *tok* protease domain, confirming that this allele did indeed correspond to a *tok* null mutation. Other *P*-element-induced deletion lines were then tested against tok^1 for complementation. All 23 lines failed to complement *tok*, indicating that they deleted the *tok* gene. These lines were molecularly characterized to determine the extent of their deletions. We find that these deletions occur both 5′ and 3′ of the *P* element. Some deletions are <300 bp and lie directly 3′ to the *P* element. These deletions remove *tok* 5′ untranslated sequences, as determined by Southern blotting analysis using *tok* 5′ sequences as a probe. We also find that some lines have deleted only portions 5′ of the *P* element, indicating that the *tok* gene has regulatory regions that extend 5′ of the *P* element or that the *P* element resides in a *tok* intron. It is interesting to note that the original P[(w^-, ry^+)G]3 strain is homozygous viable, indicating that this *P* element does not disturb important *tok* regulatory sequences, even though it is flanked on both sides by *tok* regulatory sequences.

***tok* expression:** Developmental northern analysis indicates the presence of a single transcript of approximately 5.5 kb, which is expressed throughout development. To examine the spatial expression of *tok* and its relationship to *tld* and *dpp*, we compared the patterns of expression through embryogenesis. *tok* is expressed in the dorsal part of the Drosophila blastoderm embryo (Figure 5A). At this early stage, *tok* expression patterns here overlap those of *tld* and *dpp* (Figure 5, B and C) and fade rather quickly. *tok* hybridization is not due to cross-hybridization with *tld*, because we do not see other *tld* patterns in later embryos when a *tok* probe is used. Because the transcription unit of *tok* appears to be large, it is possible that the length of the mitotic cycles at this stage is too short to permit entire *tok* transcription and thus prevents *tok* protein from being produced. That the length of mitotic cycles can provide a physiological barrier to transcript size has been suggested by SHERMOEN and O'FARRELL (1991). A similar situation has also been observed by ROTHE *et al.* (1992), who demonstrated that the 23 kb *knirps-related* gene was not fully transcribed during mitotic cycle 13 of the preblastoderm embryo.

During germ band extension, *tok* is expressed near the mesectoderm (Figure 5D). At this stage, *tld* and *dpp* are expressed in the dorsal ectoderm (Figure 5, E and F). During stage 10, three faint lateral spots of *tok* expression are seen in the thoracic segments (Figure 5D). During dorsal closure, we observe *tok* expression in the

FIGURE 1.—*tok* cDNA sequence. The N-terminal proregion of *tok* extends from position 1 to position 393. The protein sequence in the black box beginning at amino acid 520 is the metalloprotease domain. The first EGF repeat is represented by a black box beginning at amino acid 958, whereas the second EGF repeat is reperesented by a black box beginning at amino acid 1118. The beginning of each CUB domain is denoted by an open box containing the first two amino acids of each repeat. A putative proteolytic processing site at position 516, adjacent to the protease domain, is boxed.

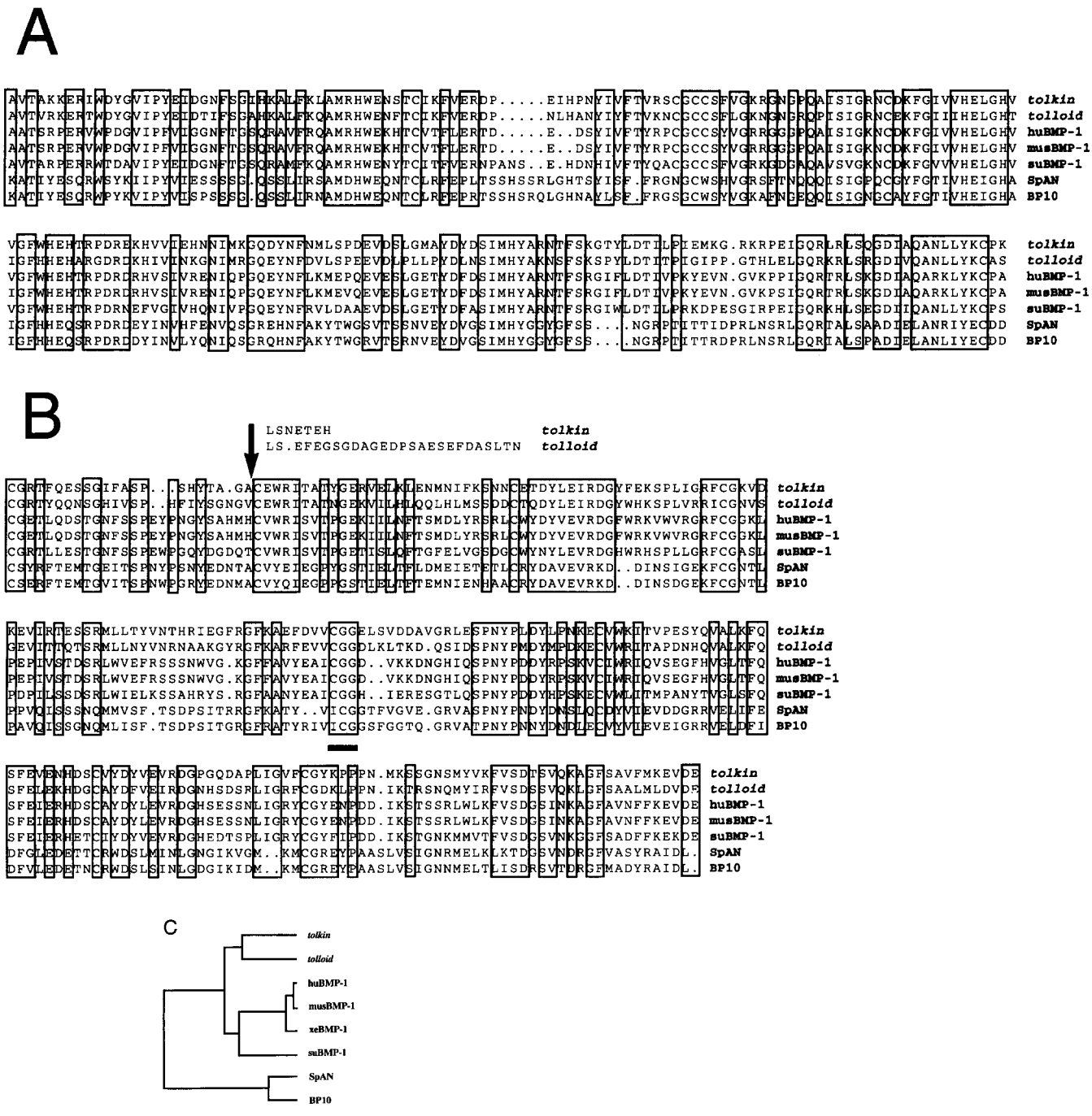


FIGURE 3.—(A) Alignment of the protease domains of the *tolloid*/BMP-1 family of zinc-binding metalloproteases. Boxes represent locations where five of seven amino acids are identical. Dots represent gaps created to maximize alignment. (B) Alignment of CUB domains 1 and 2. Both *tld* and *tok* contain amino acid inserts in the first CUB domain. The black bar indicates where the second CUB domain begins. (C) Dendrogram analysis of the protease domains of the *tolloid*/BMP-1 family members. A dendrogram of the first two CUB repeats from the same species reveals the same relationships (huBMP-1 is from WOZNEY *et al.* 1988; musBMP-1 is from FUKAGAWA *et al.* 1994; suBMP-1 is from HWANG *et al.* 1994; SpAN is from REYNOLDS *et al.* 1992; BP10 is from LEPAGE *et al.* 1992).

cells that will form the corpus allatum of the ring gland, a specialized larval endocrine complex composed of several glands that secrete hormones that regulate Drosophila growth and development (DAI and GILBERT 1991). These staining cells form two pronounced symmetric spots on either side of the embryo (Figure 5J), ultimately migrating toward each other to form a single dorsal spot in the third thoracic segment (Figure 5K).

Late patterns of *tok* staining are observed (Figure 5G). In the central nervous system, *tok* is dynamically expressed in a small number of cells. In stage 14 and 15 embryos, one or two of these cells are at the position of the RP motoneurons, or just lateral to them. In older embryos, *tok* is expressed in many large ventral cells in the nerve cord (C. DOE, personal communication).

In the larval brain, *tok* is expressed in two distinct

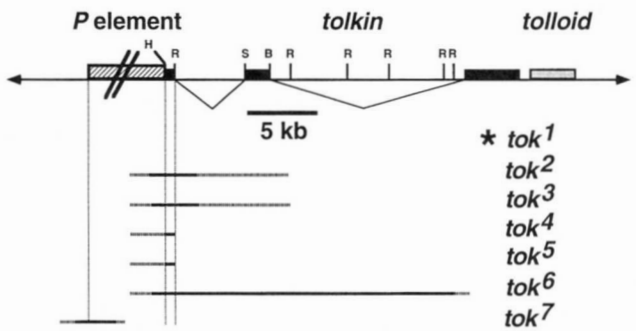


FIGURE 4.—Molecular map of the *tok*/*tld* genomic region. Black boxes represent the *tok* cDNA sequences. The third *tok* exon in the map actually contains several small introns that were not shown due to their small size. The *tld* gene is depicted as a grey box. All of the known *Eco*RI sites in the region are represented. However, only significant *Bam*HI, *Hin*dIII, and *Sal*I sites are shown. The slashes in the *P* element indicate that the *P* element is not drawn to scale. Hatch marks represent the extent of deleted genomic sequences. The proximal endpoint of *tok7* has not been molecularly identified but is known to extend a few hundred bases from the *P* element. The asterisk denotes a point allele.

rings in both hemispheres, one of which corresponds to the outer proliferation center of the brain (Figure 6, A and B). It is here where cells undergo cell divisions before they differentiate (SELLECK *et al.* 1992). For comparison, we also stained *tld* larval brains. *tld* expression patterns are both overlapping and distinct from those of *tok* (Figure 6C). Like *tok*, *tld* is also expressed in the outer proliferation center of the brain. In addition, *tld* appears to be expressed in the internal proliferation centers of the brain. We have also performed *tld lac-Z* staining on larval brains (Figure 6D). Because these patterns match those obtained for the *tld* RNA *in situ*, we can be sure there is no cross-hybridization between *tld* and *tok*. *dpp* is also expressed in the brain in two spots, rather than stripes, in each hemisphere (BLACKMAN *et al.* 1991; POSAKONY *et al.* 1991; KAPHINGST and KUNES 1994). These spots of expression may overlap *tld* and *tok* expression.

In most imaginal disks, both *tok* and *tld* stain the entire imaginal disk, with some areas staining more intensely than others. *dpp* is also expressed in specific patterns in all disks. In the eye-antennal disks, both *tld* and *tok* are expressed in all cells anterior to the morphogenetic furrow (Figure 6, E and F). *dpp* is expressed in the morphogenetic furrow of the eye-antennal disks (BLACKMAN *et al.* 1991; POSAKONY *et al.* 1991) and is critical for progression of the furrow (HEBERLEIN *et al.* 1993). *tok* and *tld* expression patterns overlap those of *dpp* in the eye-antennal disk, suggesting common roles.

Lethal phase and phenotype of *tok* mutants: Because *tok* mutations are lethal, we wanted to determine at what stage *tok* mutants die. Viable larvae from crosses between two independently induced null *tok* alleles were counted. Essentially all *tok* embryos hatch, indicating that lethality does not occur during embryogenesis. This explains why *tok* was not detected in the screens for embryonic lethals on the third chromosome (WIESCHAUS *et al.* 1984). Most *tok* mutants die during the larval stages, with <10% pupating. A possible explanation for the lethality of *tok* larvae may be defects in the central nervous system or ring gland, which specifically show *tok* expression. Up to 3% actually eclose, depending on the specific allele. To examine defects of *tok* mutant larvae, the *red* gene was recombined onto the *tok* deletion strains tok^4 and tok^5. The *red* gene, when homozygous, gives the Malpighian tubules of larvae a red color, and thus, larvae mutant for *tok* could be identified. Some *tok* larvae (30–50%, depending on the allele) possess smaller brains and imaginal disks and appear to be smaller in overall body size. In addition, although these larvae appear morphologically normal, they seem to be sickly. Smaller larvae are also found with some *dpp shortvein* (*shv*) mutations (SEGAL and GELBART 1985). *shv* is a *cis*-regulatory region of *dpp* that governs expression in several tissues and is necessary for larval viability. This raises the possibility that *tok* is involved in the regulation of *dpp* at the larval stage, although the characteristic gut abnormalities of *shv* mutant larvae are not seen in *tok* larvae. We also noted that the mutant phenotypes of *tok* animals that have one copy of *tld* removed are not worse than *tok* larvae. This would suggest that *tld* does not enhance the phenotype of a *tok* mutant and that the two genes may function independently.

***tok* escapers:** Up to 3% of progeny from crosses of heteroallelic combinations of *tok* escape to adulthood. These escapers possess wing defects in which the posterior crossvein is missing. Less frequently, escapers have been observed where both crossveins are incomplete on both wings or one wing appears to be wild-type, whereas the other is missing the posterior crossvein (Figure 6H). Otherwise, the adults appear normal. Defects in wing venation are seen with *shv* alleles of *dpp* (SEGAL and GELBART 1985), *saxophone* null alleles (*dpp* type I receptor) (BRUMMEL *et al.* 1994; NELLEN *et al.* 1994; PENTON *et al.* 1994; XIE *et al.* 1994), and *thick veins* (*dpp* type I receptor) (BRUMMEL *et al.* 1994; NELLEN *et al.* 1994; PENTON *et al.* 1994) alleles, which are all components of the *dpp* pathway. In our crosses, *trans*-heterozygotes of *shv* and *tok* do not show any genetic interaction. However, these assays may not be sensitive enough to detect interactions.

DISCUSSION

The *tolloid*/BMP-1 family is part of the larger astacin family. The astacin family is one of five families of structurally distinct zinc-binding metalloproteases (JIANG and BOND 1992). Unlike many of the other metalloprotease families, members of the *tolloid*/BMP-1 family have been implicated in a variety of developmental processes. In this article, we describe a new member of the astacin family in Drosophila, *tok*, which is most related

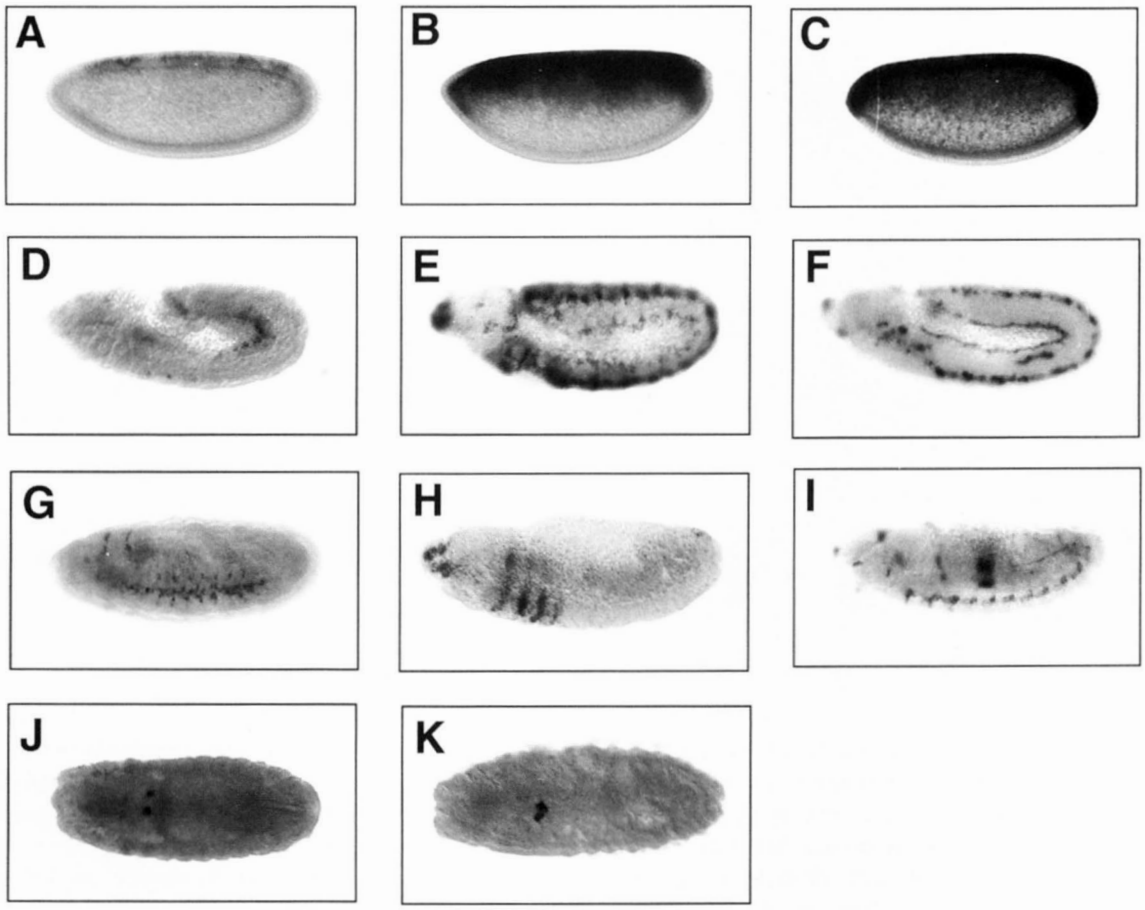


FIGURE 5.—RNA *in situ* of *tok*, *tld*, and *dpp* embryos. Embryos are positioned so that anterior is to the left and dorsal is up. *tok* (A), *tld* (B) and *dpp* (C) at the blastoderm stage, during germ band extension (D–F), and after germ band retraction (G–I). Embryos showing *tok* expression in the corpus allatum of the ring gland as two bilateral symmetric spots during migration (J) that eventually migrate to form a single dorsal spot (K). The embryo in (G) is rotated so as to expose more of the ventral aspect, moving the ring gland out of view.

to the Drosophila *tld* gene. As is true for *tld*, *tok* is mutable to a lethal phenotype. *tok* mutants die mostly as larvae. Although this mutant phenotype is not fully penetrant, some *tok* larvae have reduced brains and disks. Adult escapers of *tok* mutations possess wing defects in which the anterior and/or posterior crossvein is missing and/or reduced.

Do *tld* and *tok* have similar functions? One interesting question is whether *tld* and *tok* can function similarly or do they interact specifically with different molecules and serve different functions? Examination of similarly duplicated genes in Drosophila reveals a wide range of functions of the duplicated gene. The *engrailed* and *invected* genes are partially duplicated and reside near each other. Their expression patterns overlap, revealing a difference only in timing. However, this difference may be a consequence of the larger transcription unit of *invected* (>30 kb) versus that of *engrailed* (4 kb) (COLEMAN *et al.* 1987). The Drosophila *knirps-related (knrl)* gene has a larger transcription unit (23 kb) than its homologue, *knirps (kni)* (3 kb). A *knrl* minigene, with the large intron removed, can rescue *kni* embryos (ROTHE *et al.* 1992). The *sloppy paired 1 (slp1)* and *sloppy paired 2 (slp2)* genes of the *sloppy paired* locus are similarly transcribed. Mutations in the *slp* genes do not behave in an additive fashion because they show a genetic interaction. Removal of one copy of *slp2* in a *slp1* background gives an intermediate phenotype (GROSSNIKLAUS *et al.* 1992). The duplicated genes of the *gooseberry* locus have expression patterns that overlap but are distinct. It remains to be demonstrated whether or not both genes are functional and contribute to the phenotype (BAUMGARTNER *et al.* 1987; CÔTÉ *et al.* 1987). In the case of *z1* and *z2*, two closely linked homeobox genes in the *zen* region of the Antennapedia complex, *z2* appears to be dispensable. *z1* can rescue a deficiency that removes *z1* and *z2* (RUSHLOW *et al.* 1987). Because the coding region and expression patterns of both genes are maintained, it may be that they partially compensate for each other, and outside the laboratory setting there may be a selective advantage for having both genes. These data suggest that the functions of duplicated genes can range from essential to nonessential.

From our data, there is no convincing evidence that *tld* and *tok* compensate for each other or that *tok* is involved with *dpp* activity. Unlike many of the dupli-

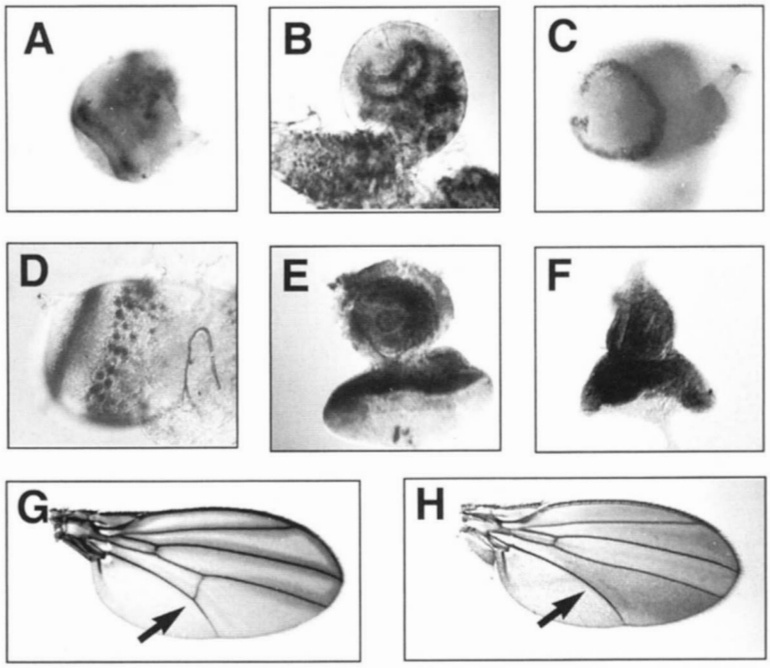


FIGURE 6.—RNA *in situ* of larval brains and eye antennal disks and mounted wing of a *tok* escaper. RNA *in situ* of *tok* (A and B) and *tld* (C) in the larval brain. *lac-Z* staining of *tld* in the larval brain (D). RNA *in situ* of *tok* (E) and *tld* (F) in the eye antennal disk. Shown is a wild-type wing (G) and the wing of a *tok* escaper (H). Note the missing posterior crossvein in the *tok* wing.

cated genes mentioned, the genetic analysis of *tld* and *tok* provides clear evidence that each is independently mutable. *tld* mutants die as early embryos, indicating that *tok* cannot substitute for *tld* at this stage. Similarly, *tld* function cannot substitute for *tok*, and *tok* mutants die as larvae. Differences in protein interactions of *tld* and *tok* could result from differences in their CUB repeats, which define these interactions. *tld* has an insert of about 26 amino acids in the first CUB domain, whereas *tok* only has seven additional amino acids. These inserts are specific to *tld* and *tok* and are not observed in the CUB domains of other proteins (BORK and BECKMANN 1993). It is, however, possible that *tok* and *tld* functions overlap in some respects, as *tld* and *tok* disk expression patterns are similar. Regarding *tok's* possible involvement with *dpp*, there are no obvious gut defects in *tok* embryos, as is observed in *dpp* embryos, suggesting that *tok* does not interact with *dpp* here. However, escapers heteroallelic for *tok* (0–3% of flies) have wing venation defects similar to those seen in alleles of *dpp*, *saxophone* and *thick veins* (BRUMMEL *et al.* 1994; NELLEN *et al.* 1994; PENTON *et al.* 1994). Because these genes are all components of the *dpp* pathway, it is possible that *tok* interacts with *dpp* in this imaginal disk, acting to define proper cell fates in the wing. In addition, because *tok* larvae sometimes have small disks and brains, as seen with *dpp* and other genes of the *dpp* pathway, it is possible that *tok* is involved in the *dpp* pathway in these structures. It is quite possible that *tok* enhances the activity of *dpp*, but our assays may not be sensitive enough to substantiate the interaction.

What is *tolkin* function? In cases where *tok's* function may be different from *tld*, what could be the function of *tok*? If the *tolloid*/BMP-1 family proteolytically processes TGF-β-like growth factors, maybe *tok* participates in this process with the *60A* or *screw* genes, or other TGF-β-like molecules that have not been reported. Perhaps *tok* forms heterodimers with *tld* at certain stages in development, contributing to the specificity of interactions. It is also possible that *tok* may interact with other proteases involved in the processing of these growth factors. *tok* is expressed generally in the imaginal disks and is expressed anterior to the morphogenetic furrow in the eye antennal disks. Because *dpp* is also expressed in these structures and is necessary for their development, it is possible that *tok* is involved in its activation. Other TGF-β-like growth factors that have not yet been characterized may be expressed in the disks as well, and it is also possible that *tok* may be involved in their activation.

Other *tolloid*/BMP-1 members: There exist other *tolloid*/BMP-1 members in different phyla. One group, consisting of the Drosophila genes *tld* and *tok*, the sea urchin suBMP-1 (HWANG *et al.* 1994), Xenopus BMP-1 (MAÉNO *et al.* 1993), mouse BMP-1 (FUKAGAWA *et al.* 1994) and human BMP-1 (WOZNEY *et al.* 1988), has five CUB repeats and two EGF repeats. In mouse and humans, there is alternative splicing, which produces a shorter version of this longer form.

The study of other genes may be helpful in deciphering the function of this family of developmental metalloproteases. We have begun examination of *tld* homologues in the nematode *C. elegans*, which were first identified by the genome sequencing project. It remains to be seen whether these *tld* homologues in the nematode are involved in a TGF-β-like signal transduction pathway, as is *tld*. Whether or not the *tolloid*/BMP-1 members function independently of TGF-β-like proteins will be addressed by further examination of members of the family.

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LITERATURE CITED

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS and D. LIPMAN, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

APPELLA, E., I. T. WEBER and F. BLASI, 1988 Structure and function of epidermal growth factor-like regions in proteins. FEBS Lett. **231:** 1–4.

BAUMGARTNER, S., D. BOPP, M. BURRI and M. NOLL, 1987 Structure of two genes at the *gooseberry* locus related to the *paired* gene and their spatial expression during Drosophila embryogenesis. Genes Dev. **1:** 1247–1267.

BLACKMAN, R. K., M. SANICOLA, L. A. RAFTERY, T. GILLEVET and W. M. GELBART, 1991 An extensive 3′ cis-regulatory region directs the imaginal disk expression of *decapentaplegic*, a member of the TGF-b family in Drosophila. *Development* **111:** 657–666.

BODE, W., F. X. GOMIS-RÜTH, R. HUBER, R. ZWILLING and W. STOCKER, 1992 Structure of astacin and implications for activation of astacins and zinc-ligation of collagenases. Nature **358:** 164–167.

BORDER, W. A., N. A. NOBLE, T. YAMAMOTO, J. R. HARPER, Y. YAMAGUCI *et al.*, 1992 Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. Nature **360:** 361–364.

BORK, P., and G. BECKMANN, 1993 The CUB domain A widespread molecule in developmentally regulated proteins. J. Mol. Biol. **231:** 539–545.

BROWN, N. H., and F. C. KAFATOS, 1988 Functional cDNA libraries from Drosophila embryos. J. Mol. Biol. **203:** 425–437.

BRUMMEL, T. J., V. TWOMBLY, G. MARQUÉS, J. L. WRANA, S. J. NEWFELD *et al.*, 1994 Characterization and relationships of *dpp* receptors encoded by the *saxophone* and *thick veins* genes in Drosophila. Cell 78: 251–261.

BUSBY, T. F., and K. C. INGHAM, 1988 Domain structure, stability and interactions of human complement C1s: characterization of a derivative lacking most of the B chain. Biochemistry **27:** 6127–6135.

BUSBY, T. F., and K. C. INGHAM, 1990 NH2-terminal calcium-binding domain of human complement C1s mediates the interaction of C1r with C1q. Biochemistry **29:** 4613–4618.

CELESTE, A. J., J. A. IANNAZZI, R. C. TAYLOR, R. M. HEWICK, V. ROSEN *et al.*, 1990 Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. Proc. Natl. Acad. Sci. USA **87:** 9843–9847.

CHIANG, C., and P. A. BEACHY, 1994 Expression of a novel *Toll-like* gene spans the parasegment boundary and contributes to *hedgehog* function in the adult eye of Drosophila. Mech. Dev. **47:** 225–239.

CHILDS, S. R., and M. B. O'CONNOR, 1994 Two domains of the *tolloid* protein contribute to its unusual genetic interaction with *decapentaplegic*. Dev. Biol. **162:** 209–220.

COLEMAN, K. G., S. J. POOLE, M. P. WEIR, W. C. SOELLER and T. KORNBERG, 1987 The *invected* gene of Drosophila: sequence analysis and expression studies reveasl a close kinship to the *engrailed* gene. Genes Dev. **1:** 19–28.

CÔTÉ, S., A. PREISS, J. HALLER, R. SCHUH, A. KIENLIN *et al.*, 1987 The *gooseberry-zipper* region of Drosophila: five genes encode different spatially restricted transcripts in the embryo. EMBO J. **6:** 2793–2801.

DAI, J.-D., and L. I. GILBERT, 1991 Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of *Drosophila melanogaster*: a cytophysiological analysis of the ring gland. Dev. Biol. **144:** 309–326.

DEVEREUX, J., P. HAEBERLI and O. SMITHIES, 1984 A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:** 387–395.

DOCTOR, J. S., D. JACKSON, K. E. RASHKA, M. VISALLI and F. M. HOFFMANN, 1992. Sequence, biochemical characterization, and developmental expression of a new member of the TGF-b superfamily in *Drosophila melanogaster*. Dev. Biol. **151:** 491–505.

FERGUSON, E. L., and K. V. ANDERSON, 1992a Localized enhancement and repression of the activity of the TGF-b family member, *decapentaplegic*, is necessary for dorsal-ventral pattern formation in the Drosophila embryo. Development **114:** 583–597.

FERGUSON, E. L., and K. V. ANDERSON, 1992b *decapentaplegic* acts as a morphogen to organize dorsal-ventral pattern in the Drosophila embryo. Cell **71:** 451–461.

FINELLI, A. L., C. A. BOSSIE, T. XIE and R. W. PADGETT, 1994 Mutational analysis of the Drosophila *tolloid* gene, a human BMP-1 homolog. Development **120:** 861–870.

FUKAGAWA, M., N. SUZUKI, B. L. M. HOGAN and C. M. JONES, 1994 Embryonic expression of mouse bone morphogenetic protein-1 (BMP-1), which is related to the Drosophila dorsoventral gene *tolloid* and encodes a putative astacin metalloendopeptidase. Dev. Biol. **163:** 175–183.

GENTRY, L. E., and B. W. NASH, 1990 The pro domain of pre-pro-transforming growth factor β1 when independently expressed is a functional binding protein for the mature growth factor. Biochemistry **29:** 6851–6857.

GOMIS-RÜTH, F. X., W. STÖCKER, R. HUBER, R. ZWILLING and W. BODE, 1993 Refined 1.8 A X-ray crystal structure of astacin, a zinc-endopeptidase from the crayfish *Astacus astacus* L. Structure determination, refinement, molecular structure and comparison with thermolysin. J. Mol. Biol. **229:** 945–968.

GROSSNIKLAUS, U., R. K. PEARSON and W. J. GEHRING, 1992 The Drosophila *sloppy paired* locus encodes two proteins involved in segmentation that show homology to mammalian transcription factors. Genes Dev. **6:** 1030–1051.

HEBERLEIN, U., T. WOLFF and G. M. RUBIN, 1993 The TGFβ homolog *dpp* and the segment polarity gene *hedgehog* are required for propagation of a morphogenetic wave in the Drosophila retina. Cell **75:** 913–926.

HEMMATI-BRIVANLOU, A., O. G. KELLY and D. MELTON, 1994 Follstatin, an antagonist of activin, is expressed in the Spemann Organizer and displays direct neuralizing activity. Cell **77:** 283–295.

HURSH, D. A., R. W. PADGETT and W. GELBART, 1993 Cross regulation of *decapentaplegic* and *Ultrabithorax* transcription in the embryonic visceral mesoderm of Drosophila. Development **117:** 1211–1222.

HWANG, S.-P. L., J. S. PARTIN and W. J. LENNARZ, 1994 Characterization of a homolog of human bone morphogenetic protein 1 in the embryo of the sea urchin, *Strongylocentrotus* purpuratus. Development **120:** 559–568.

IRISH, V., and W. M. GELBART, 1987 The *decapentaplegic* gene is required for dorsal-ventral patterning of the Drosophila embryo. Genes Dev. **1:** 868–879.

JIANG, W., and J. S. BOND, 1992 Families of metalloendopeptidases and their relationships. FEBS Lett. **312:** 110–114.

KANZAKI, T., A. OLOFSSON, A. MORÉN, C. WERNSTEDT, U. HELLMAN et al., 1990 TGF-β1 binding protein: a component of the large latent complex of TGF-β1 with multiple repeat sequences. Cell **61:** 1051–1061.

KAPHINGST, K., and S. KUNES, 1994 Pattern formation in the visual centers of the Drosophila brain: *wingless* acts via *decapentaplegic* to specify the dorsoventral axis. Cell **78:** 437–448.

KINGSLEY, D. M., A. E. BLAND, J. M. GRUBBER, P. C. MARKER, L. B. RUSSELL *et al.* 1992 The mouse *short ear* skeletal morphogenesis locus is associated with defects in a bone morphogenetic member of the TGF-β superfamily. Cell **71:** 399–410.

LEPAGE, T., C. GHIGLIONE and C. GACHE, 1992 Spatial and temporal expression pattern during sea urchin embryogenesis of a gene coding for a protease homologous to the human protein BMP-1 and to the product of the Drosophila dorsal-ventral patterning gene *tolloid*. Development **114:** 147–164.

LEVIS, R., T. HAZELRIGG and G. M. RUBIN, 1985 Separable cis-acting control elements for expression of the *white* gene of Drosophila. EMBO J. **4:** 3489–3499.

LEWIS, E. B., and F. BACHER, 1968 Method of feeding ethyl methane sulfonate (EMS) to Drosophila males. Dros. Inf. Serv. **43:** 193.

LINDSLEY, D., and G. ZIMM, 1992 *The Genome of Drosophila Melanogaster.* Academic Press, New York.

MAÉNO, M., Y. XUE, T. I. WOOD, R. C. ONG and H. KUNG, 1993 Cloning and expression of cDNA encoding *Xenopus laevis* bone morphogenetic protein-1 during early embryonic development. Gene **134:** 257–261.

MASSAGUÉ, J., L. ATTISANO and J. L. WRANA, 1994 The TGF-β family and its composite receptors. Trends Cell Biol. **4:** 172–178.

MESSING, J., 1983 New M13 vectors for cloning. Methods Enzymol. **101:** 20–30.

MIYAZONO, K., U. HELLMAN, C. WERNSTEDT and C. H. HELDIN, 1988 Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. J. Biol. Chem. **263:** 6407–6415.

MIYAZONO, K., A. OLOFSSON, P. COLOSETTI and C. H. HELDIN, 1991 A role of the latent TGF-β1-binding protein in the assembly and secretion of TGF-β1. EMBO J. **10:** 1091–1101.

NAKAMURA, T., K. TAKIO, Y. ETO, J. SHIBAI, K. TITANI *et al.*, 1990 Activin-binding protein from rat ovary is follistatin. Science **247:** 836–838.

NELLEN, D., M. AFFOLTER and K. BASLER, 1994 Receptor serine/threonine kinases implicated in the control of Drosophila body pattern by *decapentaplegic.* Cell **78:** 225–237.

NGUYEN, T., J. JOUMANA, M. J. SHIMELL, K. ARORA and M. B. O'CONNOR, 1994 Characterization of tolloid-related-1: a BMP-1-like product that is required during larval and pupal stages of Drosophila development. Dev. Biol. **166:** 569–586.

PADGETT, R. W., R. D. ST. JOHNSTON and W. M. GELBART, 1987 A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-β family. Nature **325:** 81–84.

PADGETT, R. W., J. WOZNEY and W. M. GELBART, 1993 Human BMP sequences can confer normal dorsal-ventral patterning in the Drosophila embryo. Proc. Natl. Acad. Sci. USA **90:** 2905–2909.

PANGANIBAN, G. E. F., R. REUTER, M. P. SCOTT and F. M. HOFFMANN, 1990 A Drosophila growth factor homolog, *decapentaplegic*, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. Development **110:** 1041–1050.

PEARSON, W. R., and D. J. LIPMAN, 1988 Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:** 2444–2448.

PENTON, A., Y. CHEN, K. STAEHLING-HAMPTON, J. L. WRANA, L. ATTISANO *et al.*, 1994 Identification of two bone morphogenetic protein type I receptors in Drosophila and evidence that Brk25D is a *decapentaplegic* receptor. Cell **78:** 239–250.

POOLE, S. J., L. M. KAUVAR, B. DREES and T. KORNBERG, 1985 The *engrailed* locus of Drosophila: structural analysis of an embryonic transcript. Cell **40:** 37–43.

POSAKONY, L. G., L. A. RAFTERY and W. M. GELBART, 1991 Wing formation in *Drosophila melanogaster* requires *decapentaplegic* gene function along the anterior-posterior compartment boundary. Mech. Dev. **33:** 69–82.

REUTER, R., G. E. F. PANGANIBAN, F. M. HOFFMANN and M. P. SCOTT, 1990 Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of Drosophila embryos. Development **110:** 1031–1040.

REYNOLDS, S. D., L. M. ANGERER, J. PALIS, A. NASIR and R. C. ANGERER, 1992 Early mRNAs, spatially restricted along the animal-vegetal axis of sea urchin embryos, include one encoding a protein related to *tolloid* and BMP-1. Development **114:** 769–786.

ROBERTSON, H. M., C. R. PRESTON, R. W. PHILLIS, D. M. JOHNSON-SCHLITZ, W. K. BENZ *et al.*, 1988 A stable genomic source of *P* element transposase in *Drosphila melanogaster.* Genetics **118:** 461–470.

ROTHE, M., M. PEHL, H. TAUBERT and H. JÄCKLE, 1992 Loss of gene function through rapid mitotic cycles in the Drosophila embryo. Nature **359:** 156–159.

RUSHLOW, C., H. DOYLE, T. HOEY and M. LEVINE, 1987 Molecular characterization of the *zerknüllt* region of the Antennapedia gene complex in Drosophila. Genes Dev. **1:** 1268–1279.

SEGAL, D., and W. M. GELBART, 1985 Shortvein, a new component of the decapentaplegic gene complex in *Drosophila melanogaster.* Genetics **109:** 119–143.

SELLECK, S. B., C. GONZALEZ, D. M. GLOVER and K. WHITE, 1992 Regulation of the G1-S transition in postembryonic neuronal precursors by axon ingrowth. Nature **355:** 253–255.

SHERMOEN A. W., and P. H. O'FARRELL, 1991 Progressionof the cell cycle through mitosis leads to abortion of nascent transcripts. Cell **67:** 303–310.

SHIMELL, M. J., E. L. FERGUSON, S. R. CHILDS and M. B. O'CONNOR, 1991 The Drosophila dorsal-ventral gene *tolloid* is related the human bone morphogenetic protein 1. Cell **6:** 469–481.

SPENCER, F. A., F. M. HOFFMANN and W. M. GELBART, 1982 *decapentaplegic*: a gene complex affecting morphogenesis in *Drosophila melanogaster.* Cell **28:** 451–461.

STADEN, R., 1982 Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. Nucleic Acids Res. **10:** 4731–4751.

STADEN, R., 1984 A computer program to enter DNA gel reading data into a computer. Nucleic Acids Res. **12:** 499–503.

STÖCKER, W., F.-X. GOMIS-RÜTH, W. BODE and R. ZWILLING, 1993 Implications of the three-dimensional structure of astacin for the structure and function of the astacin family of zinc-endopeptidases. Eur. J. Biochem. **214:** 215–231.

STORM, E. E., T. V. HUYNH, N. G. COPELAND, N. A. JENKINS, D. M. KINGSLEY *et al.*, 1994. Limb alterations in *brachypodism* mice due to mutations in a new member of the TGF-β superfamily. Nature **368:** 639–643.

TAKAHARA, K., G. E. LYONS and D. S. GREENSPAN, 1994 Bone morphogenetic protein-1 and a mammalian *tolloid* homolog, (*mTld*), are encoded by alternatively spliced transcripts which are differentially expressed in some tissues. J. Biol. Chem. 269:32572–32578.

TAUTZ, D., and C. PFEIFLE, 1989 A non-radioactive *in situ* hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene *hunchback.* Chromosoma **98:** 81–85.

VILLIERS, C. L., G. J. ARLAUD and M. G. COLOMB, 1985 Domain structure and associated functions of subcomponents C1r and C1s of the first component of human complement. Proc. Natl. Acad. Sci. USA **82:** 4477–4481.

WAKEFIELD, L. M., D. M. SMITH, K. C. FLANDERS and M. B. SPORN, 1988 Latent transforming growth factor-β from human platelets: a high molecular weight complex containing precursor sequences. J. Biol. Chem. **263:** 7646–7654.

WEISS, V., C. FAUSER and J. ENGEL, 1986 Functional model of subcomponent C1 of human complement. J. Mol. Biol. **189:** 573–581.

WHARTON, K. A., G. H. THOMSEN and W. M. GELBART, 1991 Drosophila 60A gene, another transforming growth factor β family member, is closely related to human bone morphogenetic proteins. Proc. Natl. Acad. Sci. USA **88:** 9214–9218.

WIESCHAUS, E., C. NÜSSLEIN-VOLHARD and G. JURGENS, 1984 Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster.* III. Zygotic loci on the X chromosome and fourth chromosome. Wilhelm Roux's Arch. Dev. Biol. **193:** 296–307.

WOZNEY, J. M., V. ROSEN, A. J. CELESTE, L. M. MITSOCK, M. J. WHITTERS *et al.*, 1988 Novel regulators of bone formation: molecular clones and activities. Science **242:** 1528–1531.

XIE, T., A. L. FINELLI and R. W. PADGETT, 1994 The Drosophila *saxophone* gene: a serine-threonine kinase receptor of the TGF-β superfamily. Science **263:** 1756–1759.

YAMAGUCI, Y., D. M. MANN and E. RUOSLAHTI, 1990 Negative regulation of transforming growth factor-β by the proteoglycan decorin. Nature **346:** 281–284.

YASUMASU, S., K. YAMADA, K. AKASAKA, K. MITSUNAGA, I. IUCHI *et al.*, 1992 Isolation of cDNAs for LCE and HCE, two constituent proteases of the hatching enzyme of *Oryzias latipes*, and concurrent expression of their mRNAs during development. *Dev. Biol.* **153:** 250–258.

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