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Toll homolog expression in the beetle *Tribolium* suggests a different mode of dorsoventral patterning than in *Drosophila* embryos

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Abstract

The gene *Toll* (*Tl*) encodes a maternally supplied interleukin 1 receptor-related transmembrane protein, a key component required to establish dorsoventral polarity in the *Drosophila* embryo. We have isolated *Tl* homologs of a primitive dipteran, *Clogmia albipunctata*, and of the beetle *Tribolium castaneum*. *Tribolium Tl* protein (Tl) lacks sequences in the C-terminal portion of the cytoplasmic domains that are conserved in the dipteran homologs. *Tl* lacking these sequences mediates the ventralizing activity when expressed as a gain-of-function variant in transgenic *Drosophila*, indicating that the sequences conserved in the Diptera are not essential for Tl signaling. In contrast to *Drosophila* where *Tl* gene expression occurs maternally and supplies uniformly distributed Tl in the egg membrane, *Tl* transcripts form a ventral-to-dorsal gradient in the *Tribolium* blastoderm stage embryo. This localized expression pattern of *Tl* transcripts, as compared with the strong maternal and ubiquitous expression in *Drosophila* and *Clogmia* embryos, suggests that dorsoventral patterning in long-germ band and short-germ band insects involves the same components but different modes of their action.

Keywords: Axis evolution; Dorsoventral patterning; *Tribolium*; Toll signaling

1. Introduction

Establishment of dorsoventral (DV) polarity in the *Drosophila* embryo is initiated by cell–cell signaling events between the oocyte and surrounding follicle cells (reviewed in Chasan and Anderson, 1993). These signaling events activate the *Toll* (*Tl*) protein (Tl), a maternally supplied, ubiquitously expressed membrane-spanning interleukin 1 (IL1) receptor-related protein (Hashimoto et al., 1988). Local Tl activation occurs in response to *spätzle* (*spz*), likely to encode a Tl ligand with spatially restricted activity in the perivitelline fluid of the embryo (Morisato and Anderson, 1994). Activated Tl mediates DV patterning through a signaling cascade, which causes the dissociation of a heterodimer composed of the NF-κB-like transcription factor Dorsal and its IκB-related inhibitor Cactus (Whalen and Steward, 1993; Bergmann et al., 1996). After their dissociation and degradation of Cactus (Belvin et al., 1995; Reach et al., 1996), Dorsal enters the nuclei and forms a ventral-to-dorsal nuclear gradient in the preblastoderm embryo (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). Dorsal acts as a transcription factor that controls localized expression of zygotic target genes which specify different cell fates along the DV axis (Ray et al., 1991). They include *twist* (*twi*) and *snail* (*sna*) in the ventral-most position of the blastoderm embryo (Thisse et al., 1991; Ip et al., 1992). *Twi* acts as a transcriptional activator of mesoderm-specific genes, whereas *sna* functions as a transcriptional repressor of non-mesodermal genes (Leptin, 1991).

The embryonic regulatory pathway comprising the gene products between the Tl ligand Spätzle and Cactus, but not upstream or downstream of them, also plays a major role in the anti-fungal immune response in *Drosophila* larvae and adults (Lemaitre et al., 1996; Ferrandon et al., 1998). Upon fungal infection, expression of the anti fungal peptide gene *drosomycin* is activated in a Tl-dependent manner. The intriguing structural and functional parallels to the NF-κB/IκB-dependent mammalian immune response suggested that the regulatory gene cassette composed of the IL1 receptor/Tl, IκB/Cactus and NF-κB/Rel/Dorsal is conserved in evolution (Wasserman, 1993). This observa-

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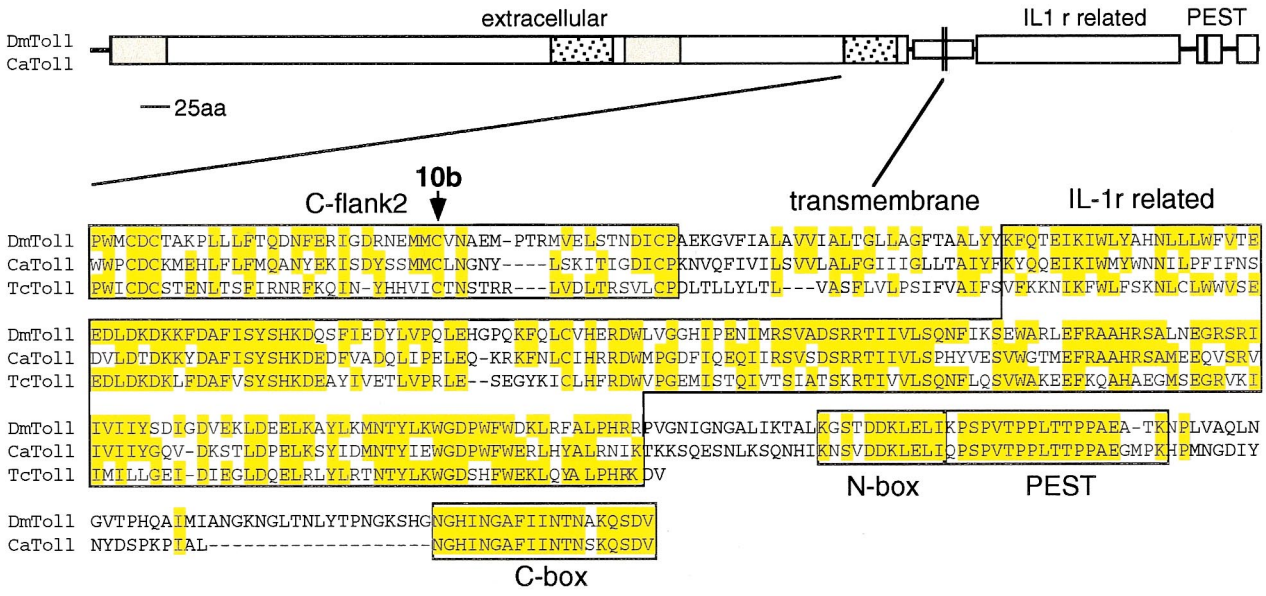


Fig. 1. Comparison of Toll proteins of *Drosophila melanogaster* (Dm), *Clogmia albipunctata* (Ca) and *Tribolium castaneum* (Tc). C-terminal sequences of the proteins were aligned using the MegAlign (DNA Star) program. Identical amino acids shared by at least two of the proteins are highlighted in yellow; dashes indicate gaps for optimal alignment. The carboxy-flanking region 2 (C-flank2) with the position of the Toll^{10b} mutation, the IL1 receptor-related signaling domain (IL-1r-related), the PEST domain and two newly identified regions N-terminal (N-box) and C-terminal (C-box) to the PEST domain are indicated.

tion and the finding that the early expression patterns of *twi* and *snail* are conserved in embryos of the flour beetle *Tribolium castaneum* (Sommer and Tautz, 1994) led to the proposal that the initial cues and the mode of DV patterning might be conserved in insects (Tautz et al., 1994).

We have addressed this proposal by characterizing the Tl homologs from a primitive dipteran, *Clogmia albipunctata* (Psychodidae, Nematocera), and from the flour beetle *Tribolium castaneum* (Tenebrionidae, Coleoptera). *Clogmia*, like *Drosophila,* undergoes long-germ band development, whereas *Tribolium* is a short-germ band insect (Sander, 1976). Our results show that Tl is conserved, but the cytoplasmic C-terminus of the protein, which is conserved in dipteran Tl, is absent in *Tribolium* Tl. *Tribolium* Tl has the same C-terminal characteristics as vertebrate Tl and IL1 receptor homologs (Rock et al., 1998). Gain-of-function experiments in *Drosophila* show that these conserved C-terminal domains of the dipteran proteins are not essential for Tl signaling. *Drosophila*-like ubiquitous Tl distribution in *Clogmia* embryos suggests that the initiation of dorsoventral polarity by localized Tl-activating components is conserved in the two long-germ insects, whereas the localized *Tl* transcripts in *Tribolium* embryos imply a different mechanism for establishing dorsoventral polarity in short-germ insects.

2. Results and discussion

2.1. Cloning of Tl homologs from Clogmia and Tribolium

PCR primers were designed to clone fragments of *Tl* homologs from the primitive dipteran *Clogmia albipunctata* (Psychodidae, Nematocera) and the beetle *Tribolium castaneum* (Tenebrionidae, Coleopterea). The choice of primers (see Section 3) was facilitated by comparing the conserved regions between the *Drosophila melanogaster and Drosophila pseudoobscura* Tl (Yamagata et al., 1994) and vertebrate IL1 receptor (Mitcham et al., 1996) proteins. After amplification, we obtained a single PCR fragment from *Clogmia* and *Tribolium* DNA, respectively, and used them to screen corresponding cDNA libraries prepared from poly(A)$^+$ RNA of early embryos. From *Clogmia*, we obtained a cDNA clone containing the entire open reading frame of a *Tl* homologous gene, whereas the open reading frame of the longest cDNA obtained from *Tribolium* lacks N-terminal sequences.

The diagnostic domains described for *Drosophila* Tl, such as the signal peptide and the leucine-rich repeats with their N- and C-terminal flanking regions (Hashimoto et al., 1988; Schneider et al., 1991), are conserved in *Clogmia* Tl. The IL1 receptor-related cytoplasmic domain (57% identity), a PEST domain and two novel islands of sequence identity, termed N- and C-box (see Fig. 1), were conserved between the dipteran Tl homologs. Interestingly, the *Tl* homolog of *Tribolium* lacks these conserved C-terminal domains; it terminates shortly after the IL1 receptor-related cytoplasmic domain (56% identity) as has been observed in vertebrate Tl and the IL1 receptor proteins (Fig. 1). The conserved C-terminal domain in the long-germ band insects *Clogmia* and *Drosophila*, and its absence from the *Tribolium* and vertebrate homologs, suggest that they may represent derived characteristics of Tl function in long germ band insects. We therefore, performed functional studies asking

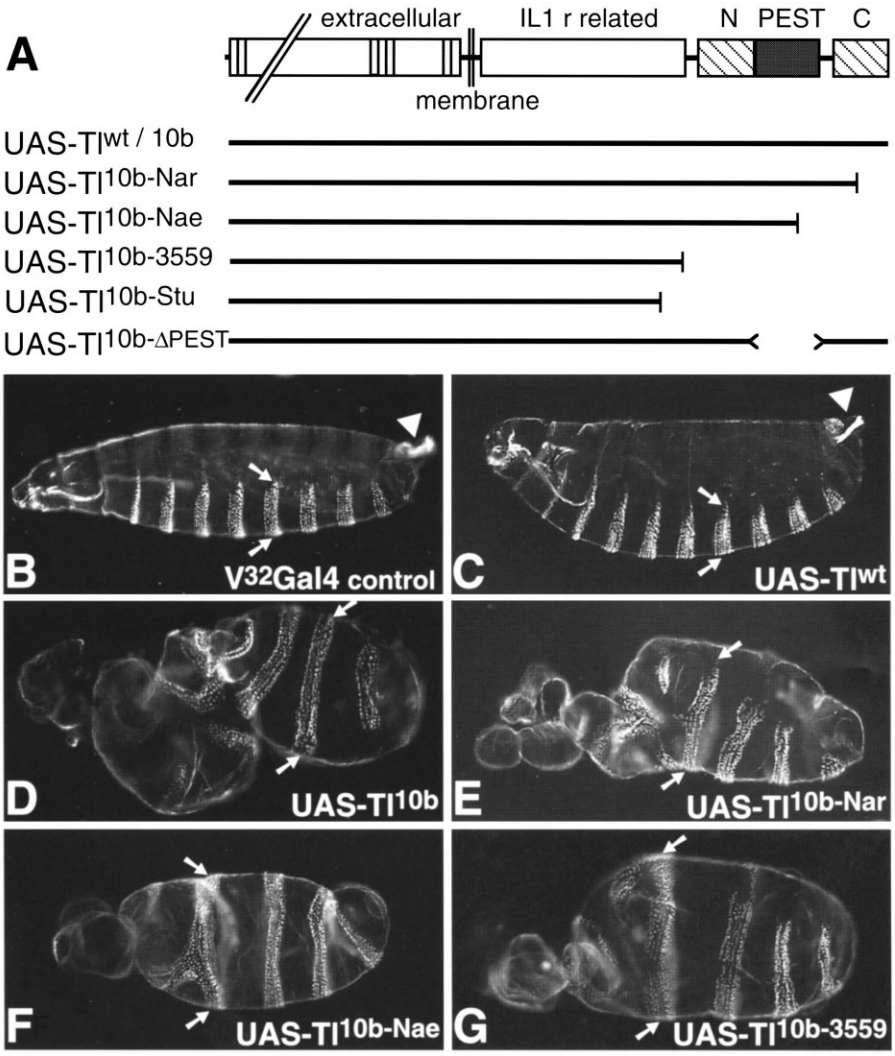
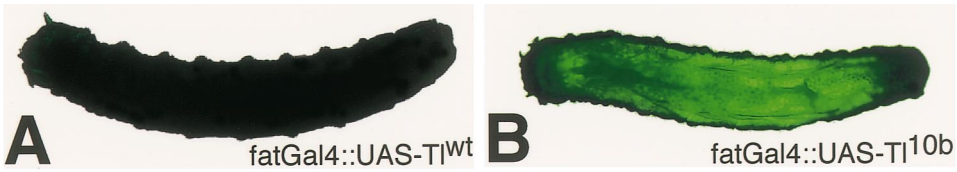


Fig. 2. Larval cuticle pattern in response to transgene expression of Tl and Tl^{10b} mutant protein variants indicating that the C-terminus is not essential for activated Tl signaling. UAS-driven wild type Tl and five truncation mutants of the Tl^{10b} C-terminus (A) were activated by the V^{32}-GAL4 driver line. Note that the drawing is not in scale. V^{32}-GAL4 expression (B) and V^{32}-GAL4-driven UAS-Tl^{wt} expression (C) had no discernable effect on the cuticle pattern, meaning that the ventral denticle belts are restricted to the ventral half of the embryo (arrows) and the dorsolateral Filzkörper are present (arrow head). UAS activated Tl^{10b}, as well as Tl^{10b} C-terminal truncations up to the IL1 receptor related signaling domain result in ventralization of the larvae (D-G), meaning that the denticle belts form circumferential rings (arrows) and Filzkörpers are strongly reduced or absent. For a detailed description of ventralized embryos see (Anderson et al., 1985).

whether the conserved C-terminal domains carry an essential function for Tl signaling in *Drosophila* embryos.

2.2. The Tl C-terminal region is not required for signaling

To address a possible function of the conserved C-terminal region of Tl in signaling, we used a gain-of-function approach, employing a point mutation ($G_{2.916} \rightarrow A$) which causes a constitutively active Tl protein, termed Tl^{10b}, due to a cystein replacement by threonin within the extracellular Tl domain (Schneider et al., 1991). Maternal inheritance of the Tl^{10b} protein, which corresponds to a previously described dominant gain-of-function mutant protein, causes a fully penetrant ventralization of embryos (Schneider et al., 1991). In order to demonstrate a possible function of the conserved C-terminal domains of *Drosophila* and *Clogmia* Tl in signaling, we used the GAL4/UAS system (Brand and Perrimon, 1993) to express Tl^{10b} variants containing C-terminal deletions during syncytial blastoderm stage. In addition, the GAL4/UAS system was used to express the Tl^{10b} variants in the fatbody of larvae bearing a *drosomycin* green fluorescent protein ($Drom^{GFP}$) transgene that can be activated in direct response to Tl signaling (Ferrandon et al., 1998). This reporter transgene allowed us to visualize whether the Tl signaling-dependent anti-fungal immune-response is activated in the larvae (Ferrandon et al., 1998). This way, we could assay for Tl signaling activity in the larval cuticle pattern and test whether the Tl variants are also able to activate the immune response in larvae.



C

genotype	number of examined larvae	Immune responsive larvae	(%)
Drom^{GFP}	174	2	<2%
fatGal4; Drom^{GFP}	130	2	<2%
fatGal4::UAS-Tl$^{10b\text{-}Stu}$; Drom^{GFP}	275	4	<2%
fatGal4::UAS-Tl^{wt}; Drom^{GFP}	440	14	<4%
fatGal4::UAS-Tl^{10b}; Drom^{GFP}	289	59	20%
fatGal4::UAS-Tl$^{10b\text{-}Nar}$; Drom^{GFP}	132	34	26%
fatGal4::UAS-Tl$^{10b\text{-}Nae}$; Drom^{GFP}	87	24	28%
fatGal4::UAS-Tl$^{10b\text{-}3559}$; Drom^{GFP}	151	38	25%
fatGal4::UAS-Tl$^{10b\text{-}\Delta PEST}$; Drom^{GFP}	131	30	23%

Fig. 3. Activation of Tl-dependent immune response in *Drosophila* larvae. Tl and Tl derivatives (see Fig. 2) were expressed in drosomycin GFP (Drom^{GFP}) reporter gene-bearing larvae using a fat body-specific GAL4 driver (fatGAL4) and UAS-driven *Tl* transgenes. Third instar larvae were examined for the absence (A) or presence of GFP activity (B) to indicate whether the immune response was activated in response to ectopic Tl signaling. Control flies carrying only the GFP transgene, GFP and GAL4 transgenes, or both in combination with UAS-Tl^{wt} showed only background level of Drom^{GFP} reporter gene activity in 0–4% of the larvae, whereas the Tl^{10b} derivatives found to provide Tl signaling activity in the embryo (see Fig. 2) also caused Drom^{GFP} activation (C). Tl$^{10b\text{-}Stu}$, a mutation deleting the C-terminal region including the last 24 amino acids of the IL-1r-related signaling domain, is inactive in signaling. Note that only about 25% of the larvae are of the fatGAL4, UAS-Tl and Drom^{GFP} transgene genotype which allows for the expression of the reporter gene in response to activated Tl signaling.

Fig. 2A shows the UAS-driven effector genes including wild type *Tl*, *Tl*^{10b} and five different *Tl*^{10b} variants which contain different C-terminal portions of Tl. C-terminal deletions of Tl^{10b} variants which lack up to the IL1 receptor-related domain caused ventralized embryos similar to those developed in response to UAS-driven *Tl*^{10b} (Fig. 2B–G) and Drom^{GFP} reporter gene expression in larval fat body (summarized in Fig. 3). These results indicate that the C-terminal domains of Tl, which are conserved in *Clogmia* and *Drosophila*, are not required for Tl signaling. Thus, their absence in the *Tl* homolog of *Tribolium* would also not interfere with this aspect of Tl function. One of the conserved C-terminal domains of dipteran Tl, the PEST domain, was previously shown to mediate ubiquitin-dependent degradation of proteins by the 26S proteasome and thereby to affect the half-life of the protein (Rechsteiner and Rogers, 1996). Since early development including blastoderm formation is prolonged in *Tribolium* as compared to *Drosophila* and *Clogmia* embryos, one may speculate that the conserved PEST domain serves to prevent long-lasting Tl activity in long-germ band embryos, whereas prolonged Tl activity might be required for short-germ band development.

Tl is also expressed in the post-blastodermal *Drosophila* embryo and has been proposed to function in cell-adhesion processes required for cell-cell interactions involved in movements during embryonic development (Gerttula et al., 1988; Keith and Gay, 1990; Hashimoto et al., 1991). We have not addressed a possible adhesion function of Tl in our studies. Thus, we cannot exclude the possibility that the conserved C-terminus of Tl participates in such a function in long germ band embryos.

2.3. Localization of Tl transcripts in Tribolium embryos

Previous results showed *Drosophila*-like expression of *sna* and *twi* homologs in *Tribolium* embryos (Sommer and Tautz, 1994). Therefore it was assumed that the modes of DV pattern formation in short and long-germ band embryos are conserved (Tautz et al., 1994, see also Brown and Denell, 1996). This assumption implies that the *Tl* transcripts are, like in *Drosophila*, maternally expressed and evenly distributed in eggs and early embryos of both *Clogmia* and *Tribolium*. We tested this proposal by visualizing the *Tl* transcript patterns in oocytes and early embryos of *Clogmia* and *Tribolium*, respectively.

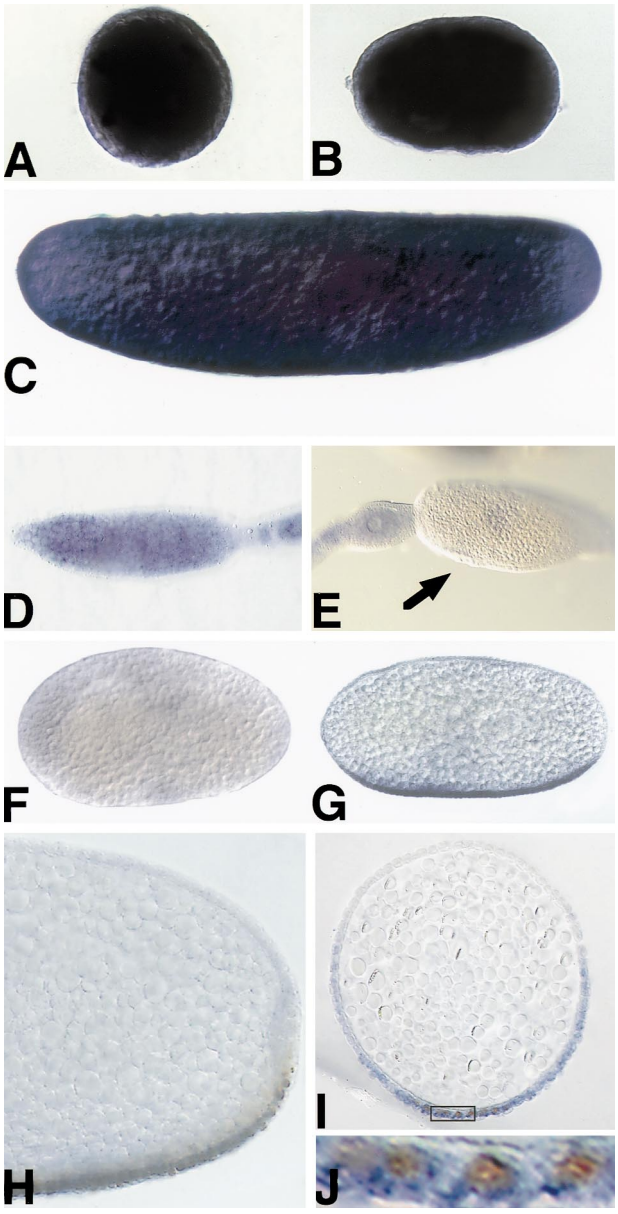


Fig. 4. Whole mount in situ detection of *Clogmia* and *Tribolium Tl* transcripts in oocytes and embryos. Like in *Drosophila* (Gerttula et al., 1988), the *Clogmia Tl* transcripts are strongly expressed in a uniform pattern during oogenesis (A, B) and preblastodermal embryonic development (C). During *Tribolium* oogenesis,*Tl* transcripts are barely detectable during early stages (D, E). However, no transcripts were observed during the late stages of oogenesis (E; arrow) and in freshly laid eggs (F). In early blastoderm stages, *Tl* transcripts are expressed in a restricted pattern at one side of the embryo (G). Double staining of *Tl* transcripts (blue) and Twi protein (anti Twist antibody staining; brown) of a blastoderm embryo shows that *Tl* transcripts form a gradient with highest concentration at the ventral side (H) as established by cross-sectioning (I, enlarged in J). Orientation C–H: anterior to the left, dorsal side up. I, J: dorsal side up.

Fig. 4 shows that the high amounts of Tl transcripts of *Clogmia* are evenly distributed in the growing oocyte (Fig. 4A,B). The ubiquitous distribution is maintained in embryos during syncytial stages (Fig. 4C) and ceases during the cellular blastoderm stage. Thus, no significant difference was observed by comparing the *Tl* transcript patterns of *Clogmia* and *Drosophila* (Gerttula et al., 1988). This implies that the DV coordinates of *Clogmia* are generated in a *Drosophila*-like fashion, meaning in response to the localized activity of a ventralizing ligand (Morisato and Anderson, 1994). In other words, localized activation of ubiquitous Toll determines where Dorsal is transferred into the nuclei serving as a prerequisite for the Dorsal-dependent regulation of the zygotic target genes involved in DV patterning.

In contrast to both *Drosophila* and *Clogmia*, the *Tl* transcripts in *Tribolium* accumulate in a restricted pattern (Fig. 4D–J). Only barely detectable levels of *Tl* transcripts were observed during early stages of oogenesis. We can not unambiguously decide whether the weak staining represents background or transcript concentrations at the detection limit. No staining was observed in mature oocytes and in embryos during the early syncytial development of the embryo (Fig. 4D–F). However, at early blastoderm stage, *Tl* transcripts appear in a localized pattern, forming a concentration gradient along the DV axis (Fig. 4G). Since morphology is not a reliable criterium to distinguish the prospective dorsal and ventral regions in *Tribolium* embryos, we performed double stainings of the embryos using antibodies directed against *Tribolium* Twist (K.H. and S.R., manuscript in preparation) and in situ hybridization to detect the *Tl* transcripts in parallel. The co-localization of the two gene products (Fig. 4H–J) establishes that the *Tl* transcripts are located at the prospective ventral side of the embryo. Also, the pattern of nuclear Dorsal expression is conserved in *Tribolium* embryos and overlaps the patterns of Twist expression and *Tl* transcripts transiently (G. Chen and S.R., unpublished results). Thus, the components known to play key roles in DV patterning of *Drosophila* (Chasan and Anderson, 1993) are conserved in *Tribolium* embryos.

2.4. Different modes for DV polarity initiation in long- and short-band insects?

The localization of *Tl* transcripts at the blastoderm stage could mean that maternal transcripts, present at undetectable levels, accumulate to detectable levels at the prospective ventral side of the embryo. Such a process would require transport within the syncytial embryo. Although transport of mRNA has been shown to localize the mRNA of maternal key factors along the anterior–posterior axis of the oocyte and early embryo, no such mechanism has been reported for mRNA along the DV axis (St Johnston and Nüsslein-Volhard, 1992). Therefore, and in view of the high amounts of transcripts appearing during the stage when zygotic expression of segmentation genes was reported in *Tribolium* embryos (Wolff et al., 1995), it appears likely that the localized *Tl* transcripts observed in embryos are the result of zygotic expression.

Irrespective of the cause of the localization, the results show that the mode of generating DV polarity in the long-

germ band embryos of *Drosophila* and *Clogmia* differs with respect to how localized Tl activity is generated in a short-germ band embryo such as *Tribolium.* In *Drosophila,* the activating ligand of Tl, Spätzle, is thought to be generated in limiting amounts at the ventral side of the embryo (Morisato and Anderson, 1994). High and uniform levels of Toll receptors in the plasma membrane bind the ligand and thereby limit its diffusion. This mechanism accounts for a sharp ventral maximum of Tl activation, resulting in the formation of the nuclear gradient of the transcription factor Dorsal (Morisato and Anderson, 1995). In this view, local expression of the receptor Tl in *Tribolium* might lead to a local sequestration of the activating ligand.

Mechanisms and factors to account for the control of a local expression of Tl in *Tribolium* embryos might be unrelated to the DV cascade in *Drosophila* or it could be that the Tl ligand upregulates its receptor by establishing a positive feedback-loop. Indeed, nuclear accumulation of Dorsal protein seems to parallel the local expression of Tl in the *Tribolium* embryo, suggesting that local Tl expression is signaling-dependent (G. Chen and S.R., unpublished results). A positive feedback mechanism, on the other hand, would generate a localized pattern only if the ligand is supplied from a localized source or if the ligand-induced feedback-loop is coupled to a lateral inhibition mechanism. In the latter case, a weak asymmetry in ligand distribution would be sufficient to produce a localized pattern, which implies that DV axis formation could be initiated with very little extraembryonic spatial information. It is interesting to note that even in *Drosophila,* where an elaborate follicle cell pattern provides asymmetric spatial cues for DV axis formation (Nilson and Schüpbach, 1998), Dorsal gradient formation involves a lateral inhibition mechanism (Roth and Schüpbach, 1994) and that the production of the Tl ligand Spätzle is under the control of an inhibitory mechanism which depends on nuclear Dorsal activity (Misra et al., 1998). In *Tribolium,* a comparable inhibition mechanism might be coupled with the transcriptional upregulation of the receptor Tl.

Coupling of positive feedback regulation and lateral inhibition mechanism would account for the regulative capacity of the DV axis which has been observed in many classical embryological studies on short and intermediate germ-band insects (Sander, 1976). Ligations separating dorsal and ventral egg halves of the leaf hopper *Euscelis* can result in two normal looking twin embryos, one in each half of the ligated egg (Sander, 1971). Furthermore, cold shock treatment of the egg of the beetle *Atrachya* causes the development of up to four embryos with normal body axes (Miya and Kobayashi, 1974; Sander, 1976). Both phenomena could be accounted for by a local activation of axis specifying factors and/or lateral inhibition mechanisms that modulate or newly generate DV axis formation in the embryo (Meinhardt, 1989; Roth, 1993). The localized *Tl* expression in *Tribolium* embryos is consistent with the proposal of an inducible and self-amplifying activation system that causes molecular asymmetry in embryos of short germ band insects. An alternative could be that there is a second *Tl* in *Tribolium* which performs the maternal function and thereby activates the Dorsal nuclear translocation on the ventral side of the *Tribolium* embryo. In turn, this could be sufficient to activate the zygotic *Tl* gene. Despite intensive searches that were based on PCR approaches and hybridization experiments under low stringency conditions (not shown), we were not successful in isolating a second *Tl* gene in *Tribolium.* Therefore, we favor the idea that the mode of initiating dorsoventral polarity in *Tribolium* is different from *Drosophila* and *Clogmia*. The localized zygotic expression of *Tl* in a short-germ band embryo provides an entry point towards the analysis of the mechanisms underlying DV patterning in such insects.

3. Experimental procedures

3.1. Cloning of Tl homologs

Genomic fragments were isolated from *Clogmia* or *Tribolium* DNA by PCR with degenerate primer-pair Tl5′ = AAR GAY AAR AAR TTY GAY GCI TT and Tl3′ = CCC CAY TTI ARR TAI GTR TTC AT (with R = A or G, Y = C or T and I = Inosin) corresponding to positions 3137–3160 and 3494–3517 of the *Drosophila Tl* sequence (Hashimoto et al., 1988). The genomic fragments were used to screen embryonic *Clogmia* and *Tribolium* (Wolff et al., 1995) cDNA libraries. The *Clogmia albipunctata* 'maternal' cDNA library was constructed from 0–2 h embryonic mRNA using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, Ca). At least three independent clones were analyzed on sequence level for both organisms.

3.2. Egg collections and staining

Clogmia embryos were fixed and stained essentially as described for *Drosophila* (Tautz and Pfeifle, 1989) with an additional sonication step after fixation to remove the vitelline membrane. Handling and staining of *Tribolium* and *Clogmia* embryos were done as published (Schmidt-Ott et al., 1994; Wolff et al., 1995). For double stainings the *in situ* hybridization was followed by an antibody stain, both according to standard protocols. For stageing *Tribolium* embryos were counter stained with DAPI before mounting them in glycerol (Wolff et al., 1995). For photography of the in situ patterns, embryos were mounted in araldite.

3.3. UAS constructs

pUAS-10b^{Nar} (cuts after base pair number 3825), pUAS-10b^{Nae} (cuts after base pair number 3681) and pUAS-10b^{Stu} (cuts after base pair number 3482) were obtained by restriction digest of the Tl^{10b} cDNA with Nar1, Nae1 and Stu1, respectively, and blunt end cloned in pUAST. The pUAS-

$10b^{3559}$ truncation was produced by introducing a stop codon in the Tl^{10b} cDNA after base pair 3559 by PCR. This truncation corresponds to the minimal version of the IL1 receptor shown to be active in signaling (Heguy et al., 1992). Detailed cloning protocols are available on request. P-element mediated transformation was done as described (Brand and Perrimon, 1993). For each construct, at least two independent transgenic lines were analysed.

3.4. Fly work

The maternal V^{32}-GAL4 driver line containing a GAL4 DNA binding domain/V16 activation domain fusion expressed under the control of the maternal α4tubulin promoter was provided by D. St Johnston (Häcker and Perrimon, 1998). Virgin V^{32}-GAL4 females were crossed to males carrying the different UAS-*Tl* transgenes and kept at 25°C. Cuticle preparations were performed from the progeny 24–36 h AEL (Roberts, 1986). An enhancer trap line driving GAL4 in the fat body (obtained from G. Technau) and a drosomycin-green fluorescent protein (GFP) reporter line (Ferrandon et al., 1998) were used for the immune system assay. Combining homozygote stocks in a two step crossing scheme, 25% of the F2 generation were expected to carry GAL4, UAS-*Tl* and the GFP reporter together. Third instar larvae were collected and GFP expression observed under an UV microscope.

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