# Conserved and divergent aspects of terminal patterning in the beetle *Tribolium castaneum*

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To infer similarities and differences in terminal pattern formation in insects, we analyzed several of the key genes of this process in the beetle *Tribolium castaneum*. We cloned two genes of the terminal pattern cascade, namely *tailless* (*tll*) and *forkhead* (*fkh*), from *Tribolium* and studied their expression patterns. In addition, we analyzed the pattern of MAP kinase activation at blastoderm stage as a possible signature for *torso*-dependent signaling. Further, we analyzed the late expression of the previously cloned *Tribolium caudal* (*Tc-cad*) gene. Finally, we used the upstream region of *Tc-tll* to drive a reporter gene construct in *Drosophila*. We find that this construct is activated at the terminal regions in *Drosophila*, suggesting that the *torso*-dependent pathway is conserved between the species. We show that most of the expression patterns of the genes studied here are similar in *Drosophila* and *Tribolium*, suggesting conserved functions. There is, however, one exception, namely the early function of *Tc-tll* at the posterior pole. In *Drosophila*, the posterior *tll* expression is involved in the direct regulation of the target genes of the terminal pathway. In *Tribolium*, posterior *Tc-tll* expression occurs only for a short time and ceases before the target genes known from *Drosophila* are activated. Thus, we infer that *Tc-tll* does not function as a direct regulator of segmentation genes at the posterior end. It is more likely to be involved in the early specification of a group of "terminal" cells, which begin to differentiate only at a later stage of embryogenesis, when much of the abdominal segmentation process is complete. Thus, there appears to have been a major shift in *tll* function during the evolutionary transition from short germ to long germ embryogenesis.

*tailless* | *forkhead* | *caudal* | *torso* | short germ insect

**T**erminal pattern formation in *Drosophila* is maternally initiated at both poles of the egg through the *torso*-mediated receptor tyrosine kinase pathway (1). Two zygotic gap genes, namely *tailless* (*tll*) and *huckebein* (*hkb*), are activated in a concentration-dependent manner in response to the signaling activity of *torso* (*tor*) (2, 3). Both *tll* and *hkb* code for transcription factors that regulate further target genes, among them *forkhead* (*fkh*), *brachyenteron* (*byn*), and the terminal *hunchback* (*hb*) and *wingless* (*wg*) domains (4–8). *fkh* and *wg* are also activated by *caudal* (*cad*) (8), which is expressed maternally as well as zygotically in the posterior half of the embryo (9, 10). Together these genes are required for patterning the terminal cuticle structures, such as the most posterior segments, the anal pads, and the telson, as well as the stomodaeum, the hindgut, and the Malpighian tubules.

Embryogenesis in the flour beetle *Tribolium* represents a more ancestral form of embryogenesis in insects (11). Nevertheless, it was possible to clone most of the early segmentation genes known from *Drosophila* also from *Tribolium*, and the analysis of their expression patterns has suggested that they may have similar functions in both species (12, 13). At the same time, because of the different modes of embryogenesis between the two species, one has to expect that differences must have evolved, with respect either to regulatory interactions or to primary function of genes. We have shown previously that the primary regulation of *hunchback* has undergone an evolutionary transition from dependence on *caudal* in *Tribolium* to dependence on *bicoid* in *Drosophila* (14). Our results presented here suggest for the posterior expression domain of *tll* that there has also been a major transition in function.

## Materials and Methods

**Cloning the *tll* and *fkh* Orthologues.** Redundant primers for the *fkh* domain (gift of S. Farrington, Biological Laboratories, Harvard) and for the *tll* DNA-binding domain were used to initially amplify fragments from genomic DNA (*fkh* primers: forward -ckd atd swr tty tgc ca, reverse -aar ccn ccn tay wsh ta; *tll* primers: forward -ggn ath tay gcn tgy gay gg, reverse -ytg nac ngc rtc ytt rtt cat). The respective fragments were then used to screen a cDNA library (*fkh*) or a genomic library (*tll*). Because only truncated sequences could be recovered, 5′ and 3′ rapid amplification of cDNA ends (using the Marathon or the SMART Kit, CLONTECH) was used to find the 5′ end (*tll* and *fkh*) and the 3′end (*tll*) of these genes. The GenBank database accession no. for *Tc-fkh* is AF217810 and for *Tc-tll* is AF219117. To isolate the upstream sequence of the *Tc-tll* gene, the initially isolated genomic fragment was used to screen a genomic library. Of the eight recovered λ phages, one (phage no. 6.1), containing approximately 10 kb upstream region, was chosen for further analysis. *Sal*I and *Eco*RI fragments were isolated, subcloned in Bluescript KS+ (Stratagene), and sequenced to get a 9.8-kb upstream sequence (accession no. AF225975).

**Transgenic Flies.** In a two-step cloning strategy (details available on request), the complete 9.8-kb upstream region was cloned in the original orientation in front of the *lacZ* reporter by using the pCasperAUG vector (15). The resulting construct was called pTc-*tll*-9.8. P-element-mediated transformation was done as described (16). The recipient strain was $y \ w^{67c23}$.

**Generation of the *tll* Antibody.** A fragment coding for part of the ligand-binding domain (position 181–305) was cloned in frame into an expression vector (pRSET C, Invitrogen). Overexpressed protein was affinity purified on a TALON column (CLONTECH). One rabbit was immunized with approximately 5 mg of protein (Eurogentec, Brussels) by using about 1 mg per boost.
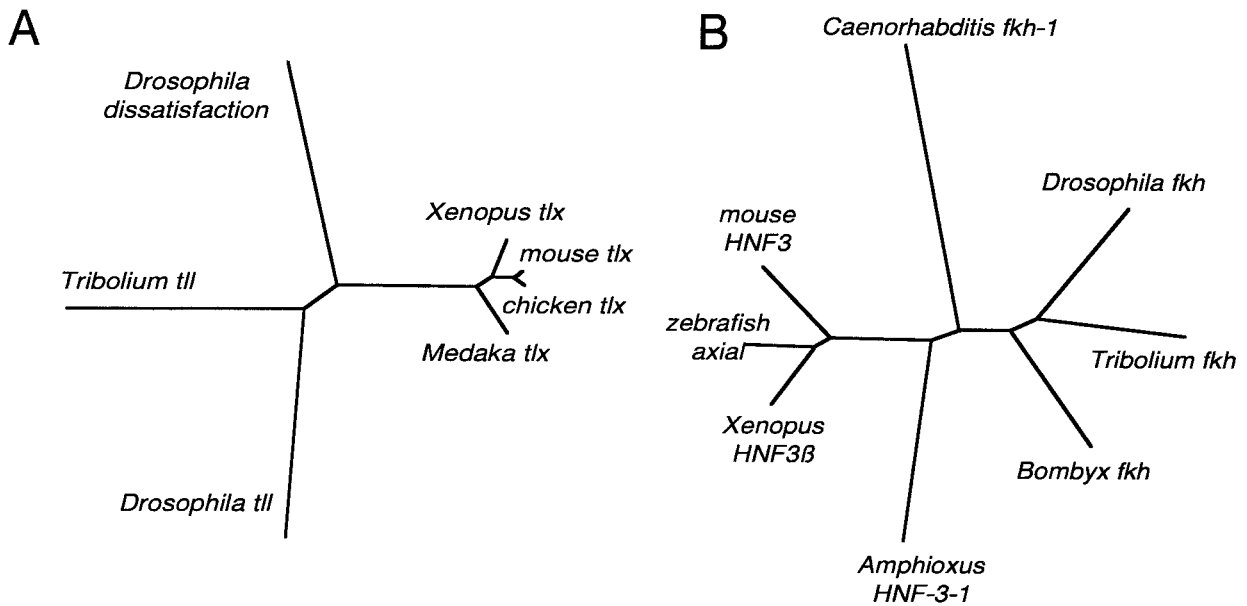
EVOLUTION

**Fig. 1.** Phylograms for *tll*-related (*A*) and *fkh*-related genes (*B*). The trees were constructed with a maximum likelihood algorithm by using PUZZLE (48) based on a CLUSTAL alignment (49). All branches receive very high support. The accession numbers for the respective sequences are: (*A*) *Drosophila tll* M34639, *Tribolium tll* AF219117, *Drosophila dissatisfaction* (as a *tll*-related gene in *Drosophila*) AF106677, *Xenopus* tlx P70052, mouse tlx Q64104, chicken tlx Q91379, medaka tlx AJ131390, and (*B*) *Drosophila fkh* P14734, *Tribolium fkh* AF217810, *Bombyx fkh* Q17241, *Caenorhabditis fkh*-1 AAA96319, amphioxus HNF-3–1 CAA65368, *Xenopus* HNF-3β I51436, zebrafish Axial Q07342, and mouse HNF-3β P35583.

After the fifth boost, the serum was affinity purified by using the same protein.

**Expression Analysis.** Whole-mount *in situ* hybridization and antibody stainings were done essentially as described for *Drosophila* (17, 18). The anti-*active MAP-K* antibody (Sigma) was used essentially as described (19).

**Results**

**tll and fkh Orthologues from Tribolium.** Candidates for *tll* and *fkh* orthologues from *Tribolium* were first obtained by PCR amplification by using degenerate primers in conserved regions. Only one type of fragment was obtained in each case, which was subsequently used to screen a cDNA library. As no full length cDNAs could be recovered, rapid amplification of cDNA ends experiments were performed to obtain the full sequences. For *tll*, we also obtained the genomic region from a genomic λ library, approximately 10 kb of which were also sequenced.

The *tll* gene displays two conserved protein motifs, namely a zinc finger DNA-binding domain and a ligand-binding domain (20). Both domains can be aligned to the respective orthologues from other species. The phylogram in Fig. 1*A* depicts the relationships between the known *tll* orthologues in different species.

Transcription factors belonging to the *forkhead* class encode a winged-helix-type DNA-binding motif, the *forkhead* domain. Orthologues of the *fkh* gene are known from many different phyla. In a detailed sequence analysis, the different *fkh* genes were grouped into 10 different classes (21). *fkh* itself belongs to class 1 and shows diagnostic amino acids within the 110-aa-long *fkh* domain. All these amino acids can also be found in the *Tc-fkh* sequence. The phylogram resulting from the alignment with the known *fkh* orthologues from other species is shown in Fig. 1*b*.

**Expression of Tc-tll.** Tailless protein expression in *Tribolium* can be detected already at blastoderm stage, where a small group of cells at the posterior pole express the gene (Fig. 2*a*). Staining is

seen in the nuclei, and a short posterior-to-anterior gradient is evident (Fig. 2*b*). It was shown previously that there is also a very early expression domain of *Tc-wingless* at the posterior pole of the *Tribolium* embryo at blastoderm stage (22), which is reminiscent of the terminal *wg* domain in *Drosophila* (23). This suggests that at least part of the posterior terminal structures are determined already at blastoderm stage in *Tribolium*. This is also in general accord with classical fate mapping experiments, which could localize the anlagen for the hindgut in short germ insects at the posterior end of the blastoderm embryo (24).

There is no expression of *Tc-tll* at the anterior pole of the *Tribolium* embryo, which is in contrast to *Drosophila* (20). However, at late blastoderm stage, *Tc-tll* expression appears in the developing head region, which lies at the ventral side of the embryo (Fig. 2*c*). It seems likely that this expression is homologous to the known expression of *tll* in the head of *Drosophila* (20, 25). With the beginning of gastrulation, *tll* expression ceases at the posterior end (Fig. 2*d*) at both the RNA and protein levels, whereas the head-specific expression domains become stronger and persist throughout development (Fig. 2 *e* and *f*).

**Expression of Tc-fkh.** The first expression of *Tc-fkh* RNA can be seen in yolk nuclei (not shown). A similar expression is also known from *Drosophila* (4) although much later in development. As the germband elongates, *Tc-fkh* expression becomes visible in the primordia of the stomodaeum and the proctodaeum (Fig. 3 *a* and *b*). Staining in these regions persists until the end of embryogenesis (Fig. 3 *b* and *c*). In the late embryo, *Tc-fkh* is expressed in the primordium of the central nervous system, in parts of the brain, and in the Malpighian tubules (Fig. 3 *b* and *c*). All these expression aspects are very similar to *Drosophila*, where *fkh* acts as a homeotic gene for specifying the stomodaeum and the proctodaeum (4). In *Drosophila*, however, the first expression is seen at blastoderm stage (4), which is not evident in *Tribolium*. But this can be reconciled with the fact that *fkh* is required not for initiating pattern formation but only for later differentiation steps.
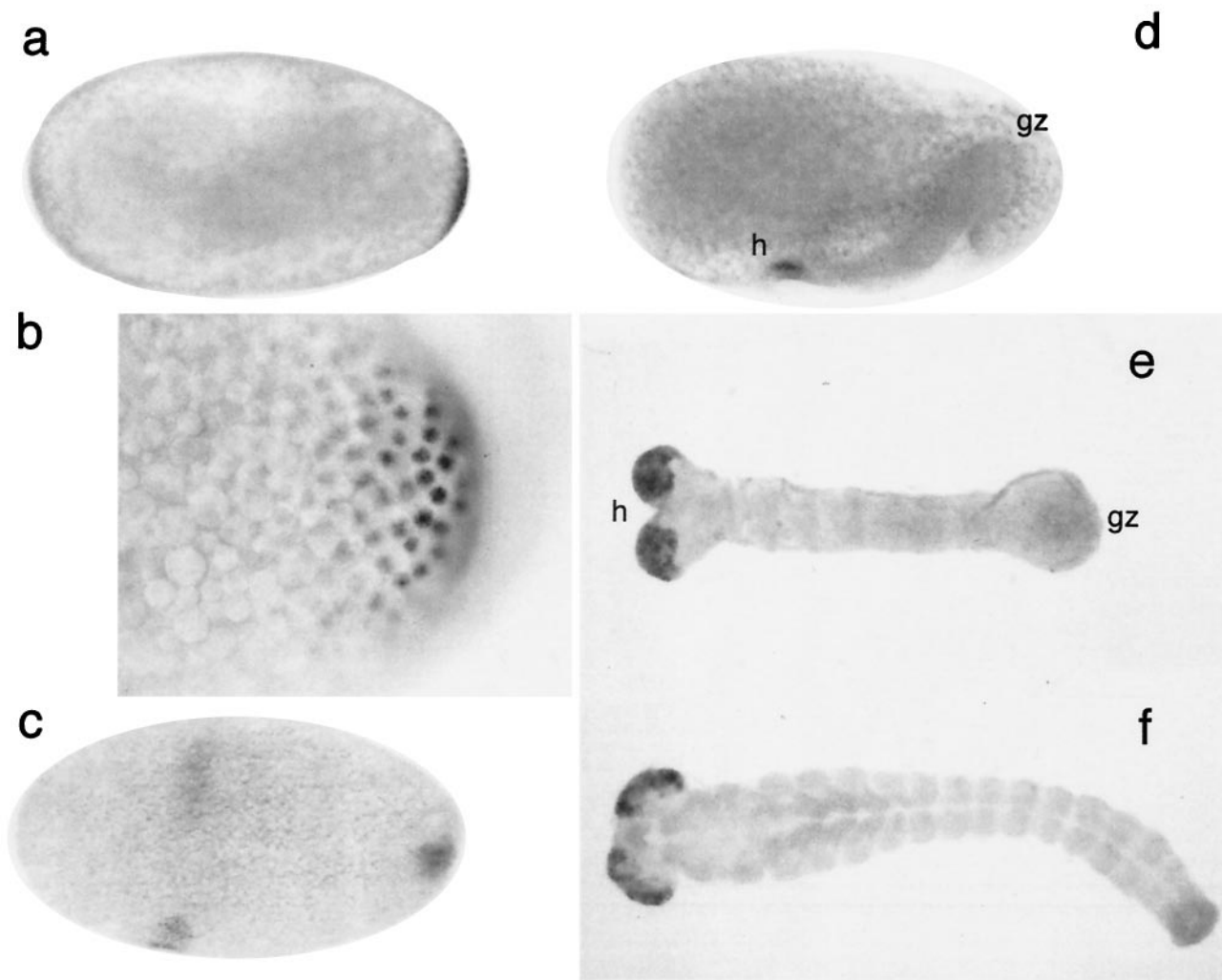
**Fig. 2.** RNA and protein expression of *Tc-tll*. Embryos were stained either with a *Tc-tll*-specific antibody (*a–d*) or by whole-mount *in situ* hybridization (*e*, *f*). *a–f* represent progressively older stages of embryogenesis. Anterior is to the left, *a*, *b*, and *d* are lateral views (dorsal is up), and *c*, *e*, and *f* are ventral views. No staining is detected in the growth zone (gz) with the beginning of germband growth (*d* and *e*), whereas the expression in the head (h) persists. See text for further details.

**Late Expression of *Tc-cad*.** The early stages of *Tc-cad* expression have been described previously (26). We have looked here more closely at those expression pattern aspects that can be related to the terminal function of *cad*. *Tc-cad* expression is present throughout development in the growth zone (26), but at the end of the segmentation process, *Tc-cad* expression becomes restricted to a subterminal stripe (Fig. 4 *a* and *b*), which occurs similarly in *Drosophila* (9, 10). Some groups of cells at the most posterior end remain *Tc-cad* positive (Fig. 4 *c* and *e*). These might correspond to the primordia of the anal plates, as described for *Drosophila* (9, 10) and the silk moth *Bombyx* (27). With the beginning of germ band retraction and hindgut formation, *Tc-cad* expression becomes detectable in the Malpighian tubules (Fig. 4 *d* and *f*), which is again in line with the function of *cad* in *Drosophila* (28).

**Regulation by *torso*.** The *tor* signal transduction pathway acts via a MAP kinase, whose activity can be monitored with a specific antibody against active MAP-K (29). We used this antibody to see whether one can detect possible signs of *tor* activity in the region where *tll* is expressed. Fig. 5*a* shows that there is indeed staining at the posterior end of the embryo at early blastoderm

stage, in the region where *tll* is activated (compare Fig. 2*a*). Interestingly, there is also staining in a broad anterior domain, which corresponds roughly to the region of the future serosa cells, which are probably specified at this stage (30, 31).

To test whether *Tribolium tll* responds to the terminal activation pathway in *Drosophila*, we fused the genomic upstream region of *Tc-tll* to a *lacZ* reporter and transformed this in *Drosophila* embryos. This reporter construct is indeed activated at the terminal regions of the embryo at blastoderm stage (Fig. 5*b*), comparable to similar constructs with the *Drosophila tll* upstream region (25). This indicates that this activation pathway is conserved between *Drosophila* and *Tribolium*.

**Discussion**

Previous comparative studies of segmentation genes in *Drosophila* and *Tribolium* have shown that differences in expression patterns can usually be correlated with the differences in the early fate map (12–14). Thus, it would seem that the general function of most segmentation genes is conserved between the species. This is also likely to be the case for the genes studied here, with the exception of posterior *tll* function. If this *tll* function were comparable in *Drosophila* and *Tribolium*, we
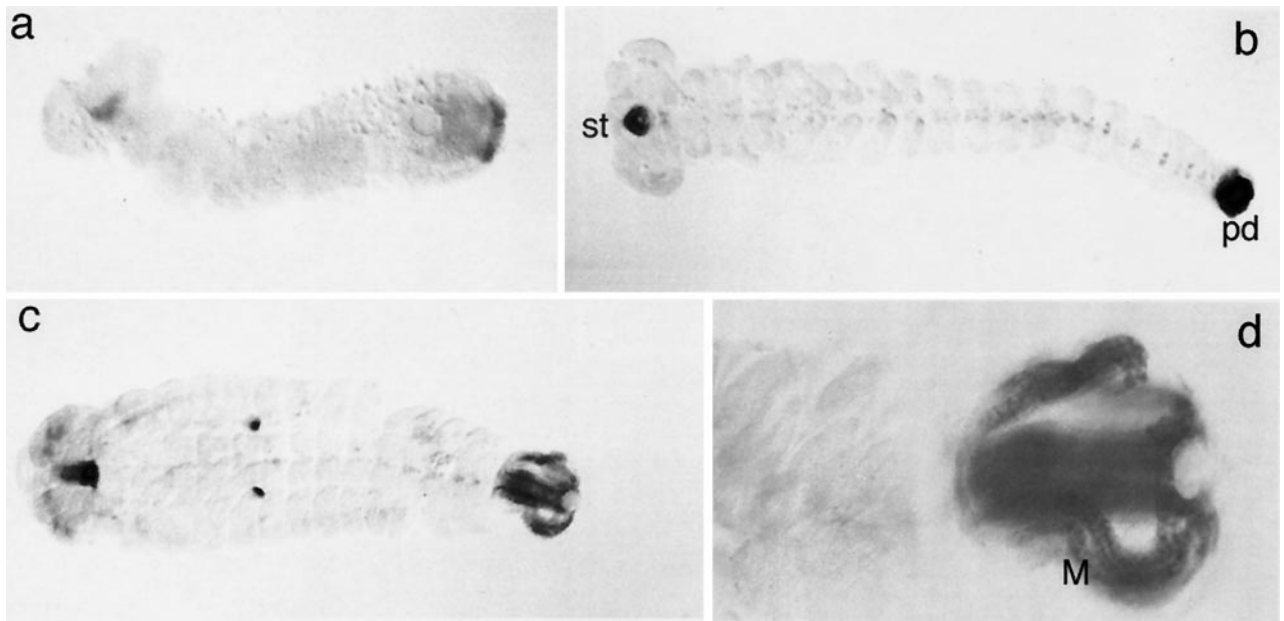
**Fig. 3.** RNA expression of *Tc-fkh*. Embryos were stained by whole-mount *in situ* hybridization. *a–c* represent progressively older stages, and *d* is an enlargement of the posterior end of an embryo that is at the same stage as the embryo in *c*. st, stomodaeum; pd, proctodaeum; M, Malpighian tubules. Note that the dots seen in the middle of the embryo in *c* are unspecifically staining cells in the pleuropodia on segment A1.

would expect *tll* in *Tribolium* to remain expressed at the posterior pole until the target genes are activated. We find, however, that posterior *Tc-tll* expression ceases quickly at both the RNA and protein levels, long before the first expression of the target genes is visible. Thus, it appears that the early function of *tll* has undergone a major evolutionary shift between *Tribolium* and *Drosophila*.

**Changing Role of Posterior *tll* Function.** There are at least three known direct target genes of *tll* that are required for the formation of the posterior terminal structures of the *Drosophila* embryo, namely *fkh*, *byn*, and the terminal *hb* stripe (4–7, 32). All three are thought to be regulated directly by *tll*, although this has been shown formally only for the terminal *hb* stripe (6). In *Tribolium*, all three of these genes (or expression domains) start to be expressed only after *tll* expression has ceased at the

posterior end. This late onset of expression of the target genes suggests that *tll* could not be their direct activator. A similar inference applies to another target gene of *tll*, namely *Krüppel* (*Kr*). *tll* acts as a repressor of *Kr* in *Drosophila* (33, 34). In *Tribolium*, *Tc-Kr* expression occurs at the posterior pole of the blastoderm embryo (35), overlapping with the early *Tc-tll* expression. This coexpression suggests that *Tc-tll* does not act as a repressor of *Tc-Kr*. Thus, *Tc-tll* does not appear to play the same role as *tll* in *Drosophila* as a direct regulator of other segmentation genes.

Nonetheless, *Tc-tll* is likely to be involved in determining the posterior terminal fate in *Tribolium*. We can infer this from the observations presented here, as well as from classic fate mapping experiments. We find that a receptor tyrosine kinase pathway is active at the same time and location where *Tc-tll* is activated at the posterior pole, suggesting that the *torso*-mediated induction
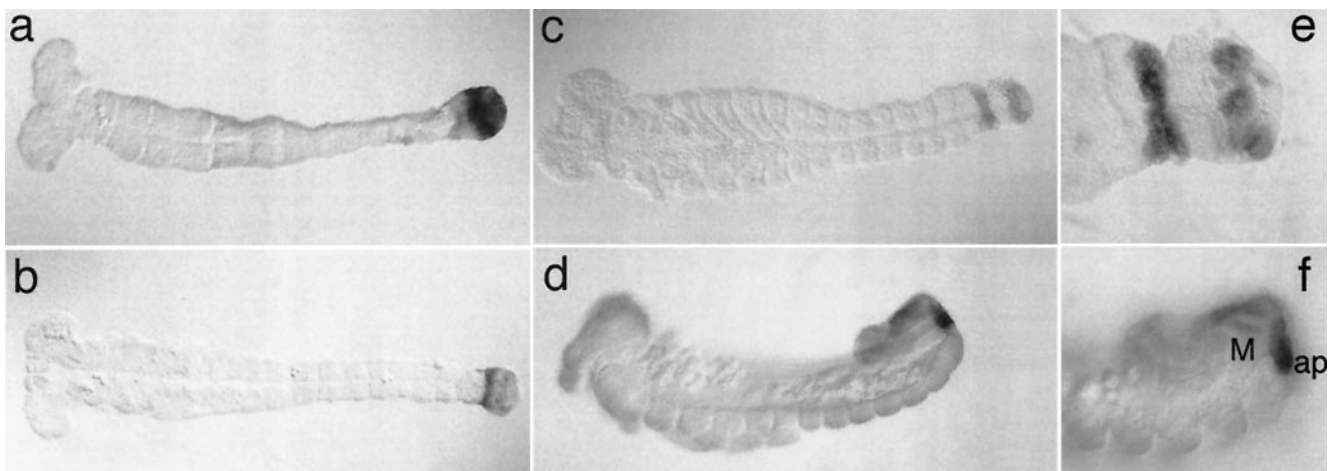


**Fig. 4.** RNA expression of *Tc-cad* during late stages of embryogenesis. Embryos were stained by whole-mount *in situ* hybridization with a probe for *Tc-cad* (26). (*a–d*) Progressively older stages of embryogenesis. (*e* and *f*) Enlarged views of the posterior ends of the embryos in *c* and *d*, respectively. ap, anal plates; M, Malpighian tubules.
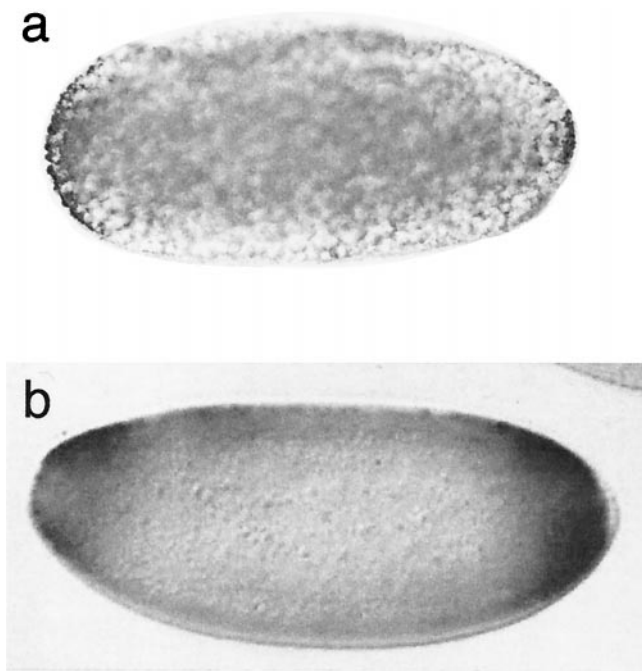
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**Fig. 5.** Evidence for *torso* activity. (*a*) Staining of *Tribolium* early blastoderm stage embryo with an antibody against active MAP kinase, an intermediate protein in the receptor tyrosine kinase pathway that is activated by the *torso*-signaling cascade. (*b*) Staining of an early blastoderm stage embryo of *Drosophila* against *lacZ* as reporter. The embryo is transformed with a construct containing the upstream sequences of *Tc-tll* in front of the *lacZ* gene. See text for further details.

of *tll* is conserved between the two species. Accordingly, we find that a reporter gene construct carrying the *Tc-tll* upstream region in *Drosophila* is activated in a very similar pattern as that known for the endogenous *tll* pattern in *Drosophila* (25). The other clue comes from the general fate mapping experiments in short germ insects. These fatemaps suggest that the future terminus, represented by the hindgut anlage, is specified already at blastoderm stage, although the structure itself develops only much later (reviewed in ref. 24). This is in contrast to the abdominal segments, for which there is no representation in the blastoderm fate map. They are sequentially generated from the posterior growth zone only after blastoderm stage. To reconcile these observations, we propose that there is a group of cells within the growth zone that is determined at blastoderm stage to produce the terminal structures, but which remains quiescent until much of the abdominal segmentation has been completed. In this scenario, the most likely role for *Tc-tll* would be an involvement in the differentiation of cells that could be called "terminal cells."

There is further evidence that such "terminal cells" do indeed exist at the posterior end of the *Tribolium* embryo. This comes from the observation of a very early activation of a *Tribolium wingless* (*Tc-wg*) expression domain in this region (22). *Tc-wg* may be involved in the differentiation process, because *wg* is also required for terminal structure formation in *Drosophila* (8). However, different from *Tc-tll*, *Tc-wg* remains expressed in the terminal region until late in embryogenesis (22), suggesting that its primary function may be conserved.

**The Role of the *torso*-Signaling Pathway.** The *torso*-signaling pathway acts symmetrically at both ends of the embryo in *Drosophila*. The same appears to apply for *torso* in *Tribolium*, as is shown by the fairly symmetrical staining for the activated MAP kinase. One might not have expected this outcome, however, because of

the differences in the fate map in the two species. Although the anterior terminal structures become specified at the anterior end of the blastoderm in *Drosophila*, this is different for *Tribolium*. In *Tribolium* the extraembryonic serosa is specified at the anterior end (30, 31), a structure that does not exist in this form in *Drosophila*. In *Drosophila*, the extraembryonic cells are determined at the dorsal side and consist of remnants of both the serosa and the amnion (36). Still, a molecular similarity between these structures is suggested by the fact that *zerknüllt* (*zen*) and *decapentaplegic* (*dpp*) are expressed in both species in these groups of cells (30, 31, 37, 38).

One can speculate that the role of the anterior *torso* pathway in *Tribolium* is the activation of *Tc-zen* and *Tc-dpp*. In this case, one would have to propose another major functional shift, because *zen* and *dpp* are activated primarily by the dorsoventral system in *Drosophila* (36). There are, however, indications that in both species *zen* and *dpp* are under the control of both the terminal and the dorsoventral system. In *Tribolium*, the expression of *Tc-dpp* and *Tc-zen* occurs initially at the anterior pole, but it shifts quickly toward the dorsal side, together with the expansion of the serosa. This shift is likely to be under the control of the dorsoventral system. In *Drosophila*, it is known that polar expression of *zen* and *dpp* require activation by the terminal system (39).

Thus, if the genes involved in extraembryonic membrane formation are regulated by the terminal and the dorsoventral system in both species, the apparent difference could be explained by heterochronic shifts and differences in the relative importance of the maternal axis determining systems for their expression. In this case, it would not be necessary to propose a major evolutionary transition to explain these differences.

**Anterior *tll* Expression.** In contrast to *Drosophila*, we do not observe an initial expression of *tll* at the anterior pole in *Tribolium*. But this is in line with the changed fate map, as discussed above. On the other hand, the brain-specific expression of *Drosophila* occurs also in *Tribolium*, albeit at somewhat later stages. At this time, the expression is at the ventral side and is therefore unlikely to be under the control of the terminal system. In *Drosophila*, the regulation of the anterior *tll* expression is also under the control of *bicoid* (40). It is, however, still unclear whether there is a homologue of *bicoid* in *Tribolium* (41), although functional evidence has suggested that it could be present (14). Still, it seems premature to make any speculation on whether this anterior expression of *tll* has a conserved regulation in both species. However, at least its function may be conserved. In *Drosophila*, the brain-specific expression is required for the formation of the anterior-most portions of the brain, including the optic lobes (25). A similar function is likely in *Tribolium*, as *tll* staining persists in the most anterior head region and also covers the ocular region. An involvement for the formation of the eyes has also been suggested for the *Xenopus* homologue of *tll* (42). Thus, at least parts of the brain-specific functions of *tll* may have been conserved even between phyla.

**Conclusions**

If the above interpretations are correct, then one would ascribe the most distinctive evolutionary changes in the terminal patterning systems of *Tribolium* and *Drosophila* to the function of the posterior *tll* expression. If we take *Tribolium* as a representative of the more ancestral form of embryogenesis in insects, then we would conclude that the ancestral function of *tll* was to differentiate the terminal cells. In *Drosophila*, *tll* has apparently lost this function but has acquired the direct control of the activation of key genes in this process, such as *byn* and *fkh*. In addition, *tll* in *Drosophila* must have acquired new regulatory interactions with the other gap genes, as well as with the enhancers driving the posterior pair rule stripes of *hairy*. *tll* acts

mainly as a repressor in this context, and these interactions are direct (43, 44). Thus, at least for one expression aspect of *tll*, one has to infer a major transition in regulatory interactions in the evolution of the long germ insects. A more detailed analysis of this evolutionary transition will become possible with the help of the emerging genetic (45, 46) and transgenic (47) techniques in *Tribolium*.

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