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Conserved and divergent expression aspects of the *Drosophila* segmentation gene *hunchback* in the short germ band embryo of the flour beetle *Tribolium*

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SUMMARY

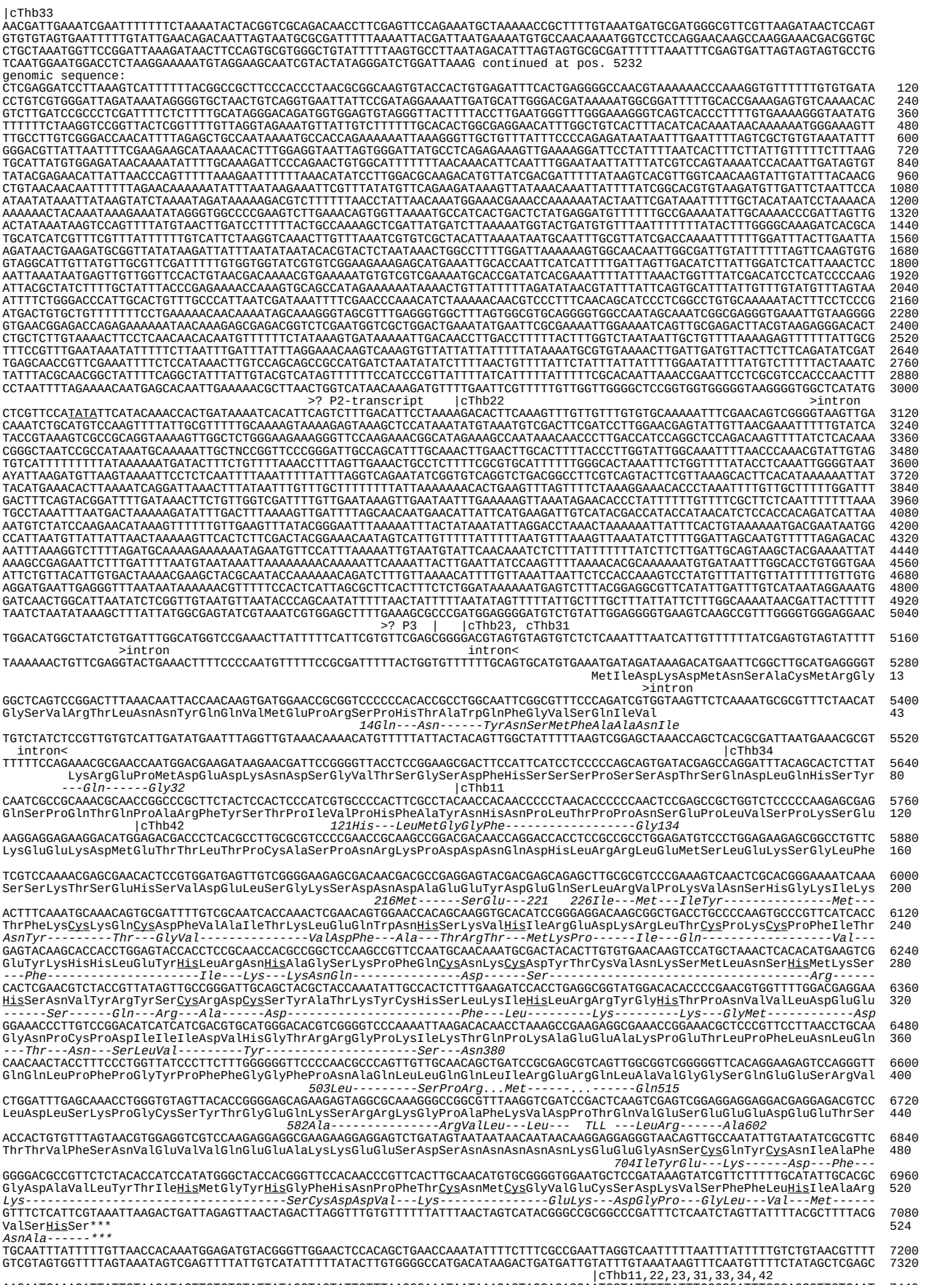
The segmentation gene *hunchback* (*hb*) plays a central role in determining the anterior-posterior pattern in the *Drosophila* embryo. We have cloned the homologue of *hb* from the flour beetle *Tribolium* and show that, on the basis of its expression pattern, most of its functions seem to be conserved between these two species. Like *Drosophila*, *Tribolium* has a maternal *hb* expression that appears to be under translational control by a factor at the posterior pole of the embryo. The maternal expression is followed by a zygotic expression in the region of the developing head and thoracic segments. During germ band extension, a posterior expression domain appears that is likely to be homologous to the posterior blastoderm expression of *hb* in *Drosophila*. These observations suggest that *hb* may have the same functions in early *Drosophila* and *Tribolium* development, despite the different types of embryogenesis in these two species (long versus short germ development). One differing aspect of *hb* expression in *Tribolium* concerns a structure that is not present in *Drosophila*, namely the serosa. An *hb* expression domain at the anterior pole precisely demarcates the border between the extra-embryonic serosa and the embryonic field in the *Tribolium* embryo at an early stage, and *hb* protein remains expressed in the serosa cells until the end of embryogenesis.

Key words: segmentation, *huncback*, short germ band embryo, embryonic pattern formation

INTRODUCTION

Drosophila segmentation is initiated by a cascade of interacting transcription factors that are provided maternally and zygotically in the early embryo (Pankratz and Jäckle, 1993). Homologues of these transcription factors can often be found in distantly related species (Tautz and Sommer, 1994). On the basis of the well known function of these transcription factors in *Drosophila*, it is possible to compare their expression patterns and to infer how far the segmentation pathways are conserved at the molecular level. Comparisons between different insect species are currently of special interest in this respect. When compared to other insects, *Drosophila* development represents a highly derived form of embryogenesis. In *Drosophila*, all segments are determined at the syncytial blastoderm stage, before cellularization. This allows the transcription factors to diffuse freely between adjacent nuclei and to excert their function by forming diffusion-controlled gradients. This situation is different for other forms of insect embryogenesis. In the more ancestral species, only the anterior pattern elements are determined at blastoderm stage, while most or all of the abdominal segments are generated in a secondary growth process (Anderson, 1972; Sander, 1976). The embryos of the flour beetle *Tribolium castaneum* represent such a form of short germ embryogenesis. Though the holometabolous beetles are still more closely related to the Diptera than some older insect orders, it can be argued that the *Tribolium* embryo may nonetheless represent the archetypical form of insect embryogenesis (Anderson, 1973; Tautz et al., 1994). *Tribolium* has thus become the focus of a number of comparative studies in molecular embryology (Sommer and Tautz, 1993, 1994; Brown et al., 1994a,b; Patel et al., 1994; Nagy and Caroll, 1994). These studies have indicated that the segmentation gene hierarchy, as it is known from *Drosophila*, is much more conserved in these different types of embryos than one would have previously assumed.

We describe here the cloning and the expression pattern of a key gene in the segmentation gene hierachy, namely *hb*. *Drosophila hb* codes for a zinc-finger type transcription factor (Tautz et al., 1987) which forms an early morphogenetic gradient in the embryo that is required to regulate the abdominal gap genes (Hülskamp et al., 1990; Struhl et al., 1992; Schulz and Tautz, 1994, 1995). *hb* expression is provided maternally and zygotically. The maternal RNA is distributed homogeneously in the embryo, but is under the translational control of the posterior maternal factor *nanos* (*nos*). Since NOS protein is distributed in a gradient from


|cThb33
genomic sequence:
>? P2-transcript
|cThb22
>intron
>? P3
|cThb23, cThb31
>intron
intron<
|cThb34
|cThb11
|cThb42
14Gln---Asn------TyrAsnSerMetPheAlaAlaAsnIle
121His---LeuMetGlyGlyPhe----------Gly134
216Met------SerGlu---221
226Ile---Met---IleTyr----------Met---
582Ala----------ArgValLeu---Leu---
TLL ---LeuArg------Ala602
704IleTyrGlu---Lys------Asp---Phe---
503Leu---------SerProArg...Met------...------Gln515
|cThb11,22,23,31,33,34,42

posterior to anterior (Gavis and Lehmann, 1992), this translational control results in an opposing gradient of HB protein distribution in the abdominal region of the embryo (Tautz, 1988). The zygotic expression of *hb* is under the transcriptional control of the anterior maternal gene *bicoid* (*bcd*), which forms an anterior-posterior gradient of protein distribution (Driever and Nüsslein-Volhard, 1988). This gradient directly activates *hb* transcription up to a certain threshold concentration of BCD protein (Driever and Nüsslein-Volhard, 1989) whereby *hb* itself appears to be synergistically involved in this process (Simpson-Brose et al., 1994). Thus *hb* becomes homogeneously expressed in the whole anterior half of the embryo (Tautz, 1988). HB protein diffuses from the border of this expression domain posteriorly and thus forms a gradient in the abdominal region. It was shown that this zygotic gradient can functionally replace the maternally provided gradient (Lehmann and Nüsslein-Volhard, 1987; Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989). At later stages, there are additional expression domains of *hb* at blastoderm stage (Tautz et al., 1987; Schröder et al., 1988; Tautz and Pfeifle, 1989). The anterior expression is replaced by two stripes of expression that are not under the direct control of the *bcd* gradient. One of these stripes, the 'parasegment 4 stripe' is under the regulatory control of *hb* and *Krüppel* (*Kr*) (Hülskamp et al., 1994). A posterior expression domain of *hb* is seen forming first a cap and then a stripe covering parasegments 13/14. This posterior expression domain is under the regulatory control of the terminal gap genes (Casanova, 1990).

We show here that most of this expression pattern of *hb* is conserved in *Tribolium*, suggesting that the functional role of *hb* may also be conserved between *Drosophila* and *Tribolium*. However, we describe one novel expression of *hb* in the prospective serosa cells of the *Tribolium* embryo that might play a role in determining between extra-embryonic and embryonic tissues. This distinction appears to be one of the first differentiation decisions that is made in the *Tribolium* embryo.

Fig. 1. Sequence of the *Tribolium hunchback* gene region. The presumptive start points of transcription (>), the intron positions (>.....<), as well as the extent of the different cDNA clones (|) are marked above the respective sequences. Note that the first four lines shown were only recovered from a cDNA and lie outside of the cloned genomic region. The numbering refers therefore only to the contigous genomic sequence. The conceptual open reading frame is given alongside with the alignment of regions that are conserved between *Drosophila* and *Tribolium*. All blocks shown are similarly conserved in *D. virilis* and *Musca domestica*. Dashes indicate identities, dots indicate gaps. The numbers flanking these conserved blocks refer to the respective amino acid positions in *Drosophila melanogaster* (Tautz et al., 1987). The stretch 14-32 corresponds to the A-Box, the stretch adjacent to the first finger domain to the C-Box and the stretch 503-515 to the D-Box as defined by Hülskamp et al. (1994). The potential TATA box for the P2 transcript, the crucial cystein and histidin residues of the Zn-finger domains and the poly(A) signal are underlined. Note that all cDNAs have a common 3′ end, indicating that only one poly(A) signal is utilized. The potential *nanos* response element (Drosophila consensus: GTTGTNNNNNATTGTA; Wharton and Struhl, 1991) in the untranslated 3′ end of the mRNA is indicated by dotted underlining. The sequence is accessible under EMBL accession number X91618.

MATERIALS AND METHODS

Cloning and sequencing

The first *Tribolium hb* clone was obtained by PCR with degenerate primers in the Zn-finger region as described by Sommer et al. (1992). The PCR fragment was used to screen a genomic library (a gift from S. Brown; Brown et al., 1990). About 7.5 kb of the genomic clone was sequenced using a combination of shot gun and primer walking procedures. To obtain information on the transcript structure, we have constructed an early cDNA library from 0- to 96-hour embryonic RNA in lambda ZAP, using the cDNA synthesis kit from Stratagene (Heidelberg). Several different types of cDNAs were recovered from

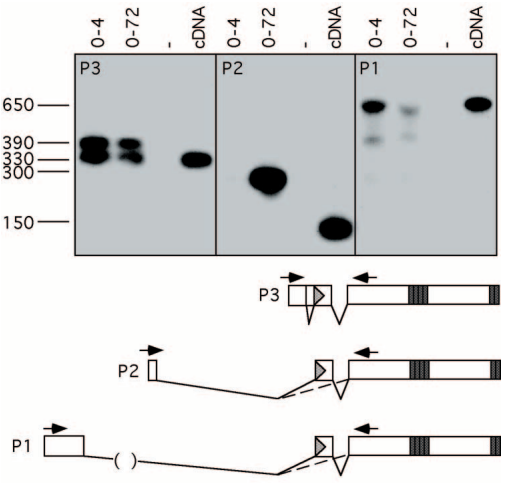


Fig. 2. Transcription structure of the *Tribolium hunchback* gene. The results of the RT-PCR experiments are shown on the top and the transcription structure as inferred from these experiments and from the structure of the different cDNAs is shown at the bottom. Top: RT-PCR was performed with an internal primer and three leader-specific primers (positions marked with arrows in the bottom diagram). A maternal (0-4 hours) and an embryonic (0-72 hours) RNA preparation was used. In addition, a control without RNA (–) and with the respective cDNA clone was included. The P3-specific primer pair yielded two equally abundant fragments of 331 bp and 386 bp. The 331 bp is equivalent to the cDNA fragment which lacks the small intron, while the 386 bp fragment still includes the intron (confirmed by sequencing). The P2-specific primer yielded a single 271 bp fragment only in the embryonic RNA preparation, indicating that this promoter is only zygotically active. The fragment is 139 bp larger than the corresponding cDNA, since it includes the respective exon (confirmed by sequencing; see diagram below). However, the fact that the cDNA clone did not contain this exon prompts us to assume that the splicing variant indicated by the dashed line exists as well, albeit at a very low frequency (a weak band is also visible in the blot after prolonged exposure). The P1-specific primer yields a strong 590 bp band and a weaker 450 bp band. The upper band corresponds to the length of the cDNA band, while the lower one would correspond to a splicing variant that does not contain the respective exon (not confirmed by sequencing), similar to that for the P2 transcript. Bottom: schematic drawing of the transcript structure. The non-coding regions are drawn as open boxes, the coding region as shaded boxes. The location of the zinc-fingers are marked (dark boxes), as well the location of the presumptive NRE sequence (arrowhead). The splicing variants are indicated by the different lines, whereby the rarer variants are indicated by dashed lines. The length of the intron separating the P1 leader is unclear (brackets). The figure is otherwise drawn approximately to scale.

this library and partially or fully sequenced. The beginning and the end of the different cDNAs are marked in Fig. 1.

Determination of transcription profile

To assess which of the different cDNA types are expressed at which time, we have performed RT-PCR experiments using the superscript kit (Life Technologies, Eggenstein) with staged RNA preparations (0-4 and 0-72 hour). The 0- to 4-hour RNA preparation represents the maternal RNA and was staged particularly carefully. To control the staging, a fraction of the eggs was stained with Hoechst 33342 to confirm that only embryos with a maximum of four nuclei were included. The first strand cDNA synthesis was done with a specific internal primer (CW856: 5′-AGAGTGCTGTAAATCCTG-3′, pos. 5638-5520). Aliquots of this cDNA were subjected to PCR using the CW856 primer as well as the following exon specific primers: exon 1 - CW859 (5′-TAAATGATGCGATGGGCG-3′), exon 2 - CW858 (5′-GTGCAAAAATTTCGAACAG-3′; pos. 3088-3106) and exon 3 - CW857 (5′-GTTCGAGCGGGGACGTAG-3′; pos. 5093-5110). The resulting fragments were resolved on agarose gels, blotted and hybridized with a *hb* cDNA probe that included all exons.

Antibody production

A *Hinc*II fragment spanning the amino acid positions 194-524 was expressed in *E. coli* using the pET vector system (Rosenberg et al., 1987). The protein was partially purified by gel electrophoresis and used to immunize a rabbit. The antiserum was affinity purified against the same protein fragment.

Embryo staining

Fixation and staining of the embryos with antibodies or whole-mount in situ hybridization was done using procedures similar to those used for *Drosophila* (Tautz and Pfeifle, 1989) and employing RNA probes according to the protocol of Klingler and Gergen (1993). In situ double staining was done with digoxigenin- and fluorescein-labelled RNA probes using a similar protocol to that described by Hauptmann and Gerster (1994). All embryos were counterstained with Hoechst 33342 before mounting in glycerol to visualize the distribution of the nuclei in the early embryos. Photographs were taken on a Zeiss axioplan microscope with Kodak Ektachrome 320T film and the slide images were transferred to Kodak photo CD. The final images were edited electronically with Photoshop (Adobe) to balance the sizes of the embryos and the colours, as well as to remove background spots.

RESULTS

Cloning of the *hb* gene

Primers from the zinc finger region of the *hb* gene were employed to amplify and clone a short fragment from *Tribolium* genomic DNA (Sommer et al., 1992). This fragment was then used to screen genomic and cDNA libraries. One genomic and several cDNA clones were recovered. The coding region as well as a large part of the upstream region was sequenced (Fig. 1). Comparison between the cDNA and the genomic sequences showed that the leaders of two different cDNA types reside within the genomic clone, while the leader of a third cDNA type must lie outside the cloned genomic region (Fig. 2). A potential TATA-box and a consensus transcription start site were found only for the P2 transcript (Fig. 1). Only one large open reading frame is evident which is interrupted by a short intron. The different leaders of the three cDNAs have a common acceptor splice site upstream of the AUG that initiates the open reading frame. The conceptually translated protein encodes the zinc finger domain that was already amplified by PCR and in addition a second zinc finger domain at the C terminus, as is typical for *hb* in other species (Treier et al., 1989; Sommer, 1992). An additional region of similarity at the amino acid level can be found downstream of the first finger domain (Fig. 1). Furthermore, a specific comparative search with the subregions that are conserved among dipteran *hb* genes (Treier et al., 1989; Sommer, 1992) reveals several additional very short conserved stretches (Fig. 1). One of these is partially encoded in the first coding exon, suggesting that this exon is indeed translated, though some splicing variants exist that exclude this exon (see below). Together, the amino acid similarities in the Zn-finger domains, as well as in the previously identified A-, C- and D-Boxes (Hülskamp et al., 1994) are sufficient to establish a likely homology of the cloned gene with the *Drosophila hb* gene.

A search for sequence regions conserved between the upstream regions of *hb* in *Drosophila* and *Tribolium* by dot plot comparisons did not yield any clear results. It was previously shown that some highly conserved regions can be

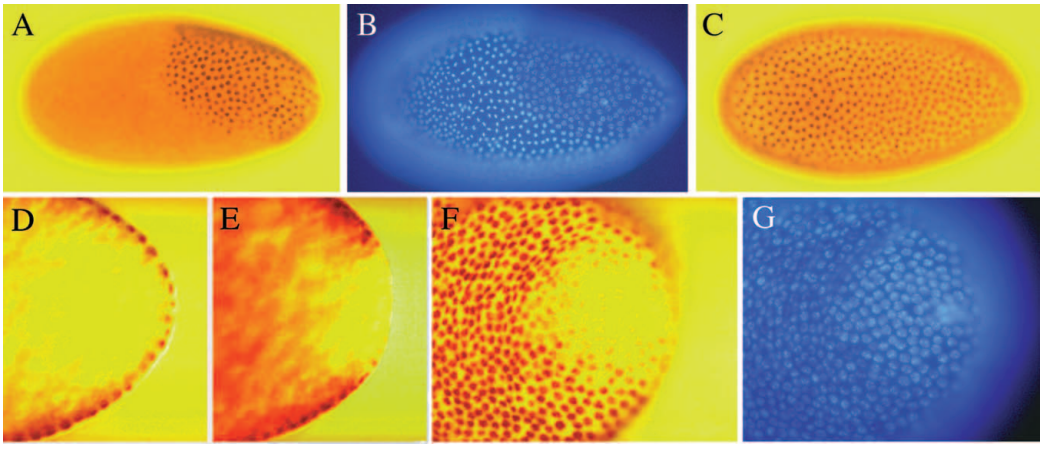


Fig. 3. Maternal HB protein expression in *Tribolium*. Early embryos were stained with a polyclonal antiserum against the *Tribolium hb* protein (A, C-F). The embryos were counterstained with Hoechst 33342 (B,G) to allow an exact determination of their stage. (A,B) The same embryo stained for protein (A) and Hoechst stained (B). A mitotic wave goes through the embryo and all nuclei that are not in mitosis stain positively with the antiserum. (C) Embryo of the same stage after completion of mitosis - all nuclei are now stained. (D) Posterior end of an embryo of the same stage as the embryo in C. (E) Posterior end of an embryo one nuclear division cycle later than that in D. (F,G) Embryo of the same stage as the one in E, but viewed from the posterior end. The comparison between the stainings in D and E-G shows clearly that the nuclei at the posterior pole all contain HB protein at early stages, but are cleared from HB protein at sligthly later stages.

detected in the upstream region between the *D. melanogaster* and *D. virilis hb* genes (Treier et al., 1989; Lukowitz et al., 1994). Given that the evolutionary time of separation between *Drosophila* and *Tribolium* is only about four times longer, one might have expected that at least some of these motifs are still conserved, but even specific searches for these motifs were unsuccessful. In spite of this, we found that some regulatory elements of the *Tribolium hb* upstream region are still functionally recognized in *Drosophila* (Wolff et al., unpublished data).

Promoter analysis

The recovery of different types of cDNA clones suggested that there are different transcripts from the gene. To study these further and to analyse whether at least one is transcribed maternally, we have employed PCR experiments with reverse transcribed RNA from maternal and zygotic stages of embryogenesis. Three primer pairs were used which can differentiate between the three different transcripts (Fig. 2). The results show that the RNAs from two of the promoters, namely the most distal one (P1) and the proximal one (P3) are already transcribed maternally, while the promoter between these (P2) is only zygotically active. Half of the P3 transcripts still contain a small intron, while a fraction of the P1 and P2 transcripts lack the first coding exon (Fig. 2). Though this transcription pattern is more complicated than in *Drosophila*, it is nonetheless reminiscent of the situation there. In *Drosophila*, the distal P1 promoter yields both maternal and zygotic transcripts, while the proximal P2 promoter yields only early zygotic transcripts (Tautz et al., 1987; Schröder et al., 1988; Treier et al., 1989; Lukowitz et al., 1994). The equivalent of an even more proximal P3 promoter was not found in *Drosophila*.

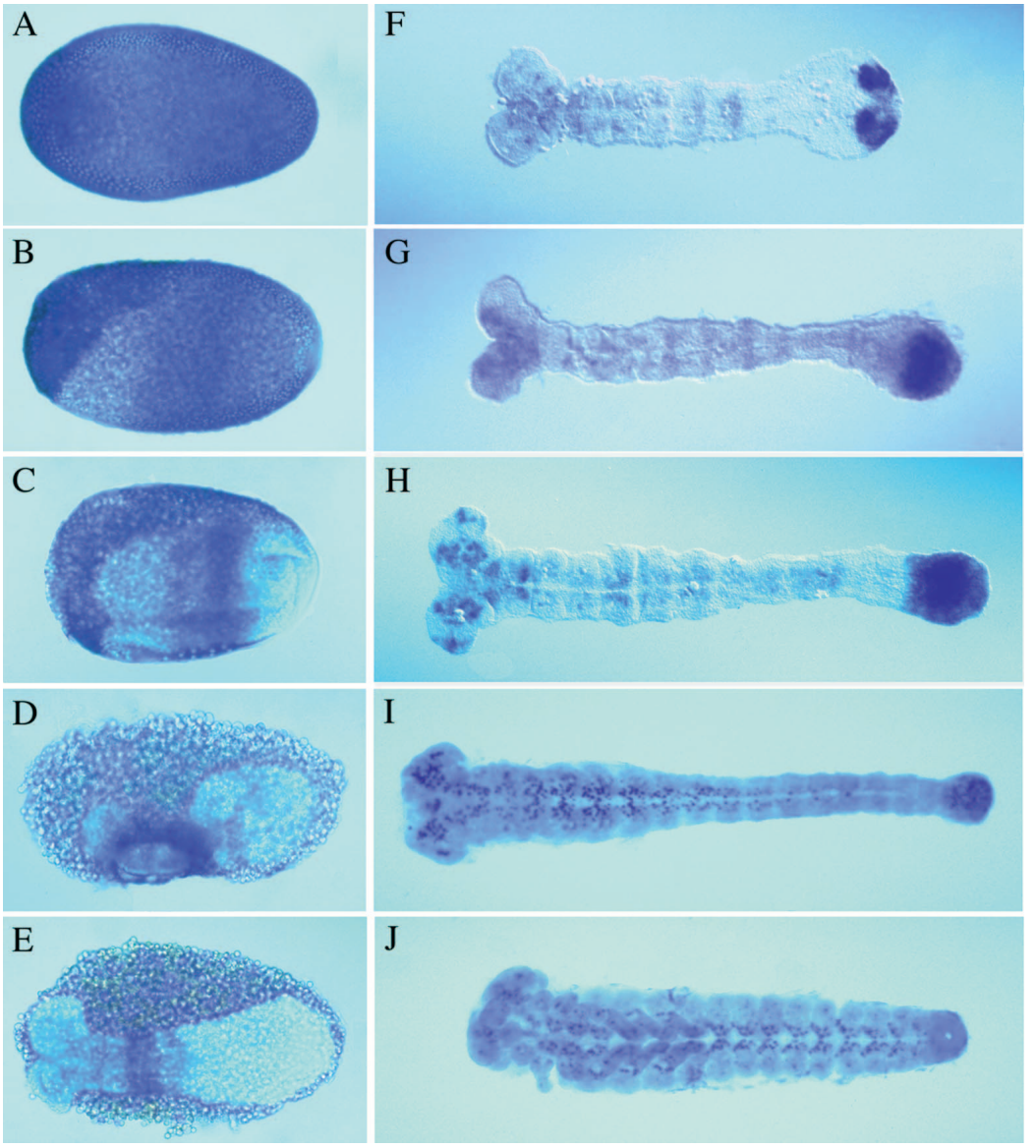


Fig. 4. Zygotic *hb* expression in *Tribolium*, (A-H) whole mount in situ hybridization, (I,J) antibody staining. The embryos in A-E were counterstained with Hoechst 33342 to visualize the distribution of the nuclei and cells within the egg. The embryos in F-J were prepared free from surrounding egg material. (A) Earliest differential zygotic expression of *hb* in an anterior cap and in the posterior two thirds of the egg. Note that the RNA staining is uniform even at the posterior pole, though the protein is already cleared from the pole region at an equivalent stage (see Fig. 5A). (B) Beginning of the formation of the germ rudiment; posterior dorsal extension of the anterior expression domain. (C) Formation of the germ rudiment and begin of gastrulation. The mesoderm starts to invaginate at the ventral side and the amnion folds over at the posterior end. (D) Stage shortly before the completion of the separation between serosa and amnion. Only a small ventral hole is still visible and *hb* expression demarcates exactly the rim of this hole. Note that the remaining nuclei of the amnion do not seem to express *hb* RNA any more, though they still express the protein (Fig. 5D). (E) Beginning of germ band extension. The serosa formation is complete, only *hb* RNA expression within the embryo is still visible. (F) First signs of the posterior *hb* expression in a U-shaped rim around the posterior end of the growth zone. (G) Full establishment of the posterior expression domain and transient formation of segmental stripes (partially visible also in F). (H) Secondary expression of *hb* in the CNS beginning in the head region. (I) End of germ band extension. Anterior-posterior establishment of the CNS expression, presumably in the segmental neuroblasts. (J) End of segmentation process, formation of the posterior hindgut invagination.

Maternal expression

To study the spatial and temporal expression of *hb* in *Tribolium*, we have utilized whole-mount in situ hybridization. In these experiments, early embryos always stain strongly and homogeneously with the *hb* probe (not shown). It seems likely therefore that the maternal *hb* mRNA that was identified in the RT-PCR experiments is distributed homogeneously in the early embryo, as is the maternal *hb* RNA in *Drosophila* (Tautz and Pfeifle, 1989). In *Drosophila*, the translation of this RNA is under the negative regulatory control of the posterior maternal system and HB protein from this RNA is therefore expressed mainly in the anterior half of the embryo, while it forms a gradient extending into the posterior half (Tautz, 1988). To study the protein distribution of *hb* in *Tribolium*, we have raised an antiserum against a large part of the coding portion of the gene. This antiserum was affinity purified and then used to stain embryos. The HB protein is first detected during the last nuclear division cycles shortly before blastoderm stage. All nuclei are homogeneously stained at this stage, only those that are in mitosis show no staining (Fig. 3A-C). Shortly after the completion of the last nuclear division cycle, the posterior terminus becomes cleared from protein expression (Fig. 3D-G). At this stage, the RNA is still present in this area (Fig. 4A), suggesting that it is translational control that causes this effect. This suggests that the maternal *hb* RNA in *Tribolium* may also be under the regulatory control of a posterior factor. Indeed, a short nucleotide motif that is reminiscent of the *nanos* response elements (NRE) that mediate the translational control in *Drosophila* (Wharton and Struhl, 1991; Murata and Wharton, 1995) can be found in the 3′-region of the mRNA (Fig. 1). Furthermore, functional homologs of *nanos* were recovered from very diverse dipteran species, indicating that its function is evolutionarily conserved (Curtis et al., 1995). The fact that at early stages all nuclei express HB protein might be explained by the fact that *nanos* itself is under translational regulatory control (Gavis and Lehmann, 1994) and that the first NOS protein is produced only somewhat later in *Tribolium*, albeit still before the onset of the first zygotic expression (see below).

Blastoderm expression

The first differential distribution of transcripts can be seen at the early blastoderm stage, after the posterior pole has become cleared of HB protein (see above). At this stage, one observes an anterior cap and a large posterior domain (Fig. 4A). It seems likely that these two domains are the result of a zygotic expression, since their level of expression is higher than that of the maternal RNA expression. The anterior cap expands dorsally towards the posterior at later stages (Fig. 4B), while the posterior domain recedes from the posterior tip and eventually forms a broad band in the central region of the embryo (Fig. 4B). This band splits into two subdomains at later stages (Fig. 4C). Concomitant with the dorsal expansion of the anterior domain, the posterior one recedes to the ventral side of the embryo (Fig. 4B,C).

These apparent movements of the expression domains reflect the movements of the nuclei in the early embryo. The early germ anlage forms ventrally in the posterior two thirds of the egg. Accordingly, the embryonic nuclei move into this region, mainly from the dorsal side, but partly also from the anterior region. This movement is compensated by the movement of the serosa nuclei that migrate dorsally from anterior to posterior. The serosa nuclei become enlarged (probably due to polyploidization) and are more scattered at later stages, which makes them clearly distinguishable from the embryonic nuclei (Fig. 5B,C). The border of the anterior *hb* domain conicides exactly with the border between these prospective extra-embryonic and embryonic fields. This border becomes established very early and quickly forms a very sharp boundary, as is evident both from RNA and protein distribution patterns (Fig. 5A-C). None of the other expression domains of segmentation genes that have so far been studied in the early *Tribolium* embryo show such a distinct border at such an early stage. This suggests that the developmental decision for the committment of extra-embryonic versus embryonic tissue is made at a very early stage of embryogenesis, possibly even before any subdivision of the anterior-posterior axis becomes established. *hb* continues to be expressed in the extra-embryonic cells until very late in development. RNA expression is seen exactly at the rim of the closing serosa (Fig. 4D) and protein expression is seen in all serosa cells almost until the end of embryogenesis (Fig. 5D). Interestingly, since *hb* RNA seems to be absent in the serosa at these later stages (Fig. 4E), one has to conclude that the HB protein in these cells has a much longer halflife than in other tissues where the protein staining pattern always matches the RNA staining pattern very closely. Given the very early and also persistent expression of *hb* in the serosa cells, one could speculate that *hb* is causally involved in the formation of these cells.

On the basis of these considerations, it is evident that the anterior *hb* expression domain in *Tribolium* cannot be homologous to the anterior *hb* expression domain in *Drosophila*, since extra-embryonic cells are not specified at such an early stage in *Drosophila*. Instead, it appears that the posterior *hb* expression domain in *Tribolium* must be homologous to the anterior domain in *Drosophila*. This is evident from the fact that it is expressed in the prospective head and thoracic region in the developing embryo (Fig. 4C,E). Furthermore, it lies in the same region where the anterior pair rule and segment polarity stripes are formed in *Tribolium* (Sommer and Tautz, 1993; Patel et al., 1994; Nagy and Caroll, 1994; Brown et al., 1994a,b) and anteriorly adjacent to the expression domain of *Tribolium Krüppel* (Sommer and Tautz, 1993), similar to the situation in *Drosophila*.

Postblastoderm expression

The posterior blastoderm expression domain of *hb* can still be seen during gastrulation and in the early germ band (Fig. 4E). At this stage it becomes clear that it is expressed in the region of the prospective gnathal and first thoracic segments. In *Tribolium*, double staining with the *hairy* probe (Fig. 6) shows that the more posterior *hb* stripe overlaps with the second *hairy* stripe, which demarcates PS 2/3 in *Drosophila*. The border of the early zygotic expression of *hb* in *Drosophila* is in a similar position (Tautz et al., 1987), though its exact determination is difficult since *hb* forms a short range gradient at this stage. Furthermore, a secondary blastoderm expression stripe of *hb* occurs in PS4 a little later, overlapping the border of the previous domain (Tautz et al., 1987; Schröder et al., 1988; Hülskamp et al., 1994). Thus, within the limits of reasonable

resolution, we conclude that the posterior border of the *Tribolium hb* domain appears to be at a homologous position to that of the early *Drosophila hb* domain.

Halfway through germ band extension, additional expression patterns are seen. At first there is a very weak expression that appears to occur segmentally (Fig. 4F,G). Such an expression pattern is not known from *Drosophila*. However, intriguingly, it occurs very similarly in the distantly related dipteran *Musca domestica* (Sommer and Tautz, 1991) as well as in the lepidopteran *Manduca sexta* (Kraft and Jäckle, 1994).

Another expression of *hb* is visible at the posterior end at this stage (Fig. 4F-I). This expression of *hb* is reminiscent of the posterior *hb* expression seen in *Drosophila* at blastoderm stage. In *Drosophila* the posterior *hb* domain is located in the region of PS 12-13 and is required for the formation of abdominal segments A7 and A8 (Tautz et al., 1987; Lehmann and Nüsslein-Volhard, 1987; Bender et al., 1987). To map the *Tribolium* domain more precisely, we have again used double staining with the *Tribolium hairy* probe. The results show that the anterior border of this *hb* domain is posterior to *hairy* stripe no. 6 and overlaps with *hairy* stripe no. 7 (Fig. 6D), which is in line with the situation in *Drosophila*. *Tribolium* forms two more abdominal segments than *Drosophila* and accordingly, an eighth *hairy* stripe develops. This stripe lies within the posterior *hb* domain (Fig. 6E,F). On the basis of these results we suggest that the posterior *hb* domain expression in *Drosophila* and *Tribolium* is at a homologous position.

In *Drosophila*, *hb* is expressed also in the developing nervous system, most notably in the early neuroblasts and later in the CNS. A similar expression of *hb* can be detected in *Tribolium* (Fig. 4H-K), though a more detailed comparison with the *Drosophila* expression needs still to be done.

DISCUSSION

Morphologically, *Drosophila* and *Tribolium* represent two very different types of early development, namely long and short germ band embryogenesis. While the early pattern formation process in long germ band embryos occurs essentially in a syncitial situation, patterning in short germ band embryos occurs at least partially in a cellular environment. This had raised the question of whether the same molecular mechanisms could govern early development, or whether there would be substantial adaptations to the special mode of syncitial development seen in *Drosophila*. However, the analysis of the expression of the gap gene *Krüppel* as well as of pair-rule gene and segment-polarity gene homologues in *Tribolium* has suggested that the molecular basis of early development might not be so different after all (reviewed by Tautz and Sommer, 1994). The expression analysis of the gap gene *hb* now provides additional evidence suggesting that the early molecular aspects of development are indeed much more conserved than the morphological comparisons had suggested.

The first conserved aspect relates to the maternal expression of *hb*. Both in *Tribolium* and in *Drosophila* there is a posterior factor that excerts a translational control on the early *hb* RNA which is initially homogeneously distributed in the egg. In *Drosophila*, this factor is *nos*, in conjunction with *pumilio* and an as yet unidentified protein (Wang and Lehmann, 1991; Gavis and Lehmann, 1992; Murata and Wharton, 1995). In *Tribolium* we find a conserved motif in the 3′ end of the mRNA where these gene products bind (Murata and Wharton, 1995). It is therefore reasonable to assume that the same factors are also active in *Tribolium*, even though homologues have not yet been identified in this species.

The earliest zygotic expression of *hb* in *Tribolium* consists of two separate domains, one demarcating the region of the prospective serosa cells and one in the region of the developing head and thoracic segments. This latter domain can be considered to represent a conserved expression between *Drosophila* and *Tribolium*, despite its different location within the egg. In *Drosophila*, the equivalent expression domain covers the whole anterior half of the embryo, while in *Tribolium* it is expressed in the posterior two thirds. Still, it seems possible that both domains could be under a similar regulatory maternal control. In *Drosophila*, *hb* is activated by the anterior BCD protein gradient, its posterior border being set by a threshold concentration below which the BCD protein cannot function as an activator. Since such a posterior boundary is not evident at the earliest stages in *Tribolium*, one would have to postulate that an activating effect by a potential *Trib-bcd* homologue would at first be active throughout the embryo. This would still leave unexplained how the anterior border of the posterior *hb* domain is determined. Either the *bcd* gradient alone, or in conjunction with a factor from the terminal system might be responsible for this. Such an interaction between *bcd* and the terminal system exists in *Drosophila* (Ronchi et al., 1993). However, a completely different regulatory control of this anterior border seems still possible.

The regulatory control of the posterior border of the zygotic *hb* domain might be more relevant for the segmentation process. A short range gradient of *hb* emanating from this border controls the expression of the more posterior gap genes in *Drosophila* (Hülskamp and Tautz, 1991). In *Tribolium*, the maternal translational control (see above) produces such a protein gradient. Interestingly, the zygotic RNA expression domain, which is seen a little later, extends at first to the posterior tip of the embryo. However, since the posterior pole remains free of HB protein even at this stage, we have to conclude that both the maternal and the zygotic RNAs are under the negative translational control of the posterior maternal factors. This is in line with the fact that both types of RNA have the same 3′ untranslated region and thus both carry the NRE motif. The apparent retraction of the zygotic domain from the pole at later stages might then be solely due to a reduced stability of the untranslated RNA. However, an additional regulatory input from the terminal system might also exist.

Independently of how this posterior border of *hb* expression might be regulated, we note that it lies eventually at a similar position to that in *Drosophila*, at least when compared on the basis of the expression in the respective segment anlagen. Most importantly, it lies anteriorly to the expression domain of *Tribolium Krüppel* (Sommer and Tautz, 1993) and could thus be involved in its regulation in a similar manner to that in *Drosophila*, namely by repressing *Krüppel* at high concentrations and activating it at lower concentrations (Hülskamp et al., 1990; Struhl et al., 1992; Schulz and Tautz, 1994).

The most anterior zygotic *hb* domain does not seem to relate to any *hb* expression domain in *Drosophila*. This may be explained by the fact that it demarcates a structure that is not

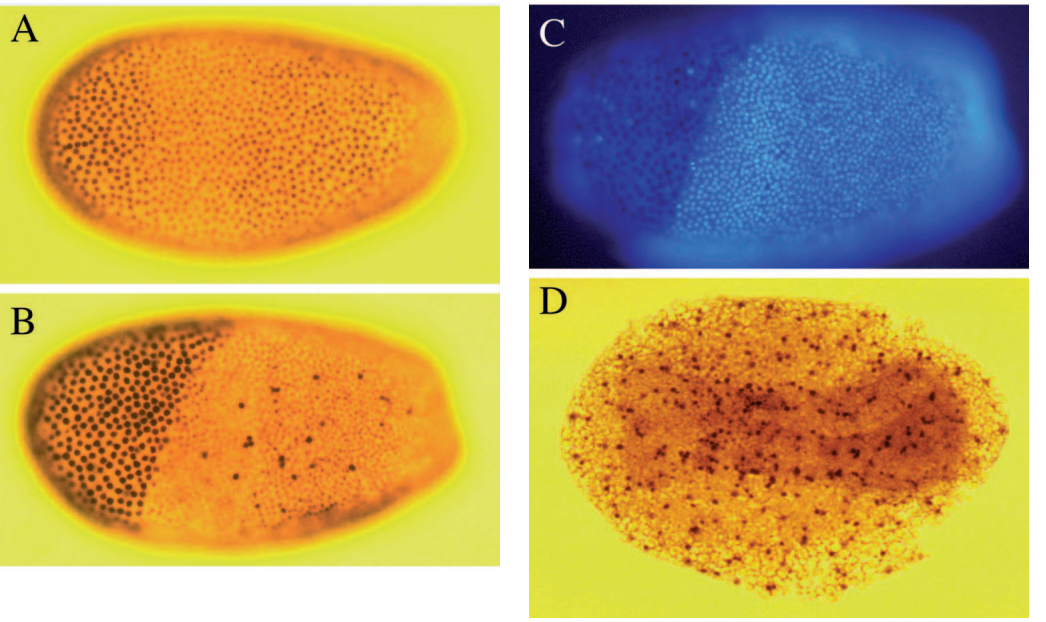


Fig. 5. Protein expression of *hb* in the serosa cells. (A) Earliest differential zygotic expression, reflecting the RNA expression at a comparable stage (Fig. 4A), apart of the posterior pole region, which is free of protein. (B) Formation of a sharp boundary distinguishing the serosa from the embryonic cells. Note the relatively weak expression of protein in the posterior half which is intermingled with strongly expressing nuclei of unknown significance. (C) Same embryo as in B, with Hoechst staining. (D) Embryo after the completion of the formation of the serosa; all nuclei show *hb* protein staining.

present in *Drosophila*, namely the serosa. The serosa is an extra-embryonic membrane that eventually surrounds the whole developing embryo and that is not directly connected to it at later stages. This is different for the other extra-embryonic tissue, the amnion, which remains attached to the embryo until dorsal closure is complete (Anderson, 1972). The extra-embryonic tissue of *Drosophila*, the amnio-serosa, remains attached to the embryo until dorsal closure. In this respect it resembles the amnion, suggesting that an equivalent of a serosa might be absent in *Drosophila*, which would explain the absence of *hb* expression in the respective tissue in *Drosophila*. However, we have obtained conflicting data on this point. We have studied the expression pattern of an apparent homologue of *zerknüllt* (*zen*) in *Tribolium* and found that it is expressed in the same anterior domain as *hb* and thus in the serosa cells, but not in the amnion which forms at later stages (unpublished data). In *Drosophila*, the *zen* gene is expressed in the amnio-serosa (Rushlow et al., 1987), which would suggest a closer

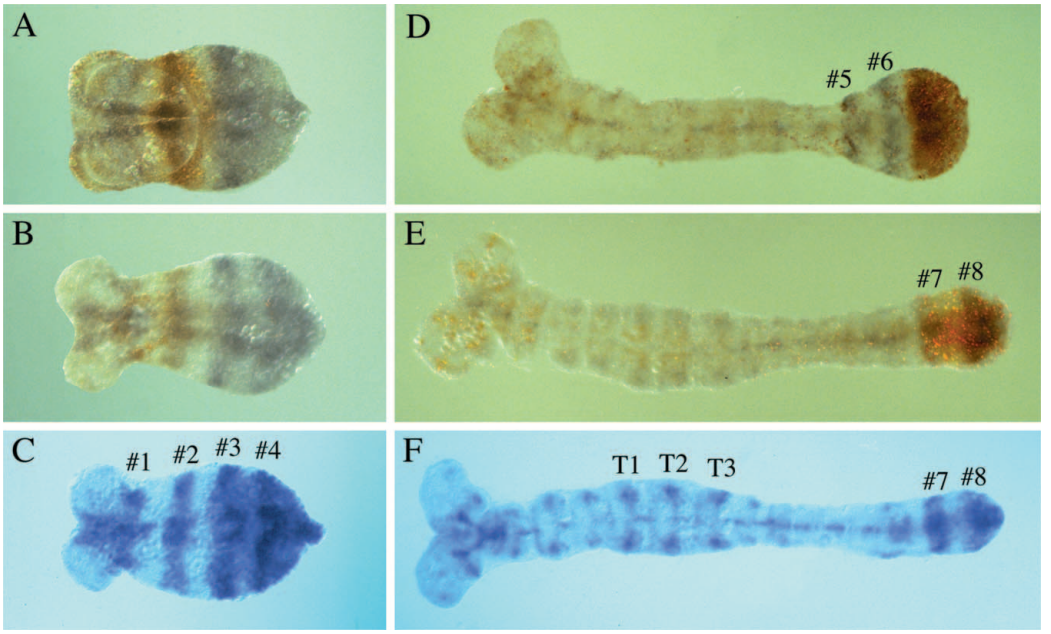


Fig. 6. Mapping of the *hb* expression domains by double staining with the *Tribolium hairy* probe. *hb* in brown and *hairy* in grey or blue. (A) Early germband stage, shortly before the completion of the serosa formation (note the hole in the anterior half). The central *hb* stripe overlaps with *hairy* stripe no. 2. (B) Beginning of germ band extension and separation of *hairy* stripes nos. 3 and 4. (C) Same stage, but *hairy* staining only, stripes 1-4 are marked. The comparison between B and C shows particularly clearly the overlap of *hb* expression with stripe no. 2. (D) Formation of the posterior *hb* domain, behind *hairy* stripe no. 6. Stripes 5 and 6 are marked. (E) Separation of *hairy* stripes nos. 7 and 8 within the *hb* domain. (F) Same stage as in E but *hairy* staining only, the thoracic segments T1-T3 and stripes nos. 7 and 8 are marked.

relationship to the serosa cells. Thus, the question of the homology status of the extra-embryonic tissues must remain open until more comparative data from other species are obtained.

However, setting this question aside, one can simply take the anterior *hb* (or *zen*) expression domain as a marker for the prospective extra-embryonic versus embryonic tissues. It then becomes clear that only a very small portion of the whole embryo is dedicated, at blastoderm stage, to become extra-embryonic tissue, while most of the nuclei/cells will eventually end up in the embryo or the amnion precursor cells which at this stage are located in a small rim around the embryonic cells (Anderson, 1972). This observation suggests that maternally derived gradients could act more or less in the same way as in *Drosophila*, namely throughout the egg (St. Johnston and Nüsslein-Volhard, 1992). Such gradients could instruct the nuclei before they move to the posterior ventral side where they form the germ anlage. Interestingly, this movement of the nuclei is characteristic for all short germ band embryos, but does not occur in long germ band embryos (Anderson, 1972). Thus, long germ band embryos must have lost the respective developmental pathway and are therefore neotenic to short germ band embryos in this respect.

Drosophila hb also shows a posterior expression domain at blastoderm stage which is regulated by the terminal system. Apparently the same expression domain is present in *Tribolium*, though not at the blastoderm stage, but towards the end of germ band elongation. This raises the interesting question of how this is regulated in *Tribolium*. The terminal maternal information in *Drosophila* is transmitted via *torso*, which codes for a receptor tyrosine kinase (Sprenger et al., 1992). Signalling from these receptor molecules (Casanova and Struhl, 1993) eventually activates the terminal gap genes *tailless* and *huckebein* which code for transcription factors that form short range gradients in the embryo (St. Johnston and Nüsslein-Volhard, 1992; Pankratz and Jäckle, 1993). *tailless* then acts as an activator for the terminal *hb* expression and *huckebein* as a repressor (Casanova, 1990). Evidently, this system, if conserved in *Tribolium*, must act somewhat differently. If a terminal signal exists and if this signal activates terminal gap genes, these would exert their regulatory function on *hb* only much later, namely when the germ band has elongated and when it is in fact physically not linked to the posterior pole any more, but has moved up to the dorsal side. This would imply that the cells that have received the terminal signal at blastoderm stage are already committed to a terminal fate, even though the relevant genes are expressed only at later stages. Interestingly, early fate mapping experiments in short germ embryos have indicated that the proctodaeum, which depends on the terminal system in *Drosophila*, can be fate mapped already at blastoderm stage (Anderson, 1972), indicating that the respective cells are indeed already committed. There is even some molecular evidence for an early terminal signal in *Tribolium*, namely from the expression pattern of the segment polarity gene *wingless* (Nagy and Carroll, 1994). Apart of its segmental expression, *wingless* also has a posterior terminal expression domain, both in *Drosophila* and in *Tribolium*. Though the function and exact regulation of this domain is not yet known, it can nonetheless be observed in *Tribolium* from blastoderm stage onwards until the end of germband extension.

We conclude that although the *hb* expression between *Drosophila* and *Tribolium* differs in some details, the general pattern is nonetheless surprisingly well conserved. Most importantly, since *hb* in *Drosophila* is more or less directly regulated by three maternal systems (*bcd*, *nos* and *tor*) and since the respectively regulated expression domains can be seen in *Tribolium*, we can speculate that these maternal systems may also be conserved in *Tribolium*.

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