Development 135, 881-888 (2008) doi:10.1242/dev.018317

Delimiting the conserved features of *hunchback* function for the trunk organization of insects

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The gap gene *hunchback* in *Drosophila* acts during syncytial blastoderm stage via a short-range gradient and concentration-dependent activation or repression of target genes. Orthologues of *hunchback* can be easily found in other insects, but it has been unclear how well its functions are conserved. The segmentation process in most insect embryos occurs under cellular conditions, which should not allow the formation of diffusion-controlled transcription factor gradients. We have studied here in detail the function of *hunchback* in the short germ embryo of *Tribolium* using parental RNAi and interaction with possible target genes. We find that *hunchback* is a major regulator of the trunk gap genes and Hox genes in *Tribolium*, but may only indirectly be required to regulate other segmentation genes. The core function of *hunchback* appears to be the setting of the *Ultrabithorax* expression border via a repression effect, and the activation of the *Krüppel* expression domain. These regulatory effects are likely to be direct and are conserved between *Drosophila* and *Tribolium*. We find no evidence for a classical gap phenotype in the form of loss of segments in the region of expression of *hunchback*. However, the phenotypic effects in *Tribolium* are highly comparable with those found for other short germ embryos, i.e. the core functions of *hunchback* in *Tribolium* appear to be the same in these other insects, although they are evolutionarily more distant to *Tribolium*, than *Tribolium* is to *Drosophila*. These results allow the disentanglement of the conserved role of *hunchback* in insects from the derived features that have been acquired in the lineage towards *Drosophila*. Given that the gap phenotype appears to occur only in long germ embryos and that the main role of *hunchback* appears to be the regionalization of the embryo, it may be appropriate to revive an alternative name for the class of gap genes, namely 'cardinal genes'.

KEY WORDS: Segmentation, Gap genes, Hox genes, *Tribolium*, Short germ embryogenesis

INTRODUCTION

As one of the major coordinators of the *Drosophila* segmentation gene cascade, *hunchback* (*Dm'hb*) is required for the proper expression of patterning genes involved in both metamerization and segment identity specification (Hülskamp and Tautz, 1991). Maternally expressed *Dm'hb* polarizes the *Drosophila* embryo by forming an anterior to posterior morphogenetic gradient in the posterior half of the syncytial blastoderm, which is required for positioning the central and posterior gap gene domains (Tautz, 1988; Hülskamp et al., 1990; Struhl et al., 1992). The anterior zygotic expression of *Dm'hb* overlaps spatially and functionally with the maternal expression and appears to act as a canonical gap gene being required for the formation of an adjacent set of segments (Lehmann and Nüsslein-Volhard, 1987).

In addition to the role during segmentation, *Dm'hb* also controls the expression of Hox genes. The anterior domain of *Dm'hb* limits the anterior borders of the *Dm'Ubx* and *Dm'Antp* expression domains (Irish et al., 1989; Lehmann and Nüsslein-Volhard, 1987; Qian et al., 1991; White and Lehmann, 1986; Zhang and Bienz, 1992). However, the effects caused by this ectopic expression of Hox genes in *Dm'hb* mutants are often concealed by the segmentation defects, as the Hox genes are ectopically expressed in the segments that are deleted in the larvae (Lehmann and Nüsslein-Volhard, 1987).

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Accepted 28 December 2007

The role of *hunchback* in segmentation has also been functionally studied in other insects with the aim to elucidate the transition from short germ to long germ embryogenesis (He et al., 2006; Liu and Kaufman, 2004; Mito et al., 2005; Schröder, 2003). Although the expression patterns of *hunchback* are well comparable, different functional roles have been ascribed to *hunchback* in the different insects. More or less canonical gap phenotypes were reported for *Tribolium* (Schröder, 2003) and *Nasonia* (Pultz et al., 2005). Different phenotypes, including transformations and loss of trunk segmentation were found in *Oncopeltus* (Liu and Kaufman, 2004), *Gryllus* (Mito et al., 2005) and *Locusta* (He et al., 2006). In addition, it is clear that the primary regulator of zygotic *hunchback* expression in *Drosophila*, *bicoid*, is a late evolutionary acquisition that emerged only in the higher Diptera (Stauber et al., 2002; Schröder, 2003). Thus, it appears that *hunchback* regulation and function has been subject to major evolutionary changes even within insects.

Even in *Drosophila*, the role of *hunchback* is more complex than it is often portrayed. Some alleles of *Dm'hb* produce directly a combination of homeotic transformations and trunk segmentation defects (Lehmann and Nüsslein-Volhard, 1987). One allele has originally been identified as *Regulator of postbithorax* and hence as a homeotic gene (Bender et al., 1988). In addition, given that phenotypic effects are always a combination of loss of the gene itself and changes in downstream genes, it is often not easy to recognize possible conserved features.

Here, we re-investigate the *hunchback* phenotype in *Tribolium* and assess its role in regulating the trunk gap genes and Hox genes. We focus the study on the anterior expression region, as this reflects the key function for *hunchback* in organizing the *Drosophila* segmentation gene cascade (Hülskamp and Tautz, 1991). We find that one can identify some core components of *hunchback* function that appear to be conserved in all insects. This includes the role in specifying anterior borders of Hox gene expression and interactions

with other gap genes. However, the major function in segmentation that leads to the typical gap phenotype in *Drosophila* appears to be limited to long germ embryos. Hence, most similarities in *hunchback* function appear to exist among insects that represent the ancestral type of embryogenesis, while more-derived types have developed additional features.

MATERIALS AND METHODS

Culture rearing

Tribolium castaneum strain San Bernardino beetles were reared on white flour supplemented with brewer's yeast at 30°C. The pupae for injections were obtained by collecting eggs within a time span of 9 hours of development and leaving them for about 25 days at 30°C to develop.

RNA interference

Parental RNA interference essays were performed as described by Bucher et al. (Bucher et al., 2002). Double-stranded RNA was injected into pupae at a concentration of 2 μg/μl. We found this concentration ideal to obtain maximum penetrance for most genes. Eclosed females were mated with wild type males and reared under standard conditions (see above). Knockdown embryos were collected every second day and one collection per week was kept at 30°C to monitor RNAi penetrance at the cuticular level. The collections were performed until the phenotypic effect had decreased significantly. Embryos for in situ hybridization were taken from females showing the highest penetrance, as judged by the parallel analysis of cuticle phenotypes.

Embryo fixation

The eggs were washed for 1 minute in 50% bleach solution and for 2 minutes in running water to remove the chorion. The fixation was performed in a scintillation vial with 3 ml PBS, 6 ml heptane and 4% formaldehyde for 30 minutes. The eggs were then devitellinized by replacing the PBS with 8 ml of methanol and by shaking thoroughly for 30 seconds. The eggs that lose the vitelline membrane become hydrophilic and move from the interphase to the hydrophilic phase. After several washes with methanol they were transferred to Eppendorf vials. The remaining eggs were passed through a 0.9 mm needle until the vitelline membrane was removed.

Expression analysis

The gene expression profile was obtained by whole-mount in situ hybridization as previously described (Lehmann and Tautz, 1994; Tautz and Pfeifle, 1989). Digoxigenin- or fluorescein-labelled probes were detected using alkaline phosphatase-coupled antibodies and INT/BCIP (red) or NBT/BCIP (blue) substrates.

Cuticular preparation

First-instar larvae were digested overnight in a 1:1 lactic acid/Hoyer's medium solution at 70°C and mounted on microscope slides. The cuticular autofluorescence in a range of 520-660 nm was detected on a Leica Confocal microscope, and maximum projection images were created from *z* stacks composed of 50 layers scanned four times each.

RESULTS

The hunchback phenotype

Parental RNA interference (pRNAi) was used to generate females lacking both, maternal and zygotic *Tribolium hunchback* (*Tc'hb*) function. As the mother transfers the RNAi effect to the eggs, the phenotype decays in relation to the age of the injected female, resulting in a phenotypic series (Fig. 1).

Schröder (Schröder, 2003) has previously shown that loss of *Tc'hb* function (*Tc'hb*^{pRNAi}) does not affect the pre-gnathal segments labrum, antenna and mandible. In the strong phenotypes, the remaining segments bear no appendages and appear to have abdominal identity. As a consequence, this could be interpreted as a gap phenotype in which the maxillary, labial and thoracic segments are missing (Schröder, 2003).

However, a reanalysis of the phenotypic series suggests that the phenotype observed is a combination of transformation and loss of segments. In weak phenotypes, all segments appear to be present, but the maxillary and the labial segments are transformed into abdominal identity (Fig. 1B). In addition, the thoracic segments look also more like abdominal segments, although T2 and T3 retain some appendage stumps, which may even look like developed legs in T3 (arrow in Fig. 1B). In more severe phenotypes, all segments beyond the mandibular one look like abdominal segments (Fig. 1C). Yet there are often more than the normal eight abdominal segments, suggesting that this cannot be interpreted as a simple loss of the remaining gnathal and thoracic segments. Instead, one sees a disruption of more posterior segments (arrowhead in Fig. 1C,D) and an increasing loss of posterior structures (Fig. 1D). Therefore, the *Tc'hb*^{pRNAi} phenotype can be described as a transformation of gnathal and thoracic segments into abdominal identity combined with a loss of abdominal segments in the more severe phenotypes. In the most extreme phenotypes, only the antennae and mandibles are left, followed by four segmental structures of abdominal identity (Fig. 1D).

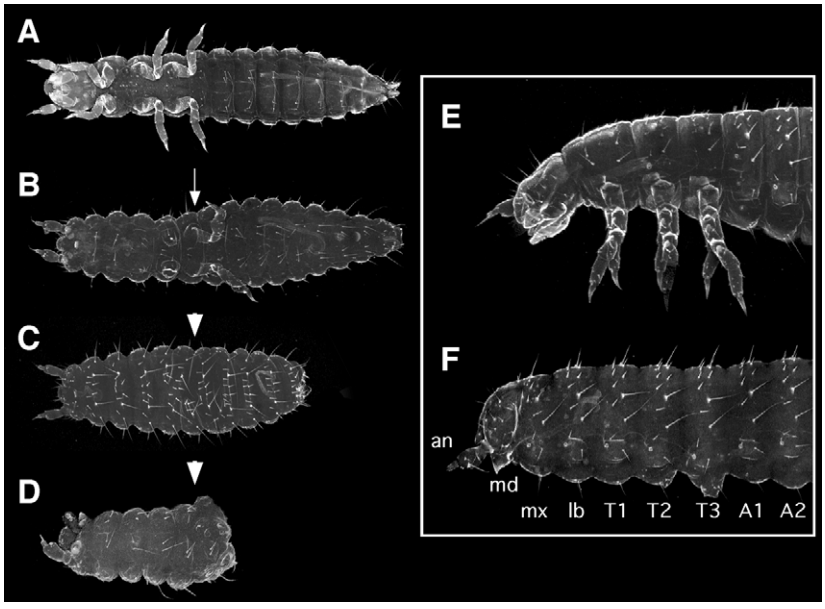

Fig. 1. *Tribolium hunchback* phenotypic series. Cuticular preparation of wild-type larvae (**A**,E) and *hunchback*-depleted larvae (B-D,F). (**B**) All body segments are formed but gnathal and thoracic segments are transformed to abdominal identity. The thoracic segments appear to be partially transformed, showing underdeveloped limbs (arrow). (**C**) Larva displaying approximately 10 segments with abdominal identity and fusion of segments (arrowhead). (**D**) Larva displaying the strongest phenotype. Antennae and mandible are still formed and the segments following these have abdominal identity up to the fusion point (arrowhead), after which no further segments are seen. (**E**,**F**) Comparison of the anterior region of a wild-type larva (E) and a larva with a weak phenotype (F). The latter shows a normal antenna (an) and mandibular (md) segment, while the prospective remaining segments (mx, maxilla; lb, labium; T1-T3, thoracic; A1, A2, abdominal) are transformed into segments of abdominal identity. Some leg stumps are still visible in T2 and T3, indicating that the transformation towards abdominal segments was not complete in these segments.

Regulation of Hox genes

The transformation of anterior segment identity into posterior segment identity suggests an ectopic expression of posteriorly acting Hox genes in *Tc'hb*^{pRNAi} embryos. In order to test this, we have compared the wild-type expression of the gnathal Hox genes, *Tc'Dfd* and *Tc'Scr*, as well as the trunk Hox genes *Tc'Antp*, *Tc'Ubx* and *Tc'AbdA* with their expression in *Tc'hb*^{pRNAi} embryos (Fig. 2). To assess the segmental register in these embryos, we have used the segment polarity gene *gooseberry* (*Tc'gsb*) as segmental marker. *Tc'gsb* was chosen instead of *engrailed* because its expression precedes the expression of other segment polarity genes (Savard et al., 2006a).

In wild-type embryos, *Tc'Dfd* is expressed in the mandibular and maxillary segment (Fig. 2A), followed by the expression of *Tc'Scr* in the labium (Fig. 2C). In *Tc'hb*^{pRNAi} embryos, we see a narrower expression domain of *Tc'Dfd* (Fig. 2B), while *Tc'Scr* is absent (Fig. 2D). This is in line with the observed cuticle phenotypes. *Tc'Dfd* is required for mandible specification in *Tribolium* and also partly for the maxilla (Brown et al., 2000). Given that the mandible is not affected in *Tc'hb*^{pRNAi} embryos, *Tc'Dfd* expression was expected to be retained in this segment. *Tc'Scr* is required for the labial segment (DeCamillis et al., 2001), but given that this is transformed into an abdominal segment, its absence is in line with the phenotype.

The three trunk Hox genes *Tc'Antp*, *Tc'Ubx* and *Tc'AbdA* are all expanded towards anterior in *Tc'hb*^{pRNAi} embryos, starting to be expressed from the mandibular segment onwards (Fig. 2E-J). Each of these genes has specific functions in specifying thoracic and anterior abdominal segments, in agreement with their specific anterior expression borders (Lewis et al., 2000). However, in the abdominal segments, they are all co-expressed in wild-type embryos (Fig. 2E,G,I) and they are likely to have a joint function in specifying abdominal segment identity. Accordingly, the fact that all three are co-expressed from the mandibular segment onwards in *Tc'hb*^{pRNAi} embryos is in line with the observation of the transformations into segments of abdominal identity (Fig. 1).

We can conclude from these observations that *Tc'hb* is required for the regulation of at least four Hox genes along the anteroposterior axis, although some of these regulatory effects may be indirect (see Discussion).

Regulation of gap genes

To understand the basis of the segment deletions observed in *Tc'hb*^{pRNAi} embryos, we have analysed *Tc'Kr* and *Tc'gt* as possible target genes. In *Drosophila*, the *hunchback* gradient is required to regulate other gap genes, in particular *Dm'Kr*, *Dm'kni* and *Dm'gt* (Hülskamp et al., 1990; Struhl et al., 1992). The homologues of *Krüppel* and *giant* have been functionally studied in *Tribolium* (Bucher and Klingler, 2004; Cerny et al., 2005) and we have therefore focused on these in the following.

Tc'Kr expression starts already at the blastoderm stage with a broad domain at the posterior end (Fig. 3A) (Sommer and Tautz, 1993), which covers the three thoracic segment primordia in the early germband (Fig. 3C) (Cerny et al., 2005). In *Tc'hb*^{pRNAi} embryos, this domain is strongly reduced or even absent (Fig. 3B,D), indicating that *Tc'hb* is required for its activation. There is also a segmental expression of *Tc'Kr*, which appears during segment differentiation (Fig. 3E) (Cerny et al., 2005). This expression is not affected in *Tc'hb*^{pRNAi} embryos, although fewer segmental stripes are generated (Fig. 3F), in line with the loss of segments in such embryos. Thus, *Tc'hb* is required for the activation of the early *Tc'Kr* domain. This is also an essential function of *hunchback* in *Drosophila* (Hülskamp et al., 1990; Struhl et al., 1992; Schulz and Tautz, 1994), i.e. this regulatory interaction appears to be conserved.

Tc'gt is initially expressed in a broad domain during blastoderm stage covering the future head and gnathal segments, but excluding the labium (Bucher and Klingler, 2004). The trunk expression appears during germband elongation (Fig. 4A) and converges to two stripes over the third thoracic and second abdominal segments (Fig. 4B) (Bucher and Klingler, 2004). In *Tc'hb*^{pRNAi} embryos, the anterior domain is not visibly affected (Fig. 4C). The expression of the trunk stripes, however, is lost (compare Fig. 4B,D). With further development, it becomes apparent that the segments that should have expressed *Tc'gt* are partially fused, as monitored by the *Tc'gsb* expression (Fig. 4D). No further segments are produced beyond this point, at least in strong phenotypes. These results suggest that *Tc'hb* acts formally as an activator of *Tc'gt*, which would be different from its role in *Drosophila*, where it acts as a repressor (Struhl et al., 1992).

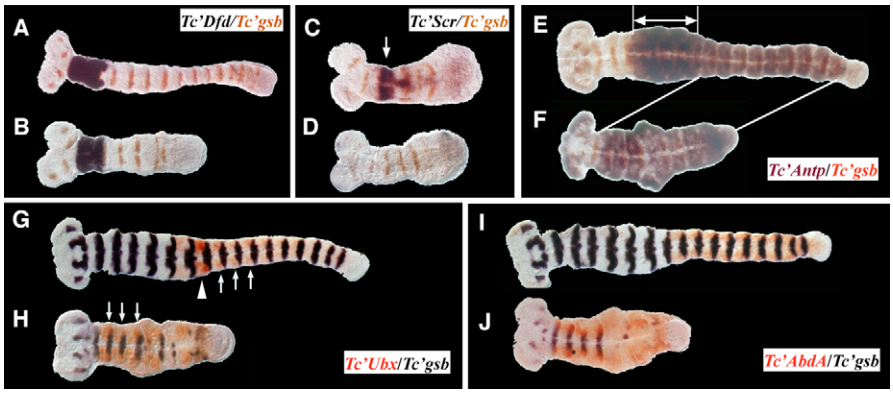

Fig. 2. Changes in Hox gene expression in *Tc'hb*^{pRNAi} embryos. Wild-type expression is shown in A,C,E,G,I; expression in loss of *hunchback* embryos (strongest phenotypes) is shown in B,D,F,H,J. All embryos are double stained with a *Tc'gsb* probe, which serves as a segmental reference marker. *Tc'gsb* staining is brown in A-F and dark purple in G-J. Hox gene staining is the reverse. (**A**,**B**) *Tc'Dfd* expression extends over the mandibular and maxillary segment in wild type (A) and is lost from the maxillary segment in *Tc'hb*^{pRNAi} embryos (B). (**C**,**D**) *Tc'Scr* expression covers the labial segment (arrow) in wild type (C) and is lost in *Tc'hb*^{pRNAi} embryos (D). (**E**,**F**) *Tc'Antp* expression covers the thoracic and abdominal region in wild type (E) with a stronger expression in the thoracic segment (double-headed arrow). In *Tc'hb*^{pRNAi} embryos, *Tc'Antp* expression is shifted towards anterior up to the mandibular segment, but its intensity is comparable with the wild-type expression of *Tc'Antp* in the abdominal segments (F). (**G**,**H**) *Tc'Ubx* expression starts in the third thoracic segment and extends throughout the abdominal region in wild-type embryos (G), with a stronger expression in the first abdominal segment (arrowhead). In *Tc'hb*^{pRNAi} embryos, *Tc'Ubx* expression is shifted towards anterior up to the mandibular segment (H), but its intensity is comparable with the wild-type expression of *Tc'Ubx* in the abdominal segments (arrows in G,H). (**I**,**J**) *Tc'Antp* expression starts in the second abdominal segment and extends throughout the abdominal region in wild-type embryos (I). In *Tc'hb*^{pRNAi} embryos, *Tc'Antp* expression is shifted towards anterior up to the mandibular segment (J).

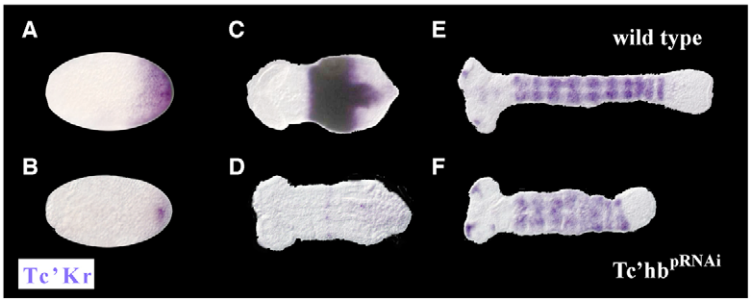

Fig. 3. Expression of *Tc'Kr*. Wild type (A,C,E) and *Tc'hb*^{pRNAi} embryos (B,D,F). (**A**,**B**) Blastoderm stage embryos. *Tc'Kr* is expressed at the posterior end in wild-type embryos (A) and this expression is strongly reduced (and often absent) in *Tc'hb*^{pRNAi} embryos (B). (**C**,**D**) Early germband stages. *Tc'Kr* is expressed in a central domain in wild-type embryos (C) and this expression is absent in *Tc'hb*^{pRNAi} embryos (D). (**E**,**F**) Extended germband stages. *Tc'Kr* is secondarily expressed in segmental stripes in wild-type embryos (E), an expression aspect that is not changed in *Tc'hb*^{pRNAi} embryos (F), but these develop fewer segments (compare with Fig. 1).

Regulation by gap genes

As cross-regulatory interactions among gap genes are known in *Drosophila*, we have also analyzed the effects of *Tc'Kr* and *Tc'gt* on the expression of *Tc'hb*.

Tc'hb is initially maternally expressed throughout the whole zygote and early embryo. At blastoderm stage, it forms an anterior cap in the extra-embryonic serosa and a domain in the prospective head region, which covers the head segments up to the labium (Wolff et al., 1995). This domain becomes weaker in the early germband (Fig. 5A). At later stages, *Tc'hb* forms a strong expression domain in the growth zone, remaining there until the end of segmentation (Fig. 5D). Finally, there is weak expression in segmental stripes (Fig. 5D).

In *Tc'Kr*^{pRNAi} embryos, the blastodermal *Tc'hb* expression domains appear not to be strongly affected, although it is possible that the head domain is extended towards the posterior pole. Given that this domain develops very dynamically, it is not possible to show this unequivocally. A major effect is seen from early germband stages onwards. The posterior domain develops much earlier and is expressed much more strongly. Its anterior boundary is initially within the maxillary segment (Fig. 5B) and overlapping the normal head domain. This boundary recesses at later stages and the domain is broadly confined to the growth zone (Fig. 5E). Thus, *Tc'Kr* acts formally as a repressor on the posterior domain, a role that is not known from *Drosophila*.

In *Tc'gt*^{pRNAi} embryos, *Tc'hb* expression is not significantly changed. There are no visible effects on the anterior domains (Fig. 5C). The posterior expression domain in the growth zone is present, but appears to be activated earlier and in a smaller area (Fig. 5F). This effect could be caused by the segment deletions observed in these embryos (arrowhead in Fig. 5F). In *Drosophila*, *giant* has a role in regulating secondary anterior *Dm'hb* expression domains, but has no role for the posterior *Dm'hb* domain (Wu et al., 1998).

Effect on pair-rule genes

Gap genes in *Drosophila* are directly required for regulating the primary pair-rule stripes. Accordingly, pair-rule stripe formation is disrupted in the area of the expression of the respective gap gene. In *Tribolium* it seems possible that the pair-rule pattern is set up only via interactions among the pair-rule genes themselves, whereby *runt* and *even-skipped* have essential functions (Choe et al., 2006). We have therefore analyzed the expression of these genes in *Tc'hb*^{pRNAi} embryos (Fig. 6). In wild type, the first *Tc'runt* stripe appears in the maxillary segment, the second in the first thoracic segment. The border of *hunchback* expression is within the labial segment, i.e. between the two stripes. Thus, if *hunchback* did have a direct effect on *Tc'runt* expression, one would expect to see the first two stripes to be affected. This does not appear to be the case. Stripe 1 and the distance to the second stripe are practically unchanged in *Tc'hb*^{pRNAi} embryos (Fig. 6A,B). Only the formation of the further stripes is disturbed. They form a large domain rather than separate stripes (Fig. 6C,D). At later stages, only a broad domain remains visible in the growth zone (Fig. 6E,F). The situation is comparable for *Tc'eve*, with the complication that the pattern is more dynamic. The first *Tc'eve* stripe overlaps the mandibular/maxillary segments and the second the labial/T1 segments. These then split into segmental stripes (Fig. 6G). In *Tc'hb*^{pRNAi} embryos, these first two stripes are almost normally formed and only subsequent stripes are less well resolved (Fig. 6G,H). An additional difference concerns the stability of the stripes. In *Tc'hb*^{pRNAi} embryos they disappear much faster than in wild type (Fig. 6I-M). Interestingly, however, separate stripes are still seen in the growth zone (Fig. 6L,M), suggesting that stripe patterning is less disrupted for *Tc'eve* than for *Tc'run*.

DISCUSSION

Studying the phenotypic series of *hunchback* effects in *Tribolium* reveals that the phenotype is not a simple loss of several adjacent anterior segments. Instead, it is a combination of transformed segments and loss of posterior segments. This does not fit the canonical definition of gap genes in *Drosophila*, but fits well with the effects seen for other gap genes in *Tribolium*, namely that they display a combination of transformation and segment loss phenotypes (Bucher and Klingler, 2004; Cerny et al., 2005; Savard et al., 2006a). Thus, regulation of Hox genes and segmentation genes are linked features for the *Tribolium* gap genes. In the following, we want to argue that this core role of *Tc'hb* can be reasonably well defined and that it is in fact not so much different between the different insects.

Regulation of Hox genes

The setting of Hox gene expression domains appears to be particularly sensitive to *hunchback* function and may be the key feature for understanding its role. Changes in Hox gene expression are also one of the hallmarks of the allelic series of *hunchback* phenotypes in *Drosophila*. In hypomorphic class III alleles (Lehmann and Nusslein-Volhard, 1987), *Dm'Ubx* is ectopically expressed in the thoracic segments (White and Lehmann, 1986). In the region where four metameres should have formed (corresponding to two thoracic and two abdominal segments), only two enlarged metameres spanning this entire region appear. Owing to a resizing process, which involves cell death, these two enlarged metameres approach wild type width later in development (White and Lehmann, 1986). Because of ectopic *Dm'Ubx* expression, they are specified as abdominal segments. Hence, the phenotype is characterized as a loss of T2 and T3 in the larvae, although the remaining resized metameres are composed of primordial cells of thoracic and abdominal segments.

Other *hunchback* alleles in *Drosophila* are directly characterized by homeotic transformations of anterior segments into abdominal identity (Lehmann and Nüsslein-Volhard, 1987; Hülskamp et al.,

Fig. 4. Expression of *Tc'gt*. Wild-type (A,B) and in *Tc'hb*^{pRNAi} embryos (C,D), at early (A,C) and late (B,D) germband stages. The embryos are double stained with a *Tc'gsb* probe (brown), which serves as a segmental reference marker. (**A**,**B**) *Tc'gt* (purple) is expressed in an anterior domain and two posterior stripes in wild-type embryos. (**C**,**D**) In *Tc'hb*^{pRNAi} embryos, the anterior domain is not significantly affected, but the posterior expression of *Tc'gt* in T3 and A2 (arrows in B) is absent in *Tc'hb*^{pRNAi} embryos. The segments where *Tc'gt* should have been expressed are partially fused in *Tc'hb*^{pRNAi} embryos, indicated by the partial fusion of the *Tc'gsb* stripes (arrowheads in D).

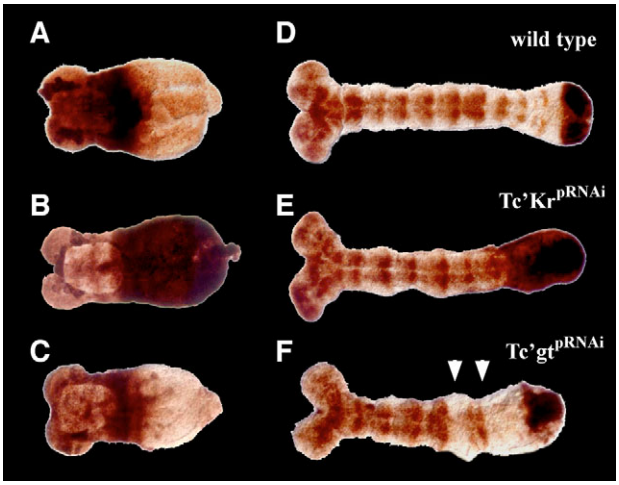

Fig. 5. Expression of *Tc'hb*. Wild type (A,D), *Tc'Kr*^{pRNAi} (B,E) and *Tc'gt*^{pRNAi} embryos (C,F). Early (**A-C**) and late (**D-F**) germband stages. In wild-type embryos, *Tc'hb* is expressed in an anterior domain at early germband stages (A) and in a segmental register and a posterior domain at later germband stages (D). In *Tc'Kr*^{pRNAi} embryos, *Tc'hb* is expressed ectopically in a strong domain posterior to the maxillary segment (B; this embryo is much less stained than the one in A, i.e. the anterior expression domain of *hunchback* shows up only weakly). At later stages, the posterior expression is restricted to the prospective growth zone (E), although it remains broader than in wild-type embryos (compare with D). In *Tc'gt*^{pRNAi} embryos, *Tc'hb* is not significantly affected (C,F), only a partial fusion of segments in the posterior region becomes visible (arrowheads in F).

1994) or act as dominant regulators of Hox genes (Bender et al., 1988). Some *Dm'Ubx* enhancers have been shown to bind HB protein, i.e. the regulatory interaction appears to be direct (Qian et al., 1991). Similar results were also obtained for *Dm'AbdA* regulation and enhancers (Casares and Sánchez-Herrero, 1995; Irish et al., 1989; Shimell et al., 2000) and there is evidence that *Dm'Scr*, *Dm'Antp* and *Dm'AbdB* are also regulated by *Dm'hb* (Casares and Sánchez-Herrero, 1995; Riley et al., 1987; Wu et al., 2001).

Our results suggest that *Tc'hb* acts formally as a repressor on *Tc'Antp*, *Tc'Ubx* and *Tc'AbdA*, as the expression domain of all three expands towards anterior in *Tc'hb*^{pRNAi} embryos, and formally as an activator of *Tc'Scr*, as its expression is lost in *Tc'hb*^{pRNAi} embryos. It seems clear, however, that some of these effects are indirect (Fig. 7). The expansion of the *Tc'Antp* domain in *Tc'hb*^{pRNAi} embryos may be the reason for the repression of *Tc'Scr*, as *Dm'Antp* is known to have an epistatic effect (posterior prevalence) on *Dm'Scr* in *Drosophila* (Carroll et al., 1988; Pelaz et al., 1993).

In *Drosophila*, *hunchback* does not act as a repressor on *Dm'Antp*. Instead, the secondary blastoderm expression of *Dm'hb*, the PS4 stripe expression, acts as an activator of *Dm'Antp* in this domain (Wu et al., 2001). An equivalent of the PS4 stripe expression is missing in *Tribolium* (Wolff et al., 1995) and a conserved regulatory interaction cannot be expected for this aspect. Thus, the repression effect of *Tc'hb* on *Tc'Antp* is not a conserved feature, at least not in *Drosophila*.

A possible direct repressor function of *Tc'hb* on *Tc'Ubx* is not obvious, as *Tc'hb* expression does not visibly reach to the anterior border of the *Tc'Ubx* trunk expression domain. However, the regulatory effect might be mediated via epigenetic regulation. It was proposed that *Dm'hb* initiates the formation of a silencing complex and that another protein, apparently dMi-2 (Kehle et al., 1998), takes over the role of Dm'HB protein when Dm'HB levels start to decline. In *Tribolium*, this mechanism would imply that only a few cells in the growth zone, which show no HB protein expression (Wolff et al., 1995), might retain the capacity to express *Tc'Ubx*, even though the actual transcription may start later.

Regulation between gap genes

The second consistent feature of *Tc'hb* function is the interaction with other gap genes, most notably *Tc'Kr* (Fig. 7A). In *Drosophila*, *Krüppel* is regulated by many other genes (Gaul et al., 1987), but the only activators that were identified are *Dm'bcd* and *Dm'hb* (Hoch et al., 1992; Hülskamp et al., 1990; Struhl et al., 1992). *Dm'bcd* is a late addition in higher Dipterans (Stauber et al., 2002), so only *Dm'hb* is a candidate for a conserved positive regulator. Moreover, it has been shown that *Dm'hb* alone is capable of establishing a functional *Dm'Kr* expression domain (Schulz and Tautz, 1994). Hence, the finding that the *Tc'Kr* domain is dependent on *Tc'hb* is in line with the activation role of *hunchback* on *Krüppel* observed in *Drosophila*. Given that *Tc'Kr* expression starts already at blastoderm stage at the posterior pole, in the region where *Tc'hb* forms a short gradient, it would seem likely that this effect is direct, i.e. this may be another conserved feature of *hunchback* function.

The regulatory interaction with *giant*, however, is clearly not conserved. In *Drosophila*, *hunchback* is a strong repressor of *giant*, i.e. the anterior expression border of the posterior domain is set by a low concentration of the HB protein gradient at blastoderm stage (Struhl et al., 1992). By contrast, in *Tribolium*, we find formally an activating effect of *hunchback* on *giant*. However, at the time where *Tc'gt* becomes expressed in the trunk, there is no contact to the *Tc'hb* domain, i.e. this effect is likely to be indirect. *Tc'Kr* cannot be the mediator of this effect, as loss of *Tc'Kr* alone does not lead to a complete loss of the trunk *Tc'gt* stripes (Cerny et al., 2005). Instead, the effect may be caused by a combination of *Tc'Kr* and *Tc'mlpt*. *Tc'mlpt* expression in the trunk is strongly reduced in *Tc'hb*^{pRNAi} embryos and *Tc'gt* expression is lost in *Tc'mlpt*^{pRNAi} embryos

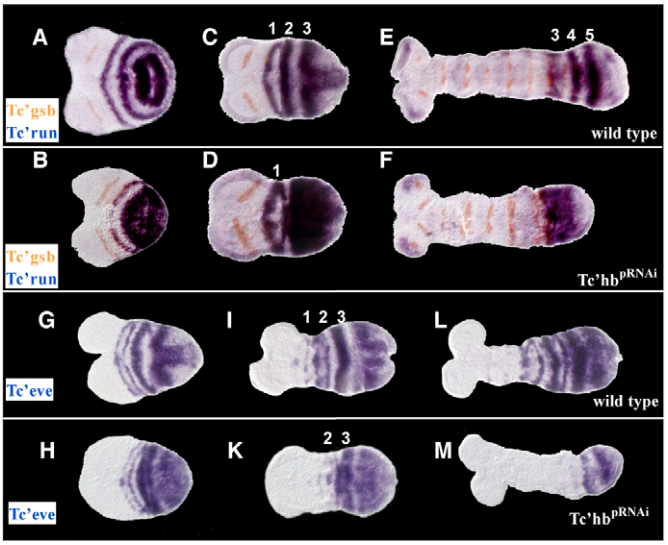

Fig. 6. Expression of *Tc'runt*. Double staining with *Tc'gsb* (**A-F**) (brown) and *Tc'eve* (**G-M**) in wild type and in *Tc'hb*^{pRNAi} embryos. It is evident that the first stripes are not affected in *Tc'hb*^{pRNAi} embryos, while the patterning beyond the second stripe appears to be disrupted for both genes. *Tc'eve* expression is very dynamic, i.e. the first stripe disappears fast and the primary stripes split directly into two secondary stripes.

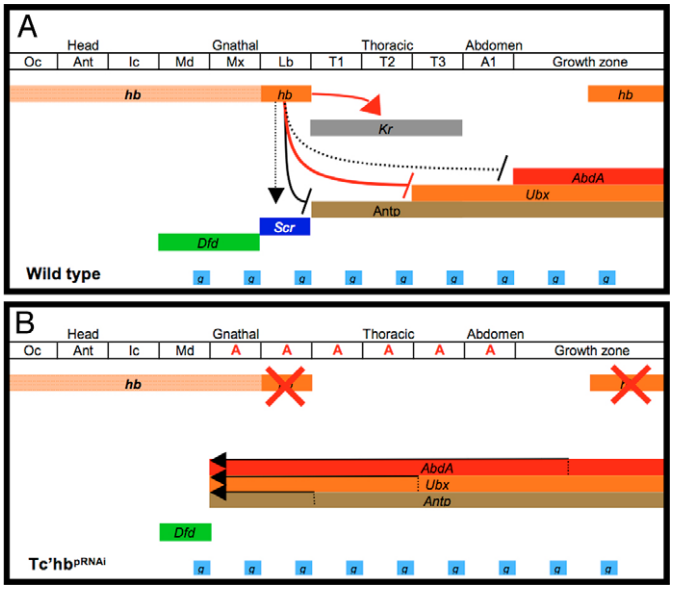

Fig. 7. Schematic drawing of the effects of *hunchback* on the expression of the target genes in *Tribolium*. (**A**) Wild type. (**B**) *Tc'hb*^{pRNAi}. Arrows indicate activation and bars indicate repression. Unbroken lines indicate a possible direct interaction and broken lines indicate a probable indirect effect. The red lines indicate the actions of *hunchback* that appear to be conserved in all insects.

(Savard et al., 2006a). This combined loss of *Tc'Kr* and *Tc'mlpt* in *Tc'hb*^{pRNAi} embryos may account for the loss of *Tc'gt* expression in the trunk. Thus, we can conclude that the apparent direct interaction between *hunchback* and *giant* in *Drosophila* is not a conserved feature of *hunchback* function, but has probably been acquired in the lineage towards the higher Diptera.

hunchback in other insects

Liu and Kaufman (Liu and Kaufman, 2004) have studied *hunchback* function in the intermediate germband insect *Oncopeltus* (*Of'hb*) using also a parental RNAi approach. Their phenotypic series is almost identical to the series we have found for *Tribolium*. Only the most extreme phenotype is stronger in *Tribolium*. They find also misexpression of Hox genes and conclude that *Of'hb* has the same dual role that we find for *Tribolium*. Hence, *hunchback* functions in *Oncopeltus* and *Tribolium* are likely to be very similar, possibly even at the level of the interactions with the other gap genes. For example, the segmental defects seen in the abdomen of intermediate strength *Of'hb*^{pRNAi} phenotypes appear to correspond to the segments that are also affected in *Tribolium* and which may be related to the loss of the two stripes of trunk *giant* expression.

Mito et al. (Mito et al., 2005) have studied *hunchback* function in *Gryllus* (*Gb'hb*). Although they suggest that there are distinctly different functions for *hunchback* in this species, it seems that the core findings are nonetheless very much in line with our results in *Tribolium* and the results in *Oncopeltus*. Again, ectopic expression of Hox genes is the first effect seen in weak *Gb'hb*^{pRNAi} phenotypes, accompanied with signs of transformation of thoracic segments. Stronger *Gb'hb*^{pRNAi} phenotypes show a progressive loss of abdominal segments. The most severe phenotypes described by these authors are not as strong as those found for *Tribolium* or *Oncopeltus*, but it is naturally difficult to ensure that the parental RNAi effect is fully penetrant. These authors have also studied expression of *Gb'Scr* and *Gb'Kr* and find, similar to our results in *Tribolium*, that expression is severely reduced or even absent in *Gb'hb*^{pRNAi} embryos.

He et al. (He et al., 2006) have shown parental RNAi phenotypes for *hunchback* in *Locusta* (*Lm'hb*^{pRNAi}) and conclude that some of these appear to be different from those found for *Tribolium*, *Oncopeltus* and *Gryllus*. In the weakest *Lm'hb*^{pRNAi} phenotype, they find only abdominal effects, but no anterior transformation effects, suggesting that the Hox gene misregulation effect is not as sensitive as in the other species. However, we interpret their most frequent *Lm'hb*^{pRNAi} phenotype (class II) as embryos where the head and thoracic segments are transformed into abdominal segments and where segmentation stops after this. This would be comparable to the strongest phenotypes in *Tribolium*, *Oncopeltus* and *Gryllus*, although even fewer segments appear to be produced in *Locusta*, possibly because the germ anlage is so extremely short in this species. The even stronger *Lm'hb*^{pRNAi} phenotypes observed by these authors (class III) appear to be related to a separate function of *hunchback* in the extra-embryonic membrane (He et al., 2006).

Pultz et al. (Pultz et al., 2005) found that the headless mutant in *Nasonia* is an apparent null allele of *hunchback* (*Nv'hb*). They find also misregulation of Hox genes in *Nv'hb* mutant embryos but the phenotype is not easily comparable with the ones found for *Tribolium*, *Gryllus* or *Oncopeltus*. Instead, the *Nv'hb* mutant phenotype mimics the *Drosophila* phenotype in showing a large deletion of anterior segments, as well as loss of posterior abdominal segments (Pultz et al., 1999). However, as hypomorphic *Nv'hb* alleles are not available, it is difficult to assess whether these would show a homeotic transformation, as we see it in *Drosophila*. The more extensive loss of head segments in *Nv'hb* mutant embryos may be explained by the fact that *bicoid* is partially redundant with

hunchback function in *Drosophila* (Hülskamp et al., 1990), i.e. might rescue some of the phenotypic effects. As there is no *bicoid* in *Nasonia*, this effect would not occur.

The role of hunchback in segmentation

Bucher and Klingler (Bucher and Klingler, 2004), Cerny et al. (Cerny et al., 2005) and Choe et al. (Choe et al., 2006) have suggested that the action of the pair-rule genes may not be as strongly coupled to the gap genes in *Tribolium* as it is known in *Drosophila*. Choe et al. (Choe et al., 2006) have even suggested that the segmentation process may largely be controlled by interactions among the pair-rule genes themselves. This inference is also supported by the analysis of *Tc'hb* function. If *Tc'hb* were directly involved in setting segmental boundaries, one would expect that the major phenotypic effect would occur in or around the domain where it is expressed. However, the first two pair-rule stripes of *Tc'runt* appear to form more or less normally in *Tc'hb*^{pRNAi} embryos and disruption of the patterning is seen only for the subsequent stripes. This is in line with the observation that four segments are still formed in the most extreme *Tc'hb*^{pRNAi} phenotypes (Fig. 1D), although they are transformed into abdominal identity. A similar pattern is seen for *Tc'eve*, although this is more complex owing to the fast splitting of the primary stripes and the fast disappearance of the anterior stripes in *Tc'hb*^{pRNAi} embryos. Thus, there is no indication that *Tc'hb* is directly involved in regulating the anterior pair-rule stripes. However, it is evident that the regulation of the Hox genes and the setting of segmental boundaries have to be coupled by some mechanism, but it is still not known how this is achieved.

The conserved core elements of hunchback function

Short (or intermediate) germ embryogenesis is the ancestral form of embryogenesis in insects (Tautz et al., 1994). The *hunchback* function found in these types of embryos should be taken as a reference when considering conserved and diverged features. Interestingly, most details of *hunchback* function are fully comparable between *Tribolium*, *Oncopeltus* and *Gryllus*, although these insects belong to different orders that have a longer evolutionary separation time than, for example, beetles and flies (Savard et al., 2006b).

The two key features of *hunchback* function are clearly the regulation of *Ultrabithorax*, as well as the activation of *Krüppel* (Fig. 7A). These features are well documented in *Drosophila* and it seems now clear that they are ancestral. By contrast, the effect on *Antp*, *Scr* and *giant* appear to be partially indirect and partially not conserved, at least not with respect to the exact type of interaction.

Most intriguingly, the name-giving 'gap' function does not belong to the conserved core elements of *hunchback* function, but appears to have evolved independently in *Drosophila* and *Nasonia* (e.g. long germ embryos). The term 'gap gene' is therefore not appropriate for the *hunchback* function in most insects and appears also not appropriate for *Krüppel* and *giant* in *Tribolium* (Bucher and Klingler, 2004; Cerny et al., 2005). Thus, one could consider to revive another name that has been used to describe the gap genes, namely 'cardinal genes'. Meinhardt (Meinhardt, 1986) has proposed this name in the context of his segmentation model for *Drosophila*. He proposed the existence of 'cardinal regions' that would be set up by maternal gradients and the genes expressed in these regions would be required for regulating pair-rule expression. Interestingly, he concluded that this mechanism would only be required for long germ embryos, because the sequential segment formation in short germ embryos could be achieved by pair-rule gene interactions alone. However, Akam (Akam, 1987) has then pointed out that gap genes regulate both segmentation genes and Hox genes in broader domains and that the term 'cardinal genes' should reflect both of these aspects. Given that the Hox gene regulation appears to be the more conserved function of gap genes, it would indeed seem appropriate to adopt the term 'cardinal genes' for this gene class, at least for other insects.

H.M. was a fellow of the International Graduate School of Genetics and Functional Genomics in Cologne. In addition, this work was supported by the SFB 572 and SFB 680.

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