Separable stripe enhancer elements for the pair-rule gene *hairy* in the beetle *Tribolium*

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Pair-rule genes in *Drosophila* integrate the positional information provided by the transcription factor gradients of gap genes and set the framework for the expression of the segment polarity genes. Pair-rule genes are also expressed during the segmentation of the short germ-band embryo of the flour beetle *Tribolium*, although this occurs under cellular conditions that should not allow the generation of transcription factor gradients by passive diffusion. To analyse the regulation of the pair-rule gene *hairy* in *Tribolium*, we have used germline transformation with reporter gene constructs based on the *piggyBac* vector. We can identify an upstream fragment that drives the full expression of the eight pair-rule stripes, which are sequentially generated during embryogenesis. Further experiments with smaller fragments reveal separable regions driving stripes three to five, and one region driving stripe three only. Our results suggest that the generation of pair-rule stripes in *Tribolium* is comparable to that in *Drosophila* despite the cellular versus syncytial mode of embryogenesis.

Keywords: segmentation; short germ-band development; gap genes; insects; germline transformation; reporter gene constructs

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INTRODUCTION

The molecular mechanism of segmentation has been particularly well studied in *Drosophila*. However, the *Drosophila* mode of segmentation must be considered as highly derived. *Drosophila* embryogenesis is characterized by a syncytial stage, during which the anlagen of all segments are generated. The early steps of segmentation are directly driven by transcription factors that form diffusion-controlled gradients within the syncytial blastoderm (for review see Pankratz & Jäckle, 1993). In contrast to *Drosophila*, segmentation in most other animals is achieved through a sequential process, in which new segments are generated in a growth zone at the posterior end of the embryo. In vertebrates, this involves cell–cell communication based on the Delta–Notch

signalling pathway (Pourquie, 2003) and Wnt signalling (Aulehla *et al*, 2003). These processes generate waves of cyclic expression of target genes that are required for generating the segmental (or somitic) borders.

Most arthropods also show a sequential formation of segments, which is at least superficially reminiscent of the vertebrate mode of segmentation. Intriguingly, in the spider *Cupiennius*, it was shown that the Delta–Notch signalling pathway also has a role in segmentation (Stollewerk *et al*, 2003), although the molecular basis of segment border formation is clearly homologous to that in *Drosophila* (Damen, 2002).

Among insects, there are essentially two separate modes of embryogenesis, the long germ-band mode as in *Drosophila* and the short germ-band mode that is found in the more basal representatives, such as beetles and grasshoppers (Davis & Patel, 2002). In short germ-band embryos, only a germlage with a few anterior segments is generated during a syncytial blastoderm stage, whereas the remainder of the segments grow from a posterior growth zone during a subsequent cellular phase. Thus, one should expect that this secondary growth process should require cell–cell communication pathways to generate the segments. Conversely, most zygotic segmentation genes from *Drosophila* are expressed in a similar way to that in *Tribolium*, although with differences in detail. A functional analysis of segmentation genes in more basal insects should therefore reveal how far the molecular mechanism of segmentation is conserved in insects and how these mechanisms relate to the vertebrate mode of segmentation.

We have focused here on the regulation of the pair-rule gene *hairy* in the beetle *Tribolium*, as the question of pair-rule patterning is of particular importance for understanding the segmentation gene network (Tautz & Sommer, 1995; Davis & Patel, 2002). In *Drosophila*, it has been shown for the primary pair-rule genes *hairy*, *even-skipped* and *runt* that individual stripes are regulated by separate enhancer elements that respond directly to the gap gene transcription factor gradients (Harding *et al*, 1989; Pankratz *et al*, 1990; Riddihough & Ish-Horowicz, 1991; Klingler *et al*, 1996). Thus, their regulation seems to be particularly specific for a long germ-band mode of embryogenesis, where the transcription factors can freely diffuse between nuclei. *Tribolium* embryos are typical representatives of the short germ-band mode of embryogenesis (Tautz *et al*, 1994; Davis & Patel, 2002) where the secondary growth process occurs under cellular conditions.

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Still, homologues of the *Drosophila* pair-rule genes are expressed in a pair-rule pattern in *Tribolium* (Sommer & Tautz, 1993; Brown et al, 1994; Patel et al, 1994; Davis et al, 2001) and there is also functional evidence for a transient pair-rule organization in the segmentation process of *Tribolium* (Schröder et al, 1996; Maderspacher et al, 1998).

Tribolium is a genetically tractable system (Beeman et al, 1989; Berghammer et al, 1999a), and germline transformation technology has been developed (Berghammer et al, 1999b; Lorenzen et al, 2003). These tools permit the study of the regulation of *Tribolium* segmentation genes in the same way as in *Drosophila*. Most notably, enhancer-reporter gene constructs can be used to ask whether stripe-specific enhancer elements can also be detected in *Tribolium*. We present here the first results of this approach, which indicates that there are indeed separable stripe-specific enhancer regions upstream of the *hairy* gene in *Tribolium*.

RESULTS

In wild-type embryos of *Tribolium*, *hairy* is initially expressed in two sequentially generated blastodermal stripes and a blastodermal posterior cap (Fig 1A–E), which can be interpreted as the forming stripes #1–#3. The following stripes #4–#8 appear one by one in the growth zone (Fig 1F–J). Shortly after the formation of the stripes in the growth zone, they split into segmental expression patterns, which persist for some time (Fig 1G–J), a feature that is not seen for *hairy* in *Drosophila*. In addition, *Tribolium hairy* shows an expression in the midline (Fig 1G–I), which is also not known from *Drosophila*. Sectioning of the embryos suggests that these latter expression features are likely to be relevant for neurogenesis in *Tribolium* (not shown).

To study the enhancer elements of *hairy* in *Tribolium*, we made use of the general insect transformation vector that is based on the *piggyBac* transposon (Berghammer et al, 1999b; Horn & Wimmer, 2000; Lorenzen et al, 2003). This allowed us to obtain germline transformants for a number of different reporter gene constructs (Fig 2) and thus to test the regulatory capacity of different upstream regions of *hairy* in *Tribolium*.

In a first series of constructs, we used progressive 5'-deletions of the *Tribolium hairy* upstream region fused to a *lacZ* coding region (Fig 2). Hence, these constructs include the endogenous transcriptional start site. We find that an 8.8 kb upstream fragment is sufficient to drive the expression of all eight *hairy* stripes (Fig 3). Stripe formation at blastoderm as well as during the growth process mimics the formation of the endogenous stripes. Thus, all necessary control elements for stripe formation are included in this region. Conversely, the construct does not include the enhancer for the wild-type midline expression (compare Fig 3D–F with Fig 1G–I).

Two progressively shorter constructs with 4.9 and 3.1 kb upstream sequences drive a subset of the eight stripes. Stripes #1 and #2 are now absent at the blastoderm stage (Fig 4A), but become weakly visible in the germlage during the early elongation process (Fig 4C). Stripes #3–#5 form normally during germ-band growth (Fig 4C), whereas the more posterior stripes #6–#8 are absent (Fig 4D). However, stripes #3 and #4 persist longer than expected (compare Fig 4D with Fig 3E) and form a broader domain. A construct with only 1.5 kb upstream sequences showed no expression.

These results prove that the *hairy* regulatory region in *Tribolium* has a modular organization. The main regulatory elements for

Fig 1 | Wild-type expression of *hairy* in *Tribolium*. Developmental series of progressively older embryos, showing the successive development of the pair-rule stripes of *hairy*. (A–C) Blastoderm stage embryos. (D,E) Early gastrulation stage. (F–J) Germ-band stages, prepared out of the egg and flattened. The forming stripes are numbered in (F–J). In contrast to *Drosophila*, which has only seven stripes, *Tribolium* develops an eighth stripe, because it forms two additional abdominal segments during embryogenesis, which later become fused. The stripes resolve into segmental expression patterns, which change dynamically during further development. Segment identities in (J): Md, mandible; Mx, maxilla; Lb, labrum; T1–T3, thoracic segments; A1ff, abdominal segments.

stripes #1 and #2 can be mapped to the region between -8.8 and -3.1 kb. The same region must also contain the elements for stripes #6–#8, whereas essential enhancers for stripes #3–#5 appear to be located in the region between -3.1 and -1.5 kb.

We then specifically tested the region that should encompass the regulatory elements for stripes #3–#5. For this, we used another type of construct that contains a basal *Drosophila* heat-shock gene transcriptional start site in front of the *lacZ* coding

Fig 2 | Scheme of the constructs used for the germline transformation experiments. The upper line depicts the genomic region of *hairy*, with the boxes indicating the exons. The arrow indicates the transcriptional start site, as inferred from the longest cDNA available. In the progressive deletion constructs, the open reading frame of the bacterial *lacZ* gene with the SV40 3'UTR was cloned into the start codon of the *hairy* gene, thus including the endogenous promoter. In the 3.1 kb construct only part of *lacZ* gene was used, whereas the rest comprises the endogenous gene sequences (see Methods). For the three smaller subconstructs, we used a *Drosophila* heat-shock promoter (P) as the basal promoter. The broken lines at the bottom indicate the approximate positions of the inferred enhancer elements. Note that these assignments are in part based only on negative evidence.

region. A 1.9 kb fragment encompassing positions -3.1 to -1.2 kb was directly cloned in front of the basal transcriptional start site. Germline transformation with this fragment indeed yielded an expression in the region where the normal stripes #3–#5 are expected. However, the resolution of the stripes is not as clear as for the -3.1 kb construct alone (compare Fig 5A with Fig 4C). Conversely, similarly to the -3.1 kb construct, they persist at later stages (compare Fig 5B with Fig 4D). Expression in the region of stripe #5 is only very weak (Fig 5B).

These results indicate that the 1.9 kb fragment between -3.1 and -1.2 kb is lacking important co-elements for appropriate stripe resolution, as well as for full expression of stripe #5. It thus seems likely that the upstream region between -1.2 and 0 kb contains these missing elements, although it did not show an activity on its own. Still, the enhancers for the region-specific expression are clearly present in the 1.9 kb fragment.

A 1.1 kb fragment between -3.1 and -2.0 kb showed the same pattern as the 1.9 kb fragment (not shown). An even smaller 0.8 kb fragment between -3.1 and -2.3 kb showed a subset of the pattern, namely an expression that starts in a similar way to the larger fragments (Fig 5D) but becomes confined to the stripe #3 region (Fig 5E).

DISCUSSION

Our deletion analysis of the regulatory region of *hairy* in *Tribolium* shows that one can separate the elements responsible for the generation of stripes #1, #2 and #6–#8 from those driving stripes #3–#5. However, a further separate analysis of the fragment that should contain the elements for stripes #3–#5 showed only a partial activity for those stripes. This indicates that the regulatory parts for these stripes are dispersed over a larger region than deletion analysis alone indicates. This type of disperse regulation is similarly found for the primary pair-rule gene *runt* in *Drosophila* (Klingler et al, 1996). Conversely, even with the partial activity for stripes #3–#5, it is possible to further separate an element that appears to be fairly specific for an expression within the stripe #3 region.

Fig 3 | Expression of the 8.8 kb *hairy-lacZ* reporter gene construct in *Tribolium*. (A) Blastoderm stage. (B–F) Successively older germ-band stages. The numbers indicate the respective stripe numbers.

Fig 4 | Expression of the 3.1 kb *hairy-lacZ* reporter gene construct in *Tribolium*. (A) Blastoderm stage. (B–D) Successively older germ-band stages. The numbers indicate the respective stripe numbers. Note that the 4.9 kb construct shows the same patterns.

Fig 5 | Expression of the subconstructs in *Tribolium* at germ-band stage. (A–C) 1.9 kb construct (the 1.1 kb construct yields essentially the same pattern). (D–F) 0.8 kb construct. The numbers indicate the respective stripe numbers.

A distinct regulation of individual stripes is typical for the primary pair-rule genes in *Drosophila*, and we can therefore infer that the *Tribolium* mode of regulation is more similar to the *Drosophila* mode than to the vertebrate mode. Conversely, at least the well-characterized individual stripe enhancers in *Drosophila* are all directly regulated by the transcription factor gradients of the gap genes. It seems unlikely that this could be achieved in the same way in *Tribolium*, as the cells in the growth zone are not syncytial and therefore would not allow the diffusion of transcription factors. Sequence comparisons of the *Tribolium* and *Drosophila* upstream regions of *hairy* also show no signs of conserved blocks. Therefore, it is possible that an additional cell–cell signalling step is required to initiate the specific stripe expression.

The *hairy* homologue *her1* in zebrafish is also expressed in stripes in the somitic growth zone (Müller et al, 1996). Using similar reporter gene constructs as in *Tribolium*, we could identify separable enhancer elements for *her1* as well (Gajewski et al, 2003). However, these separable enhancers do not relate

to specific stripes along the body axis but to different phases of the repetitive cyclic expression within the growth zone. This is therefore very different from the situation that we see for *Tribolium*.

SPECULATION

Our data imply that the generation of the pair-rule stripes is dependent on the gap genes. Indeed, most of the gap genes that are involved in generating the pair-rule stripes in *Drosophila* have also been found in *Tribolium* (Sommer & Tautz, 1993; Wolff et al, 1995; Schröder et al, 2000, 2003). However, because they are expressed in a cellular context, the generation of diffusion-controlled gradients as in *Drosophila* seems impossible. One would therefore have to propose that the *Tribolium* gap genes regulate first genes involved in cell–cell communication. This could include Delta–Notch signalling, Wnt signalling or other signalling pathways. The information from these signalling molecules would then have to be interpreted by other transcription factors, which would regulate the specific pair-rule stripes.

Thus one would have to postulate two additional tiers of regulation in *Tribolium*. Conversely, we have preliminary evidence that some of the *Tribolium* stripe regulatory elements are also active in *Drosophila*. Thus, either important genetic components of the regulation of the *Drosophila* pair-rule genes have been missed, or a direct control of pair-rule stripe regulation through transcription factor gradients also exists in *Tribolium*.

METHODS

Plasmid construction. The sequence positions refer to a 11.6 kb *XhoI* fragment of the genomic region of *Tribolium hairy* (accession no. AJ457831). The four deletion constructs depicted in Fig 2 encompass: 8.8 kb (1–8769 = start codon), 4.9 kb (3872–8769), 3.1 kb (5694–8769) and 1.5 kb (7257–8679). All constructs contain a new *NcoI* site at the start codon that was engineered from AGATGG to CCATGG. They were fused to a modified *lacZ*, with an *NcoI* site at the start codon and an SV40 3' untranslated region (UTR). In the 3.1 kb construct, the 3' end of *lacZ* and the SV40 3'UTR were replaced with a 2.8 kb (8855–11649) *Tribolium hairy* fragment including exon 1 partially, exons 2 and 3, both introns, 3'UTR and downstream sequences. The constructs were inserted into a unique *PstI* site of the *piggyBac* vector pB3xP3-eGFPaf or pB3xP3-eGFPafm (Horn & Wimmer, 2000).

The subconstructs for the #3–#5 elements were cloned in front of the general *Drosophila* heat-shock gene *hs43* promoter fused to *lacZ* with the *hs43* 3'UTR. This fragment was obtained from the pCaSpeR-*hs43-lacZ* cloning vector (accession no. X81643). The following fragments were used: positions 5694–7537 for the 1.8 kb construct, 5694–6806 for the 1.1 kb construct and 5694–6468 for the 0.8 kb construct. The constructs were inserted into a unique *AscI* site of the *piggyBac* vector pB3xP3-eGFPafm (Horn & Wimmer, 2000).

***piggyBac*-mediated transformation.** The transformants were generated by injecting 500 µg/ml *piggyBac* transformation vector and 375 µg/ml helper plasmid into *Tribolium* preblastoderm eggs. We used *pearl* mutant background (white eyes), to allow an easier detection of the transformants with *gfp* expression in the eyes. For the analysis of the deletion series, we analysed one transformant line in each case. For the subconstructs of the #3–#5 elements, up to 13 transformant lines were analysed for each, as they showed generally very weak expression.

Strain keeping and embryo staining. *Tribolium* strains were kept according to the procedure described by Berghammer *et al* (1999a). Fixation and whole-mount *in situ* hybridization were carried out essentially as described by Wolff *et al* (1995).

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REFERENCES

Aulehla A, Wehrle C, Brand-Saberi B, Kemler R, Gossler A, Kanzler B, Herrmann BG (2003) Wnt3a plays a major role in the segmentation clock controlling somitogenesis. *Dev Cell* **4**: 395–406
Beeman RW, Stuart JJ, Haas MS, Denell RE (1989) Genetic analysis of the homeotic gene complex (HOM-C) in the beetle *Tribolium castaneum*. *Dev Biol* **133**: 196–209

Berghammer A, Bucher G, Maderspacher F, Klingler M (1999a) A system to efficiently maintain embryonic lethal mutations in the flour beetle *Tribolium castaneum*. *Dev Genes Evol* **209**: 382–389
Berghammer AJ, Klingler M, Wimmer EA (1999b) A universal marker for transgenic insects. *Nature* **402**: 370–371
Brown SJ, Hilgenfeld RB, Denell RE (1994) The beetle *Tribolium castaneum* has a *fushi tarazu* homolog expressed in stripes during segmentation. *Proc Natl Acad Sci USA* **91**: 12922–12926
Damen WG (2002) Parasegmental organization of the spider embryo implies that the parasegment is an evolutionary conserved entity in arthropod embryogenesis. *Development* **129**: 1239–1250
Davis GK, Patel NH (2002) Short, long, and beyond: molecular and embryological approaches to insect segmentation. *Annu Rev Entomol* **47**: 669–699
Davis GK, Jaramillo CA, Patel NH (2001) Pax group III genes and the evolution of insect pair-rule patterning. *Development* **128**: 3445–3458
Gajewski M, Sieger D, Alt B, Leve C, Hans S, Wolff C, Rohr KB, Tautz D (2003) Anterior and posterior waves of cyclic *her1* gene expression are differentially regulated in the presomitic mesoderm of zebrafish. *Development* **130**: 4269–4278
Harding K, Hoey T, Warrior R, Levine M (1989) Autoregulatory and gap gene response elements of the *even-skipped* promoter of *Drosophila*. *EMBO J* **8**: 1205–1212
Horn C, Wimmer EA (2000) A versatile vector set for animal transgenesis. *Dev Genes Evol* **210**: 630–637
Klingler M, Soong J, Butler B, Gergen JP (1996) Disperse versus compact elements for the regulation of runt stripes in *Drosophila*. *Dev Biol* **177**: 73–84
Lorenzen MD, Berghammer AJ, Brown SJ, Denell RE, Klingler M, Beeman RW (2003) *piggyBac*-mediated germline transformation in the beetle *Tribolium castaneum*. *Insect Mol Biol* **12**: 433–440
Maderspacher F, Bucher G, Klingler M (1998) Pair-rule and gap gene mutants in the flour beetle *Tribolium castaneum*. *Dev Genes Evol* **208**: 558–568
Müller M, Weizsäcker E, Campos-Ortega JA (1996) Expression domains of a zebrafish homolog of the *Drosophila* pair-rule gene *hairy* correspond to the primordia of alternating somites. *Development* **122**: 2071–2078
Pankratz M, Jäckle H (1993) Blastoderm segmentation. In *Development of Drosophila melanogaster*. Bate M, Martinez-Arias A (eds) pp 467–516. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
Pankratz MJ, Seifert E, Gerwin N, Billi B, Nauber U, Jäckle H (1990) Gradients of *Krüppel* and *knirps* gene products direct pair-rule gene stripe patterning in the posterior region of the *Drosophila* embryo. *Cell* **61**: 309–317
Patel NH, Condrón BG, Zinn K (1994) Pair-rule expression patterns of *even-skipped* are found in both short- and long-germ beetles. *Nature* **367**: 429–434
Pourquie O (2003) The segmentation clock: converting embryonic time into spatial pattern. *Science* **301**: 328–330
Riddihough G, Ish-Horowicz D (1991) Individual stripe regulatory elements in the *Drosophila hairy* promoter respond to maternal, gap, and pair-rule genes. *Genes Dev* **5**: 840–854
Schröder R (2003) The genes *orthodenticle* and *hunchback* substitute for *bicoid* in the beetle *Tribolium*. *Nature* **422**: 621–625
Schröder R, Tautz D, Jay DG (1996) Phenocopies of even skipped in *Drosophila* produced by chromophore assisted laser inactivation (CALI). *Dev Genes Evol* **206**: 86–88
Schröder R, Eckert C, Wolff C, Tautz D (2000) Conserved and divergent aspects of terminal patterning in the beetle *Tribolium castaneum*. *Proc Natl Acad Sci USA* **97**: 6591–6596
Sommer RJ, Tautz D (1993) Involvement of an orthologue of the *Drosophila* pair-rule gene *hairy* in segment formation of the short germ-band embryo of *Tribolium* (Coleoptera). *Nature* **361**: 448–450
Stoltekerk A, Schoppmeier M, Damen WG (2003) Involvement of *Notch* and *Delta* genes in spider segmentation. *Nature* **423**: 863–865
Tautz D, Sommer R (1995) Evolution of segmentation genes in insects. *Trends Genet* **11**: 23–27
Tautz D, Friedrich M, Schröder R (1994) Insect embryogenesis—what is ancestral and what is derived? *Development* (Suppl 1994): 193–199
Wolff C, Sommer R, Schröder R, Glaser G, Tautz D (1995) Conserved and divergent expression aspects of the *Drosophila* segmentation gene *hunchback* in the short germ band embryo of the flour beetle *Tribolium*. *Development* **121**: 4227–4236