Development 111, 1121-1135 (1991)
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Mis-regulating segmentation gene expression in *Drosophila*

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Summary

We have used the *hunchback* (*hb*) gap-gene promoter to drive ectopic expression of the pair-rule genes *fushi tarazu* (*ftz*), *even-skipped* (*eve*) and *hairy* (*h*). Unexpectedly, flies transformed with such constructs are viable, despite spatial and temporal mis-regulation of pair-rule expression caused by the fusion genes. We show that fusion gene expression is transcriptionally regulated, such that ectopic expression is suppressed when pattern is established, and present evidence indicating that interstripe *hb–ftz* expression is repressed by *eve*. These results are considered in terms of redundant control of pair-rule gene striping. We also discuss the potential dangers of using mis-regulated gene expression to analyse normal function.

Key words: segmentation gene expression, embryonic pattern formation, pair-rule genes, genetic redundancy, *hunchback*, mis-regulation, *Drosophila*.

Introduction

Embryonic pattern in *Drosophila* is initiated and refined through the expression of a hierarchy of segmentation genes (Nüsslein-Volhard and Wieschaus, 1980; reviewed in Akam, 1987; Ingham, 1988). Initially, maternal genes establish coarse positional signals that define domains of gap-gene transcription (Driever and Nüsslein-Volhard, 1988*a*; Driever *et al.* 1989; Nüsslein-Volhard *et al.* 1987; Struhl *et al.* 1989). Overlapping gradients of the gap-gene proteins regulate the transcription of the pair-rule genes that are expressed in a series of stripes (Gaul and Jäckle, 1989; Pankratz *et al.* 1989; Stanojevic *et al.* 1989). Pair-rule genes are expressed in different but overlapping sets of stripes (Fig. 1A) that expose individual blastoderm cells/nuclei to different combinations of pair-rule proteins. Thus, the relative positionings of pair-rule stripes (pair-rule 'phasings') define the even more precise domains of segment-polarity gene expression, such as the one-cell-wide stripes of *engrailed* (*en*) and *wingless* (*wg*) that mark parasegmental embryonic metameric boundaries (DiNardo *et al.* 1985; Ingham *et al.* 1988; Lawrence *et al.* 1987).

We are particularly interested in the regulation and function of the pair-rule genes, whose correct expression underlies the establishment of metameric pattern. Initial pair-rule transcription is first detected in broad domains during nuclear cleavage stage 12, and evolves into stripes following the final blastoderm cleavage (stage 14). This occurs over a period of 15–30 min, consistent with the extreme instability of pair-rule transcripts and proteins ($t_{0.5}$ ~6 min; Edgar *et al.* 1986; Weir and Kornberg, 1985). Pair-rule gene striping is predominantly transcriptionally controlled such that transcript levels are enhanced within stripe domains and diminished between them (i.e. are repressed in the 'inter-stripes').

The pair-rule genes have been classified according to their principle striping mechanism (Howard and Ingham, 1986; Ingham and Gergen, 1988). The 'primary' pair-rule genes (*h*, *eve* and *runt*) are thought to respond directly to gap-gene positional cues *via* extensive upstream promoters with independent regulatory elements ('stripe elements') for individual stripes (Howard *et al.* 1988; Pankratz *et al.* 1989; Stanojevic *et al.* 1989). Deletion of upstream *h* sequences leads to the loss of specific stripes, and upstream regions can drive striped expression of β-galactosidase (*lacZ*) reporter genes in individual *h* stripe domains (Howard *et al.* 1988; Pankratz *et al.* 1990; G. Riddihough and M. Lardelli, personal communication). Similarly, individual *eve* stripes are independently regulated (Goto *et al.* 1989; Harding *et al.* 1989). Less is known about the negative control of *h* and *eve* transcription, although individual stripe elements must include repressor sites to prevent interstripe expression.

In contrast, patterning of 'secondary' pair-rule genes (e.g. *ftz*) is largely a response to the striping of the primary pair-rule genes. *ftz* striping depends on a small upstream transcriptional control element (the 'zebra' element) that confers striped expression on a *lacZ* reporter gene, suggesting that all *ftz* stripes respond to a similar signal (Hiromi *et al.* 1985; Hiromi and Gehring,

1987; but see Dearolf *et al.* 1989*a*; Ueda *et al.* 1990). *h* and *eve* are both implicated in repressing *ftz* expression; indeed *ftz* stripe domains correspond to the cells that express neither *h* nor *eve* (Fig. 1A; Frasch and Levine, 1987; Carroll *et al.* 1988; Hooper *et al.* 1989; Ish-Horowicz *et al.* 1989). The sites through which *h* and *eve* act have not yet been defined although putative negative control elements have been defined within the *ftz* zebra element (Dearolf *et al.* 1989*b*).

ftz and *eve* are further subject to positive autoregulatory control, each promoter including a domain that activates positive feedback of transcription (Hiromi and Gehring, 1987; Goto *et al.* 1989; Harding *et al.* 1989). Ectopic *ftz* expression can transactivate endogenous *ftz* expression in specific cells (Ish-Horowicz *et al.* 1989). However, the normal role of such feedback may lie in ensuring persistent expression during germ-band extension to preserve the metameric boundaries that are initially defined by the anterior margins of *eve* and *ftz* expression (Lawrence *et al.* 1987).

The major task is to distinguish direct and indirect interactions between segmentation genes. The initial hierarchy was inferred from mutant cuticular phenotypes and from patterns of segmentation gene expression in mutant embryos. However, the large number of interacting genes makes it difficult to define direct genetic pathways, and has led to studies of ectopic segmentation gene expression using an inducible heat shock promoter to drive generalised segmentation gene expression during the blastoderm stage. Ectopic expression can be induced in precisely staged embryos, allowing immediate responses to be distinguished through their kinetics. In this manner, the effects of generalised *ftz*, *h* or *hunchback* (*hb*) expression at blastoderm have been explained in terms of direct effects on the expression of other segmentation genes (Struhl, 1985, 1989; Ish-Horowicz and Pinchin, 1987; Ish-Horowicz *et al.* 1989). For example, ectopic *h* leads to rapid extinction of *ftz* expression, consistent with *h*'s role as a primary repressor of *ftz* expression (Ish-Horowicz and Pinchin, 1987).

More restricted spatial mis-expression would allow investigations of the finer mechanisms that must underly the precision of final blastoderm pattern, e.g. pair-rule stripe phasing. More precise disruptions could be achieved by using promoters that themselves display spatial regulation, i.e. segmentation gene promoters. Different patterns of mis-expression would arise according to the heterologous promoter chosen.

Previous experiments indicate that such mis-expression leads to pattern disruptions and dominant lethality. For example, uncontrolled expression of *ftz*, *h* or *runt* causes pattern defects, indicating that expression in inter-stripe domains is deleterious and causes embryonic lethality (Struhl, 1985; Gergen and Wieschaus, 1986; Ish-Horowicz and Pinchin, 1987; Ish-Horowicz *et al.* 1989). Thus, the precise spatial and temporal patterns of segmentation gene expression are crucial in defining the embryonic body plan, and ectopic expression of segmentation gene products can redirect the fates of cells inappropriately expressing these genes. Nevertheless, several schemes might permit the recovery of flies transformed with predicted dominant-lethal constructs. For example, protein levels could be reduced by depressing translational efficiency. Alternatively, functional expression might be conditional on combining two constructs that are individually viable (e.g. nonsense mutation+tRNA suppressor; inducible promoter+trans-activator – cf. Kakidani and Ptashne, 1988; Webster *et al.* 1988).

As a preliminary to such experiments, we have generated three gene fusions that express the pair-rule genes, *ftz*, *eve* and *h* under the control of a gap-gene promoter (*hb*). These constructs should drive anterior mis-expression of the pair-rule proteins within the *hb* domain, the anterior half of the embryo. Such disruptions of segmentation domains should be dominant lethal although the exact effects on pattern will depend on the individual pair-rule gene and the degree of its mis-expression (i.e. the extents to which its endogenous domains overlap that of *hb* – Fig. 1A).

This paper describes and discusses our unexpected findings that flies transformed with such constructs are viable. We show that the fusion constructs are active and mis-express pair-rule genes in *hb*-like patterns, but that interstripe ectopic expression diminishes when pair-rule genes begin to stripe. These results illustrate the importance of timing in segmentation gene function, and indicate that pair-rule genes have transcriptional control regions downstream of their transcription start sites. We suggest that interstripe *hb–ftz* expression is repressed by *eve*. We also describe an unexpected effect of ectopic *h* expression on sex determination, which illustrates the potential dangers of analysing gene function through gene mis-expression (see also Parkhurst *et al.* 1990).

Materials and methods

Fly stocks

Flies were cultured on yeast, maize meal, molasses, malt extract, agar medium, at 25°C unless otherwise stated. The null alleles used in this study are: *Df(2R)eve*$^{1.27}$, *cn sca bw sp/CyO*, *Df(3R)4Scb/TM3* (*ftz*), and *Df(3R)h*^{i22}, *Ki roe p*^{p}*/TM3*. The *FG2 ftz–lac Z* transformant stock expresses a *ftz–lacZ* fusion protein that localises in the nucleus (Y. Hiromi, personal communication). The *eve–lac Z* transformant stock is described in Lawrence *et al.* (1987).

Constructs

The 4.7 kb *Bam*HI–*Xba*I fragment containing the *hb* promoter and all but 10 bases of the 5′ untranslated leader sequences was subcloned into the blunt-ended *Sal*I site of *pUChsneo* (Steller and Pirrotta, 1985). This vector, *hbneo*, drives anterior zygotic expression of coding sequences inserted at unique *Bam*HI or *Sma*I polylinker sites adjacent to the *hb* promoter. For *hb–ftz*, the *Ava*II (−75 bp) to *Hin*dIII (+2.5 kb) genomic fragment including all of the 5′ untranslated leader from pFK1 (Hiromi *et al.* 1985) was subcloned into the blunt-ended *Bam*HI site of *hbneo*. For *hb–eve*, the 4.7 kb *Xho*I genomic fragment including all of the 5′-untranslated leader sequences of p48-X4.7 (Macdonald *et al.* 1986) was subcloned into the blunt-ended *Bam*HI site of

hbneo. For *hb–h*, the 6.5 kb *Xba*I genomic fragment including 230 bases of 5′ untranslated leader sequences (Rushlow *et al.* 1989) was subcloned into the blunt-ended *Bam*HI site of *hbneo*. The appropriate orientation for all clones was determined by restriction analysis.

Embryo analysis

Embryos were prepared and analysed as described by Wieschaus and Nüsslein-Volhard (1986). Immunohistochemical detection of *h*, *ftz*, *eve*, *en* and *β-gal* was performed essentially as described by Macdonald and Struhl (1986), using biotinylated secondary antibodies and avidin-biotin-HRP complexes (Vector Laboratories, Inc.). The antibodies used in this study were generously provided by: H. Krause, rabbit anti-*ftz* antibodies (Krause *et al.* 1988); M. Frasch, rabbit anti-*eve* antibodies (Frasch *et al.* 1987); M. Wilcox, monoclonal anti-*en* antibodies (Patel *et al.* 1989); D. Tautz, rabbit anti-*hb* antibodies (Tautz, 1988); K. Hooper, rabbit anti-*h* antibodies (Hooper *et al.* 1989) and H. Durbin, 4C7 monoclonal anti-*β-gal* antibodies (Imperial Cancer Research Fund). All secondary antibodies were obtained from Jackson ImmunoResearch Labs (West Grove, PA). The stained embryos were dehydrated in 100% ETOH and mounted under a coverslip in methacrylate mounting medium (JB-4, Polysciences) that was polymerised under CO_2 for 1–2 h at room temperature.

In situ *hybridisation*

Immunohistochemical whole-mount *in situ* hybridisation was performed according to the protocol of Tautz and Pfeifle (1989). The probes used for the random priming were: the 4.7 kb *Xho*I genomic fragment for *eve* (p48-X4.7; Macdonald *et al.* 1986), the 3.5 kb *Eco*RI genomic fragment for *ftz* (pFK1; Hiromi *et al.* 1985), and the 1.8 kb *Eco*RI cDNA fragment of *h* (ThΔ1; Rushlow *et al.* 1989).

Germline transformation

bw;st embryos were transformed by injection with a mixture of recombinant plasmid (500 $\mu g\,ml^{-1}$) and helper plasmid (100 $\mu g\,ml^{-1}$), as described by Spradling (1986). The *bw;st* G_0 adults were outcrossed to wild-type and selected on standard medium supplemented with Geneticin G418 Sulphate (Gibco – 1.5 $mg\,ml^{-1}$ but varied according to batch). G_1 *bw/+; st/+* progeny were mapped by back-crossing crossed to *bw;st* on G418 food. This retested their drug resistance and assigned the insert to a specific chromosome, allowing construction of homozygous or balanced stocks. All *neo*-resistant transformants were confirmed by Polymerase Chain Reaction (Erlich, 1989) using primers specific to *neo* portion of the P-element transformation vector.

Assignment of ectopic eve *stripes in* hb–eve; eve^- *embryos*

We measured the position (anterior margin) of the ectopically expressed stripes in *hb–eve; eve*$^-$ embryos and compared them to the endogenous *ftz* stripe 1 and *eve* stripes 1 and 2 in wild-type embryos. Ten embryos were measured for each stripe and the results are expressed below in percentage egg length, where 0% is the posterior end:

genotype	*eve* stripe 1	*ftz* stripe 1	*eve* stripe 2
+/+	70.6±1.4	67.1±1.4	61.3±1.5
hb–eve; eve$^-$	71.2±1.7		62.5±1.3

Thus, the two ectopically expressed *hb–eve* stripes are coincident with the endogenous *eve* stripes.

Genetic interactions

We analysed all three fusion gene constructs for dominant interactions (eg., *hb–ftz/+; Kr*$^-$*/+*) with the gap alleles Kr^1, hb^{PTX15} and kni^{IID48} and for dominant as well as recessive (eg., *hb–ftz/+; h*$^-$*/h*$^-$) interactions with the pair-rule deletions $Df(2)eve^{1.27}$, *Df(3)4Scb* (*ftz*$^-$), $Df(3)h^{i22}$ and $Df(1)run^{IIIB}$. The only interaction identified was *hb–ftz/+; eve*$^-$*/+*. (The recessive interaction (*hb–ftz/+; eve*$^-$*/eve*$^-$) could not be tested due to the lethality of the trans-heterozygotes.)

Scanning electron microscopy

Adult males were etherised, mounted on metal discs with double-sided tape, sputter-coated with ionised gold, then viewed with a Phillips 515 scanning electron microscope.

Results

Flies transformed with hb–*pair-rule fusion genes are viable*

We used the *hb* promoter to examine the effects of mis-expressing pair-rule genes in the anterior of the embryo. Three fusion genes – *hb–ftz*, *hb–eve*, and *hb–h* – were generated by fusing a 4.7 kb *hb* promoter fragment to genomic coding sequences from the *ftz*, *eve* and *h* genes (Fig. 1C). The three fusion genes retain most of the *hb* and pair-rule gene 5′-untranslated leader sequences, while excluding the 5′ flanking sequences of the pair-rule genes that are known to function in striping (Fig. 1C; see Materials and methods). Reporter gene constructs indicate that these *hb* sequences should be sufficient to mis-express pair-rule genes in the anterior 45% of the embryo, through about 2 pair-rule stripes (Fig. 1A,B – Driever *et al.* 1989; Hülskamp *et al.* 1989). Anterior zygotic *hb* expression derives from the proximal of two promoters that is first active at stage 11/12, preceding pair-rule expression by about two cleavage-cycles (Fig. 1C – Tautz *et al.* 1987; Schröder *et al.* 1988).

The 4.7 kb *hb* fragment also includes part of the distal promoter that is first expressed during oogenesis, depositing maternal transcript into the oocyte (Fig. 1A – Tautz *et al.* 1987; Schröder *et al.* 1988). This *hb* promoter is also zygotically active during blastoderm stage 14 in two major stripes, one abutting the anterior zygotic domain, and one in the posterior of the embryo (Fig. 1A,B – Tautz and Pfeifle, 1989). The posterior *hb* stripe overlaps and extends posterior to *h*/*eve* stripes 7 (which share approximately similar phasings). *ftz* domains are reciprocal to those of *eve* (Frasch and Levine, 1987) so the posterior *hb* stripe also overlaps *ftz* interstripe 6/7. The 4.7 kb fragment drives maternal expression, but previous experiments have not revealed whether this fragment is sufficient for the posterior stripe expression.

Each of these constructs were introduced into the fly germ-line (see Materials and methods). Unexpectedly, transformed lines were readily recovered for each construct: 10 *hb–ftz*; 6 *hb–eve*; 16 *hb–h*, indicating that these fusion genes do not give rise to significant degrees

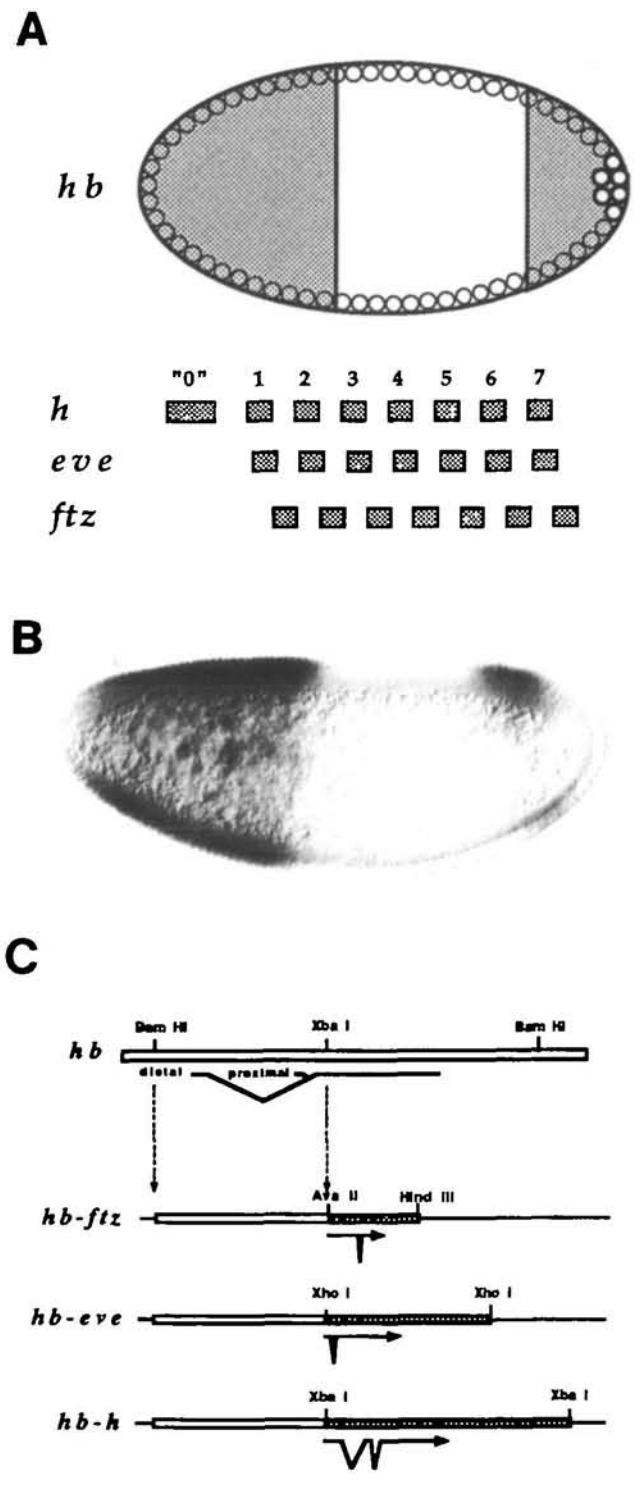

Fig. 1. (A) Relative overlap of the *hb* expression domains with those of *h*, *eve* and *ftz*. The anterior *hb* domain includes *h*/*eve* stripes 1 and 2 and *ftz* stripe 1 and part of stripe 2. The *hb* posterior domain overlaps and extends posterior to *h*/*eve* stripes 7 while overlapping *ftz* interstripe 6/7. (B) Wild-type expression pattern of *hb* protein in a late stage 14 embryo. For this and subsequent figures, anterior is to the left, dorsal is uppermost. (C) Restriction map of the *hb* gene/promoter fragment used and the fusions to *ftz*, *eve* and *h*. Both the proximal and distal promoters of *hb* are included in these fusion gene constructs. The restriction sites delimiting the fragments used have been lost in the cloning steps (see Materials and methods).

of dominant lethality. The viability of the transgenic flies suggested either that the constructs do not cause pair-rule gene mis-expression (e.g. because of an inactive *hb* promoter), or that such mis-expression is either tolerated or repressed. We therefore examined expression of the fusion genes and determined the effects of ectopic segmentation gene expression on embryonic pattern.

We shall consider each construct in turn.

ftz *is ectopically expressed in* hb–ftz *embryos and partially rescues* ftz$^-$ *larvae*

We analysed four independent *hb–ftz* stocks (in the presence of their endogenous *ftz* genes) and all show the *hb–ftz* gene directs ectopic *ftz* expression. Very weak overall *ftz* expression is first seen at stages 10/11, before the onset of zygotic *hb* transcription (Fig. 2A). We have not investigated this phase of *ftz* protein expression further, but appropriate genetic crosses for *hb–eve* (below) predict that the overall *ftz* mis-expression is derived from the distal (maternal) *hb* promoter. Thereafter, *hb–ftz* embryos express ectopic *ftz* protein zygotically in the anterior *hb* domain during nuclear cycle 12, two cleavage cycles before the endogenous *ftz* protein is normally seen (Fig. 2B). At the beginning of cycle 14, endogenous *ftz* expression begins which is superimposed on the ectopic *hb–ftz* pattern (Fig. 2C,D). The anterior domain of *hb* protein expression extends into *ftz* stripe 2 (Fig. 2C–E), and the posterior *hb–ftz* stripe results in continuous *ftz* expression between stripes 6 and 7 (Figs 1A, 2D,E).

Surprisingly, *ftz* mis-expression fails to persist through the blastoderm stage, although *hb* expression is detectable until the onset of gastrulation (Tautz and Pfeifle, 1989). Most ectopic *ftz* staining decays during blastoderm stage 14, the time at which endogenous *ftz* striping becomes prominent (Fig. 2C–F). *ftz* expression is reduced in interstripe domains (i.e. between stripes 1/2, and 6/7 and anterior of stripe 1 – Fig. 2E,F). By the end of the blastoderm stage, ectopic *ftz* expression is restricted to a novel stripe, 3–4 cells anterior to *ftz* stripe 1, that does not correspond to a normal *hb* domain (Fig. 2F).

Most *hb–ftz* lines are completely homozygous viable and show no significant embryonic cuticular pattern defects (not shown). Nevertheless, *hb–ftz* encodes an active protein. ~20% of homozygous *hb–ftz* adults lack external genitalia that derive from anlagen of segments A8–11 (Schüpbach *et al.* 1978; Tautz *et al.* 1987 – Fig. 3A–C). Although the missing structures derive from within the posterior *hb* stripe domain, we cannot unambiguously demonstrate ectopic *ftz* expression in this region. The altered pattern in such embryos indicates that the *hb–ftz* gene encodes functional *ftz* protein.

Indeed, *hb–ftz* partially rescues the pattern defects of

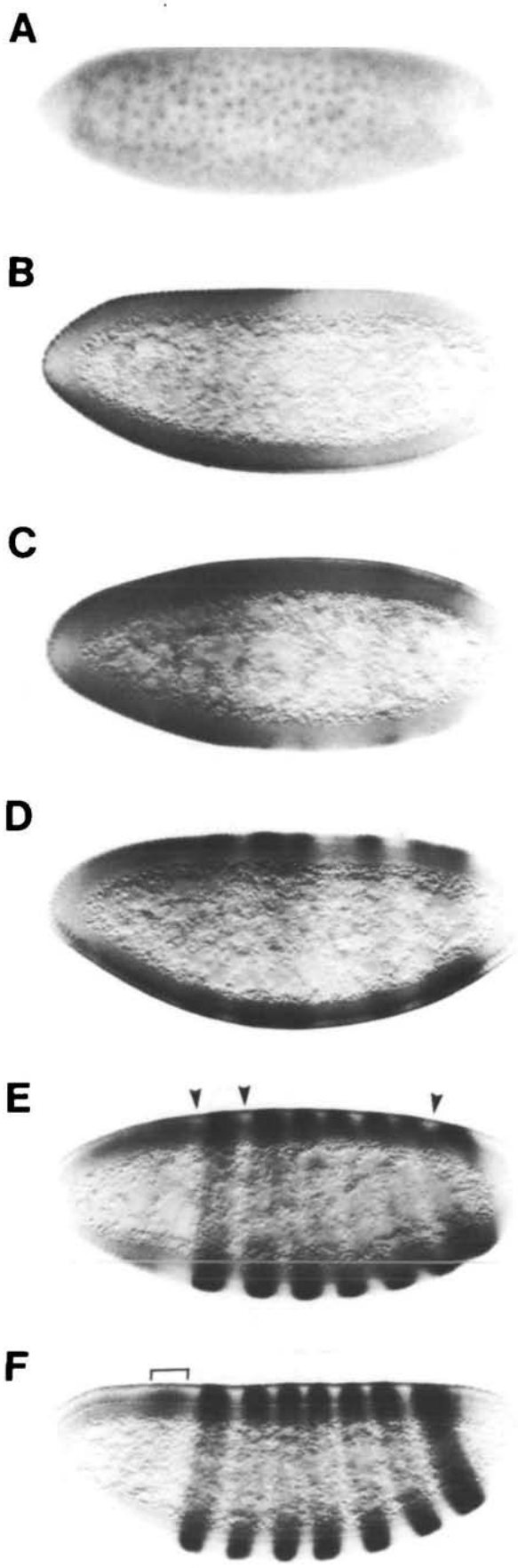

Fig. 2. *ftz* protein expression in *hb–ftz* embryos. Embryos containing two copies of the *hb–ftz* construct in an otherwise wild-type background were stained with an anti-*ftz* antibody (Krause *et al.* 1988). (A) Stage 10/11 *hb–ftz* embryo showing ectopic *ftz* protein in all nuclei, derived from the distal (maternal) *hb* promoter. (B) Stage 12 embryo showing ectopic *ftz* protein in the *hb* domain (anterior half of the embryo). (C–F) Successively older embryos showing the emergence of endogenous *ftz* protein stripes in addition to the ectopic expression in the *hb* domain. As the endogenous *ftz* stripes begin to resolve, the ectopic expression starts to clear between stripes 6 and 7, stripes 1 and 2, and just anterior of stripe 1 (E; arrowheads). At late stage 14, the final *ftz* protein pattern has been achieved, with the removal of all ectopic *ftz* protein except a small (mostly dorsal) stripe anterior to stripe 1 (F; brackets). For all embryos, anterior is to the left and dorsal is uppermost. The embryo in A is a bright-field photograph of the embryo surface. The embryos shown in B–F were photographed using Nomarski optics.

ftz$^-$ embryos. The T1 'beard' is restored, as are various chitinised mouthpart structures (Fig. 3E). This implies that the *hb* promoter is still active during cycle 14 when *ftz* is needed for patterning. Nevertheless, ectopic *ftz* is no longer expressed between *ftz* stripes, indicating that zygotic expression from *hb–ftz* is negatively regulated in the head and *ftz* interstripe regions where it would cause pattern defects. The remaining *ftz* mis-expression in the head region appears not to cause significant pattern abnormalities.

hb–eve *drives ectopic* eve *expression and causes homozygous lethality*

We analysed *eve* in three transformed lines with autosomal insertion sites and all behave similarly. *eve* protein expression in *hb–eve* embryos is first detectable as generalised nuclear staining at blastoderm stage 10/11 (Fig. 4A). This protein derives from maternal transcript as it is only seen in embryos from *hb–eve* mothers, but not from wild-type mothers. Such maternal staining is only transitory, soon being replaced by the zygotic *hb* pattern of expression.

Strong ectopic anterior *eve* protein expression is first evident at blastoderm stage 12/13, and persists until stage 14 when it overlaps endogenous *eve* stripes 1 and 2 (Fig. 4B–D). A weak posterior *eve* stripe is seen during early stage 13/14 (not shown), but its expression is soon masked by the endogenous *eve* stripe 7. During blastoderm stage 14, *hb–eve* expression decays until, by the end of blastoderm, mostly endogenous protein expression exists with very low level ectopic expression in a small anterior cap (Fig. 4D,E). *eve* is thought to act at the late blastoderm stage to regulate segment-polarity gene domains and to define the odd-numbered parasegmental boundaries (Lawrence *et al.* 1987; Ingham *et al.* 1988). The lack of ectopic *eve* expression at this stage explains the viability of heterozygous *hb–eve* embryos.

However, all five autosomal *hb–eve* lines are homozygous and *trans*-heterozygous lethal indicating that two doses of *hb–eve* are unconditionally lethal. A

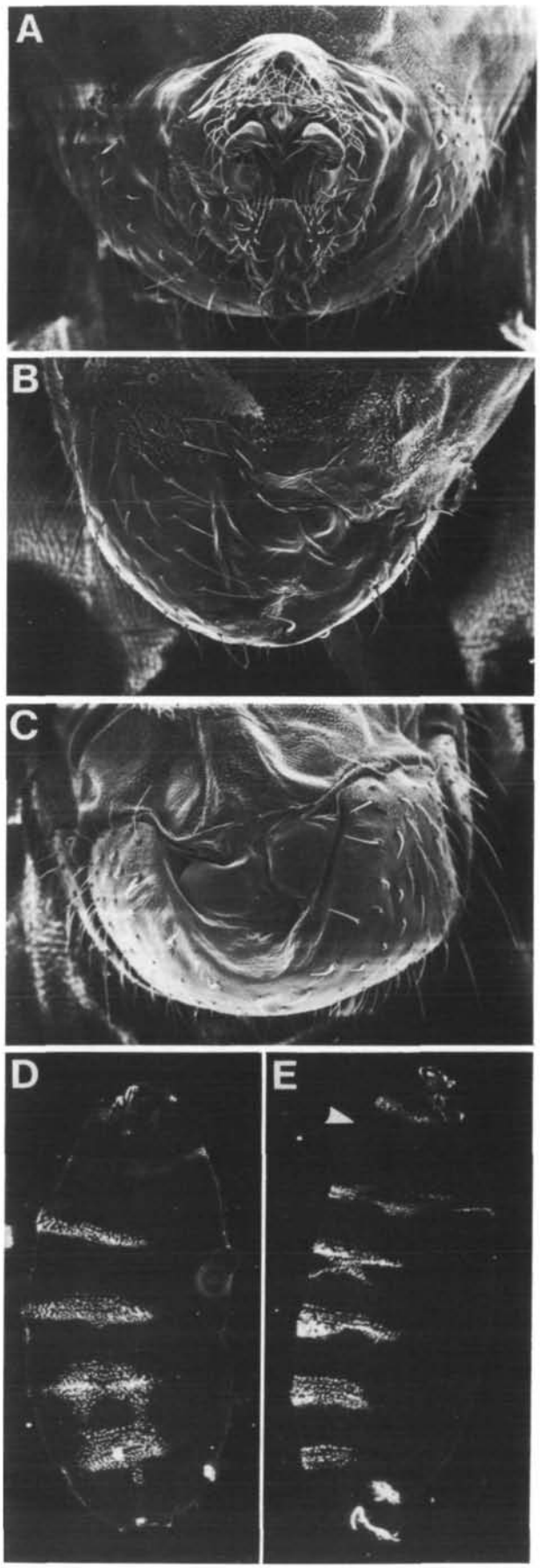

Fig. 3. Posterior defects in *hb–ftz* flies and partial rescue of *ftz*$^-$ larvae by ectopic *ftz* expression. (A–C) Posterior defects in *hb–ftz* flies. Scanning electron micrographs of wild-type (A) and *hb–ftz* (B,C) adult male genitalia. Approximately 20% of *hb–ftz* homozygous adults lack posterior structures. While most of the flies with posterior defects include the loss of structures associated with abdominal segments A6–A10 (B), some flies have rudiments of A6 structures (C). Although only males are shown, females are similarly affected. (D,E) Cuticle phenotype of larvae homozygous for *Df(3R)4Scb* (*ftz*$^-$), with zero (D), and two *hb–ftz* copies (E). The latter shows more extensive chitinised mouthparts and the T1-associated ventral hairs ('beard'– arrowhead) is restored. Abdominal segmentation is also somewhat affected although we do not currently understand why the anterior *hb* promoter affects posterior patterning or why this should only be evident in *ftz*$^-$ embryos.

sixth line, in which *hb–eve* is X-linked, is weaker and can be made homozygous. In *hb–eve* balanced stocks, about 25% (presumably the homozygous embryos) show a consistent cuticular phenotype including fusions of T2/3, A1/2 and A3/4, and loss of the A6 denticle band (Fig. 5A,B). Strikingly, pattern abnormalities arise outside the *hb* domain where little or no ectopic *eve* is expressed. These could be due either to non-autonomous action of zygotic *eve* protein, or to generalised maternal expression from the distal promoter (see Discussion).

hb–eve also exerts a weak dominant effect on segmentation and on viability. Most hemizygous (single-copy) *hb–eve* embryos survive, but about 20% (37/183) die with weak and occasional fusions of adjacent denticle bands (Fig. 5C,D). The frequency and character of the defects is independent of whether the *hb–eve* gene is maternally or paternally inherited, indicating that they are due to zygotic, not maternal, *eve* mis-expression.

Cross-regulatory interactions among primary pair-rule genes indicate that *eve* regulates the pattern of other pair-rule genes (Ingham and Gergen, 1988) and implies that the *hb–eve* pattern defects may be due to ectopic *eve* affecting expression of other segmentation genes. We therefore analysed the patterns of *h*, *ftz* and *en* expression in *hb–eve* embryos.

Segmentation gene patterning is disorganised in hb–eve *embryos*

ftz and *h* patterns are indeed affected by *hb–eve*. We analysed embryos from balanced *hb–eve* stocks in which one half of the eggs contain a single *hb–eve* copy and one quarter are homozygous for *hb–eve*. Homozygous *hb–eve* embryos (24/79) show partial or complete fusions of *ftz* stripes 3 to 6 (Fig. 5E). Hemizygous embryos show a weaker phenotype in which stripes 3 to 6 are present but compressed (Fig. 5F).

hb–eve also disrupts *h* expression, stripes 1, 2, 3 and 7 becoming stronger and broader relative to the other bands (Fig. 5G,H). More strikingly, anterodorsal *h* expression (stripe '0' in Fig. 1A) is completely missing

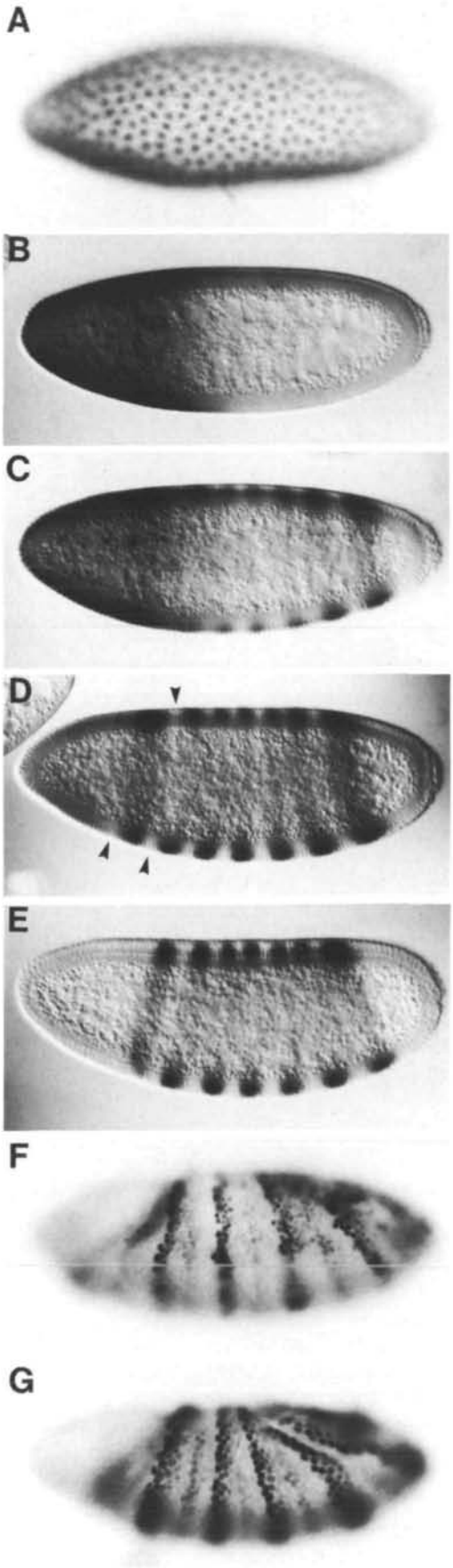

Fig. 4. *eve* protein expression in *hb–eve* embryos. Embryos from a balanced *hb–eve* stock in an otherwise wild-type background were stained with anti-*eve* antibodies (Frasch *et al.* 1987). (A) Stage 10/11 *hb–eve* embryo showing *eve* protein in all nuclei derived from the distal (maternal) *hb* promoter. (B) Stage 12/13 embryo showing ectopic *eve* protein in the *hb* domain (anterior half of the embryo). (C) Slightly older embryo showing the emergence of endogenous *eve* protein stripes in addition to the ectopic expression in the *hb* domain. (D) As the endogenous *eve* stripes begin to resolve, the ectopic expression starts to clear between stripes 1 and 2, and just anterior of stripe 1 (arrowheads). (E) At late stage 14, the final *eve* protein pattern has been achieved, with the removal of all ectopic *eve* protein except very low-level anterior expression not visible in the photograph. (F,G) During germ-band extension, *eve* protein stripes are disrupted in *hb–eve* containing embryos (F) compared to their wild-type siblings (G). The embryos in A, F–G are bright-field photographs of the embryo surface. The embryos shown in B–E were photographed using Nomarski optics.

in 35 % (21/60) of these embryos. This suggests that the ectopic *eve* suppresses the regionalised activation of *h* stripe 0. *h* stripes 1 and 2 are not eliminated, indicating that *eve* is interfering with regional signals specific for stripe 0. The *cis*-regulatory region for *h* stripe 0 has not yet been characterised, but may be responding directly to elevated levels of *bicoid* and/or *dorsal* morphogens (Driever and Nüsslein-Volhard, 1988*b*; Steward *et al.* 1988).

Initial metameric patterning is roughly normal in *hb–eve* as judged by the earliest pattern of *en* expression (not shown). However, *eve* expression at gastrulation, which should be similar to that of *en*, is somewhat abnormal in homozygous *hb–eve* embryos. 25 % of *hb–eve* embryos, show 14 *eve* stripes whose domains appear correctly positioned but whose anterior margins (which parallel those of *en*) appear less well defined (Fig. 4E,F). *eve* expression is also weaker than wild-type. This altered pattern is found in all three lines examined as well as in *trans*-heterozygous lines containing two different *hb–eve* copies.

Drastic effects on metameric patterning become apparent about 1 h later when some embryos begin to show low-level *en* expression in all cells. By 6–7 h post-fertilisation, the generalised *en* expression becomes stronger and seen in 37 % (41/112) of embryos (Fig. 5I). In some embryos, the endogenous *en* stripes are still visible above the generalised expression and are disorganised in about half such embryos (Fig. 5J). Thus, the pattern defects in homozygous *hb–eve* embryos are due to an inability to maintain metameric subdivisions.

hb–h *embryos show normal segmentation but aberrant sex determination*

Fig. 6A shows ectopic expression of *h* in the anterior region of *hb–h* embryos at about nuclear cycle 12. This expression begins to clear during early cycle 14 (Fig. 6B–D), although the *hb* promoter remains active in a stripe of cells anterior to stripe 1 (Fig. 6E). *hb–h*

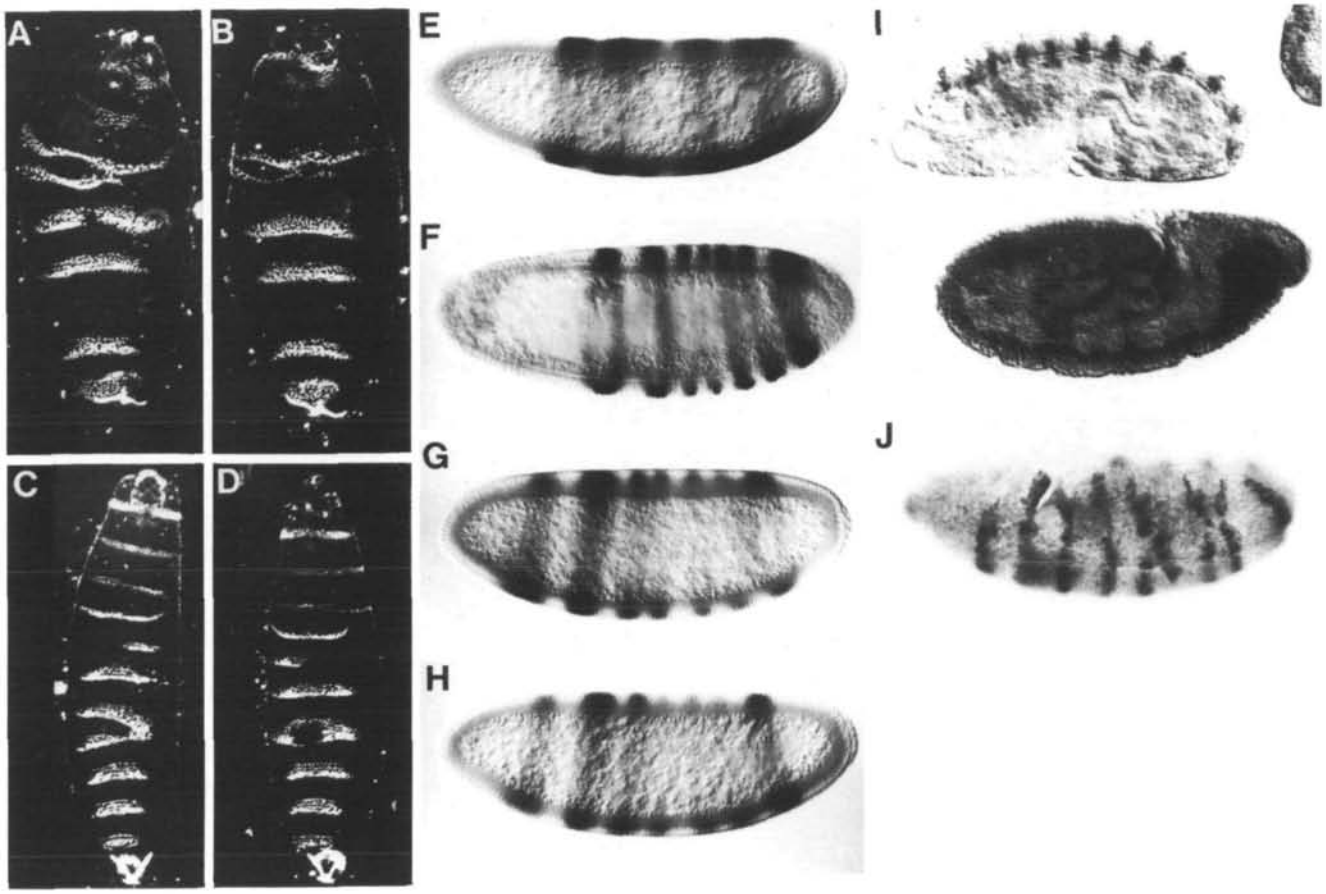

Fig. 5. *hb–eve* containing embryos have pattern defects and disrupted segmentation gene expression. (A,B) Severe cuticular phenotypes of homozygous *hb–eve* larvae. A6 is mostly missing while A1/A2 and A3/A4 are fused. (C,D) The cuticular phenotypes of hemizygous *hb–eve* embryos, showing loss/fusion of segments most commonly involving A2, A4–5. (E,F) *hb–eve* embryos stained with anti-*ftz* antibodies. (E) Homozygous *hb–eve* embryo showing fused *ftz* stripes. This pattern is not seen in crosses that yield only hemizygous *hb–eve* embryos. (F) Hemizygous *hb–eve* embryo showing compressed *ftz* stripes 3 to 6. (G,H) *hb–eve* embryos stained with anti-*h* antibodies. The anterodorsal headpatch ('stripe 0') is absent with a anterior shifting and broadening of stripes 1 and 2. The embryos in E–H were photographed with Nomarski optics. (I) *en* expression in *hb–eve* embryos, stained with anti-*en* antibodies (Patel *et al.* 1989). Two similarly staged embryos from a balanced *hb–eve* stock, are shown in the same optic field. Compare the upper embryo (with normal *en* staining) to the lower embryo showing the generalised *en* expression that is seen only in crosses yielding homozygous *hb–eve* embryos. (J) In some embryos, disorganised endogenous *en* stripes can still be visualised above the generalised expression. (Weaker photographic exposure than I to reveal the *en* stripes).

embryos show no obvious segmentation defects, consistent with the cessation of ectopic *h* expression before it would inhibit *ftz* expression. Ectopic *h* expression in a h^- background partially rescues *h* pattern defects in the anterior of the embryo, indicating that *hb–h* is active while metameric pattern is being established. Mouthparts become more organised, and anterior structures including the maxillary sense organs and T1 denticle band are restored (Fig. 6G).

Unexpectedly, *hb–h* interferes with sex determination, a process in which *h* does not normally function. *hb–h* males are fully viable and fertile whereas more than 99% of *hb–h* females die as embryos whose head defects correlate with the domain of *h* mis-expression. See Parkhurst *et al.* (1990) for a detailed examination and explanation of this phenotype.

Fusion gene transcripts are regulated in the interstripes

The above results show that homozygous *hb–ftz, hb–h* and most hemizygous *hb–eve* transformants can tolerate early ectopic expression of the respective pair-rule gene, but that later expression does not lead to pattern defects. This is not merely due to lack of promoter activity as *hb–ftz* and *hb–h* partially rescue embryos lacking endogenous *ftz* and *h*, respectively. (The variable *hb–eve* cuticular phenotype prevents unambiguous identification and analysis of *hb–eve; eve*$^-$ embryos.) Rather, interstripe expression is eliminated before it can affect pattern, either by regulation of transcript levels or by inhibition of translation of the hybrid mRNAs.

We excluded the latter explanation by showing that transcript patterns mirror those of the mis-expressed

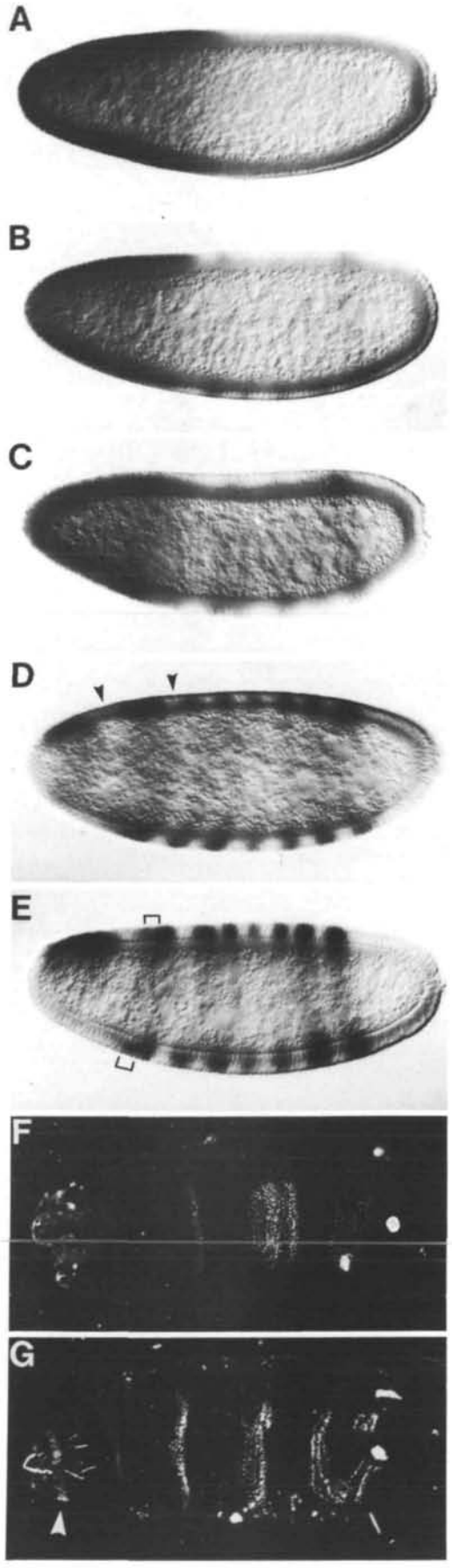

Fig. 6. *h* protein expression in *hb–h* embryos and partial rescue of h^- larvae by the ectopic *h* expression. (A) Stage 12 embryo showing ectopic *h* protein expression in the *hb* domain (anterior half of the embryo). (B–D) Successively older embryos showing the emergence of endogenous *h* protein stripes in addition to the ectopic expression in the *hb* domain. As the endogenous *h* stripes begin to resolve, the ectopic expression starts to clear between stripes 1 and 2, and just anterior of stripe 1 (D; arrowhead). At late stage 14, the final *h* protein pattern has been achieved, with the removal of all ectopic *h* protein except a small stripe adjacent to stripe 1 (E; brackets). All embryos contain one copy of the *hb–h* construct in an otherwise wild-type background, and were photographed using Nomarski optics. (F,G) *hb–h* partially restores pattern to h^- larvae. Cuticle phenotype of homozygous $Df(3R)h^{i22}$ larvae, (F) lacking *hb–h*, and (G) including one copy of *hb–h*. Mouthparts, as well as the T1 denticle band are restored (G; arrowhead).

pair-rule protein. In *hb–ftz* embryos, *ftz* transcripts initially accumulate in the anterior half of the embryo, but are then repressed between the normal stripes, leaving only a band of ectopic transcripts anterior to *ftz* stripe 1 (Fig. 7A–D). *eve* and *h* transcription in *hb–eve* and *hb–h* embryos, respectively, mimic the patterns of protein accumulation (Fig. 7E–L), showing that negative regulation of interstripe expression from the *hb* fusion genes is transcriptionally/post-transcriptionally (but not translationally) regulated. The different expression patterns of the three fusion genes shows that the regulation must act through pair-rule sequences present in the fusion constructs.

hb–ftz *and* hb–eve *retain negative regulatory elements and their expression does not require autoactivation*

Analysis of the *hb–ftz;* ftz^- and *hb–eve;* eve^- embryos also show that the fusion gene constructs retain control sequences that repress their expression in the interstripe regions. We find that the initial fusion-gene-staining patterns are not altered in ftz^- *or* eve^- mutant embryos. All stage 13 embryos in a balanced *hb–ftz;* ftz^- stock show high-level anterior *ftz* expression, including the 25 % of embryos that must lack endogenous *ftz* activity (not shown). Similarly, all embryos from a balanced *hb–eve;* eve^- stock, including those lacking an endogenous *eve* gene, show ectopic anterior *eve* expression that can only derive from the *hb–eve* fusion gene. During blastoderm stage 14, *hb–eve;* eve^- embryos are distinguished by their lack of endogenous striped expression. Such embryos still express *eve* in the anterior *hb* domain except within two domains that correspond in position to the two overlapping *eve* interstripes – anterior to stripe 1 and between stripes 1 and 2 (Fig. 8A,B; see Materials and methods). By the late blastoderm stage, *hb* transcripts from the distal *hb* promoter accumulate in two anterior stripes, the more posterior of which corresponds to *ftz* stripe 1 (Tautz and Pfeifle, 1989); however, these expression domains do not overlap with those of *eve* ectopic expression in *hb–eve;* eve^- embryos. Thus, the control elements

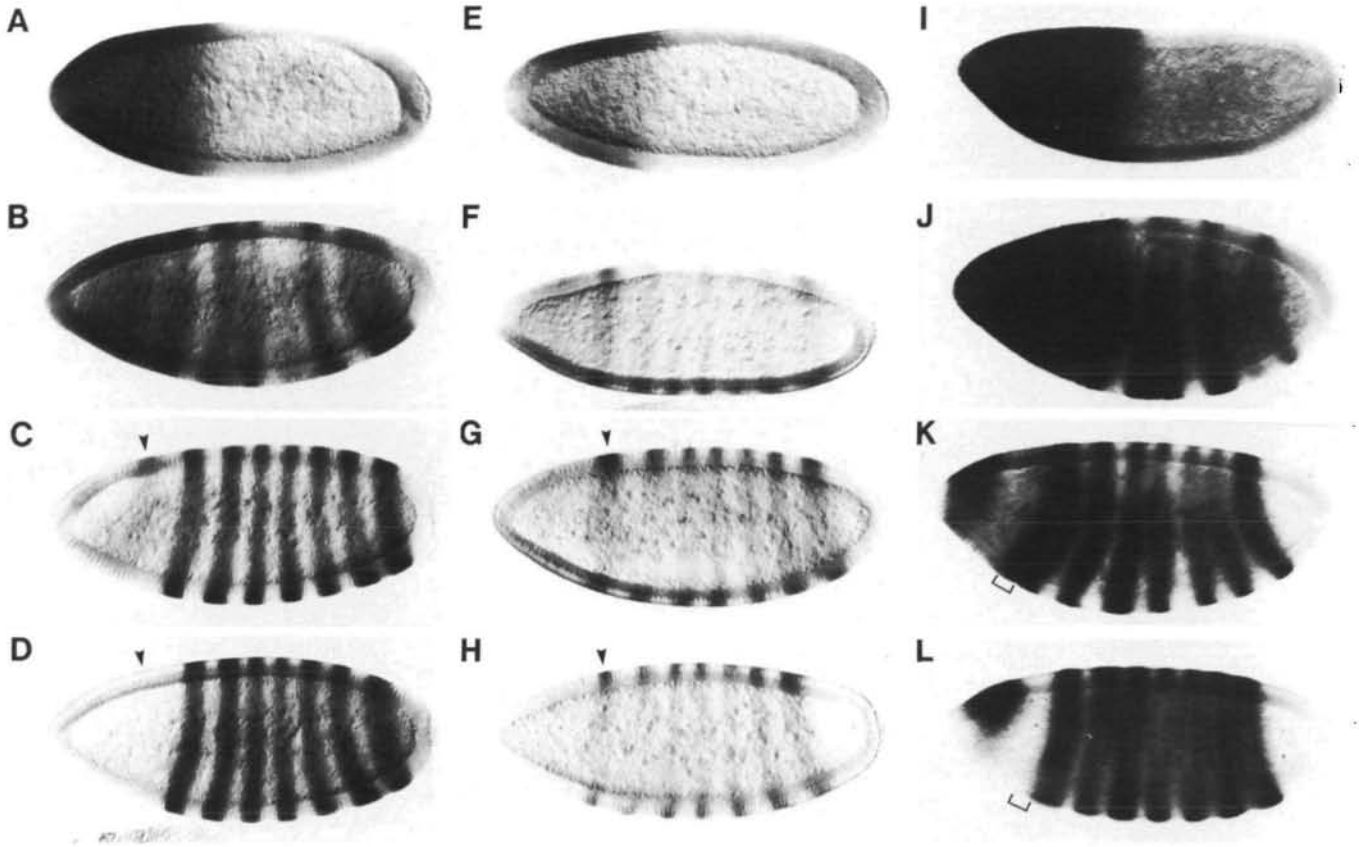

Fig. 7. Interstripe expression of fusion genes is transcriptionally regulated. *hb–ftz*, *hb–eve* and *hb–h*- containing embryos were analysed by whole-mount *in situ* hybridisation with probes specific for *ftz*, *eve* and *h*, respectively. The ectopic transcripts mimic the protein expression pattern and are lacking in the respective interstripe regions. (A–C) Successively older *hb–ftz* embryos hybridised with *ftz* sequences. At the peak of *ftz* expression (C), all ectopic expression has been cleared away except for a small (mostly dorsal) stripe just anterior of stripe 1 (arrow) when compared to a wild-type embryo at the same developmental stage (D). (E–G) Successively older *hb–eve* embryos hybridised with *eve* sequences. At the peak of *eve* expression (G), all ectopic expression has been cleared away except for a small stripe adjacent to stripe 1 (arrow) when compared to a wild-type embryo at the same developmental stage (H). Very low-level expression persists in the head region. (I–K) Successively older *hb–h* embryos hybridised with *h* sequences. At the peak of *h* expression (K), ectopic expression has been cleared away except for a stripe adjacent to stripe 1 (bracket) when compared to a wild-type embryo at the same developmental stage (L). All embryos were photographed using Nomarski optics.

mediating such negative control must exist within the *eve* sequences included in the *hb–eve* construct.

It is more difficult to visualise interstripe repression of *hb–ftz*, as endogenous *ftz* domains overlap domains of late zygotic *hb* expression. However, the interaction between *hb–ftz* and *eve* suggests that *ftz* also retains a repressor element that imposes its negative control on the *hb* promoter (see below). *hb–h* may also retain downstream negative regulatory elements that clear ectopic *h* expression between *h* stripes 1 and 2, but the female lethality of *hb–h* embryos has prevented our demonstrating this directly.

Generalised *ftz* expression from the inducible heat-shock promoter causes pattern defects by autoregulatory activation of the chromosomal *ftz* gene (Hiromi and Gehring, 1987; Ish-Horowicz *et al.* 1989). Similarly, *eve* can autoregulate its own expression (Harding *et al.* 1989). However, initial fusion-gene-staining patterns are not altered in ftz^- or eve^- mutant embryos, suggesting that *hb–ftz* and *hb–eve* are independent of endogenous *ftz* or *eve* activity. We confirmed this by using *lacZ* fusions to the *ftz* and *eve* promoters to monitor endogenous promoter activities (Hiromi *et al.* 1985; Lawrence *et al.* 1987). *hb–ftz; ftz–lacZ* embryos display no ectopic *lacZ* expression (not shown), indicating that endogenous *ftz* transcription is not autoactivated by the *hb*-encoded ectopic *ftz* protein. Similar results are obtained using *hb–eve* and a *eve–lacZ* fusion gene (not shown). Thus, *hb–ftz* and *hb–eve* ectopic expression do not autoactivate endogenous expression. The female lethality associated with the *hb–h* construct precludes our analysis of *hb–h;* h^- embryos, but the *h* gene appears not to be autoregulated (Hooper *et al.* 1989).

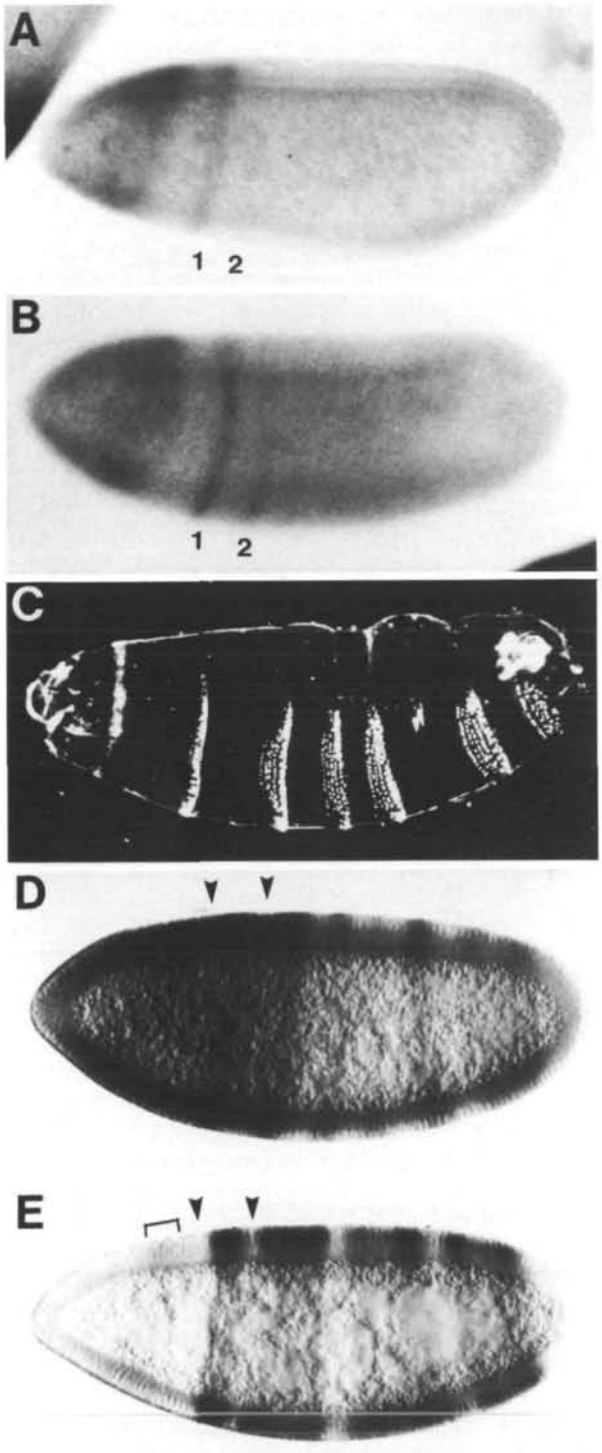

Fig. 8. Fusion gene constructs retain negative regulatory elements. (A,B) Late stage 14 *hb–eve*; eve^- embryos stained with anti-*eve* antibodies. Initial interstripe *eve* expression is repressed leaving ectopic protein expression in the normal *eve* stripes 1 and 2 positions (see Materials and methods for measurements) and in a cap at the anterior. (C–D) *eve* negatively regulates *hb–ftz*. (C) Cuticle pattern defects in *hb–ftz*/+; eve^-/+ trans-heterozygous embryos affecting T3, A2, A6. *hb–ftz*; eve^- trans-heterozygous embryos stained with anti-*ftz* antibodies show that the ectopic *ftz* expression is no longer removed from the *ftz* interstripe regions (D; arrowheads). (E) *h* does not regulate interstripe *hb–ftz* expression. *ftz* ectopic expression is still repressed in *hb–ftz*; h^- embryos (arrowheads) at the same time as *hb–ftz* drives ectopic anterior dorsal *ftz* expression (brackets).

eve *negatively regulates* hb–ftz

As interstripe repression is likely to be mediated by other segmentation genes, we analysed phenotypic interactions between the fusion genes and gap or pair-rule mutations, hoping to identify such repressors. Although most mutant combinations show no dominant interactions, *hb–ftz* and *eve* trans-heterozygotes (*hb–ftz*/+; eve^-/+) are lethal (0/187 adult progeny), dying as embryos with substantial pattern defects (Fig. 8C). Fig. 8D shows that interstripe *ftz* expression persists in *hb–ftz*/+; eve^-/+ embryos, suggesting that *eve* is responsible for repressing such interstripe expression.

In contrast, *hb–ftz* shows no dominant interactions with *h*, the other well-characterised *ftz* repressor, either because *hb–ftz* is *h*-independent, or because 50% of wild-type *h* levels is sufficient to regulate *hb–ftz*. We favour the former explanation as anterior *ftz* expression is still regulated in embryos that completely lack *h*. *ftz* expression is still repressed anterior to stripe 1 and between stripes 1 and 2 in *hb–ftz*; h^- embryos, suggesting that *h* does not regulate *hb–ftz* (Fig. 8E), although the broadened endogenous *ftz* expression in h^- embryos (Carroll and Scott, 1986; Howard and Ingham, 1986; Hiromi and Gehring, 1987) precludes detection of low-level interstripe expression. This indicates that *eve* is likely to be a major repressor of *ftz* expression.

Discussion

The fusion genes retain downstream transcriptional control elements

In this paper, we show that ectopic expression of *ftz*, *h* or *eve* under the control of the *hb* promoter does not necessarily result in pattern defects. Embryos containing the *hb* fusion genes initially express pair-rule genes in the anterior *hb* domain, but such ectopic expression largely ceases during blastoderm stage 14 (Figs 2, 4, 6). This is partially due to a decline in activity of the proximal *hb* promoter, as well as to its regulation by residual pair-rule sequences. Several lines of evidence demonstrate that the *hb* promoter in these fusion genes is still active at the time of pair-rule gene function and is regulated in the head and interstripe regions. First, the fusion gene constructs partially rescue the mutant pair-rule phenotype (Figs 3B and 6G). Second, at blastoderm stage 14, there are ectopic stripes of *eve* in a *hb–eve*; eve^- background that overlap the positions of the endogenous *eve* stripes (Fig. 8A,B). Third, although the same promoter is used for all three fusion gene constructs, each is expressed ectopically in head domains that differ between constructs (Figs 2F, 4E, 6E and 7C,G,K). Finally, interstripe expression is no

longer regulated in *hb–ftz/+; eve/+* embryos (Fig. 8D), and persists through the time of pair-rule gene expression.

For all three fusion genes, the patterns of transcript and protein localisation are similar, indicating that the lack of interstripe expression is not due to a failure of translation (Fig. 7). Clearing of interstripe transcripts through their differential stability is also unlikely as segmentation gene transcripts are extremely unstable (Edgar *et al.* 1986, 1989). Although post-transcriptional mechanisms cannot be excluded, lack of interstripe transcripts is most likely due to repression of interstripe transcriptional initiation.

The most likely candidate for a *ftz* interstripe repressor is *eve*. The lethality of *hb–ftz/+; eve/+* embryos shows that reduced *eve* levels exaggerate the effects of ectopic *ftz* expression, i.e. that *eve* normally acts to inhibit *ftz*. This is consistent with previous suggestions that *eve* repression defines the anterior boundaries of each *ftz* stripe (Ish-Horowicz *et al.* 1989), and would be mediated, at least in part, through downstream *eve*-responsive elements. In contrast, *hb–ftz* shows no interactions with *h*, the other characterised *ftz* repressor. *hb–ftz* expression is repressed between *ftz* stripes 1 and 2, even in *hb–ftz* embryos lacking *h* (Fig. 8E), suggesting that *h* does not act on *hb–ftz* and that *h* regulation of *ftz* striping might operate through upstream *ftz* sequences.

We do not know which genes repress *eve* and *h* expression in *hb–eve* and *hb–h*. The best candidate is the *runt* pair-rule gene whose stripe domains are roughly complementary to those of *eve* and *h* (Gergen and Butler, 1988; Ingham and Gergen, 1988). Although there is no direct evidence for such regulation, we consider it more likely that the downstream control regions react to a single pair-rule regulator than to alternative combinations of differing gap-genes.

Timing requirements for segmentation gene function

The timing of pair-rule gene expression is crucial: embryos are unaffected by generalised anterior mis-expression during blastoderm stages 10 to 13. Only late in cleavage cycle 14 do the pair-rule genes act to regulate segment-polarity gene expression and metameric pattern, by which stage expression from the fusion genes is restricted to functionally irrelevant domains.

Further indications of the importance of timing in patterning the early embryo comes from the temporal specificity of pair-rule autoregulation. Neither *hb–ftz* nor *hb–eve* autoactivate their endogenous genes, despite the presence of autoregulatory elements within each promoter. In contrast, late blastoderm and early gastrula-staged embryos are susceptible to *ftz* autocatalytic activation (Struhl, 1985; Ish-Horowicz *et al.* 1989). Autoregulation appears to be important for persistent expression during gastrulation and germ-band extension, but not during the earlier phases when pair-rule domains are being established (in contrast to reaction–diffusion models for pair-rule striping – Meinhardt, 1982). We note that *h* is not autoregulated and that its expression decays immediately following blastoderm (Hooper *et al.* 1989).

Pattern defects caused by ectopic pair-rule expression

Although all three constructs are viable, each has specific effects on development.

hb–ftz

Homozygous *hb–ftz* embryos show no obvious embryonic cuticular defects, but a proportion of adult flies lack terminal structures. The exact basis for this pattern abnormality is unclear, although it might be due to a weakly expressed posterior stripe of ectopic *ftz* expression, which extends into the A8–11 genital primordia. Only occasional cells can be affected as most adults are viable and the embryonic *en* pattern appears normal.

hb–eve

Unlike the other two constructs, *hb–eve* causes significant pattern abnormalities, with two copies being almost completely lethal and leading to metameric instability and subsequent segmentation defects. *eve* stripes during gastrulation are weak and irregular, and a high proportion of older *hb–eve* embryos display a generalised pattern of *en* expression in which clear *en* boundaries are lacking (Fig. 5I). Nevertheless, *hb–eve* embryos retain considerable metameric organisation (Fig. 5A,B), indicating that parasegmental boundaries can be maintained even in the absence of clear *en* boundaries. We presume that metamerisation initially requires *en* stripes, but thereafter other segment-polarity genes can contribute to intrasegmental patterning.

The major surprise is that the pattern abnormalities in homozygous *hb–eve* embryos are not restricted to the *hb* domain, i.e. to the domain of *eve* mis-expression. Thus, *eve* stripes at gastrulation are disrupted throughout the embryo (Fig. 4F; see also Fig. 5A,B). Such non-autonomy is unexpected as *eve* encodes a nuclear homoeobox protein whose direct actions should be local, i.e. restricted to the *hb* domain. Such defects are seen (albeit rarely and more weakly) in heterozygous embryos (even when from wild-type mothers), indicating that they are due to zygotic expression, presumably in the posterior domain. Although we do not directly detect such expression, we note that the domains of gap-gene action extend into domains where protein levels are immunologically undetectable (Gaul and Jäckle, 1989; Pankratz *et al.* 1989, 1990; Stanojevic *et al.* 1989; Hülskamp *et al.* 1990).

hb–h

Although *hb–h* causes female lethality by interfering with sex determination (Parkhurst *et al.* 1990), the viability of *hb–h* males shows that the ectopic *h* does not cause segmentation defects (even in two doses, unpublished observations). This is unexpected as *h* and *eve* are both primary pair-rule genes that can affect each others patterning (Ingham and Gergen, 1988). Indeed, *hb–eve* affects *h* patterning, although *eve* pattern is

normal in *hb–h* embryos. This may indicate that *h*'s role in embryonic patterning is subsidiary to that of *eve*. Alternatively, the embryo could be less sensitive to ectopic *h* because *h*'s targets are under more redundant control (see below).

Redundancy

Previous experiments have demonstrated roles for upstream sequences in regulating pair-rule striping. The *ftz* zebra element is able to direct striped expression of reporter genes, albeit predominantly in the mesoderm (Hiromi and Gehring, 1987), and putative negative regulatory elements have been defined within this region, including potential *eve* binding sites (Dearolf *et al.* 1989*b*).

h and *eve* striping appear to be regulated differently from *ftz*. Upstream domains appear to control specific individual stripes, presumably by sensing regionalised spatial cues (e.g. gap genes; Howard *et al.* 1988; Struhl, 1988). Upstream *h* elements can confer striped expression on a reporter gene construct, indicating that they include both positive and negative elements (Pankratz *et al.* 1990; G. Riddihough and M. Lardelli, personal communication). Our experiments indicate that there are also repressor sites downstream of the transcription start, i.e. that stripe repression is under redundant control. A likely reason for redundant control is to achieve the necessary precision of striping to allow precise phasing between different pair-rule genes. *h* and *eve* show similar stripe domains except that each *h* stripe is 1–2 cells anterior of each *eve* stripe (Carroll *et al.* 1988). Such displaced phasings could arise because *h* and *eve* sense similar positional cues, but with slightly differing affinities for their signals. Such striping would involve upstream repressor elements acted upon by gap genes proteins. The final stripe phasings would be achieved by the action of other pair-rule genes acting, at least in part, through downstream elements. Our analysis of *h* striping patterns in embryos mutant for other segmentation mutations has suggested that both mechanisms may operate, i.e. that the control is redundant (Hooper *et al.* 1989). Such redundancy might be required to define stripes with precise phase relationships.

Gene mis-expression – a final cautionary note

Two further messages come from these experiments. First, the unexpected viability of the fusion genes indicates the difficulty of predicting the outcome of simple mis-expressing constructs, and the need to test them before embarking on more complex strategies. Second, mis-expression experiments can give extremely mis-leading impressions of wild-type function. The female lethality of *hb–h* arises despite *h*'s playing no normal role in sex determination. Although *hb–h* has proved very valuable in studying helix-loop-helix proteins and mechanisms of sex determination (Parkhurst *et al.* 1990), the results would have been misinterpreted without previous genetic evidence of wild-type *h* function. Mis-regulation experiments in genetically less-well-characterised systems (e.g. vertebrates and cultured cells) should be interpreted with caution unless wild-type function is assayed independently.

We thank Diethard Tautz for sharing unpublished information and dealing patiently with our many questions; Suki Parks for suggestions and many helpful discussions. We are grateful to Paul Martin and Barbara Luke for help with and use of the scanning electron microscope. We also thank Helen Durbin, Manfred Frasch, Ulrike Gaul, Kate Hooper, Herbert Jäckle, Henry Krause, Diethard Tautz and Michael Wilcox for antibodies, and Peter Gergen, Yash Hiromi, Herbert Jäckle, Gary Struhl and Diethard Tautz for fly stocks and DNA clones used in this study. We are very grateful to Karen Downs, Peter Gergen, Yash Hiromi, Howard Lipshitz, Suki Parks and Detlef Weigel for critical reading and comments on the manuscript. We thank our colleagues in the lab (Kate Hooper, Sheena Pinchin, Mark Wainwright, Michael Lardelli, Guy Riddihough and Ilan Davis), in the ICRF Developmental Biology Unit, at Caltech and in the fly groups at Princeton for suggestions and stimulating discussions during the course of this work. S.M.P. was supported by a postdoctoral fellowship from the Helen Hay Whitney Foundation.

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(*Accepted 7 January 1991*)