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Drosophila hairy pair-rule gene regulates embryonic patterning outside its apparent stripe domains

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SUMMARY

The *hairy* (*h*) segmentation gene of *Drosophila* regulates segmental patterning of the early embryo, and is expressed in a set of anteroposterior stripes during the blastoderm stage. We have used a set of *h* gene deletions to study the *h* promoter and the developmental requirements for individual *h* stripes. The results confirm upstream regulation of *h* striping but indicate that expression in the anterodorsal head domain depends on sequences downstream of the two transcription initiation sites. Surprisingly, the two anterior-most *h* domains appear to be dispensable for head development and embryonic viability. One partial promoter deletion expresses ectopic *h*, leading to misexpression of other segmentation genes and embryonic pattern defects. We demonstrate that *h* affects patterning outside its apparent stripe domains, supporting a model in which primary pair-rule genes act as concentration-dependent transcriptional regulators, i.e. as local morphogens.

Key words: helix-loop-helix protein, *hairy*, pattern formation, stripes, transcriptional regulation, *Drosophila*

INTRODUCTION

The *Drosophila* embryonic body-plan arises from a cascade of transcriptional regulation that subdivides the blastoderm embryo into successively more precise spatial domains (reviewed by Ingham, 1988; Howard, 1990; Small and Levine, 1991). Early maternal positional cues lead to regionalised transcription of the gap genes whose overlapping protein domains direct striped expression of the pair-rule genes. Both these classes of zygotic genes encode known or putative transcription factors that directly regulate the transcriptional domains within the syncytial blastoderm embryo.

The transduction of early regionalised signals into an accurate metameric (reiterated) pattern can be considered to arise in three stages. First, gap genes define serially repeated domains of pair-rule transcription, each stripe responding to different combinations of overlapping gap proteins (Howard et al., 1988; Goto et al., 1989; Harding et al., 1989; Rushlow et al., 1989; Stanojevic et al., 1989; Howard and Struhl, 1990; Pankratz et al., 1990; Riddihough and Ish-Horowicz, 1991; Small et al., 1991; Stanojevic et al., 1991; Small et al., 1992). Three pair-rule genes, *hairy* (*h*), *even-skipped* (*eve*) and *runt* have been termed the 'primary' pair-rule genes because they respond directly to gap gene cues, not to other pair-rule genes (Carroll and Scott, 1986; Howard and Ingham, 1986; Ingham and Gergen, 1988). This view derives initially from studies of *h* chromosomal translocations that disrupt upstream *h* sequences and cause loss of specific *h* stripes (Howard et al., 1988). Further studies show that striped expression of *h* and *eve* requires about 14 kb and more than 8 kb of upstream DNA respectively, within which lie independent *cis*-acting transcriptional regulatory elements ('stripe elements') that control individual stripes (Howard et al., 1988; Goto et al., 1989; Harding et al., 1989; Rushlow et al., 1989; see below).

Second, the reiterated signals provided by primary pair-rule genes direct striped expression of 'secondary' pair-rule genes. Thus, a small regulatory region, the 0.6 kb 'zebra element' immediately upstream of the site of *fushi tarazu* (*ftz*) transcriptional initiation, is sufficient to regulate striped expression of this pair-rule gene (Hiromi et al., 1985; Hiromi and Gehring, 1987; Dearolf et al., 1989; Ueda et al., 1990). Primary pair-rule gene expression appears largely independent of other, 'secondary' pair-rule genes.

Finally, primary and secondary pair-rule genes together define the expression patterns of segment polarity genes, such as the adjacent one cell-wide stripes of *wingless* (*wg*) and *engrailed* (*en*) that establish and maintain the boundaries between the parasegmental metameres within which subsequent pattern is refined (DiNardo and O'Farrell, 1987; Ingham et al., 1988).

More definitive evidence for relatively independent stripe elements within the *h* promoter comes from recent experiments using *lacZ* reporter genes. For most *h* stripes, independent though overlapping transcriptional regulatory elements can be defined that sense regionalised signals and

direct *lacZ* transcription in positions roughly corresponding to *h* stripe domains (Howard and Struhl, 1990; Pankratz et al., 1990; Riddihough and Ish-Horowicz, 1991). Such gene fusions are sensitive to gap gene mutations, consistent with gap proteins acting directly to regulate *h* striping (Riddihough and Ish-Horowicz, 1991). *eve* striping appears similarly controlled, and an *eve* stripe 2 element has been demonstrated to include multiple binding sites for candidate gap protein regulators (Small et al., 1991; Stanojevic et al., 1991).

The above studies demonstrate the effects of upstream stripe elements on a heterologous promoter, usually that of a heat-shock gene, but are unable to examine aspects of pair-rule striping that depend on the endogenous *h* promoter or on downstream sequences. Indeed, none of the *h-lacZ* constructs express in the anterodorsal (AD) *h* domain in the head, and several *lacZ* reporter constructs show imprecise striping indicative of missing regulatory elements (Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991). Such reporter-gene constructs are also unable to reveal in vivo consequences of altered *h* patterning. h^- embryos suffer severe pattern deletions that probably result from *h*'s role in regulating secondary pair-rule genes. Sequential loss of *h* stripes leads to successively more extensive cuticular pattern deletions (Howard et al., 1988), but the function of individual pair-rule stripes has not yet been examined in detail.

In this paper, we analyse the in vivo activity of a set of *h* deletion mutations constructed in vitro. These cause loss of individual *h* stripes, consistent with striped expression being regulated by an array of independently acting upstream elements within the *h* promoter. The results also demonstrate that the AD domain of *h* expression is dependent on sequences downstream of the *h* promoter, and that the two anterior domains of *h* expression are not required for cephalic development. By analysing the requirements of individual *h* stripes for cuticular patterning and segmentation gene expression, we show that *h* affects embryonic patterning outside apparent stripe domains. We interpret these results in terms of a model where primary pair-rule genes act as local morphogens that define accurate boundaries of their target genes.

MATERIALS AND METHODS

Plasmid constructions

The techniques employed for the construction of the plasmids used in this study are documented in (Sambrook et al., 1989). The *neo** vector is a derivative of *pUChsneo* (Steller and Pirrotta, 1985) that was modified as follows (S. M. Parkhurst, personal communication). The *Xba*I site in the *hsp70* promoter of the *neo* gene was destroyed by filling-in with Klenow polymerase and religation. A polylinker (see below) was then inserted between the *Sal*I and *Bam*HI sites. The *Sal*I site was mutated during this process and can no longer be restricted by this enzyme, so the polylinker thus has the sites (in order); *Apa*I, *Nco*I, *Sfi*I, *Xho*I, *Not*I, *Xba*I, *Bam*HI. The polylinker sequence is:

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5′-TCGAGGGCCCCATGGGCCTCGAGTGCGGCCGCTTCTAGAG-3′
    3′-CCCGGGGTACCCGGAGCTCACGCCGGCGAAGATCTCCTAG-5′
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To construct Δ*h-14.0*, the 14.3 kb *Xho*I-*Xba*I fragment from the genomic clone *λh6* (Howard, 1986) was subcloned between the *Xho*I and *Xba*I sites of the *neo** polylinker to form *h5′X*. 21.4 kb of the *h* locus was reconstructed by subcloning the 7.1 kb *Xba*I fragment from *λh3* containing the *h* protein-coding sequences (Howard, 1986) into the *Xba*I site of *h5′X*.

The 5′ deletion series was then generated from *Δh-14.0*. Constructs are named according to the length of DNA they retain upstream of the upstream initiation site of *h* transcription, 492 bp from the start of *h* translation (Fig. 1; Rushlow et al., 1989). The plasmid was linearised with *Xho*I and partially restricted with either *Hpa*I (for *Δh-8.2*, and *Δh-4.9*), *Mlu*I (for *Δh-11.1*), *Kpn*I (for *Δh-6.9* and *Δh-0.2*), or *Sal*I (for *Δh-3.4* and *Δh-1.8*). The plasmid ends were blunted with Klenow or T4 DNA polymerase, as appropriate, *Xho*I linkers (12-mer, New England Biolabs) were attached, and the plasmids recircularised. To construct *Δh-9.2*, we isolated the upstream 3.8 kb *Eco*RI-*Mlu*I fragment, attached an *Xho*I linker to the filled-in *Eco*RI end, and then ligated it into *Xho*I and *Mlu*I restricted *Δh-14.0*.

MluINV and *MluIΔ* were constructed by inverting or deleting DNA between the two *Mlu*I sites in *Δh-14.0*. *KpnINV* and *KpnIΔ* were generated by inversion or deletion of DNA between the two *Kpn*I sites.

Analysis of *h* transgenes

We analysed the embryonic activity of X and 2nd chromosome transgenes in the absence of the endogenous *h* gene by crossing to *Df(3L)*h^{i22}*/TM3* flies and self-crossing the *transgene*/+; h^{i22}/+ progeny. Where possible, *h* expression patterns were examined from at least two lines of each construct, additional lines being analysed if expression from the first two lines differed. Transgenes on the 3rd chromosome were recombined with h^{i22}. To analyse the *h* expression pattern produced by *cosh* we examined embryos from *cosh*; h^{IL79}/h^{i22} flies. h^{i22} is a deletion of the *h* coding regions (Ish-Horowicz et al., 1985); h^{IL79} is an extremely hypomorphic allele (Ingham et al., 1985b; Wainwright and Ish-Horowicz, 1992).

We demonstrated that *Δh-14.0* rescues *h* embryonic lethality by mating *Δh-14.0* h^{IL79}*/TM3* and *Δh-14.0* h^{i22}*/TM3* flies and scoring for *Δh-14.0* h^{IL79}/*Δh-14.0* h^{i22} progeny. These are identified by the strong *h* bristle phenotype that is not rescued by our transposons (Rushlow et al., 1989). Rescue by *Δh-6.9* in combination with *KpnIΔ*, and by *KpnIΔ* alone, was demonstrated by generating *Δh-6.9*; *KpnIΔ* h^{i22}/*KpnIΔ* h^{IL79} and *KpnIΔ* h^{i22}/*KpnIΔ* h^{IL79} flies respectively. Rescue by *KpnINV* was demonstrated by generating *KpnINV*/+; *KpnINV* h^{i22}/h^{c1} flies (the two independent *KpnINV* inserts used are each lethal when homozygous) where h^{c1} is a null allele (Holmgren, 1984; Ingham et al., 1985b; Wainwright and Ish-Horowicz, 1992).

Analysis of embryos

Embryos were collected, handled and analysed according to standard methods (Wieschaus and Nüsslein-Volhard, 1986). Immunohistochemical staining of proteins used second antibodies directly coupled to alkaline phosphatase (Jackson ImmunoResearch Laboratories inc.) and Substrate Kit II (Vector Laboratories). Embryos were mounted in JB-4 methacrylate (Polysciences) and photographed using Nomarski optics. *runt* expression in *MluIΔ* h^{i22} embryos was visualised by whole-mount in situ hybridization performed essentially as described by Tautz and Pfeifle (1989).

PCR mapping of P-element insertions into the *h* locus

Genomic DNA was purified from adult flies by standard techniques (Ashburner, 1989). A pair of PCR reactions were performed for each insertion, each including a *h* primer directed upstream toward the upstream *h* cap site and either a left 'P_L' or right 'P_R' P-element primer directed out of the transposon (Fig.

4A). PCR was performed in 50 μl as previously described (Erlich, 1989): 50 cycles of 94°C/1 minute; 58°C/2 minutes; 72°C/3 minutes. Only one of the P element primers yields an amplified fragment, defining the orientation of the insertion; fragment length maps the site of transposon insertion. Initial mapping used *h*-#3568 (corresponding to DNA between positions +717 and +696 downstream of the upstream cap site; Rushlow et al., 1989) in combination with either the P_L or P_R primers. Mapping was refined by using closer primers and then testing whether amplified fragments included diagnostic restriction sites (see legend to Fig. 4A).

P_L	5′ GTGTATACTTCGGTAAGCTTCGG 3′
P_R	5′ AGCATACGTTAAGTGGATGTCTC 3′
h-#3568	5′ GGTTCGCTTCAGTTAATCCAC 3′
*h*5′to3′	5′ CCTACGAACCTGTCGATCAA 3′
*h*TIS^{UP}	5′ TCTCGGCGGAAGACTGACGA 3′
*h*TIS^{DN}	5′ ATACGGCAGGCGAATGAGCA 3′

Germline transformation

Germline transformants were generated using a mixture of 500 μg/ml transgene plasmid and 100 μg/ml *pΠ27.1* injected into *bw; st* embryos, selected on G418-containing food (Spradling, 1986; Parkhurst et al., 1990). Inserts were mapped and balanced as previously described (Riddihough and Ish-Horowicz, 1991).

Analysing interactions between gap genes and *MluI*Δ

MluIΔ^{1}*/TM3* virgin females (an *MluIΔ* line showing a high frequency of cuticular defects) were mated to balanced males carrying the segmentation gene mutation of interest. Flies were classified into three categories according to the extent to which tergites T2 and T3 were fused: complete, partial or normal. Flies were assessed as 'normal' if no defects in the 2nd or 3rd tergites could be observed. 'Complete' fusions of the 2nd and 3rd tergites showed continuous pigmentation at the posterior margin of the fused tergite, and no other 2nd/3rd tergite remnants. All other flies were declared 'partial' fusions.

Sequence analysis

This analysis was performed in collaboration with Tom Kidd. Sequences extending 150 bp upstream and downstream of each *h* transcriptional initiation site were compared using the Intelligenetics ALIGN program. The most significant homologies are shown in Fig. 4E.

RESULTS

Upstream *h* regulatory elements

A 28 kb DNA fragment from the *h* gene (*cosh*) rescues *h* segmentation mutations and therefore includes the regula-

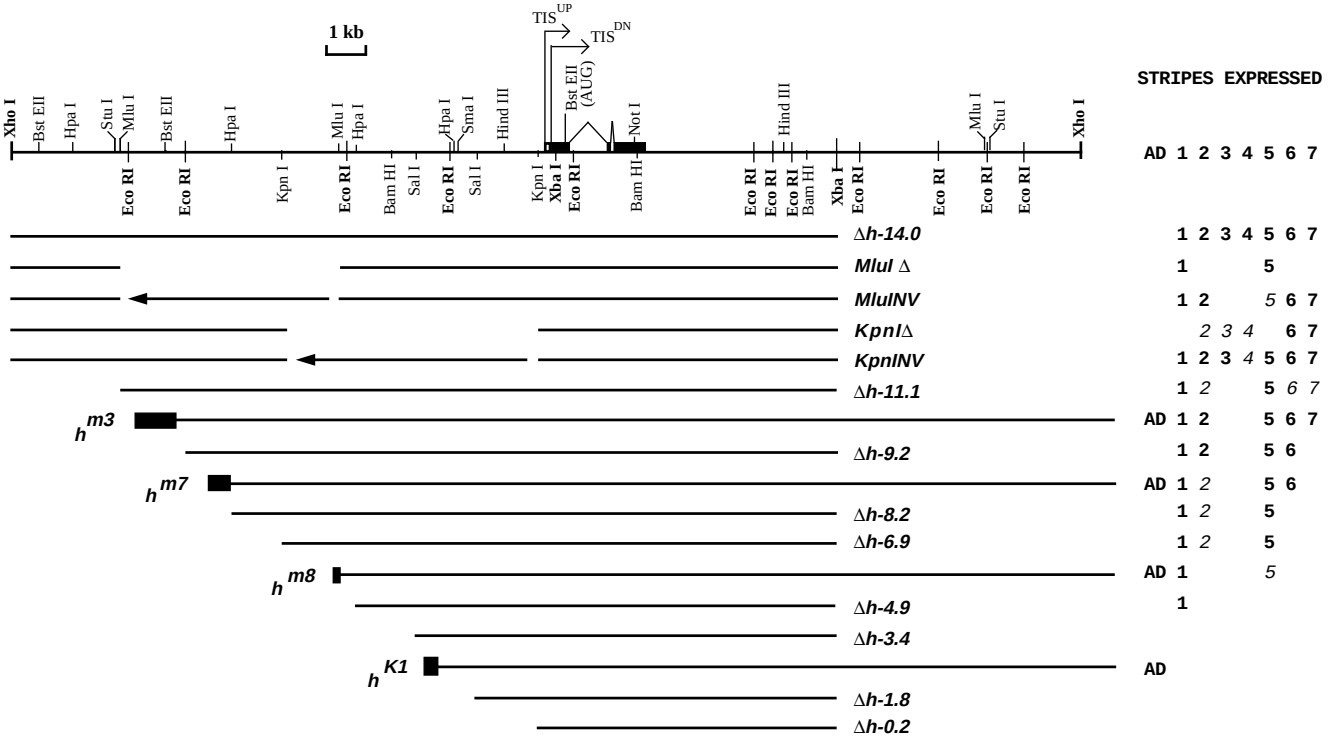

Fig. 1. Physical map of the *h* locus. The 28 kb of *h* DNA included in *cosh* that rescue embryonic *h* function are shown at the top of the diagram. The third chromosome centromere lies to the right. Not all *SalI* sites are shown, and mapping of *BstEII* and *HpaI* sites is complete only for the region of DNA included in Δ*h-14.0*. Map positions are measured from the major site of transcription initiation, which is 196 bp upstream of the minor (~10%) site of blastoderm transcription (see Fig. 4A). The bars show regions included in the constructs: inverted regions are indicated by left-pointing arrows (see also Materials and Methods). The *h* stripes produced by each construct are indicated on the right. Those in italics are expressed abnormally weakly (see text). Also presented are more precise upstream breakpoints (within the black boxes) of the previously described chromosomal rearrangements affecting *h* transcription: h^{K1}, h^{m3}, h^{m7}, and h^{m8} (Howard et al., 1988; Lardelli, 1991). TIS^{UP} and TIS^{DN} represent the upstream (major) and downstream sites of transcript initiation, respectively (Ish-Horowicz et al., 1985; Rushlow et al., 1989).

tory sequences necessary to drive expression in stripes at the blastoderm stage (Fig. 1; Rushlow et al., 1989). We defined upstream sequence requirements for *h* expression by analysing a series of 5′ deletion mutations derived from *Δh-14.0* which contains the same 14 kb of upstream DNA as *cosh*, but lacks 6.9 kb of downstream sequence (Fig. 1). Our results are generally in accord with previous studies (Howard et al., 1988; Howard and Struhl, 1990; Pankratz et al., 1990; Riddihough and Ish-Horowicz, 1991) showing that more extensive 5′ deletions cause the sequential loss of specific *h* stripes (Figs 1, 2).

Two internal deletions, *KpnIΔ* and *MluIΔ*, and two upstream inversions, *KpnINV* and *MluINV*, also lack specific *h* stripes (Fig. 2E,F). In all cases, the patterns of missing stripes are in accord with previously mapped stripe-controlling elements (Figs 1, 2C-F, 3A). With the exception of *MluIΔ* (see below), expression from repositioned or reorientated stripe elements remains accurate, as judged by the normal cuticular patterning directed by the deletion and inversion constructs (not shown; Lardelli, 1991).

Stripe 2 appears to be regulated by multiple dispersed elements, because it is expressed in two almost complementary constructs, *Δh-6.9* and *KpnIΔ* (Fig. 2C,D). This finding is consistent with the progressive reduction in stripe 2 expression in h^{m7}, *Δh-8.2*, *Δh-6.9* and h^{m8} (Howard et al., 1988), and with the reporter gene analysis.

Ectopic *h* expression in the *MluI*Δ construct is associated with pattern deletions

In embryos carrying the *MluIΔ* construct, stripe 5 is broadened anteriorly such that *h* protein extends into the region between *h* stripes 4 and 5 (‘interstripe’ 4/5; Fig. 3A-C). This ectopic expression leads to dominant pattern defects; all homozygous *MluIΔ* embryos are affected in the most strongly affected transformant line. *MluIΔ* larvae and adults are viable but show fusion of the A2 and A3 segments, correlating with the domain of *h* misexpression (Fig. 3G-I).

Expression of other segmentation genes is altered in *MluIΔ* embryos. The misexpression of *h* in interstripe 4/5 leads to complete loss of *ftz* stripe 4, and weakening of *runt* stripe 4; in contrast, *eve* stripe 4 is broadened (Fig. 3D-F). These interactions are consistent with previous studies of pair-rule interactions showing that *h* regulates *ftz* and *runt* negatively, and *eve* positively (Carroll and Scott, 1986; Howard and Ingham, 1986; Ish-Horowicz and Pinchin, 1987; Ingham and Gergen, 1988). Nevertheless, we note that *runt* and *h* are coexpressed in the broadened *h* stripe (see Discussion).

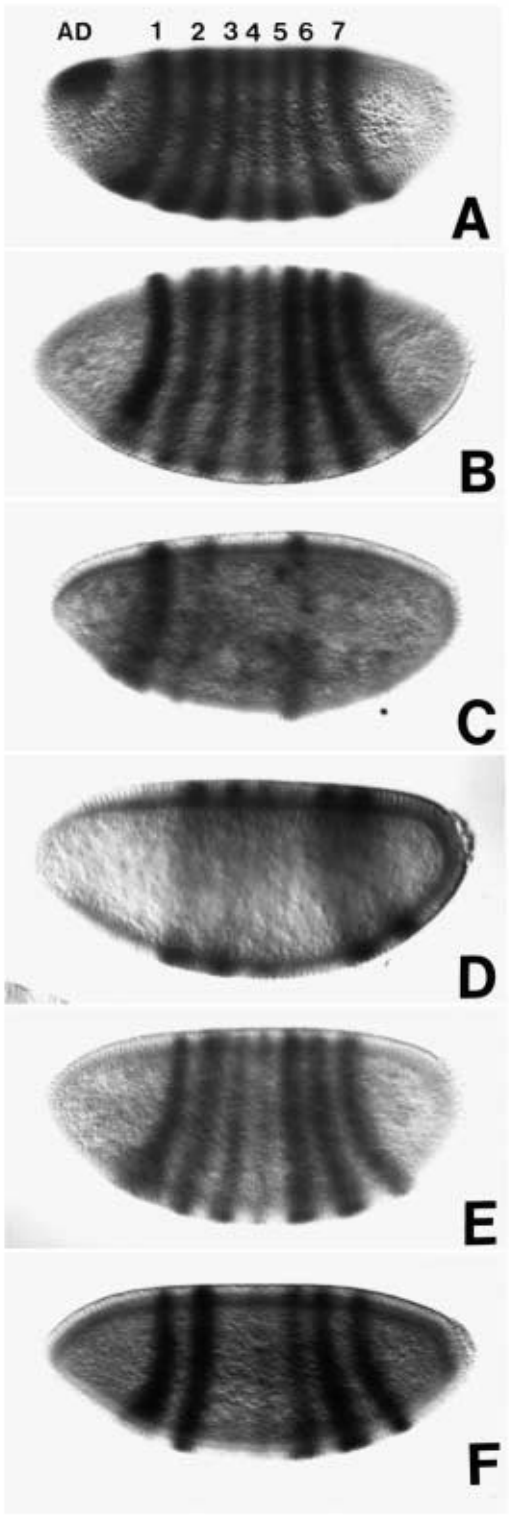

Fig. 2. *h* protein expression in Δ*h-14.0* and its derivatives. Endogenous *h* activity is eliminated with $Df(3L)h^{i22}$ or h^{IL79} (Materials and Methods). In this and subsequent figures, top-left is anterodorsal. (A) *cosh;* h^{IL79}/h^{i22} embryo showing a normal *h* pair-rule expression pattern of seven stripes (1-7) and the anterodorsal (AD) expression domain. (B) Δ*h-14.0* h^{i22} embryo showing the seven stripes of *h* expression but no AD expression. *h* expression in stripes 2, 3, and 4 is slightly reduced, probably due to the effects of the chromatin surrounding the insertion site of the transgene. (C) Δ*h-6.9;* h^{i22} embryo showing normal stripes 1 and 5 and weak stripe 2. (D) *KpnIΔ* h^{i22} embryo. Stripes 1 and 5 are absent, and stripes 2, 3, and 4 are weakened (see Results). (E) *KpnINV* h^{i22} embryo. All seven *h* stripes are present despite the inversion of DNA between the *Kpn*I sites at –0.2 kb and –6.9 kb. Stripe 4 is weak, possibly because sequences required for its full expression have been inverted. (F) *h* expression in *MluINV* h^{i22} embryo. Stripes 3 and 4 are absent because the inversion disrupts sequences required for their expression. The weak stripes 3 and 4 are due to *h* antibody cross-reactivity which is only evident after extended staining (also seen in $Df(3R)h^{i22}$ embryos; not shown).

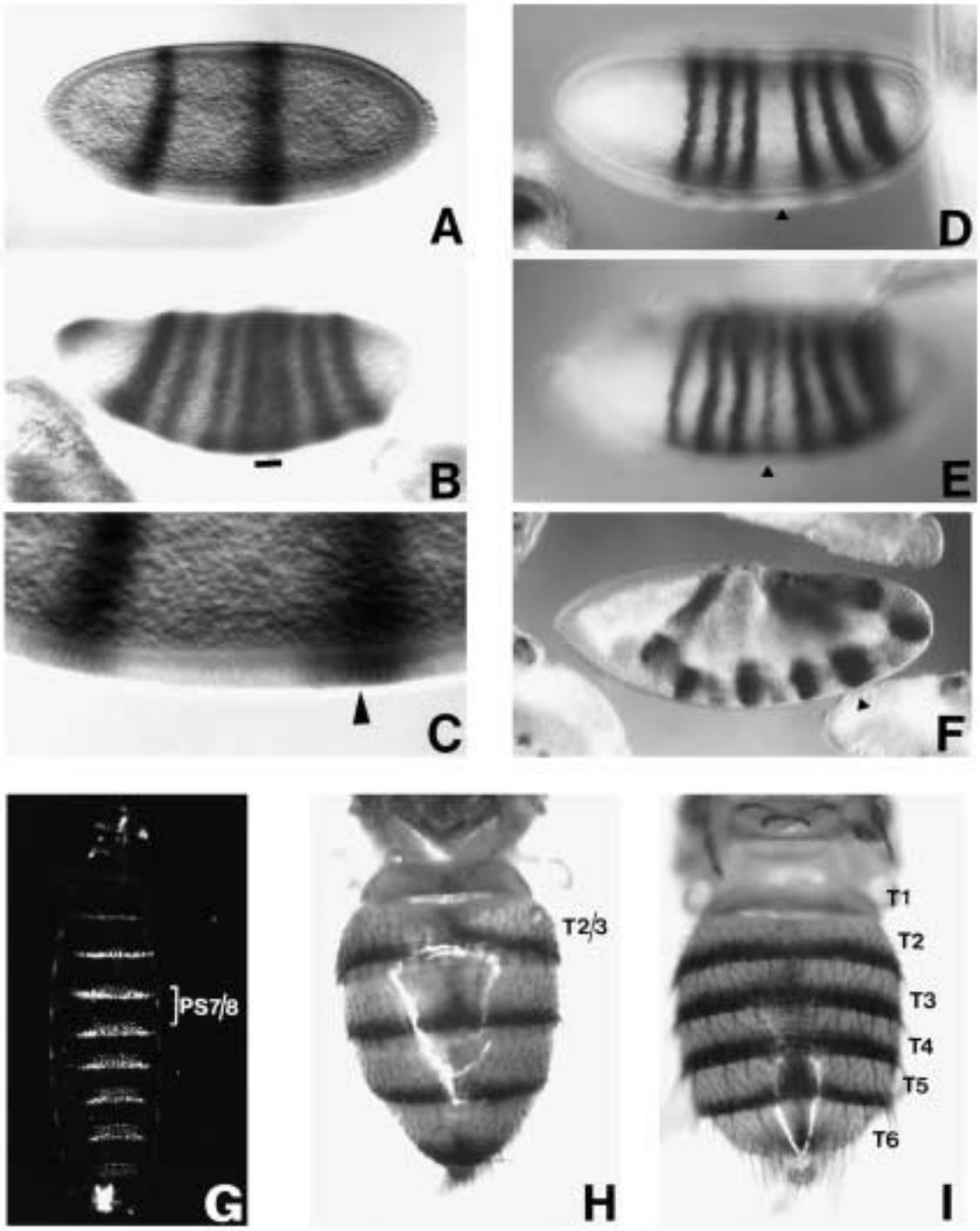

Fig. 3. Cuticular patterning and segmentation gene expression in *MluIΔ* embryos. (A) *h* expression in *MluIΔ* h^{i22} embryo: stripe 1 is normal, but stripe 5 is broadened anteriorly (see B and C). (B) Homozygous *MluIΔ* embryo retaining endogenous *h* genes. Ectopic expression from the *MluIΔ* transgene extends across the entire 4/5 *h* interstripe region (indicated by the black bar). (C) Ventral close-up of the *MluIΔ* h^{i22} embryo in A. The anterior of stripe 5 is visible as a boundary between high and low levels of *h* expression (arrow-head). Altered pair-rule expression in *MluIΔ* embryos: (D) *ftz* stripe 4 is missing (arrowhead), (E) *runt* stripe 4 is narrowed (arrowhead), and (F) slightly broader *eve* stripe 5 (arrowhead) in early extending germ-band embryo. This slight expansion is difficult to see in younger embryos and may indicate *h* action is indirect. *runt* expression was visualised using in situ hybridization. (G) *MluIΔ* embryo lacking the A3 denticle belt due to fusion of PS7 and PS8. (H) A2/3 fusions in *MluIΔ* fly, and (I) corresponding wild-type abdomen. Tergites (T) are numbered.

h expression from the *MluIΔ* construct is not uniform, being normal within the stripe 5 domain and weaker in the interstripe (Fig. 3C). As *h* is expressed at wild-type levels within the stripe, the control elements that drive stripe 5 expression are still functional. Thus, *h* expression in the interstripe region appears to be due to novel activating

Table 1. *MluIΔ* phenotypes and gap-gene dosage

Male genotype	Progeny	Normal	Partial	Complete	% fused A2/3	Probability (χ^2)
$kni^{IIV}/TM3$	$kni^{IIV}/MluI\Delta^{1}$	1	41	73	**99**	$P<<0.005$
	$MluI\Delta^{1}/TM3$	23	60	5	**73**	(80.31)
	$kni^{IIV}/TM3$	170	5	0	3	
Kr^{1}/Sco	$Kr^{1}/+;MluI\Delta^{1}/+$	46	7	0	**13**	$P<<0.005$
	$Sco/+;MluI\Delta^{1}/+$	61	56	6	**50**	(21.19)
	$Kr^{1}/+;TM3/+$	69	0	0	0	
	$Sco/+;TM3/+$	122	1	0	1	
$gt^{YA}/FM7$	$gt^{YA}/+;MluI\Delta^{1}/+$	13	91	24	**90**	$P>0.1$
	$FM7/+;MluI\Delta^{1}/+$	5	89	17	**95**	(3.64)
	$gt^{YA}/+;TM3$	143	0	0	0	
	$FM7/+;TM3$	126	0	0	0	
$hb^{PXT15}/TM3$	$hb^{PXT15}/MluI\Delta^{1}$	26	55	5	**70**	$P>0.1$
	$MluI\Delta^{1}/TM3$	44	77	2	**64**	(3.12)
	$hb^{PXT15}/TM3$	120	0	0	0	

Column 6 lists the totals of flies showing partial or complete A2/A3 fusions. The figures in bold represent the critical genotypes. The probabilities were calculated by comparing the classified distributions for the critical pair of genotypes.

sequences being transposed adjacent to the stripe 5 element, not to the disruption of repressor sequences. *MluINV* and h^{m8} share similar breakpoints to *MluIΔ* but do not expand stripe 5 (Figs 1, 2F; Howard et al., 1988).

The distal *MluIΔ* breakpoint lies within sequences required for expression of *h* stripes 3 and 4 (Howard and Struhl, 1990), so translocating them adjacent to the stripe 5 element. Such a fusion of two elements could lead to an inappropriate response to gap gene cues that regulate patterning in this region of the embryo, in particular, to *knirps* (*kni*) and *Krüppel* (*Kr*). We tested this idea by studying the effects of varying gap-gene dosage on the *MluIΔ* segmentation phenotype. The results imply that *h* misexpression in *MluIΔ* is activated by Kr^{+} and suppressed by kni^{+}, features that are consistent with the expected regulation of *h* stripes 3 and 4. The frequency and severity of A2/3 segment fusions in *MluIΔ*/+ flies is strongly enhanced by heterozygosity for *kni*, and suppressed by heterozygosity for *Kr* (Table 1). In contrast, heterozygosity for *hunchback*(*hb*) or *giant* (*gt*) does not affect *MluIΔ* penetrance (Table 1).

h transposon insertion mutations argue for downstream regulatory elements

Previous studies of upstream *h* sequences have failed to define upstream sequences that drive AD expression (Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991). Our *h* constructs indicate that downstream sequences are required. Like *cosh*, *Δh-14.0* activates *h* in all 7 stripes, and rescues *h* mutations. However, it fails to drive *h* expression in the anterodorsal AD domain (Fig. 2A,B; Materials and Methods), presumably due to the absence of 6.9 kb of downstream sequence.

Further evidence that AD expression requires downstream elements comes from several *h* mutations in which upstream P-element insertions weaken striped but not AD expression. $h^{P(Lac,ry+)L43a}$ (Fasano et al., 1988), $h^{58.2c}$ (A. Martinez Arías, personal communication), and $h^{2715.1}$, (Hiromi et al., 1986) are transposon insertions into the *h* gene (Fasano et al., 1988; Riddihough and Ish-Horowicz, 1991). $h^{58.2c}$ and $h^{2715.1}$ embryos express *h* normally in the AD domain but only weakly in the stripes, as do some but not all *P(Lac,ry+)L43a* embryos (Fig. 4B-D).

We determined the precise location of the transposons using the polymerase chain reaction (PCR; see Materials and Methods). Our results show that they are inserted very near the two *h* transcription start sites: $h^{2715.1}$ and $h^{58.2c}$ lie 105±15 bp and <12 bp, respectively, upstream of the distal initiation site; *P(Lac,ry+)L43a* is inserted <12 bp upstream of the proximal initiation site, in the 5′-leader of the longer *h* transcript (Fig. 4A). Such transposon insertions would selectively affect upstream stripe elements, rather than downstream AD regulatory elements, consistent with the observed expression patterns.

$h^{58.2c}$ and *P(Lac,ry+)L43a* retain weak *h* expression despite the adjacent transposon insertions, suggesting that both promoters depend on downstream sequences. Computer analysis reveals extensive DNA sequence similarities extending 89 bp downstream of each cap site (Fig. 4E; Materials and Methods). The first 49 bp of each transcript are 61% identical, suggesting that these downstream sequences may be functionally important, presumably recognising similar factors needed for *h* transcriptional initiation.

Expression of *h* in the anterodorsal patch and first stripe is not required for embryonic and adult viability

Despite its lack of *h* expression in the AD domain, *Δh-14.0* rescues *h* mutant embryos. *Δh-14.0* h^{-} embryos are viable and have normal head structures (Fig. 5B; see Materials and Methods). The AD domain corresponds to the labral primordium, so we re-examined head morphology in h^{i22} embryos. Contrary to a previous report (Ingham et al., 1985b), we found that the labrum is not absent (Fig. 5E), but deformed according to the ‘dorsal pouch syndrome’ described by (Jürgens et al., 1986). Thus, labral development and embryonic viability are not dependent on *h* AD expression.

We also examined embryonic viability in the absence of *h* stripe 1. Surprisingly, it also appears dispensable; indeed,

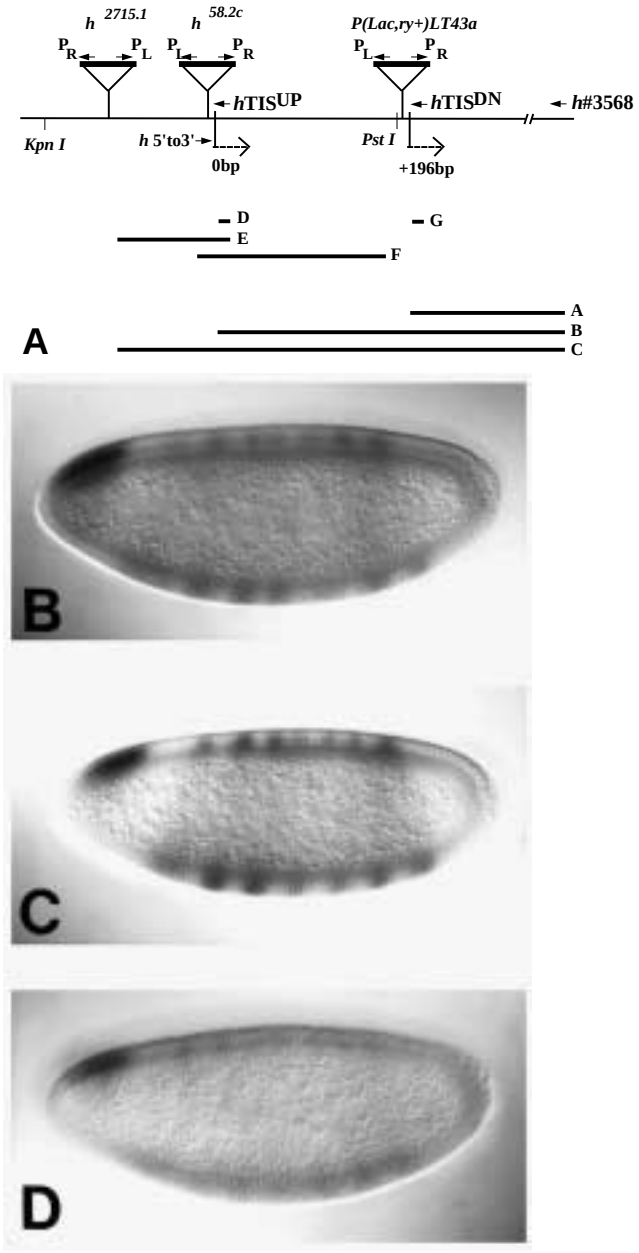

Fig. 4. Position and orientation of P-element insertions into the *h* promoter and their effect on *h* expression. (A) P-element insertion sites and orientation as determined by PCR mapping (see Materials and Methods). The P-elements are represented by the thick lines. The small arrows indicate the locations of the various PCR primers and their designations (see Materials and Methods). The large arrows indicate transcripts and their two start-sites (Fig. 1). Diagnostic fragments generated by PCR are indicated by horizontal bars labelled A-G. The generation of fragments A, B, and C using *h*-#3568 and the P-element primers P_L or P_R established the orientation and approximate position of the three transposon insertions. Primers *h*TIS^{UP} and *h*TIS^{DN} correspond to the first 20 bp downstream of the upstream and downstream cap sites, respectively. The generation of fragment G by primers *h*TIS^{DN} and P_R, and the ability of *Pst*I to cleave fragment F generated from primers *h*5′to3′ and P_L (not shown), confirms that *P(Lac,ry+)L43a* is inserted within 12 bp immediately upstream of the downstream cap site. Similarly, the $h^{58.2c}$ insertion site is within 12 bp of the upstream cap site because Fragment D, produced by primers *h*TIS^{UP} and P_R on $h^{58.2c}$ DNA, is smaller than fragment G (not shown). Fragment E, produced by primers *h*TIS^{UP} and P_L, shows that $h^{2715.1}$ is inserted 90-120 bp upstream of TIS^{UP}. *h* protein expression patterns in: (B) $h^{2715.1}$, (C) $h^{58.2c}$ and (D) *P(Lac,ry+)L43a* embryos stained with anti-*h* antibody. *h* expression appears normal, or only slightly reduced, in the AD domain but is severely reduced in the stripe domains. The majority of *P(Lac,ry+)L43a* homozygous embryos show severe reduction of *h* expression in both AD and stripe domains (not shown). (E) Sequence alignment of the two *h* leaders showing the extensive sequence homology. The Inr-like motifs are underlined (see Discussion).

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   5151      5160       5170      5180      5190       5200      5210      5220      5230
TISUP  TCGTCAGTCTTCCGCCG-AGAGCCCAGCGATAAGGTAGTCCCGCTACGCTCCGCAACATCCAGACCGAGTAAAGCAAATACTTATATATA 5239
       |  |||  ||  |  |||| |   |  |||| |  |||  |  |||| ||||  |  |  | | |  |  | |  ||  ||  |    | | |
TISDN  TGCTCATTCGCCTGCCGTATTTCGTAGCG-TGCGGTTCTATCGCTCCGCTTTGATAAACCGAATCGAAATCTAGAGAACCCCCCCAGACA 5435
   5347          5360      5370       5380      5390       5400      5410      5420      5430
```

E

embryos lacking both the AD and stripe 1 *h* domains are viable, albeit at a low frequency. 2% of h^- embryos containing two copies of the *KpnIΔ* construct hatch and survive to adulthood (6/492; see Materials and Methods), and 34% (17/50) have normal head structures. The adult flies display the *h* bristle phenotype that is not rescued by *h* transgenes (Rushlow et al., 1989; Materials and Methods), and lack one abdominal segment due to the missing stripe 5. Viability is low because most *KpnIΔ* h^- embryos express insufficient stripes 2, 3, or 4, disrupting essential head and thoracic and/or abdominal segments (Fig. 5D).

The dispensability of stripe 1 for head development is confirmed by the examination of h^{i22} embryos. Despite the grossly disorganised heads, they retain all appropriate cuticular structures derived from the mandibular and maxillary segmental anlagen that lie within *h* stripe 1 (Fig. 5E-H).

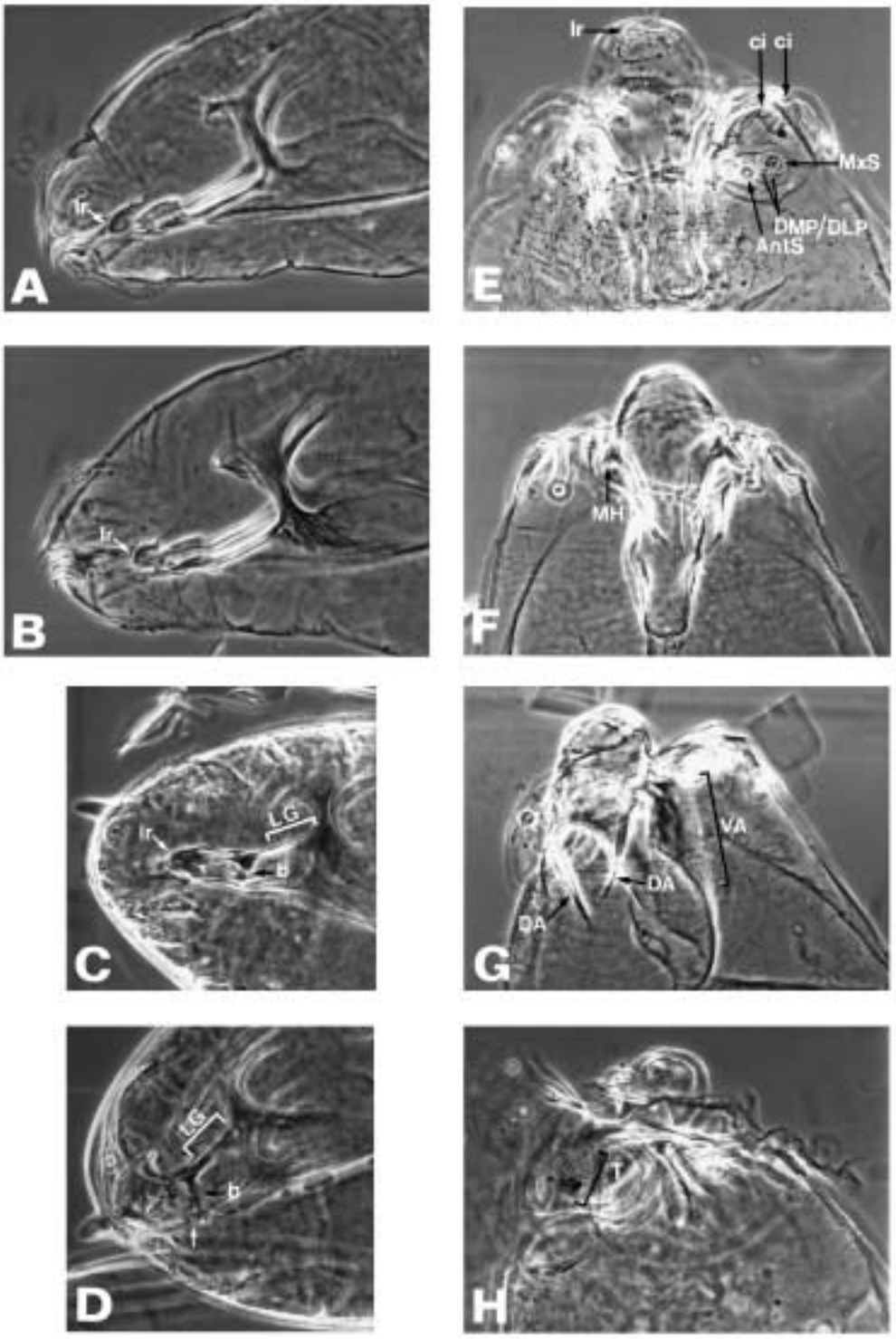

Fig. 5. Head structures in *h* mutant larvae. (A) Lateral view of the head cuticle of a wild-type first instar larva showing the labrum (lr). (B) Similar view of a $\Delta h\text{-}14.0\ h^{i22}/\Delta h\text{-}14.0\ h^{IL79}$ first instar larva showing the labrum (arrowhead) despite the absence of blastoderm AD *h* expression. (C) Lateral view of the head skeleton of a wild-type embryo in the vitelline membrane. The bridge (b) of the H-piece and the lateralgräte (LG, see white bracket) are visible. (D) Similar view of a $KpnI\Delta\ h^{i22}$ embryo showing partial breakdown (white arrowhead) of the anterior lateral bars of the H-piece due to weak *h* stripe 2 expression causing disruption of the labial/maxillary segment boundary. (E-H) The massively disrupted head cuticles of h^{i22} embryos. E shows a typical dorsal pouch syndrome (Jürgens et al., 1986; Lardelli, 1991) caused by the failure of the lateral bars and bridge of the H-piece of the cephalopharyngeal skeleton to form. A deformed labrum is evident as well as cirri (ci), the maxillary sense organ (MxS) from the maxillary segment, and the dorsomedial and dorsolateral papillae (DMP/DLP) and the antennal sense organ (AntS) from the antennal segment. F shows a mouth-hook (MH) that arises from the maxillary segment while the ventral arms (VA, bracketed) arising from the mandibulary segment and the dorsal arms (DA) arising from the acron can be seen in G. The T-ribs (T, bracketed) in the floor of the pharynx that arise in the mandibular segment can be seen in H. The lateralgräte are usually too severely disrupted by the dorsal pouch syndrome to be discernible in h^{i22} embryos, but are visible in $KpnI\Delta\ h^{i22}$ embryos (see D).

h mutations affect patterning outside the *h* stripe domains

Analysis of the cuticular pattern in embryos carrying our *h* deletions has allowed us to relate different pattern deletions associated with specific stripes of *h* expression (Table 2). Some *h* stripes affect only a single metameric boundary; e.g. loss of stripe 7 removes the PS12/13 parasegmental boundary (compare $MluINV;\ h^{i22}$ and $\Delta h\text{-}9.2;\ h^{i22}$ embryos; Fig. 6B,C; Table 2). However, certain *h* stripes are required for more than one boundary, including primordia that lie outside the stripe domain. Lack of stripe 5 ($KpnI\Delta\ h^{i22}$ embryos) removes both the PS8/9 and PS9/10 parasegmental borders (fusion of segments A3-A5; Fig. 6D). h^{m3} embryos display fusions of the T2/A1 or A1/A3 denticle bands (PS5/6 and PS 7/8; Ingham et al., 1985b; Howard et al., 1988), whose primordia also lie outside *h* stripe domains.

These pattern defects are preceded by the inappropriate

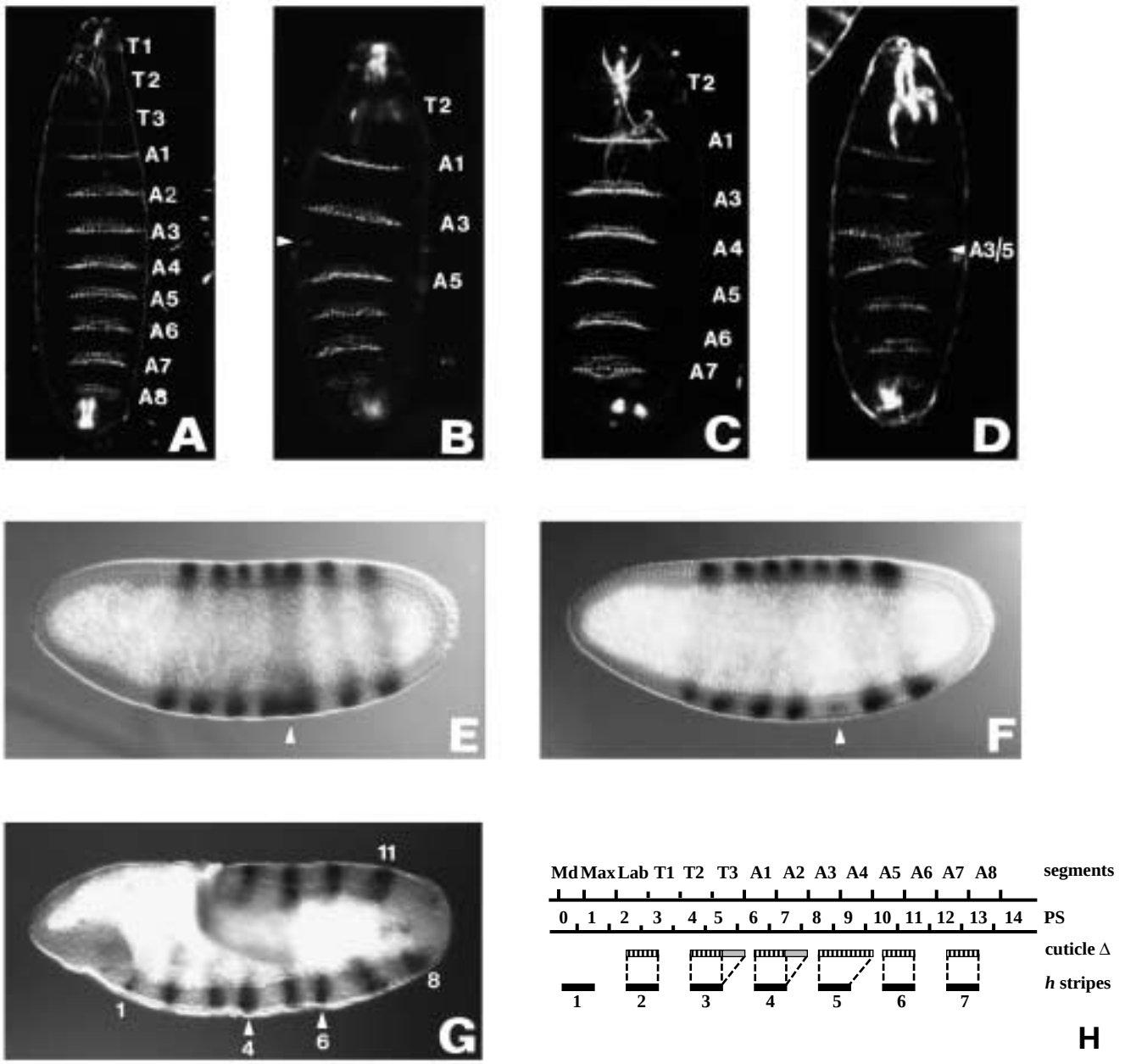

Fig. 6. Cuticular patterning and segmentation gene expression in *h* deletion larvae. (A) Normal cuticle pattern of a Δ*h-14.0* h^{i22}/Δ*h-14.0* h^{IL79} first instar larva. Thoracic and abdominal denticle belts T1-3 and A1-8 are indicated. (B) *MluINV;* h^{i22} embryo lacking the T3 and A2 denticle belts and A4 being almost completely absent (arrowhead). (C) Δ*h-9.2;* h^{i22} embryo whose cuticle pattern resembles that of the embryo in B but also lacks the A8 denticle belt. The A4 denticle belt is complete, unlike in *MluIΔ* embryos. (D) *KpnIΔ* h^{i22} embryo showing loss of A4 and partial fusion of the A3 and A5 denticle belts (arrowhead). Note the normal head cuticle. (E-G) Segmentation gene expression in *KpnIΔ* h^{i22} embryos. (E) Ectopic *ftz* expression (arrowhead) occurs between *ftz* stripes 4 and 5 where *h* stripe 5 is absent; lack of *h* stripe 1 does not lead to ectopic *ftz* expression. (F) Sagittal section showing that *eve* stripe 5 expression (arrowhead) is greatly diminished ventrally, and partially diminished dorsally because *h* stripe 5 is absent. *eve* stripe 1 is slightly weakened due to lack of *h* stripe 1. (G) *en* expression in *KpnIΔ* h^{i22} embryo. Loss of *h* stripe 5 largely abolishes *en* stripes 9 and 10. Alternate anterior *en* stripes are displaced (arrowheads), causing pairing of stripes. (H) Relationships of *h* stripes to parasegmental domains. Shown diagrammatically is the approximate correspondence between the pair-rule pattern of *h* expression in the blastoderm embryo, and the pattern of deletions developing in the cuticle of an h^{-} 1st instar larva, relative to the segmental and parasegmental domains and the ventral denticle pattern. Lightly shaded regions of cuticle deletion represent less frequent deletion events (see text). Md, Max, and Lab are mandibular, maxillary, and labial segments respectively. T1-T3 are thoracic segments. A1-A8 are abdominal segments.

expression of other pair-rule genes. *ftz* expression in *KpnIΔ* h^{i22} embryos is expanded anteriorly into the *h* interstripe 4/5 domain, often fusing with stripe 4 (Fig. 6E). *eve* stripe 5 is almost completely repressed (Fig. 6F). The disruptions of pair-rule expression lead to loss of *en* stripes 9 and 10, the latter lying within *h* interstripe 5/6 (Fig. 6G). Thus, *h*

Table 2. The cuticular effects of deleting individual *h* stripes

Stripe	Data source	Cuticular phenotype*	PS b'daries affected
7	*Δh-9.2; h^{i22}*	A8$^-$	PS12/13
6	*Δh-8.2 h^{i22}*	A6$^-$	PS10/11
5	*KpnIΔ h^{i22}*	A3/A5 fused	PS8/9 PS9/10†
3 and 4	*h^{m3}*	T3$^-$ and A2$^-$ T2/A1 and A1/A3 fusions†	PS4/5; PS6/7; PS5/6†; PS7/8†
2	*KpnIΔ h^{i22}*	Defective H piece	PS2/3
1	*KpnIΔ h^{i22}*	No cuticular effect	–
AD	*Δh-14.0 h^{i22}*	No cuticular effect	–

*Effects on ventral denticle belts.
†Frequently affected. Other boundaries are always affected.

is required to establish parasegmental boundaries that lie outside its apparent stripe domains.

Our results are most simply explained if nuclei in the interstripes domains are exposed to low levels of *h* protein which are physiologically significant but immunohistochemically undetectable. Target genes would be differentially regulated according to their sensitivity to *h* protein concentration. Such a concentration-dependent model has been proposed to explain various patterning defects induced by ectopic *runt* and *eve* expression (Gergen and Wieschaus, 1986; Manoukian and Krause, 1992; see Discussion).

Additional evidence that *h* acts in a concentration-dependent manner comes from the displacement of *en* stripes when *h* expression is altered. *h* stripes 2, 3 and 4 are severely weakened in *KpnIΔ h^{i22}* embryos (Fig. 2D). This leads to shifting and pairing of alternate *en* stripes (Fig. 6G), resembling that caused by reduced *eve* activity (Frasch et al., 1988). Such displacement of expression domains is a characteristic response to changes in morphogen concentration (Driever and Nüsslein-Volhard, 1988; see Discussion). The differential responses of *ftz* and *runt* to interstripe *h* expression in *MluIΔ* embryos (Fig. 3D,E) are also consistent with concentration-dependent *h* action.

DISCUSSION

h transcription requires both upstream and downstream control elements

Our analysis of *h* deletions complements the mapping of stripe elements in the *h* promoter, and confirms that sequences regulating blastoderm *h* transcription are spread over almost 30 kb (Fig. 1; Howard and Struhl, 1990; Pankratz et al., 1990; Riddihough and Ish-Horowicz, 1991). Upstream *h* elements confer similar expression domains on both the *hsp70* and *h* promoters, although perhaps not with the same precision (see below).

The majority of stripe elements behave discretely, with particular deletions abolishing expression of specific stripes. However, the stripe 2 element appears to be partially degenerate and dispersed over a region of several kb. A series of deletions broken in the region between –9.2 kb and –4.9 kb weaken, but do not abolish stripe 2 (Howard et al., 1988; this paper), and two essentially complementary deletions (*KpnIΔ* and *Δh-6.9*) each express stripe 2 weakly. This explains why small upstream fragments do not drive stripe 2 reporter gene expression (Howard and Struhl, 1990). Such degeneracy may not be exclusive to stripe 2 and may be inherent in defining the regulatory thresholds that appear to define stripe boundaries (e.g. Small et al., 1991; Stanojevic et al., 1991; Small et al., 1992). The molecular mechanisms of such transcriptional thresholds are likely to depend on multiple binding sites, as has been defined for transcriptional activation by *bicoid* of the *hunchback* (*hb*) promoter (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989).

While upstream elements regulate the expression of *h* in stripes, expression in the anterodorsal (AD) head-domain requires downstream sequences. Not only do constructs lacking downstream sequences fail to activate AD *h* expression, but transposon insertions adjacent to the transcription start sites (*$h^{58.2c}$*, *L43a*) selectively reduce *h* expression in the stripes (Fig. 4C,D). Such insertions displace upstream sequences away from the *h* cap site but leave downstream regions unaffected, suggesting that AD-specific expression is regulated by the latter. We have not yet succeeded in defining discrete elements that drive reporter gene expression in an AD domain (M. L., unpublished observations).

Basal *h* promoter activity probably also depends on downstream sequences. Computer analysis reveals extensive sequence conservation downstream of the two *h* cap sites, suggesting that they are recognised by similar regulatory factors (Fig. 4E). Both promoter sites lack TATA consensus sequences (Rushlow et al., 1989), and 3 bp downstream of each initiation site lies a $TCA^{G}/_{T}TC$ sequence resembling the Inr motif described for mammalian TdT and various TATA-independent *Drosophila* transcripts (Smale and Baltimore, 1989; Arkhipova and Ilyin, 1991). A requirement for downstream sequences may explain why upstream *h* elements confer slightly imprecise expression domains on an *hsp70* promoter (Riddihough and Ish-Horowicz, 1991). *h* expression from our deletion constructs must be accurate because they rescue cuticular patterning (Lardelli, 1991).

Inessential domains of *h* expression in the head

Surprisingly, the AD and stripe 1 domains of *h* expression appear dispensable for embryonic and adult viability. *KpnIΔ h^-* embryos lack these domains, yet can form normal heads and, occasionally, viable adults. This finding led us to re-examine *h* mutant embryos, and to show that all head structures, although grossly disorganised, appear to be retained (Fig. 5E-H). Either *h* expression in the AD domain and stripe 1 is not required for essential developmental processes, or pattern formation in these anterior regions is under redundant control. Genetic and evolutionary considerations have suggested that patterning mechanisms differ between the *Drosophila* head and body-trunk (Cohen and Jürgens, 1990, 1991; Finkelstein and Perrimon, 1991). Head-specific patterning might supplement or override requirements for anterior *h* expression. For example, anterior *ftz* expression is subject to 'polar repression' by the terminal co-ordinate system, preventing *ftz* expression anterior of 65%EL (Hiromi et al., 1985; Edgar et al., 1986). Thus, *h* stripe 1 would not be needed to repress *ftz* and,

indeed, *ftz* stripe 1 expression does not expand in *KpnIΔ* h^{i22} embryos that lack *h* stripe 1 (Fig. 6E).

Transposed regulatory elements misactivate *h* expression in *MluI*Δ

The *MluIΔ* construct expresses *h* ectopically in the 4/5 interstripe region and causes deletion of the A3 denticle belt in embryos (Fig. 3G). The pattern deletions result from altered pair-rule expression – reduced *ftz* and *runt* expression, expanded *eve* expression – that lead to loss of the PS7/8 boundary. These effects are consistent with previously demonstrated interactions between *h* and other pair-rule genes (Carroll and Scott, 1986; Howard and Ingham, 1986; Ish-Horowicz and Pinchin, 1987; Ingham and Gergen, 1988). The interstripe *h* expression probably arises from the juxtaposition of sequences required for stripe 3 and 4 close to the stripe 5 element (see Results), an interpretation that is supported by the sensitivity of *MluIΔ* to *Kr* and *kni* gene-dosage (Table 1). Thus, interstripe repression can be overcome by novel adjacent activating sequences, arguing that stripe boundaries are defined by combinations of activation and repression rather than exclusively the latter (Carroll, 1990).

The viability of *MluIΔ* flies indicates that restricted pattern deletions in the mid-abdomen can be tolerated, whereas equivalent deletions would be lethal in the head, thorax, or posterior abdomen. This is confirmed by our recovery of *KpnIΔ* h^- flies that lack *h* stripe 5 and A4 structures. Fused abdominal defects caused by reduced *h* expression are also not lethal (Ingham et al., 1985b). Ectopic segmentation gene expression in the mid-abdomen, e.g. driven by the *h* stripe 5 element, may allow analysis of segmentation gene interactions without associated dominant lethality.

Transcriptional regulation by *h* appears to be concentration dependent

Embryos completely lacking *h* activity suffer extensive pattern deletions, more severe than the prototypic pair-rule deletion of alternate metameres (Ingham et al., 1985b). We have shown that the pattern defects are due to direct effects of *h* on interstripe primordia. For example, lack of *h* stripe 5 (*KpnIΔ* h^-) causes loss of the PS9/10 boundary whose primordium lies within interstripe 4/5 (Fig. 6H). The mispatterning of interstripe primordia is not due to secondary effects on metameric stability because the pattern defects are associated with altered early patterns of segmentation gene expression (*ftz*, *eve*, *en*; Fig. 6E-G). Corresponding regional effects on *ftz*, *eve* and *en* patterns are found in h^- embryos (ML., unpublished observations).

The apparent nonautonomous action of *h* is presumably due to exposure of nuclei in the interstripes to low but physiologically significant levels of *h* protein. The effects of increased $runt^+$ gene-dosage on patterning in interstripe *runt* domains can be explained similarly (Gergen and Wieschaus, 1986). Before they are expressed in stripes, *h* and *runt* are both initially expressed at low levels throughout most of the embryo (Ingham et al., 1985a; Gergen and Butler, 1988). Nevertheless, we consider it unlikely that this early interstripe expression is important for patterning. Ectopic *h* expression at these stages appears not to cause pattern defects (Ish-Horowicz and Pinchin, 1987).

Rather, we suggest that primary pair-rule protein stripes extend into and regulate gene expression within 'interstripe' domains. It has recently been demonstrated that ectopic *eve* expression shows concentration-dependent repression of target genes, raising the possibility that primary pair-rule genes act as local morphogens in defining target gene domains (Manoukian and Krause, 1992). The boundaries of *h* and *runt* protein expression are somewhat diffuse (see Kania et al., 1990), so could lay down concentration gradients that would pattern locally. Concentration-dependent repression by *h* is consistent with the effects of *h* misexpression in *MluIΔ* embryos, in which ectopic *h* completely represses *ftz*, but *runt* expression is only affected near the margins of endogenous *h* expression where *h* concentrations are highest (Fig. 3D,E). Also, *ftz* expression is inhibited by immunologically undetectable levels of *h* protein in heat shocked *hsp70-h* embryos that do not affect *runt* expression (Ish-Horowicz and Pinchin, 1987; Gergen, personal communication). Further evidence comes from the sensitivity of *en* stripe-spacing to reduced *h* activity (Results).

Another attractive feature of this view is that it explains how the accurate phasing of secondary pair-rule genes is achieved. Subtle differences in stripe expression domains should be due either to direct interpretation of gap protein gradients, or to interactions between pair-rule genes. The former model appears unlikely because it demands that gap proteins directly establish relative positional distinctions that are accurate to the single-cell. In contrast, local gradients of primary pair-rule proteins would readily define the precisely phased secondary pair-rule stripes that specify segment-polarity domains, and thereby establish sharp metameric boundaries.

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