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In vivo interactions of the *Drosophila* Hairy and Runt transcriptional repressors with target promoters

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The Hairy and Runt pair-rule proteins regulate *Drosophila* segmentation by repressing transcription. To explore the ability of these proteins to function as promoter-bound regulators *in vivo*, we examined the effects of Hairy and Runt derivatives containing heterologous transcriptional activation domains (Hairy^{Act} and Run^{Act}). Using this approach, we find that Hairy and Runt efficiently target such activation domains to specific segmentation gene promoters, leading to rapid induction of transcription. Our results strongly suggest that Hairy normally acts as a promoter-bound repressor of *fushi tarazu*, *runt* and *odd-skipped*, and that Runt directly represses *even-skipped*. We also show that expressing Hairy^{Act} in early blastoderm embryos causes ectopic *Sex-lethal* expression and male-specific lethality, implying that the Hairy-related denominator element Deadpan represses *Sex-lethal* during sex determination by directly recognizing the early *Sex-lethal* promoter.

Keywords: basic helix–loop–helix/embryonic patterning/segmentation/sex determination/transcriptional repression

Introduction

The *Drosophila* Hairy segmentation protein is a member of a sub-family of basic helix–loop–helix (bHLH) transcriptional repressors, that also includes proteins encoded by the *deadpan* (*dpn*) gene and the *Enhancer of Split* complex (ES-C) which are involved in sex determination and neurogenesis, respectively (Klämbt *et al.*, 1989; Rushlow *et al.*, 1989; Delidakis *et al.*, 1991; Bier *et al.*, 1992; Delidakis and Artavanis-Tsakonas, 1992; Knust *et al.*, 1992; Schrons *et al.*, 1992; Younger-Shepherd *et al.*, 1992). bHLH proteins are characterized by two adjacent subdomains: a cluster of basic residues required for sequence-specific binding to DNA, and an HLH domain that mediates protein dimerization (Murre *et al.*, 1989a,b). Hairy-related transcriptional repressors show similar basic and HLH domains, and all terminate with an identical C-terminal tetrapeptide (WRPW), mutations of which largely or completely abolish repressor activity (Wainwright and Ish-Horowicz, 1992; Dawson *et al.*, 1995; this paper). They also include a putative amphipathic helical domain (Knust *et al.*, 1992), the so-called 'Orange' domain that may contribute to promoter specificity (Dawson *et al.*, 1995).

hairy (*h*) is a pair-rule gene which is expressed in stripes and regulates segmentation by subdividing the embryo into reiterated (metameric) spatial domains (reviewed in Pankratz and Jäckle, 1993). Hairy behaves as a repressor of another pair-rule gene, *fushi tarazu* (*ftz*): *ftz* stripes are broadened in *h* mutant embryos, and ubiquitous *h* expression abolishes *ftz* transcription (Carroll and Scott, 1986; Howard and Ingham, 1986; Ish-Horowicz and Pinchin, 1987). Hairy's embryonic patterning activity requires an intact basic domain (Wainwright and Ish-Horowicz, 1992), arguing that repression occurs via sequence-specific DNA binding. This is supported by the observation that consensus DNA binding-sites of homodimers of Hairy and related proteins can mediate transcriptional repression in cultured cells and in patterning adult sensory organs (bristles) (Akazawa *et al.*, 1992; Sasai *et al.*, 1992; Tietze *et al.*, 1992; Ishibashi *et al.*, 1993; Oellers *et al.*, 1994; Ohsako *et al.*, 1994; Takebayashi *et al.*, 1994; van Doren *et al.*, 1994; Hoshijima *et al.*, 1995).

Nevertheless, it has proved difficult to define the precise molecular mechanism of Hairy action during segmentation. Deletion analysis of the *ftz* promoter has defined sequences whose removal leads to partial derepression of reporter gene transcription (Dearolf *et al.*, 1989; Topol *et al.*, 1991), but none of these includes consensus Hairy-binding sites. Indeed, Hairy appears to repress transcription *in vivo* from a synthetic promoter that lacks consensus binding-sites (Tsai and Gergen, 1995). Also, *in vitro* physical associations between Hairy and Ftz (H.Krause, personal communication), and their antagonistic interactions in an assay affecting sex determination (Dawson *et al.*, 1995; see below), both raise the possibility that Hairy interferes with *ftz* autoregulation (Hiromi and Gehring, 1987) by binding Ftz protein. Thus, it remains possible that Hairy represses *ftz* independently of DNA binding. Finally, it is unclear what other segmentation genes are targets of regulation by Hairy.

In this paper, we describe a general approach for identifying direct regulation by transcriptional repressors, and use it to help distinguish between direct and indirect target genes within complex regulatory networks. We show that fusing a heterologous transcriptional activation domain to Hairy converts it from a repressor to an activator (Hairy^{Act}) that promotes transcription of specific target genes. In particular, expressing Hairy^{Act} during the blastoderm stage disrupts embryonic segmentation by driving ectopic expression of the *ftz*, *runt* (*run*) and *odd-skipped* (*odd*) pair-rule genes. Activation depends on an intact basic domain, indicating that direct regulation occurs via sequence-specific binding to DNA. We use a similar strategy to study regulation of pair-rule genes by Run, which behaves both as a positive and a negative regulator during segmentation (Manoukian and Krause, 1993; Tsai

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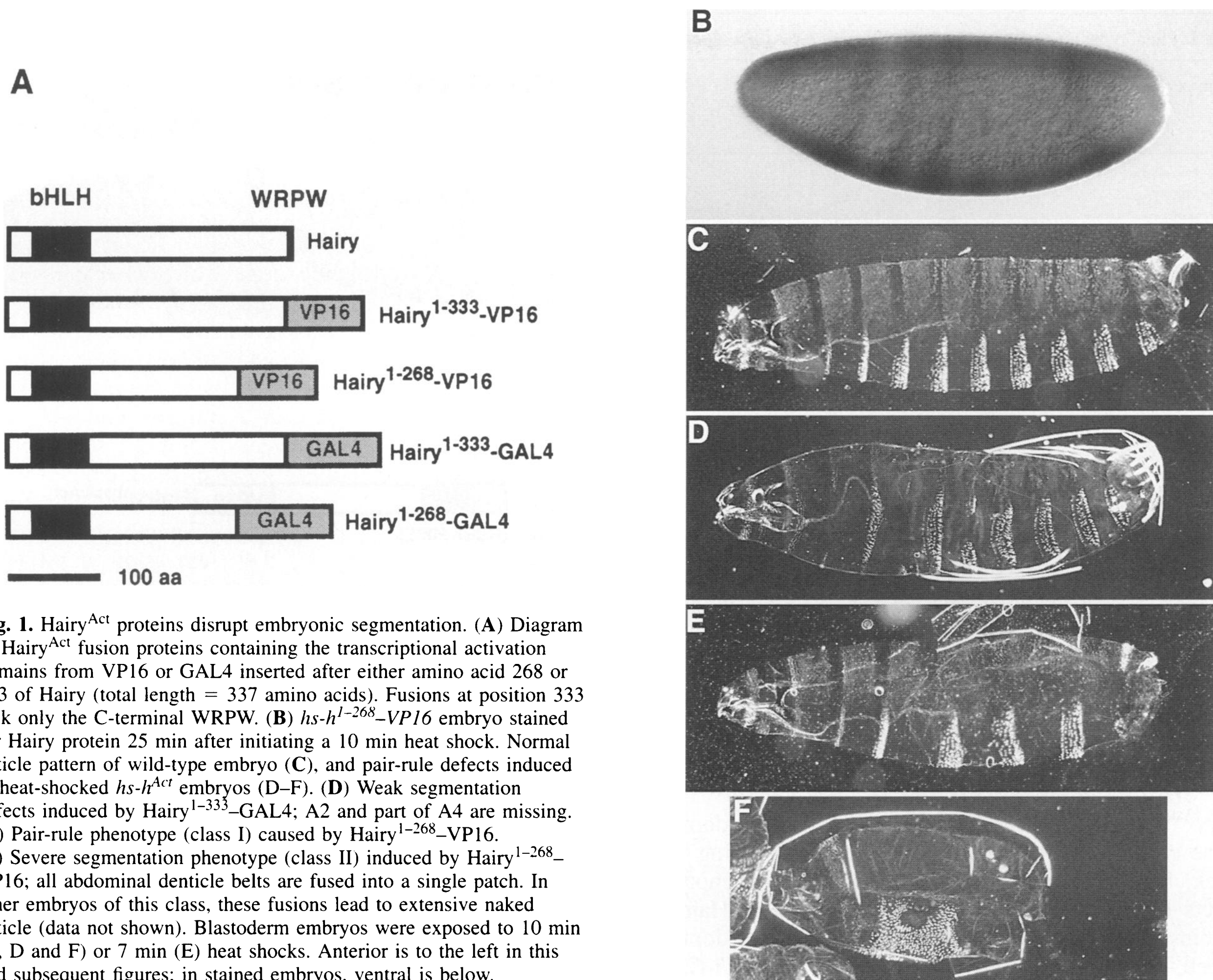


Fig. 1. Hairy^{Act} proteins disrupt embryonic segmentation. (**A**) Diagram of Hairy^{Act} fusion proteins containing the transcriptional activation domains from VP16 or GAL4 inserted after either amino acid 268 or 333 of Hairy (total length = 337 amino acids). Fusions at position 333 lack only the C-terminal WRPW. (**B**) *hs-h$^{1-268}$–VP16* embryo stained for Hairy protein 25 min after initiating a 10 min heat shock. Normal cuticle pattern of wild-type embryo (**C**), and pair-rule defects induced in heat-shocked *hs-h^{Act}* embryos (D–F). (**D**) Weak segmentation defects induced by Hairy$^{1-333}$–GAL4; A2 and part of A4 are missing. (**E**) Pair-rule phenotype (class I) caused by Hairy$^{1-268}$–VP16. (**F**) Severe segmentation phenotype (class II) induced by Hairy$^{1-268}$–VP16; all abdominal denticle belts are fused into a single patch. In other embryos of this class, these fusions lead to extensive naked cuticle (data not shown). Blastoderm embryos were exposed to 10 min (B, D and F) or 7 min (E) heat shocks. Anterior is to the left in this and subsequent figures; in stained embryos, ventral is below.

and Gergen, 1994, 1995). Our results argue that repression of the *even-skipped* (*eve*) pair-rule gene by Run is direct, and that activation of *ftz* is indirect, suggesting that Run acts predominantly as a repressor. Finally, we employ the Hairy^{Act} assay to study sex determination. Ectopic Hairy mimics the activity of Dpn in repressing early *Sex-lethal* (*Sxl*) transcription (Parkhurst *et al.*, 1990). We show that Hairy^{Act} activates *Sxl*, implying that Dpn recognizes the *Sxl* promoter directly, and excluding models for *Sxl* regulation in which Dpn functions as a passive repressor.

Results

Expression of Hairy^{Act} in blastoderm embryos induces pair-rule phenotypes

In order to convert Hairy into a transcriptional activator (Hairy^{Act}), we built four constructs in which two different Hairy C-terminal truncations were fused to two alternative activation domains: one of medium strength from the yeast GAL4 protein (Ma and Ptashne, 1987), and the very strong activation domain from the herpes simplex virus VP16 protein (Triezenberg *et al.*, 1988). These were inserted at position 269 or 334 of Hairy (Figure 1A; see Materials and methods), such that all chimeric proteins retain the bHLH and downstream helical/Orange domains but lack the C-terminal WRPW motif required for the repressive function of Hairy. *hs-h^{Act}* genes were constructed in which inducible Hairy^{Act} expression is directed by the ubiquitous *hsp70* heat-shock promoter (Materials and methods). These constructs drive Hairy^{Act} expression efficiently; the protein reaches levels similar to those of endogenous Hairy 15–25 min after the start of the heat shock (Figure 1B).

If Hairy normally represses *ftz* by binding to its promoter, Hairy^{Act} might activate *ftz* transcription, leading to preferential deletion of even-numbered segments (T1, T3, A2, A4, A6, etc.; Struhl, 1985; Ish-Horowicz *et al.*, 1989). In contrast, ectopic Hairy suppresses *ftz* expression and yields the complementary pair-rule phenotype (loss of T2, A1, A3, etc.; Ish-Horowicz and Pinchin, 1987). All four Hairy^{Act} proteins behave similarly, causing preferential deletion of even-numbered segments (Figure 1D–F and Table I), as expected if Hairy^{Act} proteins act as transcriptional activators of *ftz* expression. The two *h–GAL4* constructs cause relatively mild phenotypes in which generally one or two even-numbered segments are deleted (Table I and Figure 1D). The two *h–VP16* lines cause stronger phenotypes, and were used for subsequent experiments that are described in more detail below.

Embryos are most sensitive to ectopic Hairy–VP16 expression during the late blastoderm stage (data not shown), the time when endogenous Hairy is active. A 10 min heat shock at 36.5°C causes extensive segmental defects in >90% of 140–170 min *hs-h^{Act}* embryos. The

Table I. Phenotypes induced by Hairy^{Act}

Genotype	Number of segments deleted				Total
	WT	1–2	3–5	>5	
hs-h$^{1-333GAL4}$ a	33	41[b]	8	2	84
hs-h$^{1-268VP16}$ a	0	1	51	67	119
hs-h$^{1-268VP16}$ c	22	23	101	25	171

[a]Embryos were heat-shocked at 36.5°C for 10 min.
[b]A small proportion of embryos within this class (total = 8) showed deletions of odd-numbered segments.
[c]Embryos were heat-shocked at 36.5°C for 6 min.
WT, wild-type.

embryos can be subdivided into two classes: (I) pair-rule embryos lacking at least three even-numbered segments (Figure 1E), the phenotype expected if Hairy^{Act} activates *ftz* transcription; and (II) embryos with further loss of denticle bands (Figure 1F). Phenotypic severity depends on the length of heat shock: a 6 min heat shock predominantly generates class I or weaker embryos, whereas a 10 min heat shock induces mostly class II embryos (Table I).

An alternative explanation of these phenotypes would be that Hairy^{Act} proteins behave as antimorphic truncations which interfere with repression by endogenous Hairy protein. We excluded this possibility by expressing equivalent Hairy truncations (Hairy$^{1-268}$ and Hairy$^{1-333}$ = Hairy$^{\Delta WRPW}$) lacking the heterologous activation domain. These constructs are efficiently produced following heat shock, but their expression has little or no phenotypic effects (data not shown). Thus, the activity of Hairy^{Act} proteins depends on their transcriptional activation domain. Indeed, the relative abilities of *hs-h-VP16* and *hs-h-GAL4* to perturb segmentation parallel the efficiencies with which VP16 and GAL4 activation domains stimulate transcription in cultured cells and yeast (Sadowski *et al.*, 1988; Cousens *et al.*, 1989).

Hairy^{Act} directly activates ftz transcription

To test if Hairy^{Act} indeed activates endogenous *ftz* transcription, we followed *ftz* expression in heat-shocked 140–170 min *hs-h^{Act}* embryos. *ftz* stripes are expanded in virtually all embryos; in 50% (72/136) of embryos, the posterior five stripes are fused into a single broad domain (Figure 2B). These perturbations in *ftz* expression arise rapidly, being detectable within 25–30 min of starting the heat shock. This delay is consistent with the necessity to transcribe and translate *h^{Act}*, implying that *ftz* is a primary target of Hairy^{Act}. Activation of *ftz* is restricted to the trunk region of the embryo (Figure 2B), indicating that Hairy^{Act} is unable to overcome repression mediated by the terminal coordinate system.

These experiments suggest that Hairy^{Act} directly activates *ftz* transcription, but do not distinguish whether it recognizes the *ftz* promoter by sequence-specific contact with DNA or by interactions with other promoter-bound factors. Thus, we analysed Hairy^{Act} proteins with mutations in the basic domain that should affect sequence-specific DNA binding. First, we examined the activity of Hairy$^{\Delta basicAct}$, a Hairy-VP16 protein in which an eight amino acid deletion removes most of the basic domain (Figure 2E). This mutant protein accumulates normally

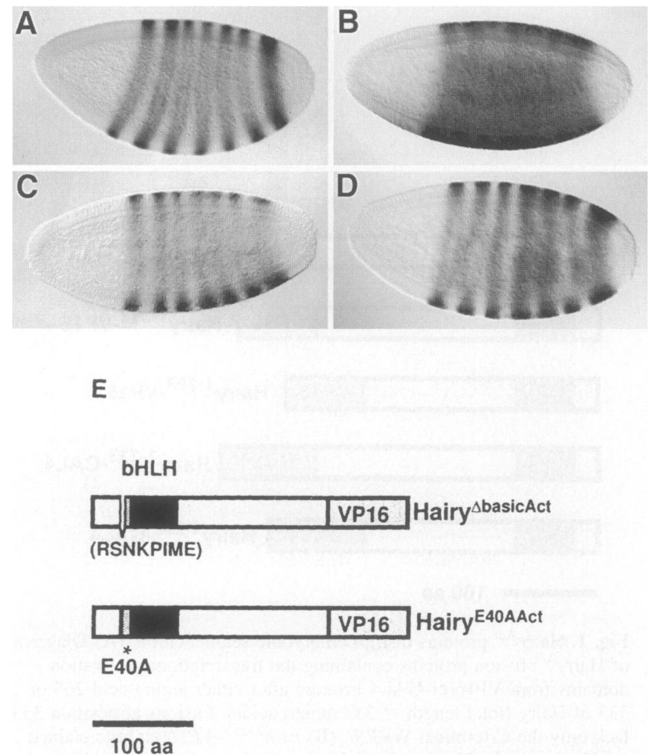
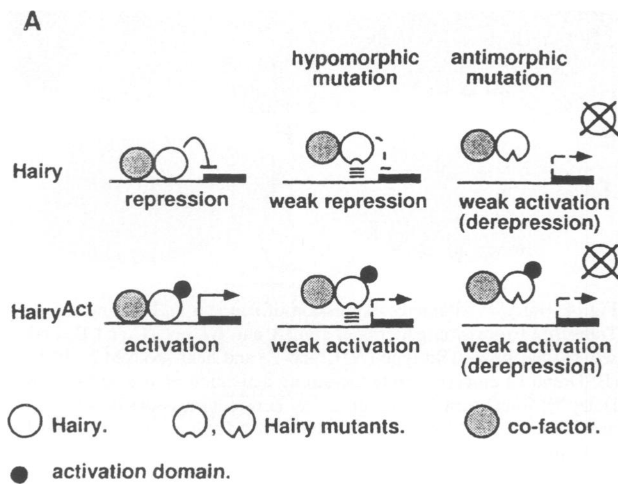


Fig. 2. Hairy^{Act} stimulates *ftz* expression and requires an intact basic region. (A–D) Effects of Hairy^{Act} proteins on *ftz* transcript expression. *ftz* is dramatically expanded in a heat-shocked *hs-h*$^{1-268Act}$ embryo (**B**) compared with wild-type (**A**). Expression is only slightly broadened in *hs-h*$^{\Delta basicAct}$ (**C**) and *hs-h*^{E40AAct} (**D**) embryos. The embryos in (B–D) were heat-shocked for 10 min and fixed after a further 20 min. (**E**) Diagram of two Hairy^{Act} derivatives with mutated basic domains: Hairy$^{\Delta basicAct}$ in which the indicated eight amino acids in the basic region have been deleted, and Hairy^{E40AAct}, which contains a single amino acid substitution of a conserved Glu.

after heat-shock induction and is efficiently translocated into the nucleus (data not shown). Nevertheless, its activity is significantly impaired: ectopic expression of Hairy$^{\Delta basicAct}$ in blastoderm embryos causes relatively weaker pattern defects (data not shown) and *ftz* expression never shows the dramatic broadening observed in *hs-h^{Act}* embryos (c.f. Figure 2B and C).

The eight amino acid deletion in Hairy$^{\Delta basicAct}$ is extreme and might also disrupt interactions between Hairy and other proteins. Indeed, the basic domain in bHLH proteins has been implicated in recognizing transcriptional cofactors (Davis *et al.*, 1990; Brennan *et al.*, 1991; Weintraub *et al.*, 1991; Davis and Weintraub, 1992). We therefore analysed Hairy^{E40AAct}, in which a single glutamic acid residue in the basic region is mutated to an alanine (Figure 2E). This Glu residue is conserved in all bHLH proteins, and appears to interact directly with DNA (Ma *et al.*, 1994), so it is unlikely to be available to mediate specific protein–protein recognition. Moreover, it is mutated in the *h^{5H}* mutation, a strong *h* allele (Wainwright and Ish-Horowicz, 1992). Hairy^{E40AAct} causes only a slight broadening in *ftz* stripes (Figure 2D), resembling that induced by Hairy$^{\Delta basicAct}$. These results suggest that Hairy^{Act}, and Hairy during normal development, regulate *ftz* by direct DNA binding to promoter regulatory sequences.



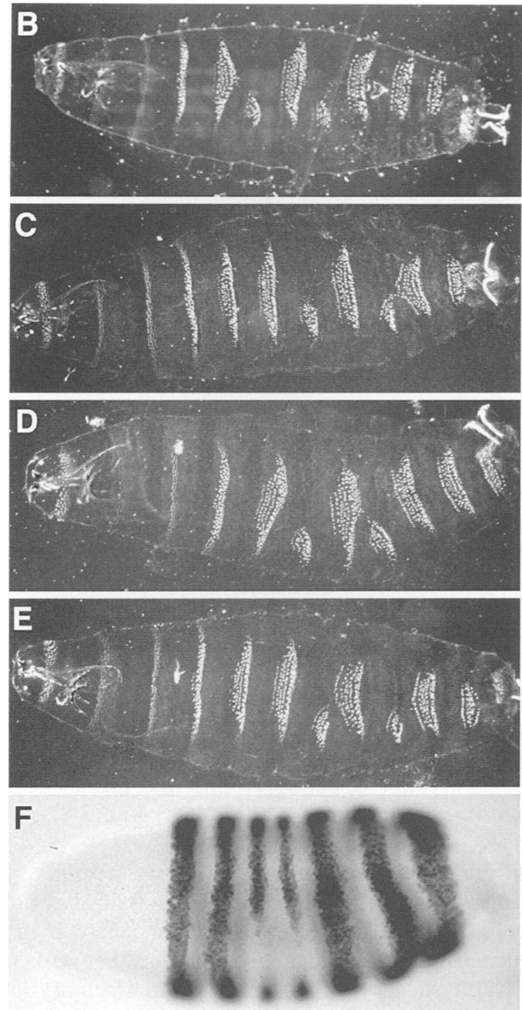


Fig. 3. Segmental defects induced by injecting synthetic *h* transcripts. (**A**) Diagram of possible outcomes induced by basic domain mutations in Hairy and $Hairy^{Act}$ proteins. Hypomorphic mutations would bind DNA less effectively; antimorphic mutations would sequester Hairy-interacting factors so that endogenous Hairy cannot act.
(B–E) Cuticular phenotypes induced by injection of *h* (**B**), h^{Act} (**C**), h^{E40A} (**D**) and $h^{\Delta basic}$ (**E**) mRNAs. *h* and h^{E40A} cause deletions of odd-numbered abdominal segments, whereas h^{Act} and $h^{\Delta basic}$ affect even-numbered segments. Injection of buffer alone causes no systematic defect (data not shown; S.M.Pinchin and D.Ish-Horowicz, manuscript in preparation). (**F**) Lack of ventral *ftz* transcript expression at site of *h* mRNA injection.

Disruption of the Hairy basic region causes antimorphic phenotypes

Both the E40A and Δbasic $Hairy^{Act}$ proteins retain a weak ability to enhance *ftz* transcription. One possibility is that the mutant proteins are hypomorphs with reduced DNA-binding activity. Alternatively, they could be antimorphic, interfering with endogenous Hairy repressor activity (e.g. by forming non-productive HLH dimers) (Figure 3A). In contrast, hypomorphic and antimorphic Hairy proteins would have opposing effects: the former would still repress *ftz* expression whereas the latter would enhance (derepress) *ftz* transcription (Figure 3A).

To test the effects of mutant Hairy proteins further, we adopted a novel rapid assay for *h* activity during segmentation (S.M.Pinchin and D.Ish-Horowicz, in preparation). Synthetic *h* mRNAs were injected from the dorsal side and deposited ventrally into 140–170 min blastoderm embryos, and their effects on ventral patterning and *ftz* expression were assayed (Materials and methods). The injected mRNAs do not diffuse significantly during the time-course of the experiment, leading to localized ectopic Hairy accumulation that peaks at ~10–15 min after the injection (data not shown; S.M.Pinchin, manuscript in preparation).

Injection of *h* mRNA leads to loss of odd-numbered denticle bands at the site of injection (Figure 3B and Table II). In these embryos, *ftz* becomes repressed locally within 20 min (Figure 3F), soon after significant levels of Hairy are translated. Injecting h^{Act} mRNA causes the complementary phenotype, defects in even-numbered ventral denticle bands (Figure 3C), showing that the mRNA injections offer a simple and faithful assay for Hairy activity during segmentation.

Table II. Frequencies of cuticular defects induced by injecting *h* mRNA

mRNA (ng/μl)	Total scoreable embryos	Embryos with deleted denticles (%)[a]	Embryos with fused denticles (%)[b]
0	128	2.3	1.6
20	123	8.9	0.8
50	93	50.5	21.5
100	148	33.8	62.8
200	128	20.4	78.1
500	100	11.0	85.0

[a]Percentage of embryos with one or two ventral denticle bands deleted but without fusion of the remaining bands.
[b]Percentage of embryos with fusions of two or more adjacent ventral denticle bands.

We used this test to study the effects of the E40A and Δbasic mutations on Hairy repressor activity. $Hairy^{E40A}$ and $Hairy^{\Delta basic}$ accumulate normally after RNA injection and are efficiently translocated into the nucleus (data not

Table III. Frequencies of cuticular defects induced by injecting h^{E40A} mRNA

mRNA (ng/μl)	Total scoreable embryos	Embryos with deleted denticles (%)[a]	Embryos with fused denticles (%)[b]
20	119	26.0	5.9
50	201	41.2	15.9
100	131	47.2	31.3
200	88	35.2	52.3

[a]Percentage of embryos with one or two ventral denticle bands deleted but without fusion of the remaining bands.
[b]Percentage of embryos with fusions of two or more adjacent ventral denticle bands.

shown), but they behave differently. Hairy^{E40A} retains weak repressor activity, preferentially affecting odd-numbered denticle bands (Figure 3D and Table III). In contrast, Hairy$^{\Delta basic}$ induces phenotypes complementary to those caused by Hairy and Hairy^{E40A}, affecting the even-numbered segments (Figure 3E). These effects are relatively mild; at 200 ng/μl only 33% of the embryos show alterations in two or more abdominal segments. Nevertheless, these results indicate that Hairy$^{\Delta basic}$ has an antimorphic action that mimics a reduction of *h* function. Thus, the similar phenotypic effects of Hairy^{E40AAct} and Hairy$^{\Delta basicAct}$ have opposite origins, the former being an hypomorph, the latter an antimorph (Figure 3A).

Hairy^{Act} also activates run and odd-skipped

ftz is unlikely to be the only direct target of Hairy during embryonic segmentation. In particular, there is genetic evidence suggesting mutual interactions between *h* and *run*. Their stripes are roughly complementary at the blastoderm stage (Kania *et al.*, 1990), and *h* mutant embryos express low levels of inter-stripe *run* (Ingham and Gergen, 1988; Klingler and Gergen, 1993). We therefore tested whether Hairy^{Act} is able to activate *run* transcription. *run* expression is expanded by 25 min after the beginning of heat shock and, by 30 min, ectopic *run* extends into the remaining interstripe regions and most *run* stripes are at least 1–2 cells wider than normal (Figure 4B). These results indicate that *run* is directly activated by Hairy^{Act}, implying that Hairy normally acts as a promoter-bound repressor of *run*.

In contrast, *eve* behaves as an indirect target of regulation by Hairy^{Act}. *eve* expression in heat-shocked *hs-h*^{Act} embryos is unaltered 25–30 min after the beginning of heat shock (data not shown). Rather, *eve* stripes 2, 4, 5 and 6 become repressed about 10 min later (Figure 4D). The extra delay in the *eve* response, and the fact that it becomes repressed rather than activated, suggests that Hairy^{Act} activates an intermediary gene which in turn represses *eve*. This gene is probably *run* because the same *eve* stripes are preferentially repressed in *hs-run* embryos (Manoukian and Krause, 1993).

We also analysed the effects of Hairy^{Act} on the *odd-skipped* (*odd*) pair-rule gene, which is expressed in similar domains to *ftz*, at least during mid-cycle 14 when both genes first become striped (Coulter *et al.*, 1990; Manoukian and Krause, 1992). *odd* expression is clearly expanded within 25–30 min of inducing Hairy^{Act} expression (Figure 4F), implying that Hairy normally represses *odd* directly.

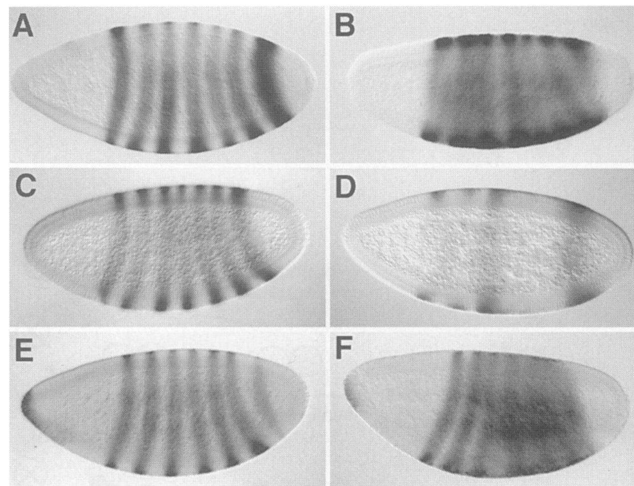


Fig. 4. Hairy^{Act} stimulates expression of *run* and *odd*, but not of *eve*. Transcript expression patterns of *run* (**A** and **B**), *eve* (**C** and **D**) and *odd* (**E** and **F**) in wild-type (A, C and E) and heat-shocked *hs-h*^{Act} (B, D and F) embryos. Note the strong activation of *run* and *odd* by Hairy^{Act}; in contrast, *eve* is repressed. Blastoderm embryos were heat-shocked for 10 min and stained after a further 20 min (B and F) or 35 min (D).

This idea is further supported by the broadening of *odd* expression in *h* mutant embryos (G.Jiménez, unpublished observations).

Finally, *h* expression is unaffected in heat-shocked *hs-h*^{Act} blastoderm embryos (data not shown), consistent with previous evidence that *h* does not directly autoregulate itself (Hooper *et al.*, 1989).

Run^{Act} activates transcription of eve, but not of ftz

During segmentation, Run also behaves as a transcriptional repressor, inhibiting expression of *eve*, *engrailed*, *giant* and *empty spiracles* (Manoukian and Krause, 1993; Tsai and Gergen, 1994). However, it has also been suggested that *ftz* transcription might be activated by Run (Tsai and Gergen, 1994, 1995). Moreover, the vertebrate Run homologue PEBP2α has been characterized as a sequence-specific DNA-binding protein that activates transcription in tissue culture cells (Ogawa *et al.*, 1993). To test whether Run acts as a repressor or an activator during segmentation, we constructed an *hs-run*^{Act} gene that directs expression of Run^{Act}, a protein chimera of the N-terminal 506 amino acids of Run (lacking the three C-terminal amino acids, RPY), and the VP16 activation domain (Figure 5A; Materials and methods). If Run normally represses its direct targets, generalized expression of Run^{Act} should have opposing effects to those of Run.

Expressing Run^{Act} in 140–170 min blastoderm embryos leads to dramatic cuticular defects, not induced by an equivalent truncated Run protein which lacks the activation domain (J.P.Gergen, personal communication). The major class of affected embryos are *ftz*-like (Figure 5B), complementary to the pair-rule phenotypes induced by ectopic Run expression (Manoukian and Krause, 1993). Most of the other embryos have more severe denticle band fusions (Figure 5C). Indeed, Run^{Act} has an opposite effect on *eve* expression to that of wild-type Run. Ectopic Run represses *eve* expression (Manoukian and Krause, 1993; Tsai and Gergen, 1994), whereas *eve* is activated in *hs-run*^{Act} embryos within 30 min of starting a heat shock (Figure

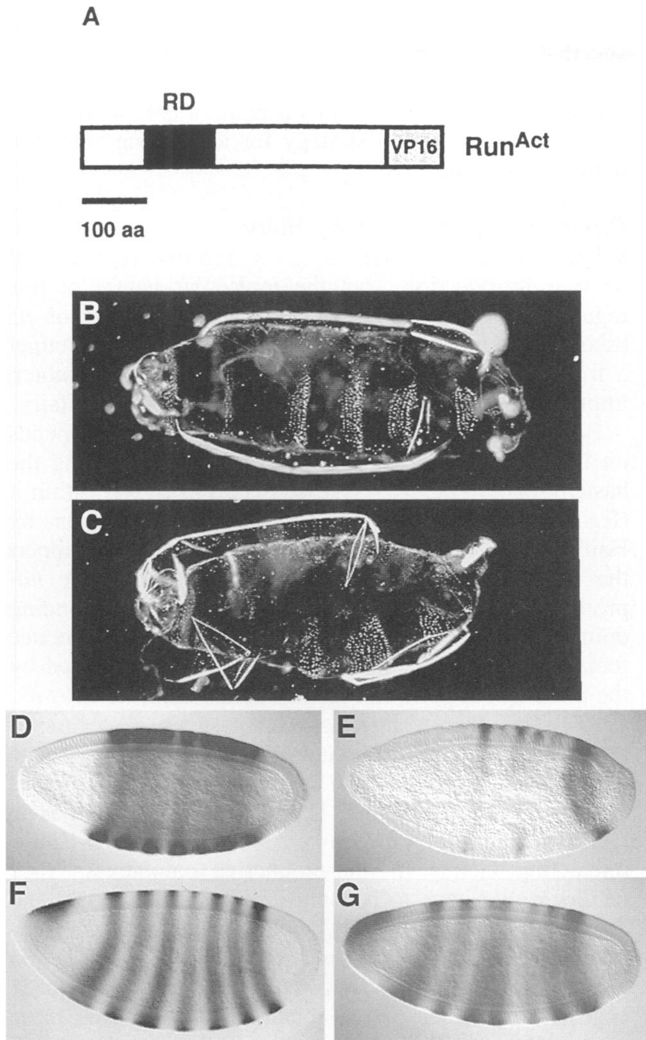


Fig. 5. Cuticle phenotypes and altered patterns of pair-rule transcription induced by Run^{Act}. (**A**) Diagram of Run^{Act} protein in which the VP16 activation domain is fused C-terminal of Run amino acid 506. The Run domain (RD) (Kagoshima *et al.*, 1993) is depicted in black. (**B** and **C**) Cuticular defects induced by heat shock in *hs-run*^{Act} embryos: (B) *ftz*-like pair-rule phenotype; (C) stronger segmentation defects in which further abdominal segments are fused. mRNA expression patterns of *eve* (expanded, **D**), *ftz* (repressed, **E**) and *h* (**G**) in heat-shocked *hs-run*^{Act} embryos. (**F**) Wild-type *h* pattern. Blastoderm embryos were heat-shocked for 10 min and fixed after 20 min (D and G) or 35 min (E).

5D). We also find that two *h* stripes (3 and 4) are broadened in *hs-run*^{Act} embryos (Figure 5F and G). This argues that Run behaves as a direct repressor of *h*, albeit only of two stripes.

Strikingly, Run^{Act} does not activate *ftz*. Rather, it causes delayed repression (Figure 5E), suggesting that Run regulates *ftz* indirectly, presumably by inhibiting expression of a repressor of *ftz*.

Hairy^{Act} *causes ectopic Sxl activation and male lethality*

We have used our strategy to probe the role of bHLH repressors in *Drosophila* sex determination. Expression of the *Sxl* sex-determining gene is initiated from a specific early promoter that is activated according to X:A ratio, the delicate balance between X-linked activator genes

Table IV. *hb-h*^{Act} causes male lethality

Genotype[a]	No. of females	No. of males
Gl/TM3	95	84
hb-h^{Act}*/TM3*	100	1
hb-h^{Act}*/Gl*	109	0

[a]Progeny of the cross: *hb-h*^{Act}*/TM3* ♀ × *Gl/TM3* ♂

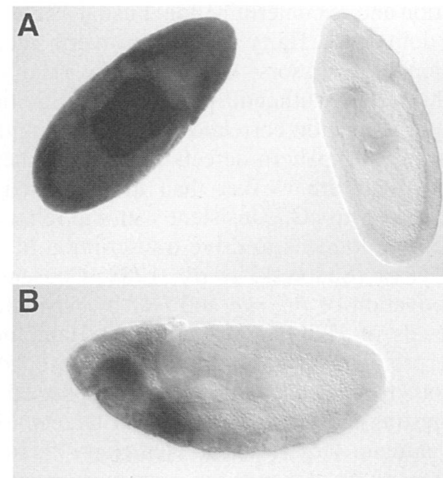


Fig. 6. $Hairy^{Act}$ induces ectopic Sxl activation in male embryos. (**A**) Sxl antibody stains wild-type presumed female (left) but not male (right) embryos (Bopp *et al.*, 1991). (**B**) Ectopic Sxl protein in the anterior domain of a male *hb-h*^{Act} embryo.

('numerators') and autosomal repressor genes ('denominators') (reviewed in Parkhurst and Meneely, 1994). *Sxl* is activated in XX females, not XY males, because females have a double dose of activator genes. Although Hairy does not normally act during sex determination, its ectopic expression during early blastoderm stages mimics denominator action, in particular that of Dpn, inhibiting *Sxl* activation in female embryos and causing female-specific lethality (Parkhurst *et al.*, 1990; Younger-Shepherd *et al.*, 1992).

If Hairy (and, by implication, Dpn) represses *Sxl* by direct interaction with *Sxl* regulatory sequences, early $Hairy^{Act}$ expression might activate *Sxl* transcription and cause male-specific lethality. We expressed $Hairy^{Act}$ under the control of the *hb* promoter, which drives ectopic expression in the anterior half of the blastoderm embryo during nuclear cycles 11–13, when X:A ratio is being specified (Parkhurst *et al.*, 1990, 1993; see also Materials and methods). In contrast to the female lethality caused by *hb-h*, *hb-h*^{Act} selectively reduces the viability of male individuals (Table IV). The degree of male lethality differs between individual transformant lines, presumably due to variable efficiencies of ectopic expression but, at its strongest, a single copy of *hb-h*^{Act} reduces the viability of male flies to $<1\%$ of that of females (Table IV).

To test whether the male-specific lethality caused by *hb-h*^{Act} is due to ectopic *Sxl* activation, we stained embryos with a monoclonal antibody that recognizes active Sxl protein which is normally present only in female embryos (Figure 6A; Bopp *et al.*, 1991). About 50% of male embryos inherit the *hb-h*^{Act} gene and show ectopic Sxl staining in the anterior (30/69; Figure 6B), indicating that

Hairy^{Act} drives *Sxl* expression. These results imply that Hairy represses *Sxl* transcription by directly recognizing the early, initiator promoter, and that Dpn acts likewise during normal development.

Discussion

In this paper, we investigate interactions between transcriptional repressors and the promoters of target genes during segmentation and sex determination. Fusing a heterologous activator domain to Hairy or Run converts them from transcriptional repressors to activators (Hairy^{Act} and Run^{Act}) that retain authentic promoter specificities. The efficiency of activation correlates with the strength of the activation domain: pattern defects induced by the Hairy–GAL4 constructs are weaker than those caused by the Hairy–VP16 constructs, consistent with the relative abilities of the two domains to drive transcription in cultured cells and yeast (Sadowski *et al.*, 1988; Cousens *et al.*, 1989). Activation of *ftz*, *run* and *odd* by physiologically normal levels of Hairy^{Act} indicates that Hairy normally acts as a promoter bound-repressor of these genes. In contrast, other genes are unaffected or repressed (e.g. *h*, *eve*), suggesting that they are not direct targets. Mutating the basic domain largely inactivates Hairy^{Act}, indicating that Hairy activity depends on sequence-specific DNA binding. These results were extended by a second, transient expression assay in which wild-type and mutant *h* mRNAs were injected into blastoderm embryos. We also show that Run^{Act} activates transcription of *eve* but not of *ftz*, implying that *eve* is a direct target of repression by wild-type Run. Finally, ectopic Hairy^{Act} activates *Sxl* expression, showing that Hairy can directly recognize the early *Sxl* promoter.

Transcriptional activation by Hairy^{Act} and Run^{Act}

There is now considerable evidence that transcriptional activation *in vivo* depends on synergistic combinations of regulatory sites and factors (Goodrich *et al.*, 1996 and references therein). Thus, it is striking that our synthetic activator proteins stimulate transcription of target genes in the developing organism. We suppose that both Hairy^{Act} and Run^{Act} retain the protein–protein and protein–DNA interactions that ensure the specificity of endogenous Hairy and Run. The Hairy^{Act} proteins we tested lack the WRPW domain, but retain the Orange domain that contributes to specificity during sex determination (Dawson *et al.*, 1995). The bHLH domain is required for dimerization and DNA binding, but may also mediate interactions with cofactors involved in promoter specificity (Davis *et al.*, 1990; Brennan *et al.*, 1991; Weintraub *et al.*, 1991; Davis and Weintraub, 1992). An ability to activate transcription may be facilitated by activator factors present on target promoters. Thus, positive factors that Hairy or Run normally antagonize (either directly or indirectly) when repressing transcription, may synergize with Hairy^{Act} or Run^{Act} in activating transcription. Inactivation of such activators by the terminal co-ordinate system may explain why Hairy^{Act} and Run^{Act} are unable to induce pair-rule expression at the termini of the embryo.

Other repressors may be less well-suited to analysis by conversion to activators, e.g. if the addition of an activation domain impairs their ability to interact with associated factors. Nevertheless, it is likely that complex networks of repression are a common developmental mechanism, and that the approach described in this paper, combined with the related approach of converting transcriptional activators to repressors (Badiani *et al.*, 1994; John *et al.*, 1995), offers a general strategy for identifying authentic regulatory interactions.

Direct regulation of ftz by Hairy

h has long been recognized as a negative regulator of *ftz* (see Introduction), but the molecular nature of this regulation has remained elusive. The activation of *ftz* transcription by Hairy^{Act} shows that Hairy is able to target a transcriptional activation domain to the *ftz* promoter, implying that the latter is normally recognized by Hairy.

Both of our assays argue that Hairy activity depends on DNA binding. Thus, either substantially deleting the basic domain (Hairy$^{\Delta basicAct}$), or mutating Glu40 within it (Hairy^{E40AAct}) sharply reduces *in vivo* activation by Hairy^{Act} (Figure 2C and D). These experiments support the work of Dawson *et al.* (1995) who used the *hb*-promoter assay to show that an intact DNA binding domain is required for Hairy to repress *Sxl* and induce female lethality. The related bHLH proteins encoded by the ES-C also require an intact basic domain (Tietze *et al.*, 1993). The mRNA injection assay shows that loss of the basic domain inactivates Hairy and generates an antimorphic protein which interferes with endogenous Hairy activity (Figure 3E). This probably results from an ability of Hairy$^{\Delta basic}$ (and Hairy$^{\Delta basicAct}$) to sequester Hairy-interacting factors in non-productive complexes. Such factors would have to become limiting in the assay, and could include Hairy itself, which forms DNA-binding homodimers *in vitro* (Ohsako *et al.*, 1994; van Doren *et al.*, 1994). Dominant-negative effects have also been observed for other bHLH mutations altering the basic region (Davis *et al.*, 1990; Brennan *et al.*, 1991; Hemesath *et al.*, 1994). In contrast, Hairy$^{\Delta WRPW}$ is not antimorphic in either the germ-line (see Results) or transient assays (S.M.Pinchin, unpublished results), suggesting that it is unable to form dysfunctional complexes. Perhaps, dimers of Hairy are functional even if they only include a single WRPW motif.

The transient expression assay shows that the E40A mutation reduces Hairy activity, presumably due to an impaired affinity for DNA. h^{5H}, in which the same Glu residue is mutated, is a severe *h* allele that retains little patterning activity (Ingham *et al.*, 1985; Wainwright and Ish-Horowicz, 1992). However, h^{5H} was induced on a h^{l}-bearing chromosome in which expression of *h* stripes 2–7 is substantially reduced due to the insertion of a *gypsy* transposon into the *h* promoter (Holmgren, 1984; Hooper *et al.*, 1989). Homozygous h^{l} embryos are viable, so the strong h^{5H} phenotype must be due to lowered expression of a partially active Hairy protein.

If Hairy regulates *ftz* by binding to specific DNA sites, some of these should lie within the 'zebra' element, a region in the proximal *ftz* promoter that drives *ftz* striping (Hiromi and Gehring, 1987). Indeed, preliminary experiments show that Hairy^{Act} can activate transcription of a reporter construct driven by the zebra element (G.Jiménez and S.M.Pinchin, unpublished observations). The zebra element DNA sequence contains two canonical Hairy binding sites that lie near the distal end of the element, but

promoter constructs lacking these sites are still expressed in stripes (Dearolf *et al.*, 1989). Perhaps the effects of removing these sites in reporter constructs is masked by repression by other factors, e.g. by Eve, which also acts as a repressor of *ftz* (Frasch and Levine, 1987; Manoukian and Krause, 1992). Alternatively, Hairy might bind different DNA sites *in vivo* from those it binds *in vitro*, e.g. as a result of associating with as yet unknown partner proteins. In either case, the Hairy^{Act} system should help detail the sites through which Hairy acts in regulating *ftz* and other target genes.

We have recently proposed that Hairy-related proteins act as repressors of transcription by recruiting Groucho (Gro) to specific promoters, a model supported by our results showing direct interactions between Hairy and target promoters. Hairy and related bHLH proteins bind Gro *in vitro* and in yeast, via their WRPW motifs (Paroush *et al.*, 1994). Gro is a nuclear protein which contains reiterated WD motifs also found in the yeast transcriptional co-repressor TUP1, but no known DNA-binding domain (Hartley *et al.*, 1988; Delidakis *et al.*, 1991). Maternal *gro* activity is required for sex determination, segmentation and neurogenesis, processes that are regulated by Hairy-related bHLH proteins (Schrons *et al.*, 1992; Paroush *et al.*, 1994). The WRPW motif is unlikely to be required for Hairy to interact with target genes because Hairy^{Act} retains accurate promoter recognition. However, the WRPW is required for Hairy activity (Wainwright and Ish-Horowicz, 1992; Dawson *et al.*, 1995; this paper), presumably to recruit Gro.

Transcriptional regulation by Run

Although Run behaves as a positive regulator of *ftz* and *Sxl*, and the vertebrate Run homologue PEBP2α activates early polyoma virus transcription (Ingham and Gergen, 1988; Duffy and Gergen, 1991; Ogawa *et al.*, 1993; Tsai and Gergen, 1994, 1995), our results suggest that Run acts predominantly as a repressor during segmentation. Were Run a direct activator of *ftz* transcription, one would have expected that introducing a VP16 activation domain would generate an even more potent activator. Rather, Run^{Act} causes delayed repression of *ftz* expression (Figure 5E), implying that it activates an intermediate gene which in turn inhibits *ftz*. *eve* is a likely candidate for such an intermediate, being activated by Run^{Act} (Figure 5D), and a known repressor of *ftz*. Manoukian and Krause (1993) have analysed the time-course of *ftz* activation by ectopic Run, and similarly concluded that Run acts indirectly on *ftz*. Indeed, Run terminates with the C-terminal tetrapeptide WRPY (Kania *et al.*, 1990) which strikingly resembles the WRPW Gro-binding motif in Hairy, raising the possibility that the two proteins share a common mechanism of transcriptional repression.

Nevertheless, Run's role as a numerator during sex determination may be due to direct activation of early *Sxl* transcription. The alternative, that Run represses a *Sxl* repressor(s) as outlined above for its 'activation' of *ftz*, appears less likely because at least one other numerator (Scute/Sisterless-b) indeed behaves as a transcriptional activator (Cabrera and Alonso, 1991), and because it is unclear if there is time for two successive rounds of transcriptional regulation before sex is determined. Further experiments with Run fusion genes will clarify whether Run activates *Sxl* directly, or if its action is indirect.

Cross-regulatory interactions among primary pair-rule genes

In classical models of the segmentation hierarchy, positional information is relayed from the gap genes to the so-called 'primary' pair-rule genes (*h*, *eve* and *run*), and thence to other pair-rule genes. Consistent with this view, expression of primary pair-rule genes largely depends on stripe-specific enhancers regulated by the gap gene products (reviewed in Pankratz and Jäckle, 1993). Genetic analyses indicate, however, an extra level of control imposed by the primary pair-rule proteins themselves, and our results imply direct recognition of *run* and *eve* regulatory sequences by Hairy and Run, respectively. These observations argue that such classical models are only a first approximation to the actual regulatory network, and that correct patterning requires additional cross-interactions to ensure precise domains of gene expression. Indeed, *run* appears also to regulate gap gene expression in the trunk of the embryo (Tsai and Gergen, 1994).

Repression between *h* and *run* has previously been proposed on the basis of their approximately reciprocal domains of expression (Ingham and Gergen, 1988; Kania *et al.*, 1990). Our results show that Hairy and Run directly repress each other's expression. However, such regulation is not strictly mutual: Hairy^{Act} has an overall effect on *run* transcription (Figure 4B), but Run^{Act} only activates *h* stripes 3 and 4 (Figure 5G). A general role for Hairy in controlling *run* stripes is consistent with the inter-stripe derepression of *run* in *h* mutant embryos (Ingham and Gergen, 1988; Klingler and Gergen, 1993). However, this ectopic *run* expression is rather weak, indicating that repression by Hairy is a minor determinant of *run* pattern. Equally, the establishment of *h* periodicity is largely independent of Run, although our results suggest that it directly controls *h* stripes 3 and 4, as has been inferred from analysis of *h* promoter elements (Hartmann *et al.*, 1994). Run may also play a role in patterning *h* stripe 1: this stripe is partially repressed by ectopic *run* (Manoukian and Krause, 1993; Tsai and Gergen, 1994). However, this stripe is unaffected in *run* mutant or *hs-run^{Act}* embryos (Hooper *et al.*, 1989). Thus, cross-regulation between *h* and *run* appears to be a secondary mechanism of control that presumably refines their regulation by gap genes.

Mutual repression also plays a role in regulating *run* and *eve* expression. Our results argue for direct interactions of Run with *cis*-controlling sequences of *eve* (Figure 5D), and ectopic Eve is a potent repressor of *run* (Manoukian and Krause, 1992). It appears that primary pair-rule cross-regulation occurs largely through transcriptional repression, a common theme throughout the segmentation gene hierarchy (Carroll, 1990; Manoukian and Krause, 1992, 1993). Although pair-rule transcription must also require activating factors, these are less well-characterized (many presumably being maternally inherited), and it is unclear to what extent they play roles in defining stripe boundaries.

Dpn denominator action is due to active repression

The ectopic activation of *Sxl* by *hb-h^{Act}* in the anterior of male embryos shows that Hairy^{Act}, and by implication

Dpn during normal development, directly recognize the early *Sxl* promoter. These results exclude models for determining X:A ratio in which Dpn functions as a passive repressor by sequestering numerator proteins (e.g. Cline, 1993; Parkhurst and Meneely, 1994; King *et al.*, 1995). Rather, it would actively participate in repression, presumably by recruiting Gro to the early *Sxl* promoter. Synergistic interactions between numerators attempt to activate the early *Sxl* promoter, and will be antagonized by synergistic interactions between denominators that set a threshold for repression. Together, these opposing influences lead to an on/off response of the *Sxl* promoter to the 2-fold difference in activator concentration between females and males.

Materials and methods

Constructs

Plasmid manipulations were performed essentially according to Sambrook *et al.* (1989). Coding segments generated by PCR were sequenced to ensure fidelity during the amplification reaction. Sequences of the primers and linkers used in the cloning are available on request.

A 0.8 kb *Hin*dIII–*Bam*HI fragment including Hairy coding sequences from amino acids 1 to 268 was isolated from *pHSH1* (Ish-Horowicz and Pinchin, 1987) and cloned into *pBluescriptII* (*pBS*; Stratagene) to generate *pBShHB*, which was used as the starting material for all Hairy derivatives expressed under heat-shock control. To generate constructs expressing Hairy$^{1-268}$–GAL4 and Hairy$^{1-268}$–VP16, PCR fragments coding for the GAL4 (amino acids 768–881) and VP16 (amino acids 412–490) activation domains were cloned into *pBShHB* as *Bam*HI–*Xba*I inserts. For *Hairy*$^{1-333}$*GAL4* and *Hairy*$^{1-333}$*VP16* constructs, Hairy coding sequences between positions 268 and 333 were joined to GAL4 or VP16 sequences by recombinant PCR (Higuchi *et al.*, 1988), and cloned into *pBShHB* as *Bam*HI–*Xba*I fragments. Hairy$^{1-268}$ was made by inserting a synthetic *Bam*HI–*Xba*I linker containing a stop codon into *pBShHB*. Hairy$^{1-333}$ was generated by cloning a PCR fragment including Hairy sequences from position 268 to 333 followed by a stop codon as a *Bam*HI–*Xba*I insert in *pBShHB*. Hairy^{E40AAct} and Hairy$^{\Delta basicAct}$ were obtained by two-step PCR mutagenesis (Higuchi *et al.*, 1988), replacing a *Hin*dIII–*Bbs*I fragment from *pBSHairy*$^{1-268}$*VP16* with its mutagenized equivalent. In all above cases, protein coding sequences were excised as *Hin*cII–*Xba*I fragments and cloned into *pCaSpeR-hs* (Pirrotta, 1988) digested with *Hpa*I–*Xba*I.

Run^{Act} coding sequences were constructed by cutting *pBSHairy*$^{1-268}$ *VP16* with *Hin*dIII–*Bam*HI (removing all Hairy sequences), and inserting a linker containing *Bgl*II and *Bbs*I sites. The resulting plasmid was digested with *Bgl*II–*Bbs*I and ligated to a *Bam*HI–*Bbs*I fragment from *pB:EDΔKS#2A* (provided by J.P.Gergen) encoding Run sequences from amino acids 1 to 506. Run^{Act} fusion sequences were recovered as a *Eco*RV–*Xba*I fragment and cloned into *pCaSpeR-hs* digested with *Hpa*I–*Xba*I.

To construct *hb-h*^{Act}, 1.5 kb of genomic *h* 3′ sequences were generated by PCR, and placed downstream of the *Xba*I site present in *pBSHairy*$^{1-268}$*VP16*. From this plasmid, a *Not*I–*Eco*RV fragment including Hairy$^{245-268}$, the VP16 domain and *h* 3′ sequences was isolated and used to replace an equivalent segment in the *hb-h* plasmid (Parkhurst *et al.*, 1990).

Germ line transformation and fly stocks

P-element-mediated transformation was carried out as described previously (Steller and Pirrotta, 1985; Spradling, 1986), selecting for rescue of *w* eyes (*phs* constructs) or G418-resistance (*hb-h*^{Act}). In general, *hs-h*$^{1-268Act}$ and *hs-h*$^{1-333Act}$ lines behaved similarly. Usually, two or more independent transformant lines were analysed for each construct, and results were confirmed using related constructs. The effects of *hb-h*^{Act} on male viability are unstable, becoming less penetrant as stocks are propagated over many generations, and were scored as soon as individual stocks permitted.

Embryo analysis

Embryos were collected on apple juice plates at 25°C. 6–10 min heat shocks were administered by placing the embryos onto damp tissue inside a humidified chamber at 36.5°C, conditions that do not cause significant pattern abnormalities in wild-type embryos (data not shown; Ish-Horowicz and Pinchin, 1987). After heat shocks, embryos were allowed to continue development for cuticle analysis (Wieschaus and Nüsslein-Volhard, 1986), or dechorionated and fixed at various times in heptane/4% formaldehyde/PBS. Appropriate expression of the constructs was confirmed using antibodies against Hairy, Run (gift of J.P.Gergen), and the VP16 activation domain (gift of P.O'Hare). Segmentation gene expression was determined by whole-mount *in situ* hybridization using digoxygenin-labelled RNA probes (Boehringer) as described (Tautz and Pfeifle, 1989; Klingler and Gergen, 1993). Sxl protein was detected using a monoclonal antibody specific for the full-length active protein (Bopp *et al.*, 1991). Appropriate secondary antibodies coupled to alkaline phosphatase (Jackson Immunoresearch Laboratories, Boehringer-Mannheim) were used to detect signals, and embryos were mounted in methacrylate (JB-4; Polyscience) and photographed under Nomarski optics.

mRNA injections

Wild-type and mutant *hairy* coding sequences were cloned in the *pSP64T* expression vector (Kreig and Melton, 1984), and used to synthesize capped RNA (Stratagene). RNA precipitates were taken up in water, and diluted accordingly in injection buffer (final concentration: 25 mM KCl, 2.5 mM PIPES, 0.5 mM EDTA, 25% glycerol; Anderson and Nüsslein-Volhard, 1984). RNA quantitation was by OD measurement and comparative analysis on ethidium bromide gels.

Embryos were dechorionated and dehydrated, and the RNA directed to the mid ventral surface by injection through the dorsal side of the embryo. Injected embryos were incubated for 15–20 min, and fixed in heptane saturated with 35% formaldehyde in PBS for 1 h (*in situ* hybridization), or 10 min (antibody staining), rinsed with fresh heptane, and transferred in a drop of heptane to a glass slide. On evaporation of the heptane, they were mounted on tape, covered with 0.1% Tween-20 in PBS, and gently hand-peeled from the vitelline membrane using 25-gauge needles. They were stored at –20°C in methanol before further processing.

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