The EMBO Journal Vol.16 No.10 pp.2883–2891, 1997

hairy mediates dominant repression in the *Drosophila* embryo

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***hairy* encodes a bHLH repressor that regulates several developmental processes in *Drosophila*, including embryonic segmentation and neurogenesis. Segmentation repressors such as Kruppel and knirps have been shown to function over short distances, less than 50–100 bp, to inhibit or quench closely linked upstream activators. This mode of repression permits multiple enhancers to work independently of one another within a modular promoter. Here, we employ a transgenic embryo assay to present evidence that hairy acts as a dominant repressor, which can function over long distances to block multiple enhancers. hairy is shown to repress a heterologous enhancer, the rhomboid NEE, when bound 1 kb from the nearest upstream activator. Moreover, the binding of hairy to a modified NEE leads to the repression of both the NEE and a distantly linked mesoderm-specific enhancer within a synthetic modular promoter. Additional evidence that hairy is distinct from previously characterized embryonic repressors stems from the analysis of the gypsy insulator DNA. This insulator selectively blocks the hairy repressor, but not the linked activators, within a modified NEE. We compare hairy with previously characterized repressors and discuss the consequences of short-range and long-range repression in development.**

Keywords: basic helix-loop-helix/development/*Drosophila*/long-range dominant repressor/neuroectodermal enhancer

Introduction

hairy (*h*) regulates several developmental processes in *Drosophila*. It is expressed in a periodic pattern in the early embryo, and helps define the seven-stripe pattern of *fushi tarazu* (*ftz*) expression (Nusslein-Volhard and Weischaus, 1980; Ish-Horowicz *et al.*, 1985; Carroll and Scott, 1986; Howard and Ingham, 1986; Ish-Horowicz and Pinchin, 1987). Later, h restricts sensory bristle formation by repressing the proneural gene *achaete* (*ac*) (Falk, 1963; Botas *et al.*, 1982; Moscoso del Prado and Garcıa-Bellido, 1984; Orenic *et al.*, 1993; Ohsako *et al.*, 1994; Van Doren *et al.*, 1994). *h* is also expressed in the developing eye, where it functions as an inhibitor of morphogenetic furrow progression (Carroll and Whyte, 1989; Brown *et al.*, 1995).

The h protein belongs to the hairy-related class of basic helix-loop-helix (bHLH) transcription factors, which includes deadpan and members of the Enhancer-of-split complex [E(spl)-C] (Rushlow *et al.*, 1989; Akazawa *et al.*, 1992; Bier *et al.*, 1992; Sasai *et al.*, 1992; Feder *et al.*, 1993; Ishibashi *et al.*, 1993). Many of these proteins have been shown to act as transcriptional repressors (Akazawa *et al.*, 1992; Sasai *et al.*, 1992; Ishibashi *et al.*, 1993; Ohsako *et al.*, 1994; Van Doren *et al.*, 1994; Dawson *et al.*, 1995; Fisher *et al.*, 1996). The hairy-related proteins share several regions of homology, including a basic DNA-binding region containing a signature proline residue, a helix-loop-helix dimerization domain, a hydrophobic domain of unknown function and the C-terminal tetrapeptide sequence, WRPW, which interacts with the groucho co-repressor (Knust *et al.*, 1992; Wainwright and Ish-Horowicz, 1992; Paroush *et al.*, 1994; Dawson *et al.*, 1995; Fisher *et al.*, 1996; Grbavec and Stifani, 1996). These repressors bind DNA sequences (‘class C sites’) that are distinct from the E-box motifs recognized by most bHLH transcription factors (Ohsako *et al.*, 1994; Van Doren *et al.*, 1994).

Transcriptional repression is essential for establishing localized patterns of gene expression during embryogenesis (Small *et al.*, 1992; Studer *et al.*, 1994; Kirchhamer and Davidson, 1996). In *Drosophila*, most of the spatially localized regulatory proteins present in the early embryo function as repressors. Four modes of transcriptional repression have been proposed (reviewed by Levine and Manley, 1989; Johnson, 1995). First, non-DNA-binding proteins can antagonize the function of transcriptional activators by preventing them from binding DNA. Members of the emc/Id class of HLH proteins, which lack a DNA-binding domain, dimerize with bHLH activators to form inactive complexes (Benezra *et al.*, 1990; Van Doren *et al.*, 1991; Cabrera *et al.*, 1994). Second, repressors can prevent activators from binding to DNA by occupying their binding sites (‘competition’). This type of repression is seen for the chicken ovalbumin regulatory factor, COUP-TF, which inhibits the binding of retinoic acid and retinoid X receptors (Tran *et al.*, 1992; Liu and Chiu, 1994). Homeodomain-containing proteins, which as a group have relatively poor DNA-binding sequence specificity, have been proposed to mediate repression by competing for ‘generic’ homeodomain recognition sequences that are also bound by homeodomain activators (e.g. Han *et al.*, 1989).

A third proposed form of repression is ‘quenching’, whereby a repressor works over short distances, usually $<$100 bp, to inhibit closely linked activators. Repressors and activators are thought to co-occupy nearby sites, but the repressor prevents the bound activator from interacting with the transcription complex. The *Drosophila* proteins snail (sna), Kruppel (Kr), giant (gt) and knirps (kni) were first shown to bind DNA elements that overlap activator

sites in native promoters, prompting the suggestion that they repress transcription via competition (Small *et al.*, 1991; Hoch *et al.*, 1992; Ip *et al.*, 1992). However, in more recent studies the repressor sites have been uncoupled from activator sites, and repression is observed even when they bind 50–100 bp from upstream activators (Gray *et al.*, 1994; Arnosti *et al.*, 1996a,b; Gray and Levine, 1996a). Direct protein–protein interactions between repressor and linked activator have not been demonstrated. An alternative model invokes transient inhibitory interactions between the repressor and one or more components of the transcription complex (see Gray and Levine, 1996b). Regardless of mechanism, this form of repression is 'local', since the repressors function only within the vicinity of their binding sites.

A fourth model for repression, silencing, differs from competition and local repression with respect to range of action. The *Drosophila* gradient morphogen, dorsal (dl), can function as a long-range silencer. dl is inherently an activator, but can repress heterologous enhancers and promoters over distances of several kilobases when bound near appropriate 'co-repressors' (Doyle *et al.*, 1989; Lehming *et al.*, 1994; Huang *et al.*, 1995; Cai *et al.*, 1996). Silencers may interact directly with the transcription complex or recruit heterochromatin to the promoter region, thus blocking access of basal transcription factors (see Herschbach and Johnson, 1993a).

In order to determine how h functions as a repressor, we analyzed a variety of fusion genes containing synthetic h binding sites in transgenic embryos. These studies suggest that h is a silencer, which can repress upstream activators over distances of at least 1 kb. h mediates dominant repression and can silence multiple enhancers in a modular promoter. These results suggest that h may repress transcription through a mechanism that is distinct from the local mode of repression employed by most other repressors present in the early *Drosophila* embryo. Further support for this view stems from the analysis of fusion promoters containing the gypsy insulator DNA. The insulator selectively blocks h, but not closely linked activators, suggesting that h might directly interact with one or more components of the basal transcription complex. We discuss the implications of dominant repression in development.

Results

Synthetic h binding sites were inserted in the *rhomboid* neuroectodermal enhancer (*rho* NEE). This enhancer is 700 bp in length and directs reporter gene expression in lateral stripes within the presumptive neuroectoderm of the early embryo (Ip *et al.*, 1992). The NEE is activated by dorsal (dl) and bHLH proteins in ventral and lateral regions, but is repressed by sna in the ventral mesoderm (Ip *et al.*, 1992). Many of the experiments involved the use of a modified *rho* NEE, whereby the sna repressor sites were eliminated, resulting in expression in both ventral and lateral regions (e.g. Figure 1B; Ip *et al.*, 1992). Transgenic embryos were hybridized with either a *lacZ* or *white* digoxigenin-labeled antisense RNA probe to visualize reporter gene expression (see Materials and methods).

h mediates transcriptional repression

The *rho* NEE contains four high-affinity dl binding sites that are clustered within a central 300 bp region of the enhancer. There are also five bHLH activator sites (E boxes) that are interspersed among the dl sites. Only the four dl sites are depicted in the diagrams accompanying the figures (Figure 1), but both dl and bHLH binding sites are essential for robust expression (Ip *et al.*, 1992). We inserted two high-affinity h binding sites in the *rho* NEE; these are located 50 bp from the central cluster of dl binding sites (see diagrams in Figure 1C and E). This modified NEE directs a segmental pattern of expression (Figure 1C). Sites of interstripe repression appear to coincide with regions of h expression (data not shown).

Interstripe repression persists when the h binding sites are moved 150 bp from the nearest dl sites (Figure 1D). The ability of h to repress transcription over this distance distinguishes it from sna, Kr and kni, which must map within 50–100 bp of the dl activators (Gray *et al.*, 1994; Arnosti *et al.*, 1996b; Gray and Levine, 1996a). We also assayed expression of the divergently transcribed *white* reporter gene. This was done to investigate the possibility that the downstream h site (see Figure 1D diagram) might block basal transcription factors within the *lacZ* promoter. The *white* transcription start site is over 300 bp from the nearest h site, beyond the range of 'basal quenching' (see Gray and Levine, 1996a). The *white* expression pattern is similar to the *lacZ* pattern, suggesting that h can repress NEE activators over a distance of at least 150 bp (Figure 1E and F).

Modified NEEs were expressed in various h mutants; an example is shown in Figure 2. This embryo is homozygous for the h^{m8} mutation, which contains a deletion in the *h* promoter region that eliminates all of the stripe-specific enhancers, except stripes 1 and 5 (Howard *et al.*, 1988). The modified *rho* NEE is repressed in just two domains, corresponding to h stripes 1 and 5 (Figure 2B). No repression is observed in embryos homozygous for h^{IL79K}, a point mutation which introduces a stop codon after the bHLH motif of *h* (data not shown).

h is a dominant repressor

We tested the ability of h to repress transcription in an 'enhancer-autonomous' fashion, whereby a repressor selectively inhibits only the enhancer to which it is bound (reviewed by Gray and Levine, 1996b). h binding sites were inserted in a modular promoter containing the *rho* NEE, as well as two tandem copies of the proximal enhancer (2xPE) from the *twist* (*twi*) promoter region, which mediates expression in the presumptive mesoderm (Jiang *et al.*, 1991; Pan *et al.*, 1991). The NEE used here contains the native sna repressor sites, which exclude expression from the ventral mesoderm and restricts the pattern to lateral stripes in the neuroectoderm (see Figure 3A). The NEE–2xPE fusion promoter directs an additive pattern of expression that includes lateral stripes (mediated by the NEE) and a band of staining in the presumptive mesoderm (mediated by 2xPE).

The NEE–2xPE fusion promoter directs a very different pattern of expression when two h sites are placed within the NEE (Figure 3B). The modified NEE mediates lateral stripes that are repressed in a pair-rule pattern. Interstripe repression is also observed for the 2xPE enhancer, even

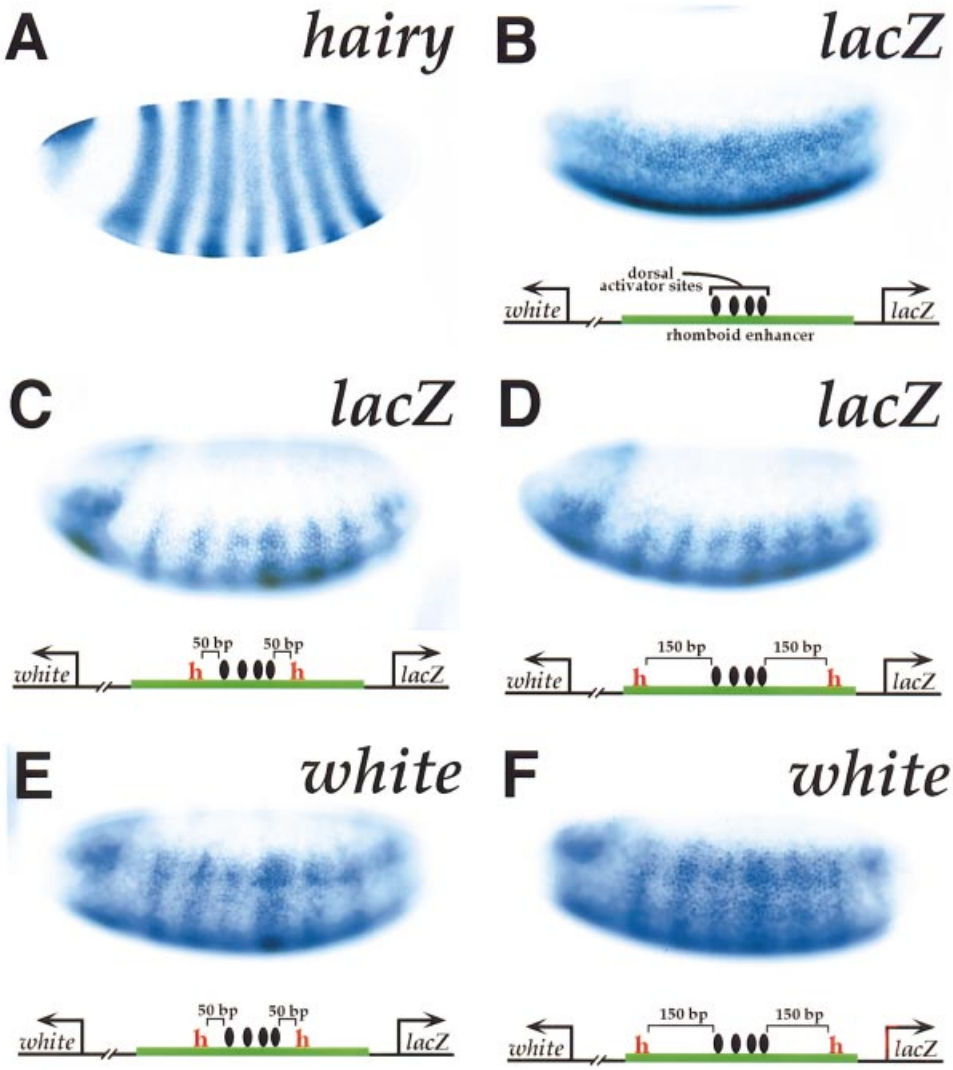

Fig. 1. hairy can repress the *rho* NEE. Transgenic embryos are oriented with anterior to the left and dorsal up. Modified NEEs were inserted between divergently transcribed *white* and *lacZ* reporter genes. All embryos are in mid- to late nuclear cleavage cycle 14 (~3 h post-fertilization). Expression patterns were visualized after hybridization with a digoxigenin-labeled antisense RNA probe; the reporter gene being assayed is indicated above each embryo. (**A**) Expression pattern of the native *hairy* gene. The staining pattern consists of seven pair-rule stripes, as well as an anterodorsal head patch. (**B**) *lacZ* expression pattern generated by the indicated fusion gene. The 700 bp *rho* enhancer lacks the four native sna repressor sites, and consequently, *lacZ* expression is observed in both ventral and lateral regions. (**C**) Same as (B) except that the *rho* enhancer was modified to include two high-affinity h binding sites (each indicated by a red 'h'). These h sites map ~50 bp from the cluster of activator sites. The *lacZ* pattern now exhibits interstripe repression. (**D**) The h sites have now been moved to 150 bp from the activator sites. Stripes of repression are still observed. (**E** and **F**) Same as in (C) and (D), respectively, except that the expression of the leftward *white* reporter gene is being assayed. The white transcription start site is located >300 bp from the closest h sites, presumably eliminating any short-range interactions between h and the transcription machinery.

though the closest h repressor site maps 290 bp from the distal-most dl activator site within the PE (see diagram in Figure 3B). h repressor sites within the modified NEE continue to repress both the *rho* lateral stripes and the 2xPE pattern when spacer sequences separate the two enhancers by either 630 bp (Figure 3C) or 1370 bp (Figure 3D). In the latter configuration, the nearest h repressor site maps ~2 kb away from the *lacZ* transcription start site. This long-range action contrasts with the local repression mediated by the four native sna sites contained within the NEE. In this case, *rho* expression is excluded from the presumptive mesoderm, but the neighboring 2xPE is unaffected. These experiments suggest that h is a dominant repressor, while sna functions in a local fashion (see Gray and Levine, 1996a).

h is a long-range repressor

The preceding experiments suggest that h can repress two enhancers even when bound only within the NEE. The next series of experiments addresses the possibility that this dominant repression depends on close linkage of the h sites with NEE activators. A single h repressor site was placed within a defective NEE lacking sna repressor sites (Figure 4).

A single h site placed 50 bp upstream of the nearest dl activator provides significant repression of the *rho* NEE

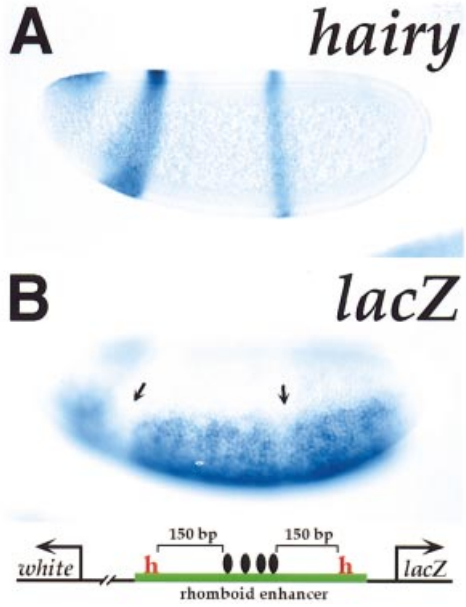

Fig. 2. h mediates transcriptional repression of the modified NEEs. Transgenic embryos are oriented as in Figure 1. (**A**) Expression of the *h* gene in an embryo that is homozygous for h^{m8}, a deletion in the *h* promoter which eliminates expression of all stripes except numbers 1 and 5. (**B**) *lacZ* expression in a h^{m8} homozygous embryo containing the *rho* enhancer with h sites spaced at 150 bp from activator sites. Only two stripes of repression are observed (arrows), corresponding to *h* stripes 1 and 5 (compare with Figure 1D and F, which show the activity of the same construct in wild-type embryos).

(Figure 4B; compare with 4A). Repression is still seen when this site is placed 150 bp upstream of dl (Figure 4C). However, the single h site has little effect on the activity of the enhancer when placed 250 bp upstream of dl (Figure 4D). These findings raise the possibility that h must bind near upstream activators in order to mediate efficient repression. However, the preceding experiments represent a rather stringent test of the repressor since only a single h binding site was used. Additional experiments were done with multiple h sites (Figure 5).

Predictably, a single h site has no effect on NEE activity at a distance of 1 kb upstream of the nearest dl activator (Figure 5A). However, efficient repression is observed when two tandem h sites are used in this experiment (Figure 5B). This result provides additional evidence that h is distinct from previously characterized local repressors. For example, four clustered sna binding sites are unable to repress the *even-skipped* (*eve*) stripe 2 or stripe 3 enhancers over a distance of just 150 bp (Gray and Levine, 1996a).

The h repressor is selectively blocked by an insulator DNA

The preceding results suggest that h functions as a long-range, dominant repressor. Previous studies have shown that the gypsy insulator DNA can block a variety of enhancers, but fails to inhibit the dl–corepressor complex within the *zerknullt* (*zen*) silencer element (VRE; Cai and Levine, 1995). Additional experiments were done to determine whether the gypsy insulator can block h.

The 340 bp gypsy insulator DNA contains 12 closely linked binding sites for the zinc finger protein, suppressor of Hairy wing [su(Hw); Spana *et al.*, 1988]. The insulator selectively blocks distal, not proximal, enhancers in transgenic embryos. A variety of enhancers have been tested, including the *eve* stripe 2 and stripe 3 enhancers, the *hairy* H1 enhancer and the *rho* NEE (Cai and Levine, 1995, 1997). Among these enhancers, the NEE is relatively refractory to the gypsy insulator, as shown in Figure 6.

The fusion promoter used for these experiments contains a modified NEE that lacks sna repressor sites but contains two h sites. A defective *eve* stripe 2 enhancer was also included (see diagrams in Figure 6A and B), but it mediates sporadic expression that is not relevant to the analysis of NEE–insulator interactions. When a spacer sequence is placed between the modified NEE and *lacZ* promoter, staining is detected in ventral and lateral regions. The pattern is subdivided into pair-rule repeats due to repression by h (Figure 6A), as seen previously (e.g. Figure 1C). The leftward *white* gene exhibits a similar, segmental staining pattern (Figure 6B). A distinct *lacZ* pattern is observed when the spacer sequence is replaced with the gypsy insulator DNA (Figure 6C). The NEE activators are not blocked, but instead, continue to drive *lacZ* expression in ventral and lateral regions. However, the staining pattern is continuous along the anteroposterior axis, and does not include pair-rule repeats of interstripe repression (compare with Figure 6A). This observation suggests that the h repressor is selectively blocked, while the NEE activators are unaffected. As a control, the leftward *white* reporter gene continues to exhibit h-mediated repression since the insulator is not interposed between the enhancer and *white* promoter (see diagram in Figure 6C and D). We note that there is only a transient failure of the insulator to block NEE activators (with respect to *lacZ*). The embryos shown in Figure 6 are undergoing cellularization. By the completion of this process the insulator blocks the NEE, so that staining in ventral regions is essentially lost (data not shown).

Discussion

We have presented evidence that h can repress heterologous enhancers in the early *Drosophila* embryo. Repression is observed even when h binding sites map far (1 kb or more) from both upstream activators and the target promoter. Moreover, h binding sites contained within a modified *rho* NEE also repress a second, distantly linked mesoderm-specific enhancer (*twi* 2xPE) within modular promoters. This long-range, dominant repression is distinct from the short-range, local repression observed for previously characterized embryonic repressors such as sna. The analysis of fusion promoters containing the gypsy insulator DNA suggests that h interacts with one or more components of the basal transcription complex. We discuss the developmental implications of long-range, dominant repression.

h is a long-range, dominant repressor

h can repress the *rho* NEE even when bound 1 kb upstream of the closest dl activator sites (see Figure 5). In contrast, previously characterized embryonic repressors such as sna, Kr and kni, must bind within 50–100 bp of activators

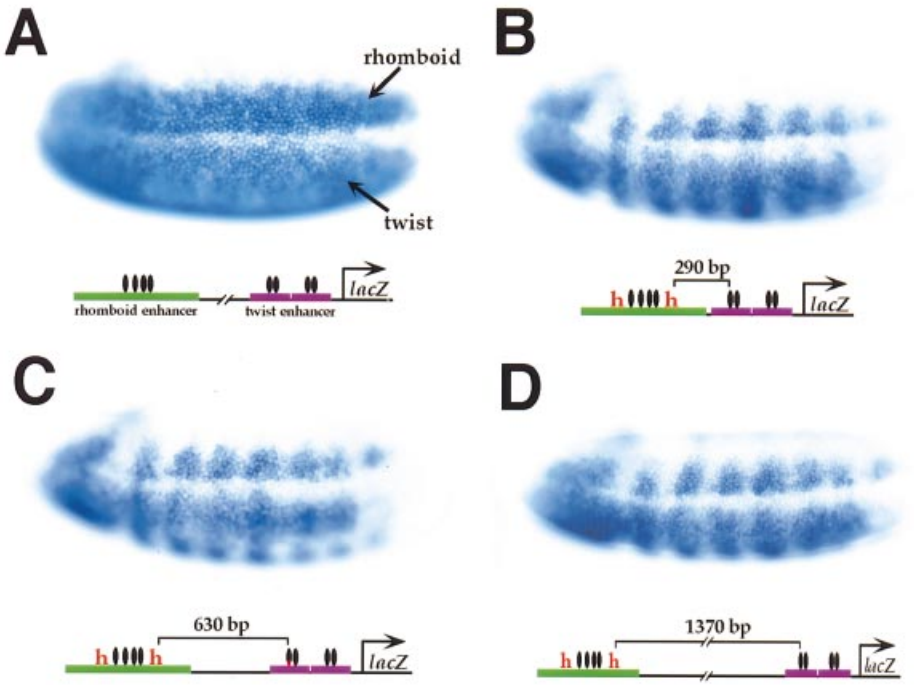

Fig. 3. h is a dominant, long-range repressor. Expression of the *lacZ* reporter gene is visualized in all embryos. (**A**) Staining pattern driven by a modular promoter containing two enhancers: a wild-type *rho* NEE (all four sna repressor sites are intact) and the *twist* 2xPE, which is expressed in ventral regions of the embryo. (**B**) The *rho* NEE, containing two h binding sites, has been placed directly upstream of the *twi* 2xPE. The downstream h site is 290 bp from the nearest activator site in the *twi* 2xPE, and over 700 bp from the *lacZ* transcription start site. Both enhancers are repressed by h. (**C**) The *rho* enhancer has been moved upstream via insertion of a CAT spacer sequence. The downstream h site is now located 630 bp from the nearest activator site in the *twi* 2xPE. Both enhancers are still repressed by h. (**D**) The *rho* enhancer has been moved further 5′ using an additional spacer sequence. The downstream h site is now 1370 bp from the nearest activator sites in the *twi* 2xPE, and over 2 kb from the *lacZ* transcription start site. h continues to repress both enhancers, so that the *rho* NEE and *twi* 2xPE patterns exhibit anteroposterior stripes.

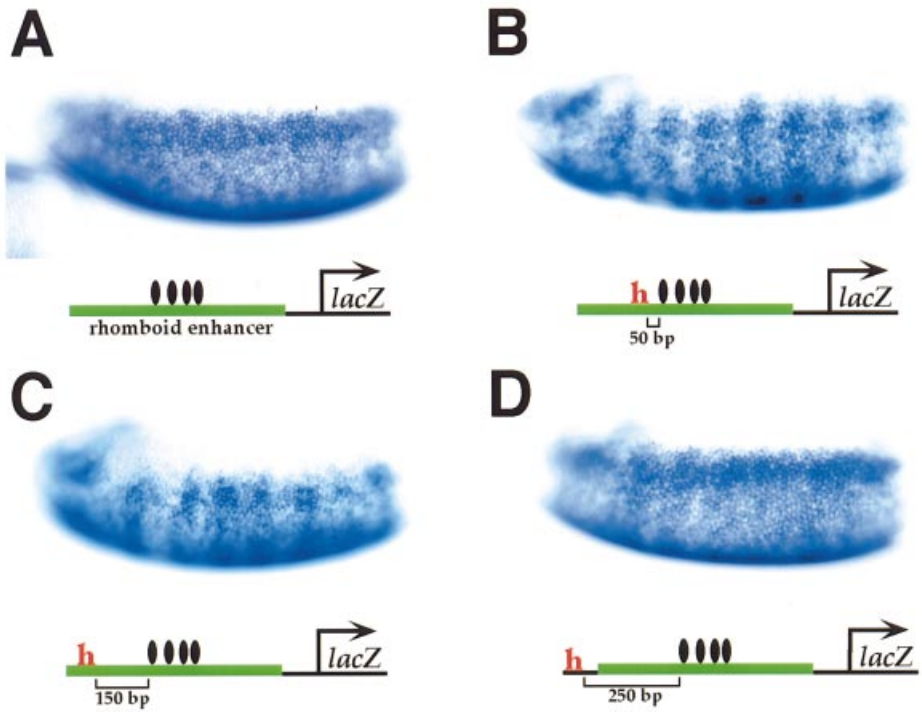

Fig. 4. The effect of distance on repression from a single h site. Transgenic embryos express modified *rho* NEEs containing a single h site. (**A**) A defective *rho* NEE that lacks all four sna repressor sites. *lacZ* expression is detected in ventral and lateral regions. (**B**) A single h binding site has been placed 50 bp upstream of the nearest dl activator site. Pair-rule repression is observed. (**C**) The h site has been moved to 150 bp from activators. Repression is still seen, but slightly reduced. (**D**) The h binding site is now 250 bp from the nearest activator site, and over 750 bp from the *lacZ* transcription start site. Repression by h is greatly reduced.

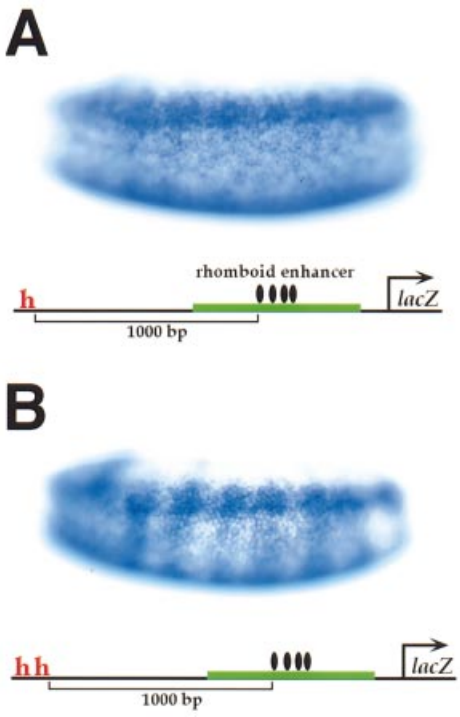

Fig. 5. Long-distance repression by h. Transgenic embryos express modified NEEs containing either one or two h binding sites. (**A**) A single h binding site, located 1000 bp upstream from the activator sites in the *rho* NEE, is unable to repress the activity of the *lacZ* reporter gene. (**B**) A pair of h sites at 1000 bp from activators, and over 1.9 kb from the *lacZ* transcription start site, mediate substantial repression.

in order to inhibit transcription (Gray *et al.*, 1994; Arnosti *et al.*, 1996b; Gray and Levine, 1996a). Several different mechanisms can account for this long-range repression. Perhaps h blocks distantly linked upstream activators. This type of mechanism has been invoked for the repression mediated by E2F–Rb complexes in mammalian cells (Weintraub *et al.*, 1995). E2F is inherently an activator, but mediates repression by recruiting Rb, which in turn, can function over long distances (>1 kb) to inhibit specific upstream activators bound within the proximal promoter. In this particular example, the long-range repressor exhibits regulatory specificity, and blocks just a subset of activators.

An alternative possibility is that h interacts directly with one or more components of the basal transcription complex. Previous studies suggest that the short-range Kr repressor can interact with the β subunit of TFIIE (Sauer *et al.*, 1995). However, this interaction must be weak and transient since Kr functions in a short-range, local fashion and permits enhancer autonomy within the modular *eve* promoter (see Gray and Levine, 1996b). Perhaps h functions in a similar manner, but binds TFIIE with a higher affinity, thereby resulting in a general silencing of the promoter. Repressor–TFIIE interactions might impede procession of the pol II transcription complex.

Repression by h is not entirely unaffected by proximity to upstream activators. A single h binding site, which is probably recognized by a h homodimer (Ohsako *et al.*, 1994; Van Doren *et al.*, 1994), fails to mediate efficient repression when bound either 250 bp or 1 kb upstream of NEE activators (Figures 4 and 5). However, the use of two, tandemly linked h sites greatly extends the range of h-mediated repression. Repression is seen even at a distance of 1 kb upstream of the NEE activators (see Figure 5). One interpretation of these results is that the occupancy of h binding sites is limiting. Efficient occupancy might depend on an 'open' chromatin state, which may be facilitated by the binding of nearby dl and bHLH activators to the NEE. When h sites are far from upstream activators, occupancy might depend on cooperative DNA binding interactions among h homodimers to linked sites.

h does not function in a local fashion within the modified *rho* NEE. Instead, it works in a dominant manner and blocks both the NEE and a distantly linked mesoderm-specific enhancer (the *twi* 2xPE). This repression is distinct from that mediated by short-range repressors, such as sna. Indeed, the contrast between h and sna is highlighted in the experiments presented in Figure 3. The *rho* NEE used in these experiments contains four native sna repressor sites, which exclude expression from the ventral mesoderm and restrict the pattern to lateral stripes in the presumptive neuroectoderm (Ip *et al.*, 1992). The sna repressor functions solely within the limits of the NEE and has no effect on the ventral expression mediated by the linked 2xPE enhancer. Thus, the NEE–2xPE pattern is strictly additive (Figure 3A) due to the local action of the sna repressor. In contrast, both h and a second long-range repression element, the *zen* VRE, mediate dominant repression of the *twi* 2xPE (Jiang *et al.*, 1992).

Targets of h-mediated repression

As discussed above, it is possible that h interacts with either upstream activators or the basal transcription complex. The difference between the dominant repression mediated by h and the local repression exhibited by sna (and other 'short-range' repressors) might correspond to the strength of the interactions between the repressors and target activators. Perhaps h makes stronger, more stable, contacts with these targets than does sna. A key issue regarding the mechanism of repression concerns the identities of the targets.

It is conceivable that h blocks upstream activators within the *rho* NEE and *twi* 2xPE. Both of these enhancers are thought to be activated, in part, by bHLH proteins, such as daughterless (da) and achaete-scute (Jiang *et al.*, 1991; Ip *et al.*, 1992). It is conceivable that h bound to the modified *rho* NEE blocks bHLH activators located within both the NEE and 2xPE through specific protein–protein interactions (Dawson *et al.*, 1995).

Dedicated interactions between h and bHLH activators are also consistent with the normal, endogenous *rho* and *twi* expression patterns seen during embryogenesis. We have treated h as a heterologous repressor, but in fact, both patterns are refined into a series of anteroposterior segmental repeats following cellularization (Jiang *et al.*, 1991; Bier *et al.*, 1992; Ip *et al.*, 1992). It is conceivable that these refinements are mediated, in part, by the h repressor. Our analysis has been restricted to precellular embryos, prior to the time when the endogenous genes may be subject to h-mediated repression. None the less,

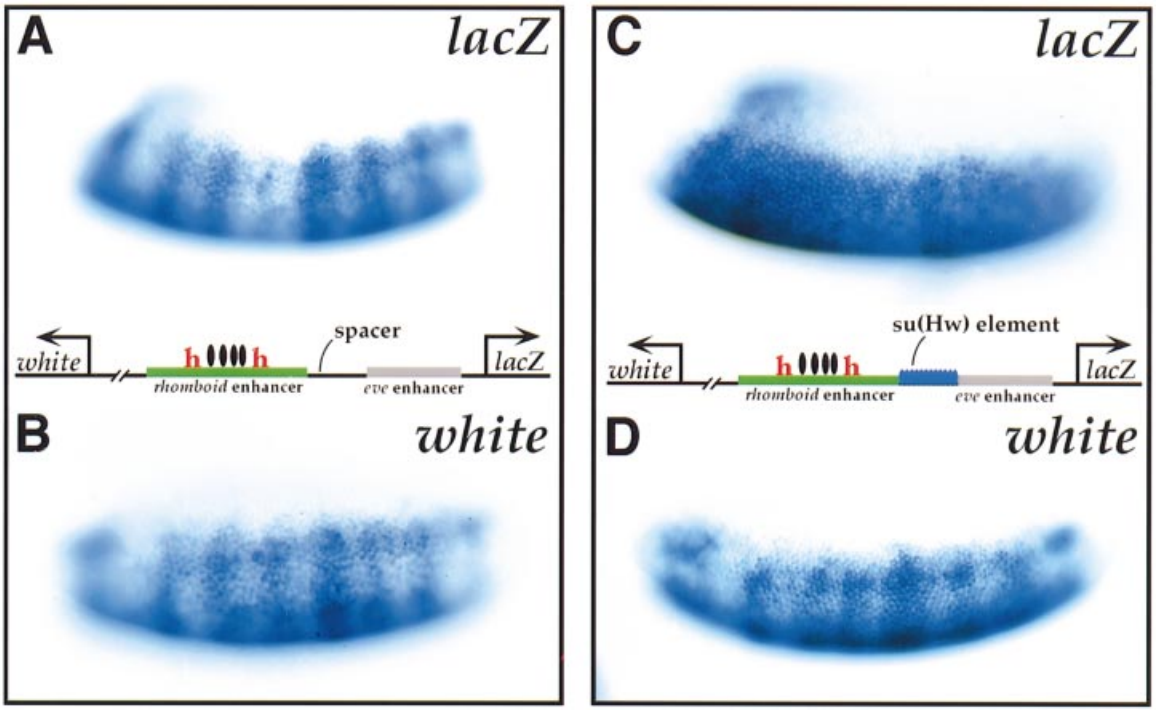

Fig. 6. The gypsy insulator blocks the h repressor. Transgenic embryos carry fusion promoters that contain a modified NEE and the gypsy insulator DNA. (**A**) The *rho* NEE, containing two h binding sites, is separated from the *lacZ* gene by a 340 bp spacer and a modified *eve* stripe 2 enhancer, which drives very weak and sporadic expression. h mediates interstripe repression of the modified *rho* NEE. (**B**) Same as (A), except that expression of the leftward *white* reporter gene is being monitored. h also mediates interstripe repression of the *white* gene. (**C**) *lacZ* expression driven by a similar fusion gene, except that the 340 bp spacer has been replaced by the 340 bp gypsy insulator DNA. Uniform staining is observed along the anteroposterior axis, suggesting that h no longer mediates interstripe repression of the pattern. However, the NEE activators are unaffected and continue to direct expression in ventral and lateral regions. The slight repression of the pattern seen in central regions is probably due to a proximal Kr repressor site within the eve stripe 2 enhancer (see Gray and Levine, 1996a). (**D**) *white* expression driven by the same construct. h mediates repression of the *white* reporter gene, suggesting that the gypsy insulator does not interfere with the occupancy of h binding sites within the modified *rho* NEE.

it is possible that both the *rho* NEE and *twi* 2xPE are 'sensitized' for repression by h.

Studies with the gypsy insulator (Figure 6) do not exclude this type of mechanism, but strongly suggest that h makes direct contact with one or more components of the transcription complex. The insulator selectively blocks h-mediated repression of a modified *rho* NEE (Figure 6C), although the dl and bHLH activators are unaffected and continue to direct expression in ventral and lateral regions of early embryos. If h worked solely by blocking upstream bHLH activators, then the insulator should have no effect on interstripe repression. The simplest interpretation of this result is that h contacts the basal transcription complex independently of the dl and bHLH activators.

Mechanism of repression

h and hairy-related bHLH repressors have been shown to interact with the co-repressor protein groucho (gro) through the C-terminal WRPW motif (Paroush *et al.*, 1994; Fisher *et al.*, 1996; Grbavec and Stifani, 1996). gro is not known to bind DNA, but fusions of gro with heterologous DNA binding domains have revealed that gro can act as a transcriptional repressor (Fisher *et al.*, 1996). *gro* is required for proper neurogenesis, segmentation and sex determination, all of which involve hairy-related bHLH repressors (Paroush *et al.*, 1994). The gro protein and its mammalian homologs contain several repeats of a 40-residue motif, termed the WD40 repeat, which is thought to mediate protein–protein interactions (Hartley *et al.*, 1988; Stifani *et al.*, 1992; for review, see van der Voorn and Ploegh, 1992). Tup1, a yeast co-repressor protein that also contains WD40 repeats, is recruited to DNA by the α2 repressor in α-type cells for the silencing of **a**-specific genes (Keleher *et al.*, 1992). Similarly, h and its relatives may recruit gro for silencing specific genes in the *Drosophila* embryo.

The yeast mating-type repressors α2 and Tup1 have been reported to interact with histones. This observation raises the possibility that Tup1 mediates transcriptional silencing by influencing chromatin structure (Roth *et al.*, 1992; Cooper *et al.*, 1994; Edmondson *et al.*, 1996). There is also evidence that Tup1 interacts with basal transcription factors (Herschbach and Johnson, 1993b). Perhaps h–gro and α2–Tup1 complexes mediate repression through similar mechanisms. Strong and stable interactions between these repressors and the basal transcription complex would be expected to cause dominant silencing of complex promoter regions.

Short-range repression is a flexible form of gene regulation that permits enhancer autonomy within complex, modular promoters (see Gray *et al.*, 1996b). In contrast, long-range silencing represents a stringent form of gene control that appears to be employed by promoters which must be unequivocally on or off. An example is sex determination in *Drosophila*. The hairy-related protein deadpan (dpn) represses the early promoter of the *Sex-lethal* (*Sxl*) gene, thereby ensuring that Sxl is off in male embryos (Younger-Shepherd *et al.*, 1992; Barbash and Cline, 1995; Hoshijima *et al.*, 1995).

Materials and methods

P-element transformations and in situ hybridization

P-elements were introduced into the *Drosophila* germline by injection of yw^{67} embryos as described by Small *et al.* (1992). *In situ* hybridizations were performed as described by Jiang *et al.* (1991), using digoxigenin-UTP-labeled antisense RNA probes to *hairy*, *lacZ* or *white*. At least three independent transgenic lines were generated and tested for each construct. To generate the embryos shown in Figure 2, transgenic flies were crossed into a hm^{8} background (Howard *et al.*, 1988) and offspring carrying both the mutation and the transgene were mated with one another. Embryos were analyzed as described above.

Construction of transgenes

The 700 bp *rho* NEE (Ip *et al.*, 1992; Gray *et al.*, 1994) and the 520 bp *twi* 2xPE (Jiang and Levine, 1993) were inserted into the polylinker of the C4PLZ transformation vector (Wharton and Crews, 1993). Two versions of the *rho* NEE were used: the wild-type enhancer (in Figure 3) and one with mutations in the four sna binding sites as described in Ip *et al.* (1992) (in Figures 1, 2, 4, 5 and 6). A 340 bp *Dra*I fragment of the chloramphenicol acetyl transferase (CAT) gene coding sequence was used as a spacer in the constructs shown in Figures 3C and D, and 6A and B. A 750 bp fragment containing the coding region of the green fluorescent protein (GFP) was used as a spacer in the constructs shown in Figures 3D, and 5A and B. Neither the CAT nor GFP sequences were found to affect reporter gene transcription in embryos (data not shown). The su(Hw) element shown in Figure 6 is a 340-bp fragment of the gypsy retrotransposon, which was isolated by PCR (Cai and Levine, 1995). The constructs shown in Figure 6 were made by inserting the 700 bp *rho* NEE, the CAT spacer and the su(Hw) element into a derivative of the CaSpeR-AUG-βgal transformation vector (Thummel *et al.*, 1988) containing the *eve* basal promoter, starting at –42 bp and continuing through codon 22 of *eve*, fused to the *lacZ* gene (Small *et al.*, 1992), and also containing a 480 bp *eve* stripe 2 enhancer, with deletions in three gt binding sites (described in Arnosti *et al.*, 1996a).

Site-directed mutagenesis of the rho NEE

The h binding site used in these experiments corresponds exactly to the optimal site determined by Van Doren *et al.* (1994) in their random binding site selection experiments: gcggCACGCGacat (capitals indicate strongly selected bases). Binding sites were added to the *rho* NEE by oligonucleotide-directed mutagenesis using the Mutagene kit (Bio-Rad, CA) as described in Small *et al.* (1992). h sites were placed 50 bp 5′ and 3′ of the d1 and d4 dl sites, respectively, in the constructs shown in Figures 1C and E, 3B–D and 6A–D. h sites were placed 150 bp 5′ and 3′ of the d1 and d4 dl sites, respectively, in the constructs shown in Figures 1D and F, and 2B. A single h site was placed 50, 150 and 250 bp upstream of the d1 dl site in the constructs shown in Figure 4B–D, respectively. The 750 bp GFP spacer was inserted into the construct shown in Figure 4D, between the h site and the *rho* NEE to create the construct shown in Figure 5A. A double-stranded oligonucleotide containing a second h binding site was inserted into the previous construct, 5 bp downstream of the existing h site, to create the construct shown in Figure 5B.

Acknowledgements

We thank David Arnosti for valuable discussions, Haini Cai, Susan Gray, Keith Maggert, Paul Szymanski and Bob Zeller for providing DNAs, and Mark Van Doren and Jim Posakony for providing the hairy binding sequence prior to publication. We especially thank Jim Posakony for his generosity and continuing support. This work was supported by an NIH grant (GM 34431), and by a Markey predoctoral fellowship to S.B.

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Received on December 5, 1996