Local action of long-range repressors in the *Drosophila* embryo

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Previous studies have identified two corepressors in the early *Drosophila* embryo: Groucho and dCtBP. Both proteins are recruited to the DNA template by interacting with short peptide motifs conserved in a variety of sequence-specific transcriptional repressors. Once bound to DNA, Groucho appears to mediate long-range repression, while dCtBP directs short-range repression. The short-range Krüppel repressor was converted into a long-range repressor by replacing the dCtBP interaction motif (PxDLSxH) with a Groucho motif (WRPW). The resulting chimeric repressor causes a different mutant phenotype from that of the native Krüppel protein when misexpressed in transgenic embryos. The different patterning activities can be explained on the basis of long-range silencing within the hairy 5' regulatory region. The analysis of a variety of synthetic transgenes provides evidence that Groucho-dependent long-range repressors do not always cause the dominant silencing of linked enhancers within a complex *cis*-regulatory region. We suggest a 'hot chromatin' model, whereby repressors require activators to bind DNA.

Keywords: CtBP/*Drosophila*/embryo/Groucho/long-range repression

Introduction

Complex enhancers direct stripes and bands of gene expression in the early *Drosophila* embryo. These enhancers are typically 300 bp–1 kb in length and contain clustered binding sites for transcriptional activators and repressors (e.g. Hoch *et al.*, 1992; Ip *et al.*, 1992; Small *et al.*, 1992). Different enhancers can work independently of one another within a common *cis*-regulatory region to direct composite patterns of gene expression. For example, the seven-stripe *even-skipped* (*eve*) expression pattern is activated by five separate enhancers located 5' and 3' of the transcription unit (Stanojevic *et al.*, 1991; Small *et al.*, 1992, 1996; Fujioka *et al.*, 1999). The ability of these enhancers to function in an autonomous fashion depends on short-range transcriptional repressors that work over distances of <100 bp to inhibit, or quench, upstream activators (Gray *et al.*, 1994). The binding of the Krüppel repressor to the stripe 2 enhancer does not interfere with the activity of the stripe 3 enhancer since Krüppel

mediates repression only when positioned near upstream activators (Small *et al.*, 1993; Gray and Levine, 1996). Consequently, Krüppel quenches Bicoid activators within the stripe 2 enhancer without interfering with the D-Stat activators bound to the stripe 3 enhancer (Small *et al.*, 1996; Yan *et al.*, 1996).

There are several short-range repressors in the early embryo, including Krüppel, Snail, Knirps and Giant (Gray *et al.*, 1994; Arnosti *et al.*, 1996; Gray and Levine, 1996; Hewitt *et al.*, 1999). Most or all of these repressors interact with a common corepressor protein, dCtBP (Nibu *et al.*, 1998a,b; Poortinga *et al.*, 1998), which is the *Drosophila* homolog of a human protein that was found to attenuate the oncogenic activities of the adenovirus E1A protein (Sollerbrant *et al.*, 1996; Schaeper *et al.*, 1998). dCtBP is maternally expressed and ubiquitously distributed throughout early embryos. A variety of studies suggest that the dCtBP corepressor protein is recruited to the DNA template by interacting with a conserved sequence motif contained in most or all sequence-specific short-range repressors: PxDSLxK/R/H (Nibu *et al.*, 1998a,b). There is emerging evidence that mammalian CtBP proteins also function as corepressors, although it is not known currently whether the mammalian repressors (e.g. bKLF, Ikaros and ZEB-1) only function over short distances (Turner *et al.*, 1998; Koipally *et al.*, 2000; Postigo *et al.*, 2000).

A number of repressors can work when positioned far from upstream activators and the core promoter. For example, the binding of the Hairy repressor to a modified *rhomboid* lateral stripe enhancer (NEE) can cause the dominant silencing of a linked mesoderm-specific enhancer, even when the two enhancers are separated by >1 kb in the 5' *cis*-regulatory region (Barolo and Levine, 1997). Hairy interacts with a second ubiquitous corepressor protein, Groucho (Paroush *et al.*, 1994). Hairy–Groucho interactions depend on a conserved sequence motif at the Hairy C-terminus: WRPW (Fisher *et al.*, 1996). These studies suggest that the dCtBP corepressor protein mediates short-range repression, while Groucho mediates long-range repression. The present study provides additional support for this possibility.

The long-range action of the Groucho corepressor poses a potential problem with regard to enhancer autonomy in complex promoter regions. In principle, the binding of a Groucho-dependent repressor could result in the dominant silencing of all enhancers located in the 5' and 3' regulatory regions of a target gene. This imposes a potentially severe constraint on the evolution of complex patterns of gene activity. To investigate this issue, we have examined the activities of chimeric repressor proteins that contain the DNA-binding domains of the short-range Krüppel or Snail repressors and the Groucho interaction sequences in the long-range Hairy repressor. These

Fig. 1. Summary of expression vectors. *Krüppel* and *snail* coding sequences were placed under the control of heterologous *twist* and *eve* enhancers, respectively. The *twist* enhancer ('twi' in the diagrams) corresponds to two tandem copies of a modified 250 bp PE enhancer sequence that contains optimal Dorsal operator sites and Twist bHLH-binding sites. Two tandem copies of a modified *eve* stripe 2 enhancer ('st2' in D) were used to misexpress Snail. An FRT–stop–FRT cassette was inserted between the promoter and coding region to circumvent lethality resulting from the misexpression of these regulatory genes. (A) The wild-type *Krüppel* coding region was placed under the control of the modified *twist* enhancer. The encoded protein is composed of 502 amino acids and contains a dCtBP corepressor interaction motif, PEDLSMH, at position 464. (B) Same as (A) except that the *Krüppel* coding region was mutagenized to disrupt the dCtBP motif. The first three amino acids were converted into alanines. This protein is unable to bind dCtBP *in vitro* and has only limited repressor function *in vivo* (Nibu *et al.*, 1998b). (C) Same as (B) except that a C-terminal portion of the *Hairy* repressor was attached in-frame to the 3' end of the *Krüppel* coding region. The *Hairy* sequence contains the weak dCtBP-binding motif, PLSLVIK, and also contains the strong Groucho corepressor-binding motif, WRPW. (D) The *snail-hairy* fusion gene was placed under the control of the *eve* stripe 2 enhancer. Both dCtBP motifs in the *snail* coding sequence were left intact. The same portion of the *Hairy* protein as used in (C) was attached to the 3' end of the *snail* coding region.

chimeric repressors were expressed in specific regions of transgenic embryos using defined, heterologous enhancers. The *Krüppel-Hairy* fusion protein causes altered patterns of segmentation gene expression that are consistent with the notion that *Hairy-Groucho* interactions convert *Krüppel* into a long-range repressor. However, the abnormal *rhomboid* expression pattern obtained with a similar *Snail-Hairy* fusion protein suggests that it does not function as a dominant silencer, but instead causes the local repression of a single enhancer. The subsequent analysis of a number of synthetic transgenes provides direct evidence that the long-range *Hairy* repressor does not always cause the dominant silencing of linked enhancers. We propose that repressors require linked activators to function and, consequently, long-range repressors can sometimes function in a short-range fashion.

Results

Krüppel-Hairy and *Snail-Hairy* chimeric repressors were expressed in transgenic embryos. The entire *Krüppel* coding sequence (composed of 502 codons) was fused in-frame with the *hairy* 3' coding region (codons 255–337) in an effort to convert *Krüppel* into a long-range repressor (Figure 1). The *Hairy* sequence includes the Groucho interaction motif, WRPW, and a weak dCtBP motif, PLSLVIK (Poortinga *et al.*, 1998). The strong dCtBP interaction motif in *Krüppel*, PEDLSMH, was mutagen-

ized in order to circumvent potential competition between the dCtBP and Groucho corepressors (Zhang and Levine, 1999). The resulting *Krüppel-Hairy* fusion protein was expressed in the ventral mesoderm of transgenic embryos using a modified PE enhancer from the *twist* promoter region (Jiang *et al.*, 1991; Jiang and Levine, 1993). A similar *Snail-Hairy* fusion protein was misexpressed using a modified *eve* stripe 2 enhancer (Kosman and Small, 1997; Figure 1). In both cases, an FRT–stop–FRT cassette was inserted between the promoter and coding region in order to block the expression of potentially deleterious proteins that might cause dominant lethality and prevent the selection of transgenic strains (Struhl and Basler, 1993). Once the lines were established, the stop cassette was removed by crossing the transgene into a strain that expresses the FLP recombinase under the control of a testis-specific tubulin promoter (Kosman and Small, 1997).

The Krüppel-Hairy fusion protein mediates long-range repression

The *twi-Krüppel-hairy* transgene causes the fusion of the fifth to eighth abdominal segments, whereas the *twi-Krüppel* transgene results in the fusion of the sixth and seventh abdominal segments (data not shown). A number of segmentation genes were examined to determine the basis of the distinct patterning activities of the chimeric and native repressors.

Fig. 2. Misexpression of *Krüppel* alters the *hairy* expression pattern. Embryos were collected from the indicated transgenic strains and hybridized with either a digoxigenin-labeled *Krüppel* antisense RNA probe (A–C) or a *hairy* probe (D–F). The different *Krüppel* coding sequences exhibit similar levels of expression in ventral regions of transgenic embryos. Note that these ectopic patterns are considerably weaker than the endogenous *Krüppel* pattern, which is seen as a broad band in central regions. Activated forms of the transgenes were obtained from transgenic males carrying the FLP recombinase under the control of the testis-specific tubulin promoter. (A and D) *Krüppel* and *hairy* expression patterns, respectively, in transgenic embryos carrying the mutant *Krüppel* coding region lacking the dCtBP repression domain (Figure 1B). The *hairy* expression pattern is essentially normal, although the spacing between stripes is sometimes irregular, and there may be a slight reduction in the ventral expression of stripe 7 (red arrowhead in D). The repression of *hairy* stripe 1 in ventral regions is also observed in wild-type embryos (not shown). (B and E) *Krüppel* and *hairy* expression patterns, respectively, in transgenic embryos carrying the wild-type *Krüppel* coding sequence. There is consistent ventral repression of stripes 2, 6 and 7 (red arrowheads). Stripe 5 sometimes exhibits an expanded pattern, possibly due to the repression of the endogenous Giant repressor (not shown). (C and F) *Krüppel* and *hairy* expression patterns, respectively, in transgenic embryos carrying the *Krüppel*–*hairy* fusion gene. There is a consistent repression of stripe 5, in addition to stripes 2, 6 and 7, as seen with the wild-type *Krüppel* transgene (red arrowheads in F; compare with E). Stripe 5 is not repressed by the wild-type *Krüppel* transgene.

hairy is expressed in a series of seven transverse stripes along the length of the embryo (Howard *et al.*, 1988). There is also a patch of staining near the anterior pole (e.g. Figure 2D). The first stripe is repressed in ventral regions prior to the onset of gastrulation. The *twi*–*Krüppel* transgene lacking the dCtBP interaction motif (PEDLSMH; see Figure 1) leads to a slight alteration in the *hairy* expression pattern. Stripes 5, 6 and 7 are sometimes shifted, and there may be a slight reduction in the expression of stripe 7 (arrowhead, Figure 2D). The *twi*–*Krüppel* transgene containing the wild-type *Krüppel* coding region causes a far more severe alteration in the *hairy* expression pattern (Figure 2E). Stripes 2 and 6 are consistently repressed in ventral regions (arrowheads). There is also an expansion of stripe 5 (bracket). These changes in stripes 2, 5 and 6 appear to require the dCtBP interaction motif since the wild-type and mutant transgenes exhibit similar levels of expression in ventral regions (Figure 2A–C).

Fig. 3. A Snail–Hairy fusion protein mediates local repression. Wild-type and transgenic embryos were hybridized with either a digoxigenin-labeled *rhomboid* or *snail* RNA probe. Embryos are oriented with anterior to the left and dorsal up. (A) *snail* expression pattern in a wild-type embryo. Staining is restricted to ventral regions that will invaginate to form the mesoderm. (B) *snail* expression pattern in a transgenic embryo carrying the *snail*–*hairy* fusion gene under the control of the *eve* stripe 2 enhancer (see Figure 1). Staining is detected in both the ventral mesoderm and an ectopic stripe (arrowhead). (C) *rhomboid* expression pattern in a wild-type embryo. In this lateral view, staining is detected in both ventral regions (the lateral neurogenic stripes) and in the dorsal ectoderm. (D) *rhomboid* expression pattern in a transgenic embryo carrying the *st2*–*snail*–*hairy* fusion gene. The ectopic stripe of *snail*–*hairy* expression creates a gap in the neurogenic stripes (arrowhead). However, staining in the dorsal ectoderm is not affected (arrow).

Previous studies have shown that low levels of *Krüppel* are sufficient to repress the stripe 6 enhancer, while higher concentrations are required to repress stripe 5 (Langeland *et al.*, 1994). The *twi*–*Krüppel* transgene directs the expression of relatively low levels of *Krüppel* repressor in ventral regions (Figure 2A–C). These low levels may be sufficient to bind the stripe 6 enhancer, but are insufficient to bind the low-affinity operator sites within the stripe 5 enhancer. Once bound to the stripe 6 enhancer, *Krüppel* functions as a short-range repressor and does not influence other enhancers in the *hairy* 5' regulatory region (see Discussion).

The *twi*–*Krüppel*–*hairy* transgene results in the consistent repression of stripe 5 in addition to stripes 2, 6 and 7 (arrowheads, Figure 2F). Unlike the native *Krüppel* protein, it would appear that the *Krüppel*–*Hairy* fusion protein does not work solely within the limits of the stripe 6 enhancer, but instead functions over long distances to repress the stripe 5 enhancer and/or core promoter. Repression of stripe 5 does not appear to depend on augmented levels of the *Krüppel*–*Hairy* fusion product since the transgene is expressed at about the same levels as the wild-type transgene (Figure 2C; compare with B).

A Snail–Hairy fusion protein does not mediate dominant repression

The *rhomboid* expression pattern was used to distinguish between short- and long-range repression along the dorsoventral axis of the early embryo (Figure 3). *rhomboid* is expressed in two different cell types: the neurogenic ectoderm and the amnioserosa (Bier *et al.*, 1990). These two patterns are probably controlled by separate enhancers. Lateral stripes in the neurogenic ectoderm are regulated by a 300 bp enhancer, the NEE, located ~1.7 kb upstream of the *rhomboid* transcription start site (Ip *et al.*, 1992). Expression in the amnioserosa depends on

Fig. 4. Local action of the Hairy repressor. Transgenic embryos express the indicated *lacZ* reporter genes (see diagrams below embryos). Embryos were stained with a digoxigenin-labeled *lacZ* antisense RNA probe and are oriented with anterior to the left. (A) Lateral view of an embryo expressing the NEE-2×PE (*twist*) transgene. A composite *lacZ* staining pattern is observed, including lateral stripes in the neurogenic ectoderm (arrowhead 'NEE') and expression in the ventral mesoderm (arrowhead 'twist'). The binding of the short-range Snail repressor to the NEE (indicated by 'S' in the diagram) does not interfere with the expression of the *twist* enhancer. (B) Same as (A) except that the 5' NEE was modified to include two synthetic Hairy repressor sites ('h', see diagram). The Hairy repressor mediates periodic repression of the NEE pattern (arrowhead 'NEE'). The binding of Hairy to the modified NEE also causes periodic repression of the mesoderm pattern (arrowhead 'twist'). (C) *lacZ* staining pattern obtained with a transgene that contains the modified h-NEE-h enhancer placed 5' of the *race* enhancer. As seen in (B), Hairy represses the modified NEE (arrowhead 'NEE'), but fails to repress the *race* staining pattern in the dorsal ectoderm (arrowhead 'race'). (D) *lacZ* staining pattern obtained with a transgene that contains tandem copies of a modified *race* enhancer placed 5' of the *twist* enhancer sequences. The h-race-h enhancer exhibits periodic stripes of repression, presumably due to the binding of the Hairy repressor (arrowhead 'race'). However, the binding of Hairy to h-race-h does not influence the staining pattern directed by the linked *twist* enhancer (arrowhead 'twist').

cis-regulatory sequences located either 5' of -2.2 kb or >2 kb downstream of the *rhomboid* transcription unit (Bier *et al.*, 1990).

A Snail-Hairy fusion protein was misexpressed in transgenic embryos using the *eve* stripe 2 enhancer (Figure 3B; summarized in Figure 1). These embryos exhibit an abnormal *rhomboid* expression pattern, including a gap in the neurogenic pattern in the vicinity of stripe 2 (arrowhead, Figure 3D). This gap probably results from the binding of the ectopically expressed Snail-Hairy repressor to the *rhomboid* NEE.

Despite the fact that the Snail-Hairy fusion repressor contains the same region of Hairy that mediates long-range repression by the Krüppel-Hairy fusion protein, only the NEE appears to be repressed; the amnioserosa pattern is unaffected (arrow, Figure 3D, compare with C). A similar pattern of *rhomboid* repression was obtained with a *stripe 2*-*snail* transgene that expresses the wild-type, short-range Snail repressor (Nibu *et al.*, 1998a). Thus, it would appear that the Snail-Hairy fusion protein does not cause the dominant silencing of amnioserosa regulatory sequences.

Hairy can permit enhancer autonomy

The failure of Snail-Hairy to mediate long-range repression within the *rhomboid* *cis*-regulatory region was unexpected since the Krüppel-Hairy fusion protein appears to work over long distances within the *hairy* locus (Figure 2). Perhaps the Snail-Hairy fusion protein is

somehow defective due to competitive interactions between the strong dCtBP interaction motifs in the Snail moiety and the WRPW motif in Hairy (Zhang and Levine, 1999). Alternatively, it is possible that long-range repressors can sometimes permit enhancer autonomy. To investigate this issue, we examined the ability of the native Hairy repressor to work over long distances within synthetic *cis*-regulatory regions containing defined enhancers (Figure 4).

The *rhomboid* NEE was placed 340 bp upstream of the 2×PE *twist* enhancer (Jiang *et al.*, 1991; Pan *et al.*, 1991; Jiang and Levine, 1993), and attached to a *lacZ* reporter gene (see diagram below Figure 4A). An additive *lacZ* staining pattern is observed in transgenic embryos (Figure 4A). There is staining in both the lateral neurogenic ectoderm (arrowhead 'NEE') and the ventral mesoderm (arrowhead 'twist'). Snail repressor bound to the distal NEE (Figure 4A, 'S' in the diagram) does not interfere with the ventral expression mediated by the proximal 2×PE. As shown previously (Barolo and Levine, 1997), this staining pattern is altered when two synthetic Hairy repressor sites are placed within the distal NEE (Figure 4B; see diagram). The *rhomboid* lateral stripes exhibit periodic sites of repression that coincide with the *hairy* expression pattern (arrowhead NEE, Figure 4B). The ventral mesoderm staining pattern also exhibits periodic repression (arrowhead 'twist'), which suggests that the binding of Hairy to the modified NEE represses both enhancers.

Additional assays were performed with a similar *lacZ* transgene except that the proximal 2×PE was replaced with two tandem copies of the minimal 533 bp *race* enhancer (see diagram below Figure 4C). The *race* enhancer directs weak, uniform expression in the presumptive amnioserosa in the dorsal-most regions of early gastrulating embryos (Rusch and Levine, 1997; e.g. Figure 4C). Both the *race* and the *rhomboid* amnioserosa patterns appear to be regulated by a Dpp–Smad signaling pathway that is activated only in the dorsal ectoderm (e.g. Rusch and Levine, 1997; Ashe and Levine, 1999; reviewed by Podos and Ferguson, 1999). The modified NEE containing the synthetic Hairy sites directs abnormal lateral stripes that exhibit periodic repression along the anteroposterior axis (Figure 4C, arrowhead ‘NEE’). However, the *race* staining pattern is uniform in the amnioserosa (Figure 4D, arrowhead ‘race’), thereby raising the possibility that Hairy does not always silence linked enhancers. Alternatively, the activators that bind the *race* enhancer may be insensitive to repression by Hairy. To distinguish these possibilities, Hairy-binding sites were inserted in the *race* enhancer.

The resulting transgene contains two copies of the modified *race* enhancer placed 5’ of tandem *twist* enhancer sequences (see diagram, Figure 4D). The *lacZ* staining pattern directed by *race* exhibits periodic sites of repression (arrowhead ‘race’). This altered pattern (compare with Figure 4C) suggests that Hairy binds to the modified *race* enhancer and represses the associated activators. The

irregularities in the pattern of repression probably reflect the varying widths of the *hairy* expression stripes in dorsal versus ventral regions of early embryos (e.g. Figure 2D). The *twist* enhancers direct uniform expression of the *lacZ* reporter gene in ventral regions (Figure 4D, arrowhead ‘twist’). This suggests that the binding of Hairy to the *race* enhancer does not lead to the dominant silencing of the linked *twist* enhancers. In contrast, a comparable modified h-NEE-h enhancer mediates efficient repression of *twist* (Figure 4B, arrowhead ‘twist’).

Discussion

A comparison of the altered patterns of *hairy* expression obtained with the *twi*–*Krüppel* and *twi*–*Krüppel*–*hairy* transgenes provides evidence that dCtBP and Groucho mediate short- and long-range repression, respectively. The *twi*–*Krüppel* transgene causes the repression of *hairy* stripe 6, but not stripe 5. Previous studies have shown that the stripe 6 enhancer contains optimal, high-affinity Krüppel operator sites that can be occupied by the low levels of Krüppel produced in ventral regions by the *twi*–*Krüppel* transgene (Langeland *et al.*, 1994). These low levels appear to be insufficient to bind the low-affinity sites within the *hairy* stripe 5 enhancer and, consequently,

Fig. 5. Summary of the hot chromatin model. The diagrams in (B–D) present the hypothetical on/off states of several enhancers located in different positions along the dorsoventral axis of the early embryo. (A) Summary of the *hairy* 5’ cis-regulatory region. *hairy* stripes 2, 5, 6 and 7 are regulated by four different enhancers in the 5’-flanking region. The stripe 5 and stripe 6 enhancers are separated by >2 kb. Previous studies (Langeland *et al.*, 1994) have shown that the stripe 6 enhancer contains a series of high-affinity Krüppel-binding sites. Consequently, low levels of the Krüppel repressor gradient (see diagram on left) are sufficient to bind these operator sites and repress stripe 6 expression. The stripe 5 enhancer is active in regions containing low and intermediate levels of Krüppel since the enhancer contains low-affinity sites that are occupied only by high concentrations of the Krüppel protein. The repression of *hairy* stripe 5 by the Krüppel–Hairy fusion protein (Figure 2) suggests that the binding of this repressor to the stripe 6 enhancer can work over a long range to repress the stripe 5 enhancer. (B) Summary of the synthetic h-NEE-h–2×PE (*twi*) transgene. The Snail repressor normally binds to the NEE and represses its activity in the ventral mesoderm. Snail recruits the dCtBP corepressor and works locally, within the limits of the NEE, and does not interfere with the activity of the linked *twi* (2×PE) enhancer. In contrast, the Hairy repressor recruits Groucho, and the Hairy–Groucho complex not only represses the modified NEE, but can also work over a distance to repress the linked *twi* enhancer. (C) Summary of the h-NEE-h–2×*race* transgene. Hairy fails to repress the *race* enhancer in dorsal regions of transgenic embryos. We propose that the h-NEE-h enhancer might be inaccessible for the binding of Hairy in dorsal regions where the *race* enhancer is active. The NEE is activated by the maternal Dorsal gradient. There is no Dorsal activator present in the dorsal ectoderm and, consequently, the h-NEE-h and NEE enhancers might not be available for binding Hairy (or Snail–Hairy) in these regions where the *race* and *rhomboid* amnioserosa enhancers are active. (D) Summary of the h-*race*-h–*twist* transgene. The *race* enhancer is probably activated by Mad and Medea, and other transcription factors that are restricted to the dorsal ectoderm (reviewed by Podos and Ferguson, 1999). The h-*race*-h enhancer might be condensed in ventral regions due to the absence of these activators. As a result, Hairy is unable to bind the modified *race* enhancer in ventral regions where the *twist* enhancer is expressed.

the native Krüppel protein works as a short-range repressor to inhibit stripe 6 expression without affecting stripe 5 expression (Figure 5A). In contrast, the *twi*-Krüppel-hairy transgene leads to the repression of both stripes 5 and 6. The binding of the Krüppel-Hairy fusion repressor to the stripe 6 enhancer appears to cause the dominant silencing of the neighboring stripe 5 enhancer over a distance of ~2.5 kb in the hairy 5' regulatory region (Figure 5A). An implication of these observations is that different repression domains exert distinct influences on embryonic patterning. Replacing the PxDSLxH motif (native Krüppel) with WRPW (Krüppel-Hairy) changes the regulatory activity of the Krüppel repressor.

The Snail-Hairy fusion protein represses the *rhomboid* lateral stripes, but fails to repress the amnioserosa pattern. In contrast, the same Hairy repression domain permits Krüppel to function as a dominant silencer within the hairy 5' regulatory region. There are several possible explanations for the failure of the Snail-Hairy repressor to silence *rhomboid* expression in the amnioserosa. Perhaps there is competition between dCtBP bound to the Snail moiety and Groucho bound to the Hairy moiety within the fusion protein (see Figure 1). The Krüppel-Hairy fusion protein was mutagenized to eliminate the dCtBP motif (PEDLSMH), whereas the Snail-Hairy fusion protein retains both dCtBP sequences. Previous studies suggest that the conversion of the weak dCtBP interaction motif near the Hairy C-terminus, PLSLVIK, into an optimal motif, PLDSLIIK, disrupts the repressor function of an otherwise normal Hairy protein (Zhang and Levine, 1999). This result was taken as evidence that the dCtBP and Groucho corepressors interfere with one another when bound to closely linked motifs within the Hairy C-terminus. An argument against this explanation for the behavior of the Snail-Hairy fusion protein stems from the observation that the binding of Hairy to a modified NEE is sufficient to repress a linked mesoderm enhancer (*twist* PE), but not a similarly spaced *race* enhancer (Figure 4). Similarly, the binding of Hairy to a modified *race* enhancer fails to silence the mesoderm enhancer.

We propose that Hairy can only bind active or 'open' enhancers (summarized in Figure 5). The NEE is activated by the maternal Dorsal nuclear gradient (Ip *et al.*, 1992) and, consequently, it might contain activator proteins in both ventral and lateral regions of early embryos (Figure 5B). As a result, the binding of Hairy to the modified h-NEE-h enhancer can lead to the dominant silencing of a linked mesoderm enhancer (*twist* PE). In contrast, there is no Dorsal activator in dorsal regions of the early embryo, thereby rendering the h-NEE-h enhancer in a closed or condensed state (Figure 5C). This absence of activator might preclude the binding of Hairy so that the *race* enhancer is not silenced. Similarly, the *race* enhancer is probably activated by transcription factors that are restricted to dorsal regions, such as Zen and Smads (Figure 5D; Rusch and Levine, 1997). These activators are absent in ventral regions and, consequently, Hairy may be unable to bind the h-race-h enhancer and silence linked enhancers such as the *twist* PE.

The altered pattern of hairy expression caused by the Krüppel-Hairy fusion protein can be interpreted in the context of this 'hot chromatin' model (Figure 5A). There is

evidence that hairy stripes 5, 6 and 7 are activated by a posterior gradient of the Caudal activator (Hader *et al.*, 1998). The binding of the Krüppel-Hairy fusion protein to the optimal Krüppel operator sites in the stripe 6 enhancer would be expected to silence the neighboring stripe 5 enhancer due to the open conformation of the stripe 6 enhancer in those regions of the embryo where stripe 5 is expressed. Thus, the Caudal activator might bind to both enhancers in the position of stripe 5, thereby rendering the stripe 6 enhancer accessible to the Krüppel-Hairy fusion protein.

The dependence of repressors on activators might restrain long-range repressors and permit enhancer autonomy. This dependence might reflect the inherent properties of activators and repressors. Some activators recruit enzymes that decondense chromatin, and this may be essential for the binding of repressors *in vivo* (e.g. Mannervik *et al.*, 1999). Short-range repression has been put forward as an important mechanism for enhancer autonomy (e.g. Small *et al.*, 1993; Gray *et al.*, 1994). We suggest that a second mechanism involves the reliance of repressors on activators for binding to target enhancers.

Materials and methods

In situ hybridization assays and transgenic embryos

Embryos were collected from transgenic adults, fixed and then hybridized with digoxigenin-labeled antisense RNA probes as described by Jiang *et al.* (1991). Transgenic strains were obtained by injecting γw^{67} embryos with various P-element transformation vectors as described by Rubin and Spradling (1982).

Preparation of expression vectors

Many of the experiments described in this study involved the misexpression of different Krüppel-coding sequences in ventral regions of pre-cellular transgenic embryos using a modified version of the PE enhancer from the *twist* 5' regulatory region (Jiang and Levine, 1993). An FRT-stop-FRT cassette was inserted between the promoter and the Krüppel-coding sequence in order to circumvent dominant lethality and permit the isolation of transgenic lines (Struhl and Basler, 1993). A 1 kb *NotI*-*NotI* DNA fragment containing two tandem copies of the *eve* stripe 2 enhancer was removed from a previously described pCaSpeR transformation vector (Kosman and Small, 1997). This vector contains the *eve* stripe 2 enhancers upstream of an FRT-stop-FRT cassette. The 1 kb *NotI*-*NotI* fragment was replaced with a 0.5 kb *NotI*-*NotI* fragment containing two tandem copies of the PEeEt enhancer, which contains nucleotide substitutions that create optimal Dorsal operator sites and Twist basic helix-loop-helix (bHLH) E boxes (Jiang and Levine, 1993). This enhancer directs expression in the ventral-most 22–26 cells, which include the entire presumptive mesoderm and ventral regions of the neurogenic ectoderm.

A wild-type Krüppel cDNA encoding the full-length protein was isolated on a 2.1 kb *PstI*-*EcoRI* fragment. This DNA also contains ~60 bp of the 5'-untranslated region (5'-UTR) and ~460 bp from the 3'-UTR. This fragment was cloned into a modified Bluescript SK+ plasmid, pBSK+/Asc2, which contains two *AscI* sites in place of unique *HincII* and *SacI* sites in the polylinker.

A mutant form of the Krüppel coding sequence, Krüppel Δ PED, lacks the dCtBP interaction motif. The PEDLSMH motif was converted into AAALSMH, which fails to mediate binding to dCtBP (described by Nibu *et al.*, 1998b). The mutant Krüppel Δ PED coding region was also isolated on a 2.1 kb *PstI*-*EcoRI* DNA fragment and cloned into the modified Bluescript vector (pBSK+/Asc2).

Unique *NdeI* and *BglII* sites were inserted between codon 502 and the stop codon within the Krüppel Δ PED cDNA. The following mutagenic oligonucleotide was used: 5'-CGGTGTGGTACTGGCctAGATCT-TGCCATATGTTGTTGATGGCC-3' (cta, stop codon; **ATG**, codon 502; underlined sequence corresponds to the synthetic *NdeI* restriction site; double underlines correspond to a *BglII* site). A 250 bp *NdeI*-*BglII* fragment containing hairy codons 255–337 was prepared by PCR using

appropriate primers. The 250 bp *NdeI*–*Bgl* II fragment was inserted into the *NdeI* and *Bgl* III sites of the mutagenized *Krüppel* Δ *PED* cDNA.

The wild-type, Δ *PED* and Krüppel–Hairy fusion sequences were isolated as *AscI*–*AscI* DNA fragments and inserted into the unique *AscI* site within the 2 $\times$ PEeEt expression vector. The *AscI* site is located between the FRT–stop–FRT cassette and the 3′-UTR from the *eve* gene.

An *EcoRI*–*XbaI* DNA fragment containing codons 255–337 of the hairy coding region was inserted into synthetic *EcoRI* and *XbaI* sites created at the 3′ end of the *snail* coding sequence (residue 390) within a modified pBluescript vector. The encoded fusion protein contains the entire *Snail*-coding sequence and amino acids residues 255–337 of the Hairy C-terminus. An *AscI* DNA fragment containing the *snail*–*hairy* coding sequence was inserted into the stripe 2–FRT–stop–FRT expression vector described by Kosman and Small (1997).

Synthetic reporter genes

The embryos shown in Figure 4A and B were obtained from transgenic strains described by Barolo and Levine (1997). The *lacZ* reporter genes were placed under the control of synthetic modular promoters containing the 300 bp *rhomboid* lateral stripe enhancer (NEE) placed upstream of the two tandem copies of the 250 bp PE enhancer from the *twist* promoter (Jiang and Levine, 1993). A synthetic Hairy repressor site was placed at each of the NEEs (Figure 4B).

The modified h-NEE-h enhancer described by Barolo and Levine (1997) was isolated as a 600 bp *Bam* HI–*Bst* XI DNA fragment. Synthetic *Bst* XI–*Bgl* II restriction sites were created within the *Bam* HI–*Bgl* III sites of a modified pBluescript II KS+ plasmid, pBlueG, which contains a unique *Bgl* III site in place of *Sma* I. A 340 bp *Pst* I–*Bam* HI (originally *Dra* I–*Dra* I) DNA fragment containing the CAT coding sequence was inserted into the *Bgl* II–*Pst* I sites of the h-NEE-h of the pBlueG plasmid. Two tandem copies of the minimal *race* enhancer (Rusch and Levine, 1997) were isolated on a 1 kb *Not* I–*Not* I DNA fragment. The *Not* I sites were blunted and inserted into the unique *Eco* RV site of the Bluescript plasmid containing the h-NEE-h enhancer. The *Hinc* II site in the polylinker was replaced with a *Not* I site. A 2.1 kb *Not* I–*Not* I fragment containing h-NEE-h, the CAT spacer and two copies of the *race* enhancer was inserted into the *Not* I site of a CaSpeR-AUG- β gal transformation vector (Thummel *et al.*, 1988) containing the *eve* basal promoter, starting at –42 bp and continuing through codon 22 fused in-frame with *lacZ* (Small *et al.*, 1992).

Hairy-binding sites were introduced into the minimal, 533 bp *race* enhancer via PCR amplification using two primers containing synthetic Hairy sites: 5′-TTAAGATCTGCGGCACGCGACATATCGATTGTTG-TCTCATCGGCGGG-3′ and 5′-ATTGGATCCATGTCGCGTGCCGC-CGCGAGCATTATTTATTTTAATGCGAG-3′ (underlined sequences correspond to the Hairy-binding site).

The PCR product was digested with *Bgl* II and *Bam* HI, and cloned into pBlueG. Head to tail two tandem copies of h-*race*-h were prepared by digestion of the recombinant plasmid with either *Sca* I–*Bam* HI or *Sca* I–*Bgl* III; the two DNA fragments were ligated to each other and isolated on a 1.1 kb *Hind* III–*Xba* I DNA fragment. This fragment was inserted into the *Hind* III–*Not* I sites of the pBSK+/*Asc* 2 vector along with a 340 bp *Xba* I–*Not* I DNA fragment containing the CAT coding sequence (used as a spacer). A 0.5 kb *Not* I–*Not* I DNA fragment containing two tandem copies of the 250 bp PE enhancer was inserted into the *Not* I site. A 1.9 kb *Asc* I–*Asc* I fragment containing two tandem copies of the h-*race*-h enhancer, the CAT spacer sequence and two copies of the *twist* PE enhancer was inserted into the *Asc* I site of the CaSpeR-AUG- β gal transformation vector (Thummel *et al.*, 1988) containing the *eve* promoter fused in-frame with *lacZ* (Small *et al.*, 1992).

Acknowledgements

We thank Drs Satoru Kobayashi and Akira Nakamura for providing the yw strain and advice on the injection of P-element transformation vectors. Y.N. is a fellow of the Japan Society for the Promotion of Science (JSPS). H.Z. is a fellow of the NIH. This work was supported by an NIH grant (GM 34431).

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Received September 11, 2000; revised February 9, 2001;
accepted March 6, 2001