Requirements for transcriptional repression and activation by Engrailed in *Drosophila* embryos

Cyrille Alexandre* and Jean-Paul Vincent

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

*Author for correspondence (e-mail: calexan@nimr.mrc.ac.uk)

Accepted 11 November 2002

SUMMARY

Genetic analysis shows that Engrailed (En), a homeodomain-containing transcription factor, has both negative and positive targets. Negative regulation is expected from a factor that has a well-defined repressor domain but activation is harder to comprehend. We used VP16En, a form of En that had its repressor domain replaced by the activation domain of VP16, to show that En activates targets using two parallel routes, by repressing a repressor and by being a bona fide activator. We identified the intermediate repressor activity as being encoded by *sloppy paired 1* and 2 and showed that bona fide activation is dramatically enhanced by Wingless signaling. Thus, En is a bifunctional transcription factor and the recruitment of additional cofactors presumably specifies which function prevails on an individual promoter. Extrardenticle (Exd) is a cofactor thought to be required for activation by Hox proteins. However, in thoracic segments, Exd is required for repression (as well as activation) by En. This is consistent with in vitro results showing that Exd is involved in recognition of positive and negative targets. Moreover, we provide genetic evidence that, in abdominal segments, Ubx and Abd-A, two homeotic proteins not previously thought to participate in the segmentation cascade, are also involved in the repression of target genes by En. We suggest that, like Exd, Ubx and Abd-A could help En recognize target genes or activate the expression of factors that do so.

Key words: *Drosophila*, Transcriptional repression and activation, Engrailed, Hedgehog, Wingless signaling, Extrardenticle, Homeotic genes

INTRODUCTION

Engrailed (En) comprises a homeodomain that recognizes specific DNA sequences (Desplan et al., 1986; Kissinger et al., 1990) and a domain that confers repressive activity to heterologous DNA-binding proteins in a variety of systems (Jaynes and O’Farrell, 1991; Han and Manley, 1993; Badiani et al., 1994; John et al., 1995). As expected therefore, during normal *Drosophila* development, several genes are repressed by En. These include *cubitus interruptus (ci)* (Schwartz et al., 1995), *wingless (wg)* (Heemskerk et al., 1991), and *patched (ptc)* (Hooper and Scott, 1989). At the same time as being a repressor, En is also involved in the activation of target genes. One probable positive target is *en* itself as genetic evidence suggests that En autoregulates positively after its initial activation by pair-rule gene products (Heemskerk et al., 1991). Another genetically defined positive target is *hedgehog (hh)*, whose expression faithfully tracks that of *en* and decays in *en* mutants (Lee et al., 1992; Tabata et al., 1992). Finally, *polyhomeotic (ph)* also requires En for continued expression and is activated by ectopic En (Serrano and Maschat, 1998). In summary, three genes appear to be positive targets of En. How could a molecularly characterized repressor activate transcription? One possibility is that it does so via an intermediate, by repressing a repressor, as proposed by Smith and Jaynes (Smith and Jaynes, 1996). This possibility was also thought to explain transcriptional activation by the repressor Eve (Manoukian and Krause, 1993). Alternatively, En could, within the appropriate sequence context, act as a true activator, perhaps by recruiting a specific set of cofactors (Pinsonneault et al., 1997).

To investigate the activation function of En in vivo, we engineered a form of En that can only function as an activator by removing its repressor region and replacing it by the transactivation domain of VP16 (thus making VP16En). Using this tool we show that one mode of activation by En involves the repression of a repression. However, we show that, in parallel, En also functions as a true activator and that such activation requires Wg signaling. Because no clear activation domain is recognizable in En (Han and Manley, 1993), we presume that positive targets can recruit cofactors that provide an activation function (reviewed by Mannervik et al., 1999). One possible cofactor is the homeodomain-containing protein encoded by *extradenticle (exd)*, given that it is required for positive autoregulation by En (Peifer and Wieschaus, 1990). Moreover, activation of *ph* by En also requires Exd (Serrano and Maschat, 1998) and in vitro binding experiments have shown that Exd increases the binding of En on specific ‘activation sites’ (Peltenburg and Murre, 1996; Serrano and Maschat, 1998). Overall, these analyses have led to the view that Exd could be a DNA binding specificity factor that operates on positively regulated genes (Chan et al., 1994). One
problem with this model is that the vertebrate homologs of Exd, the PBX family members, have been implicated in negative (as well as positive) target recognition, at least in vitro (Asahara et al., 1999; Saleh et al., 2000).

We found that VP16En requires Exd to activate positive targets at the anterior of the germ band. In other words, the VP16 activation domain does not override the need for Exd. This reinforces the view that the role of Exd is in target recognition and not in providing an activation domain. Indeed, at the anterior of the germ band, Exd is required for repression, as well as activation, by En. Thus, in this instance at least, Exd is not an activation-specific cofactor. In the abdominal region, Exd is dispensable for repression. Instead, in this domain, the homeotic proteins Ubx and Abd-A contribute to repression by En. We suggest that these two homeodomain-containing proteins (or a target thereof) could play the role of Exd in a region of the embryo where En levels are low (Rauskolb and Wieschaus, 1994; Mann and Abu-Shaar, 1996; Aspland and White, 1997).

**MATERIALS AND METHODS**

**Drosophila stocks**

The following mutants were used: \( w^{+}\) (Baker, 1987), \( en^{+}\) (Heemskerk et al., 1991), \( Df(2R)\) (Tabata et al., 1995), \( ci^{+}\) (Hochman, 1974), \( slp\) deficiencies \( Df(2L)\) (Tabata et al., 1995), \( Cyo\) (Grossniklaus et al., 1992), double mutant \( slp^{+}\) (Poole et al., 1985) was generated at the University of Michigan, Ann Harbor. Likewise, ectopic En activates expression of the endogenous \( en\) gene (detectable with a specific probe, see methods; Fig. 1E,F). Thus, formally, both \( en\) and \( hh\) are positive targets of En.

The En protein harbors a domain, located between residues 168 and 298, that mediates potent repression in *Drosophila* cells and a variety of heterologous systems (see Introduction). This well-defined repressor activity suggests that En might exert its positive transcriptional effects indirectly, by repressing a repressor. To address this possibility, we sought to invert the activity of En by replacing its repressor region [region ABCD as defined by Han and Manley (Han and Manley, 1993)] with the strong activator domain of the HSV viral protein VP16 (Triezenberg et al., 1988) (Fig. 2). The resulting protein is called VP16En. As outlined in the diagram in Fig. 2, we reasoned that if wild-type En activates transcription by repressing a repressor (e.g. \( R\)), then VP16En should repress target genes such as \( hh\) and \( en\). By contrast, if En acts as an activator, whether directly or indirectly, VP16En should still activate \( hh\) and \( en\).

Before testing these hypotheses, we asked whether the activation domain of En was functional. We assessed the effect of VP16En on the transcription of \( ci\), a gene repressed by \( en\). As expected, expression of VP16En with the \( paired-Gal4\) driver leads to ectopic transcription of \( ci\) (Fig. 3A,B). This may or may not be a direct effect of VP16En.

**RESULTS**

En activates target genes by repressing the expression of a repressor, \( R\)

En represses the expression of a variety of genes including \( ci\), \( ptc\) and \( wg\). For example, Fig. 1A,B shows that \( ci\) expression is repressed by ectopic En (expressed from the \( paired-Gal4\) driver). However, the presence of En also leads to the activation of a different set of genes. For example, ectopic En activates \( hh\) transcription (Fig. 1C,D); see also Tabata et al. (Tabata et al., 1992). Likewise, ectopic En activates expression of the endogenous \( en\) gene (detectable with a specific probe, see methods; Fig. 1E,F). Thus, formally, both \( en\) and \( hh\) are positive targets of En.

**MATERIALS AND METHODS**

**Drosophila stocks**

The following mutants were used: \( w^{+}\) (Baker, 1987), \( en^{+}\) (Heemskerk et al., 1991), \( Df(2R)\) (Tabata et al., 1995), \( ci^{+}\) (Hochman, 1974), \( slp\) deficiencies \( Df(2L)\) (Tabata et al., 1995), \( Cyo\) (Grossniklaus et al., 1992), double mutant \( slp^{+}\) (Poole et al., 1985) was generated at the University of Michigan, Ann Harbor. Likewise, ectopic En activates expression of the endogenous \( en\) gene (detectable with a specific probe, see methods; Fig. 1E,F). Thus, formally, both \( en\) and \( hh\) are positive targets of En.

The En protein harbors a domain, located between residues 168 and 298, that mediates potent repression in *Drosophila* cells and a variety of heterologous systems (see Introduction). This well-defined repressor activity suggests that En might exert its positive transcriptional effects indirectly, by repressing a repressor. To address this possibility, we sought to invert the activity of En by replacing its repressor region [region ABCD as defined by Han and Manley (Han and Manley, 1993)] with the strong activator domain of the HSV viral protein VP16 (Triezenberg et al., 1988) (Fig. 2). The resulting protein is called VP16En. As outlined in the diagram in Fig. 2, we reasoned that if wild-type En activates transcription by repressing a repressor (e.g. \( R\)), then VP16En should repress target genes such as \( hh\) and \( en\). By contrast, if En acts as an activator, whether directly or indirectly, VP16En should still activate \( hh\) and \( en\).

Before testing these hypotheses, we asked whether the activation domain of En was functional. We assessed the effect of VP16En on the transcription of \( ci\), a gene repressed by \( en\). As expected, expression of VP16En with the \( paired-Gal4\) driver leads to ectopic transcription of \( ci\) (Fig. 3A,B). This may or may not be a direct effect of VP16En. However, inversion of activity by VP16En confirms that regulation of \( ci\) expression by En requires bona fide repressor activity. If this were not the case, no inversion would be seen (see diagram in Fig. 2). Two additional genes, \( wg\) and \( ptc\), are repressed by En (Yoffe et al., 1995) (and not shown). As with \( ci\), this activity is reversed by the presence of the VP16 activation domain: VP16En activates transcription of \( wg\) (Fig. 3C,D) and \( ptc\) (not shown). Because \( Ci\) is a known positive effector of \( wg\) transcription (Alexandre et al., 1999), the activating effect of VP16En on \( wg\) expression could conceivably be mediated by \( Ci\) (VP16En activates \( ci\)
Activities of Engrailed in vivo

However, as shown in Fig. 3E,F, VP16En activates wg expression, even in the absence of Ci. Thus, Ci is not a required intermediate for VP16En to activate wg transcription. Importantly for the remainder of this paper, replacement of the repression domain of En with the VP16 activation domain does reverse repression into activation.

Having established that VP16En is functional allowed us to examine its effects on the two positive targets of En. As shown in Fig. 4, VP16En driven by pair-Gal4 represses the expression of both hh and en. In such embryos, hh transcription begins to decay at stage (st) 10 and, by late st12, hh transcripts are absent in the segments where VP16En is expressed (Fig. 4B). en transcripts follow a similar kinetics of disappearance and by st12, en is completely repressed in the stripes where VP16En is expressed (Fig. 4D). It is highly unlikely that VP16En acts as a true repressor and, therefore, VP16En probably represses hh expression via an intermediate repressor (R). Accordingly, in the wild type, En would repress the expression of R, which itself would repress hh and en expression (see diagram in Fig. 2).

**R is encoded by slp**

Could the ci gene encode R? Expression of ci is clearly repressed by En (see above). And the Ci protein can be processed by cleavage into a repressor of hh transcription (Dominguez et al., 1996; Aza-Blanc et al., 1997). However, as ci mutants are rescued by a transgene encoding an uncleavable form of Ci (Methot and Basler, 1999), the repressor form of ci is dispensable for embryogenesis. Therefore, Ci is not an essential intermediate for the activating function of En.

Other potential candidates for R are Slp1 and Slp2, two homologous zinc finger proteins encoded by adjacent co-regulated genes (Grossniklaus et al., 1992). In various embryonic assays, these genes appear to be redundant. Therefore, we used deficiencies that remove both genes to assay their function, and we refer to them as one gene, slp. slp
is a candidate for R as it represses the transcription of en and hh (Cadigan et al., 1994). Conversely, it is itself repressed by En given that ectopically expressed en (with paired-Gal4) completely suppresses slp transcription as early as st10 (Fig. 5B). As expected then, VP16En activates slp expression (Fig. 5D). In summary, En represses slp, which itself represses hh (and en), implying that slp could be R, at least within the regions of the epidermis where these interactions occur.

**En is an activator (in addition to being a repressor)**

Our results so far suggest that En activates target genes via the repression of slp expression. However, we cannot exclude the possibility that, in parallel, En could perform a positive role on its own. To investigate this possibility, we assessed the effect of exogenous En on hh expression in slp-deficient embryos. In the absence of slp, en (and hh) expression decays for lack of Wg signaling, especially in odd-numbered segments (Fig. 6A,B) (see Cadigan et al., 1994). Therefore, we used the

---

**Fig. 3.** VP16En activates genes that are normally repressed by En. Embryos placed side by side are of the same genotype except that embryos on the right-hand side express VP16En under the control of paired-Gal4. (A,B) Expression of ci in the abdominal region of wild-type (A) and paired-Gal4 UAS-VP16En (B) embryos. In the wild-type (A), ci is repressed by En in every segment. paired-Gal4-driven VP16En leads to ectopic activation of ci expression thus blotting out repression in alternate segments (B). (C,D) Expression of wg in the abdominal region of wild-type (C) and paired-Gal4 UAS-VP16En (D) embryos. Expression of wg is broadened in response to VP16En expression in alternate segments (D). (E) Expression of wg in a ciCE embryo. No expression is detectable in the segmented ectoderm. This is expected because Hh signaling (hence Ci) is required for continued wg expression (Tabata et al., 1992). (F) Expression of wg in a ciCE embryo that expresses VP16En under the control of paired-Gal4. Ectopic expression of wg is induced even though Ci is absent, consistent with the possibility that VP16En could activate wg expression directly.

**Fig. 4.** VP16En represses the expression of en and hh, two positive targets of En. Wild-type embryos are shown on the left and paired-Gal4 UAS-VP16En embryos on the right. (A,B) Expression of hh in wild type (A) and in an embryo expressing VP16En (B). (C,D) Expression of en in a wild-type embryo (C) and in an embryo expressing VP16En (D). A specific probe that does not recognize VP16En was used to detect en mRNA. In B and D, expression of the target gene (hh and en, in purple) is clearly repressed in the cells that express VP16En (labelled in ochre).

**Fig. 5.** Expression of slp is repressed by En. (A) Expression of slp in a wild-type st11 embryo. (B) Expression of slp in an embryo that expresses both ectopic en and lacZ under the control of paired-Gal4 [prG4(Z) UAS-En]. slp transcripts (black) disappear where En is ectopically expressed (here the domain of ectopic expression is recognized with a lacZ probe in red). (C) Abdominal region (A5-A8) of a wild-type embryo showing the expression of slp at high magnification. (D) Similar view of an st11 embryo expressing VP16En under the control of paired-Gal4. Notice the broadening of the slp stripes in odd-numbered segments, where paired-Gal4 is active.
paired-Gal4 driver to reintroduce En in these segments and assayed the effect on hh expression. As shown in Fig. 6C, hh expression is activated (albeit not strongly, see below). Thus, En still activates hh expression in the absence of Slp. This could conceivably occur via another intermediate repressor (R'). However, in slp mutants, in contrast to the slp+ situation, VP16En activates hh expression (compare Fig. 6D with Fig. 4B) and also that of en (not shown). We conclude that no other 'dominant' repressor operates, at least in the domain defined by paired-Gal4. If another repressor existed, its expression would be activated by VP16En and this would prevent activation of hh expression in slp mutant embryos. Note that, with the Slp repressor out of the way, En and VP16En have the same effect on expression of hh (and also of en; not shown). By contrast, in the presence of VP16En and En have the same effect on expression of hh and also hh expression is weaker in odd-numbered segments (where hh decays in slp). Panels (B) and (C) are repeated from Fig. 6A,C to allow comparison with the effect of Wg. As seen, Wg and En have a similarly weak positive impact on hh expression. Together, Wg signaling (here induced with activated Armadillo, A*) and En cause strong activation of hh expression (panel D). (E) hh transcription in a wgCX4 embryo overexpressing En under the control of paired-Gal4. The histochemical reaction was allowed to proceed for a long time to reveal the weak signal. Expression is weak. Therefore, En can partially activate hh transcription in the absence of Wg signaling but Wg signaling is required for full activation. Indeed, as shown in (F), co-expression of En and activated Armadillo leads to strong hh expression. Remember that VP16En is a stronger activator than En in slp mutants. This is probably because VP16En is a stronger activator than En in slp mutant embryos. Note that the three conditions that we have shown to be required for maximal activation of hh (Fig. 7D). This additive effect explains why VP16En is more potent than En in slp− embryos as VP16En activates wg expression in addition to activating that of en and hh. Note that the three conditions that we have shown to be required for maximal activation of hh expression (Wg signaling, presence of En and absence of Slp) are fulfilled in cells that normally express hh in wild-type embryos. The contribution of Wg signaling to activation by En is also illustrated in wg mutant that express En under the control of paired-Gal4. In such embryos, only weak (barely detectable) activation of hh expression is seen while embryos co-expressing En and activated Armadillo (otherwise wild type) show strong hh expression (Fig. 7E,F).
Exd and Homothorax are required for repression – as well as activation – by En

Because En does not contain a recognizable activation domain, it is likely that cofactors modify its activity on positive targets. Indeed, it has been suggested that Exd is an activation-specific cofactor of En (Peifer and Wieschaus, 1990; Heemskerk et al., 1991; Serrano and Maschat, 1998). Furthermore, Exd’s activity is regulated by Wg signaling, at least in leg imaginal disks (Mann and Abu-Shaar, 1996) and this could potentially explain the contribution of Wg signaling in activation by En. Consistently with a role of Exd in activation by En, neither en (not shown) nor hh is activated by paired-Gal4-driven En in exd– embryos (Fig. 8B; compare with activation in the presence of exd+ in Fig. 8C). In all its known functions, Exd requires the presence of another homeobox-containing protein, Homothorax (Hth) (Kurant et al., 1998; Rieckhof et al., 1997; Pai et al., 1998). As expected then, activation of targets (like hh and en itself) by En (driven by paired-Gal4) is severely compromised in hth64-1 mutant embryos, (Fig. 8D). Therefore, the hth64-1 mutation provides an alternative way to remove exd function (although we recognize that Hth may be more than just an accessory to Exd; see Discussion).

How does Exd/Hth contribute to activation by En? It has been suggested that Exd could mask the repressor domain of Hox proteins while at the same time perhaps providing an activation domain [e.g. for Deformed (Pinsonnault et al., 1997)]. If, in the case of En, this was the sole function of Exd, VP16En would not require Exd to activate target genes because an activation domain would be provided exogenously. We tested this possibility. For positive targets, the result is simple. VP16En was expressed with paired-Gal4 in hth– slp– double-mutant embryos (Slp was removed to avoid its dominant repressive activity). No activation of either en (not shown) or hh (Fig. 9B) is seen. This shows that Exd is required for VP16En to activate transcription even though VP16En carries its own activation domain. This is consistent with in vitro data showing that Exd is required for positive target recognition. For
negative targets, one might expect the activity of VP16En to not be affected by the removal of Exd activity. Surprisingly, this is true only in parts of the germ band. In abdominal (A) segments, at least in A1, A3, A5 and A7 where paired-Gal4 is active, ci, wg and slp are activated by VP16En even in a hth− background. By contrast, at the anterior of the germ band – for example, in the second thoracic segment (T2) – activation of the same targets does require Hth. This difference is illustrated in Fig. 9D,F, using wg and slp as targets. It can be seen that, in the hth mutant, no activation occurs in T2 but it does in A1. Therefore, in T2, and also in more anterior head segments (not shown), VP16En requires exd/hth to activate both negative and positive targets of En. One probable interpretation is that Exd helps VP16En to recognize all (including negative) targets of En. Thus, we might expect repression by En to require exd/hth in anterior segments.

As expected, we find that, in embryos devoid of maternal and zygotic Exd (or lacking zygotic Hth), exogenous expression of En with the paired-Gal4 driver can only repress the expression of both ci (Fig. 10A) and slp (Fig. 10B) in abdominal segments (from A1 to A7). No repression is seen at the anterior of the germ band (compare segments T2 and A1 in Fig. 10). By contrast, Exd is required for activation by En throughout the germ band. This is illustrated in Fig. 10C, which shows that in exd− embryos, activation of hh transcription by ectopic En is severely compromised both in T2 and in A1. Importantly, the obligate role of Exd in repression at the anterior of the germ band shows that Exd is not an ‘activation-specific’ cofactor.

Role of the two homeotic proteins Ubx and Abd-A in repression by En

Exd/Hth is required for En to repress target genes in T2 (and more anteriorly) but not in the abdomen. What could be the genetic basis of this difference? One obvious possibility is that genes of the Bithorax complex are involved given that they are differentially expressed along the A-P axis and they specify segmental identity (Akam and Martinez Arias, 1985; Karch et al., 1990; Macias et al., 1990). In particular, in the absence of Ubx and Abd-A, abdominal segments such as A1 acquire a thoracic phenotype. Conversely, overexpression of either Ubx or Abd-A converts thoracic segments into abdominal ones. Consistent with a role of homeotic genes in En function, coexpression of Abd-A and En leads to the repression of ci transcription in T2 of hth− embryos (Fig. 11A), and the same is true for coexpressed Ubx and En (not shown). Coexpression is required because any factor alone fails to repress ci expression in T2 of hth− mutant embryos (see Fig. 11B for Abd-A and Fig. 10A for En; not shown for Ubx). Note also that coexpression of En and Antennapedia (Antp), a closely related Hox protein, does not lead to repression in T2 of hth mutants (Fig. 11C).

We conclude that the presence of Ubx or Abd-A specifically allows En to repress targets in T2 in hth/exd mutant embryos. One possible interpretation is that overexpressed Ubx or Abd-A gives T2 an abdominal character and thus renders repression by En independent of exd/hth (as it is in A1-A7). Alternatively, Ubx or Abd-A (or a target gene thereof) could fulfill the role of Exd/Hth in helping En repress its negative targets in areas where Exd is low.

To further confirm the role of homeotic products, we assayed the effect of Ubx on VP16En activity. As shown above, VP16En activates the expression of negative targets of En such as wg (Fig. 9C) and, in the thorax, Hth is absolutely required for this to occur (Fig. 9D). Fig. 11E shows that coexpression of En enables VP16En to activate wg expression in T2 of a hth mutant embryo.

The experiments above used ectopic expression to show the activity of Ubx and Abd-A. We next investigated the issue of requirement using a loss-of-function approach. No defect in En function has been reported in embryos lacking Ubx and abd-A and, indeed, negative targets of En (such as ci) are normally repressed in embryos homozygous for Df(3R)Ubx109, which removes both Ubx and Abd-A (not shown). Moreover, as shown in Fig. 11G, paired-Gal4-driven En represses ci expression in Df(3R)Ubx109 embryos. Superficially then, Ubx and Abd-A appear not to be required for repression by En. However, expression of exd, as well as that of hth is upregulated in the germ band of Bithorax complex mutants (Rauskolb and Wieschaus, 1994; Kurant et al., 1998) and this could therefore provide redundant cofactor activity. To address
this possibility, we assayed En’s activity in embryos lacking Ubx, abd-A and hth (Df(3R)Ubx\textsuperscript{109} hth\textsuperscript{64–1}). Note that these embryos are still segmented and continue to express paired-Gal4 in stripes (e.g. Fig. 11I). Significantly, ectopic En does not repress ci anywhere in the germ band of such embryos (Fig. 11H). This provides evidence that Ubx and Abd-A are normally part of the mechanism that allows En to act on its negative targets in the abdomen.

**DISCUSSION**

Molecular studies with minimal DNA binding sites show that En is a transcriptional repressor (Jaynes and O’Farrell, 1991). Yet, genetic evidence suggests that it is both an activator and a repressor. Here, we show that En activates target genes using two parallel modes of action: by repressing a repressor and by acting as a bona fide activator. Although Exd has been thought to be an activation-specific cofactor for various homeodomain-containing proteins, we found that it is required for both activation and repression by En. Which cofactors allow En to function as an activator is still unknown. We also provide evidence for the requirement of an additional cofactor in repression by En and show that such requirement is fulfilled by, or dependent on, the homeotic proteins Ubx or Abd-A.

**The role of Slp**

The repressor activity that lies between En and its positive targets is encoded by slp\textsubscript{1} and slp\textsubscript{2}. These two genes are repressed by En and their products repress en expression (see also Kobayashi et al., 2003). Importantly, Slp\textsubscript{1} and Slp\textsubscript{2} are the only dominant repressors that stand between En and its positive targets, hh and en – at least in the paired-Gal4 domain. If another such repressor existed, it would prevent VP16En from activating the expression of hh (or en) in a slp mutant. Expression of slp at the anterior, and of en at the posterior, of prospective parasegment boundaries is initiated by the activity of pair-rule genes (Martinez-Arias, 1993; Nasiadka and Krause, 1999). Mutual transcriptional repression ensures that neither factor can subsequently ‘invade’ the other’s domain of expression after pair-rule genes have ceased to function and when cell communication starts to dominate segmental patterning and thus contributes to the stability of parasegment boundaries. Note that slp is only expressed at the anterior of each stripe of en expression (not at the posterior). It may be that no analogous repressive function is needed at the posterior because the Wg pathway, which contributes to activation by En, is not active there. Indeed, in otherwise wild-type embryos, ectopic activation of Wg signaling is sufficient to cause posterior expansion of en stripes (Noordermeer et al., 1992).
En as an activator

The key evidence for En being a bona fide activator is that, in the absence of slp, both En and VP16En activate hh transcription. This result, and the argument outlined in Fig. 2, suggests either that En activates hh directly or that it activates an intermediate activator of hh transcription. Either way, we suggest that En must be capable of transcriptional activation (in addition to repression). Note that in otherwise wild-type embryos, VP16En formally represses the expression of hh and en (Fig. 4). This led us to believe initially that wild-type En acts solely via an intermediate repressor since we could not see any possible effect of VP16En on the expression of en or hh. As we know now, these were masked by the presence of Slp. It was therefore essential to identify the intermediate repressor and assess the effect of removing its activity in order to infer the true activation function of En.

Wg signaling and activation by En

As shown in Fig. 7, Wg signaling contributes to the activation of En’s positive targets. We have not investigated the temporal aspect of this requirement but earlier results suggest that it is probably transient (see Heemskerk et al., 1991). Note that Wg signaling is irrelevant to repression by En and that, even in cells that are within the range of Wg, repression and activation (of distinct targets) coexist. For example, in the normal domain of en expression, ci is repressed and hh is activated. Therefore, Wg signaling does not convert En from an activator to a repressor. Perhaps Wg signaling helps the recruitment, on specific targets, of a cofactor needed to mask the repressor domain of En, while at the same time providing an activation domain. One candidate cofactor that could be regulated by Wg is the homeodomain protein encoded by exd, a known cofactor of Hox gene activity in vivo (Mann and Chan, 1996; Mann and Abu-Shaar, 1996). However, as we discuss below, Exd is not an activation-specific cofactor and more work is therefore needed to understand how Wg signaling contributes to the activating function of En.

The role of Exd

Two types of activities have been ascribed to Exd (for a review, see Mann and Morata, 2000). According to the selective binding model, Exd could help En recognize positive targets and assemble a transcription complex. Alternatively, or in addition, Exd could mask the repressor domain of En and, at the same time, recruit an activator (the so-called activity regulation model). We find in our assays that adding a functional activation domain to En (as in VP16En) does not override the need for Exd. This gives in vivo support to the selective binding model and is consistent with in vitro studies, which have shown that Exd and En can dimerize and bind DNA cooperatively (van Dijk and Murre, 1994; Serrano and Maschaf, 1998). Cooperativity requires the eh2 domain of En (Peltenburg and Murre, 1996), a domain that is left intact in VP16En (see Fig. 2). Because VP16En requires Exd for in vivo activity, we conclude that the N-terminal half of En, which is absent in VP16En, is not required for the interaction with Exd (see also Serrano and Maschaf, 1998).

As we have shown, in thoracic segments, VP16En requires exd to act on all En targets, positive and negative. This is the first indication that Exd could be involved in negative (as well as positive) target recognition by En (a suggestion made independently by Kobayashi et al., 2003). Indeed, we found that, in thoracic segments, wild-type En requires Exd for repression of its natural targets. This had presumably not been noticed previously because endogenous expression of En is lost in the absence of Exd. That Exd could be involved in repression is consistent with in vitro studies with PBX proteins and earlier suggestions from in vivo work with Drosophila (Ryoo and Mann, 1999; White et al., 2000; Kobayashi et al., 2003). Because Exd is required for both repression and activation, the issue of what distinguishes activated targets from repressed ones remains unresolved. Throughout the present study, we found that the two En-positive targets, en and hh, are expressed identically in a variety of experimental conditions. It may therefore be that the regulatory regions of these two genes might contain unique features that make them positive targets.

How does En activate targets?

As we have argued, En must be capable of activating transcription in the appropriate context. Because En harbors a robust repressor domain, it is likely that one or several cofactor(s) mask this domain and recruit an activation function and, as discussed above, it is unlikely that Exd alone provides such an activity. Nevertheless, the possible role of Hth is worth discussing. In vitro, Hth binds DNA as a part of a ternary complex with Exd and a Hox protein (Jacobs et al., 1999; Ryoo et al., 1999). Intriguingly, overexpression of an activator form of Hth (VP16Hth) phenocopies the overexpression of wild-type Hth (VP16Hth mimics overactive Hth) (Inbal et al., 2001). This suggests that the normal role of Hth is to bring an activation domain to a complex – a conclusion that contradicts our own observation that Hth is required for both repression and activation by En. One way to resolve this paradox would be to suggest that Hth has two distinct roles: to help target recognition on negative and positive targets and, in addition, to bring an activation domain onto positive targets. Of course activation by En could also involve as yet unidentified activating cofactors. Further progress will require the identification, within natural targets, of enhancers that confer either activation or repression. Comparing these sites and subsequent mutational and biochemical analysis could lead to a molecular understanding of what distinguishes negative from positive targets.

The role of homeotic genes in repression by En

The most unexpected aspect of our results is that, in abdominal segments, the Hox proteins Ubx and Abd-A are involved in repression by En. In formal genetic assays, Ubx and Abd-A can substitute for Exd in helping En act on negative targets. In the absence of Ubx, Abd-A and Exd, En can no longer repress target genes. By contrast, two other Hox proteins, Antp and Abd-B appear, not to be involved in En function. Fig. 11C shows that Antp does not help En repress targets in vivo even though its homeodomain differs from that of Abd-A at only five positions. Likewise, Abd-B, a more distantly related Hox protein, is also unlikely to participate in En function (not shown). We conclude that the role of Ubx and Abd-A in repression by En is specific.

How could ectopic Ubx or Abd-A allow En to repress targets in the absence of Exd? It could be that this is mediated by wholesale transformation of segmental identity [although such
transformation would have to be exd\textit{hth}}-independent (see Rieckhof et al., 1997]). Alternatively, Ubx and Abd-A could have a more immediate involvement in En function. One can envisage that they could regulate an as yet unidentified corepressor of En (although such regulation would not require Exd). Alternatively, and more speculatively, Ubx and Abd-A could serve as cofactors themselves in regions of the embryo where Exd levels are low. Again, molecular analysis of negative targets will be needed to discriminate these possibilities.

Homoetic genes have not been previously implicated in En function despite many years of genetic analysis of the Bithorax complex. We suggest that the role of Ubx and Abd-A in En function has been overlooked previously because, in the absence of these two genes, Exd is upregulated in the presumptive abdomen and thus takes over as a repression cofactor. However, our present results establish that homoetic genes do participate in the segmentation cascade and link two regulatory networks previously thought to be independent.

We thank Alex Gould for providing numerous reagents and for discussions and Bruno Bello for giving us UAS-Antp. We also thank Josh Brickman, James Briscoe, Jose Casal, Oriane Marchand, Alex Gould and Tim Jinks for discussions and comments on the manuscript. C.A. and J.-P.V. are supported by the UK’s Medical Research Council.

REFERENCES


