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

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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. Extradenticle (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, Extradenticle, 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 Exd 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: *wg*^{CX4} (Baker, 1987), *en*^{CX1} (Heemskerk et al., 1991), Df(2R)*en*^{E} (Tabata et al., 1995), *ci*^{Ce} (Hochman, 1974), *slp* deficiencies Df(2L)*ed*^{SZ1} and *Cyo Δ34B* (Grossniklaus et al., 1992), double mutant *slp*$^{–}$ *en*$^{–}$ [*Df(2L)ed*^{SZ1}*en*^{IO} (Cadigan et al., 1994)], *exd*^{XP11} (Peifer and Wieschaus, 1990), *hth*$^{64-1}$ (strong hypomorph) (Kurant et al., 1998) and *Df(3R)Ubx*^{109} (*Ubx*$^{–}$, *abd-A*$^{–}$). (Lewis, 1978) See also FlyBase at http://flybase.bio.indiana.edu/. To generate *exd*$^{mat\text{-}zyg–}$ embryos, we followed the protocol described by Chan et al. (Chan et al., 1994). The following recombinants were created by standard genetic methods: *hth*$^{64-1}$ *paired-Gal4*, *hth*$^{64-1}$ *UAS-VP16En*, *hth*$^{64-1}$ UAS-*en*, *Df(3R)Ubx*^{109} *hth*$^{64-1}$ *paired*-Gal4 and *Df(3R)Ubx*^{109} *hth*$^{64-1}$ UAS-*en*.

The following transgenic stocks were used: *paired*-Gal4 [made by L. Fasano and C. Desplan (see Yoffe et al., 1995)], *armadillo*-Gal4 (Sanson et al., 1996), *ftz*-Gal4 (Lecourtois et al., 2001), UAS-*en* (Guillen et al., 1995), UAS-Arm* [UAS-Arm^{S10} (Pai et al., 1997)], UAS-*wg* (Lawrence et al., 1995), UAS-Ubx-IVa (Netter et al., 1998), UAS-Abd-A (Michelson, 1994), UAS-Antp (B. Bello, NIMR, London).

Design of VP16En

A PCR product corresponding to residues 282 to 522 of En [region EFGH as defined by Han and Manley (Han and Manley, 1993)] was cloned as a *Xba*I-*Bcl*I fragment in a vector containing an HA-tagged version of the HSV VP16 activation domain (YCGLVP16). The chimeric cDNA was then cut out with *Eag*I-*Asp*718 and cloned in pUAST digested with *Not*I and *Asp*718.

Cloning of the 3′ UTR region of *en*

In order to distinguish endogenous *en* transcripts from those encoded by UAS-*en*, we designed a probe that encompasses a region of the *en* gene not present in the UAS-*en* construct. The 3′UTR of *en* from the *Eco*RI site at position 2017 to position 2421 (Poole et al., 1985) was amplified with the following primers.

Forward oligo: CCGTAGC<u>GAATTC</u>GAGCTGTAAG; reverse oligo: GATC<u>TCTAGA</u>ATTTTTTTTCCCATAATTG (an *Xba*I site was added). The PCR product was subsequently cut with *Eco*RI and *Xba*I and cloned in pBS-KS.

Embryo preparation

For RNA single and double in situ hybridization, embryos were fixed and hybridized with digoxygenin- or fluorescein-labeled single-stranded RNA probe as described by Alexandre et al. (Alexandre et al., 1999). For double labeling with an antibody and an RNA probe, the same protocol as described in Alexandre et al. (Alexandre et al., 1999) was used except that the hybridization was performed at 63°C.

The following cDNAs were used: *en* (Poole et al., 1985), *hh* (gift from M. van den Heuvel, Oxford University), *wg*, *ci*, *ptc* (gift from Phil Ingham, Sheffield University), *slp1* (gift from K. Cadigan, University of Michigan, Ann Harbor) and *lacZ*. The following antibodies were used: Anti-Ubx (White and Wilcox, 1984), Anti-Abd-A (Macias et al., 1990), Anti-Antp [Mab 8C11 Condic et al., 1991)], Anti-En (gift from C. H. Girdham and P. H. O'Farrell, University of California at San Francisco) and Anti-HA (BabCo).

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.

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 grafted onto 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 VP16 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., 1996), the activating effect of VP16En on *wg* expression could conceivably be mediated by Ci (VP16En activates *ci*

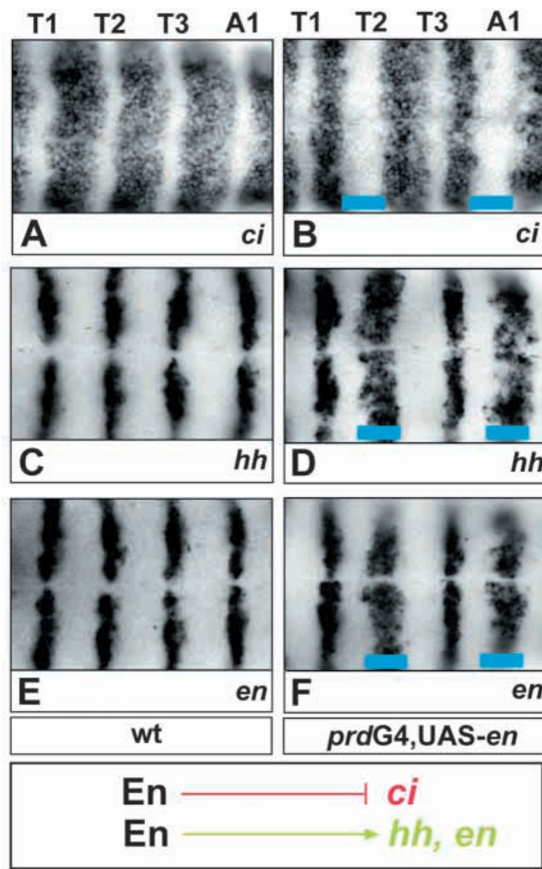

Fig. 1. Effect of *paired*-Gal4-driven En on the expression of negative and positive targets. In this and all subsequent figures the domain of *paired*-Gal4 expression is marked by a blue rectangle. Embryos are all at st11 and the identity of segments is indicated (T, thoracic; A, Abdominal). Whole embryos are shown lying on their side, whereas higher magnifications show ventral views with the ventral midline running in the middle from left to right. (A,B) Expression of *ci* in a wild-type embryo (A) and in an embryo expressing ectopic En under the control of *paired*-Gal4 (*prd-G4 UAS-en*) (B). In addition to repression in the normal domain of En expression, ectopic repression of *ci* expression is seen in alternate segments, within the paired domain. (C,D) Expression of *hh* in a wild-type (C) and *paired*-Gal4 UAS-en (D) embryo. Here, ectopic expansion of *hh* expression is seen in the paired-domain (D). (E,F) Expression of endogenous en in a wild-type (E) and *paired*-Gal4 UAS-en (F) embryo. A probe from the 3′ UTR that is not present in the UAS-en construct was used to detect endogenous expression. Endogenous en expression is activated by ectopic En. Note that repression and activation occur both in the thorax and abdomen.

expression). 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 *paired*-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*

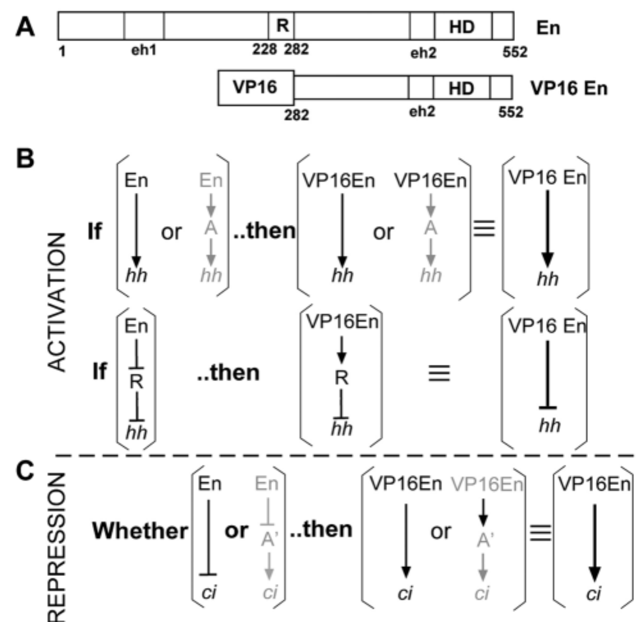

Fig. 2. Structure and possible effects of VP16En. (A) In VP16En, the repressor domain of En (as defined by Han and Manley, 1993) has been removed and replaced by the VP16 transactivating domain. Note that eh2, the domain of En implicated in cooperative binding with Exd, is still present in VP16En. (B) Two possible modes of activation by En. If En activates *hh* directly or by activating the expression of an intermediate activator (A), VP16En is expected to activate *hh* expression too. By contrast, if En activates through repression of a repressor, VP16En should activate the expression of this repressor and the net result would be repression of *hh* expression. (C) Expected effect of VP16En on negative targets of En (such as *ci*). The expected outcome is the same whether En represses *ci* expression directly or by repressing an intermediate activator (A′).

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

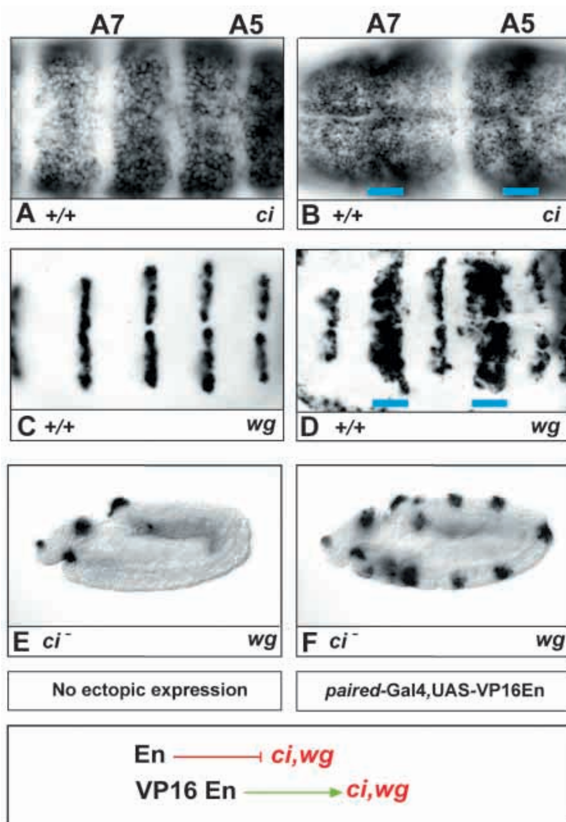

Fig. 3. VP16En activates genes that are normally repressed by En. Embryos placed side by side are of the same genotype except that embryos on the right-hand side express VP16En under the control of *paired*-Gal4. (A,B) Expression of *ci* in the abdominal region of wild-type (A) and *paired*-Gal4 UAS-VP16En (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 ci^{CE} 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 ci^{CE} 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.

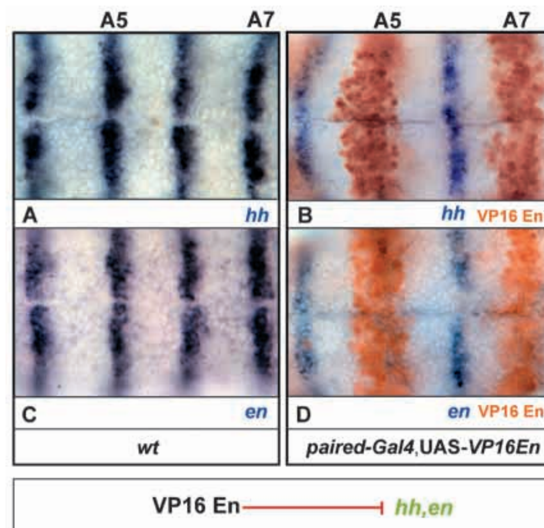

Fig. 4. VP16En represses the expression of *en* and *hh*, two positive targets of En. Wild-type embryos are shown on the left and *paired*-Gal4 UAS-VP16En embryos on the right. (A,B) Expression of *hh* in wild type (A) and in an embryo expressing VP16En (B). (C,D) Expression of *en* in a wild-type embryo (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).

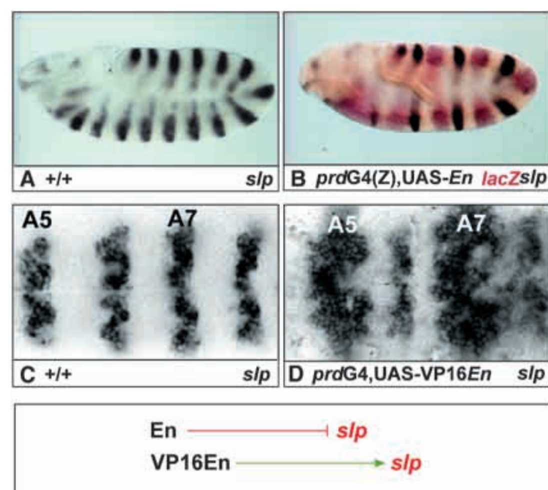

Fig. 5. Expression of *slp* is repressed by En. (A) Expression of *slp* in a wild-type st11 embryo. (B) Expression of *slp* in an embryo that expresses both ectopic *en* and *lacZ* under the control of *paired*-Gal4 [*prd*G4(Z) UAS-En]. *slp* transcripts (black) disappear where En is ectopically expressed (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.

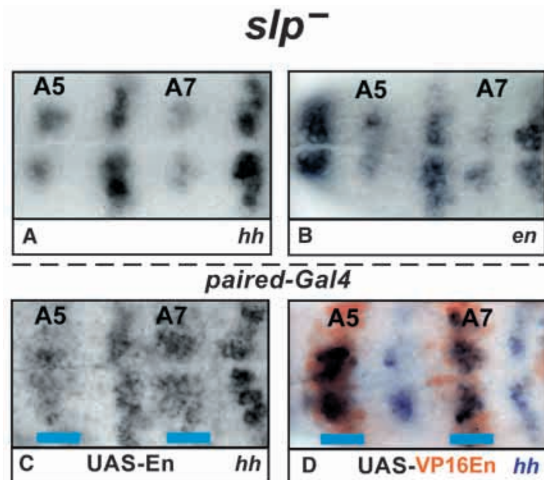

Fig. 6. Evidence for bona fide activation by En. (A,B) Expression of *hh* (A) and *en* (B) in the abdominal region of *slp* (Δ34B) mutant embryos. The expression patterns are identical. Note that expression widens in even-numbered segments, whereas it is decaying in odd-numbered segment (see Cadigan, 1994). (C,D) Expression of *hh* in *slp* (Δ34B) mutant embryos that express either En (C) or VP16En (D) in the *paired* domain. Expression of *hh* is in purple and that of VP16En is in ochre. In both cases, *hh* expression is activated, especially in the ventral region (compare with homologous segments in plain *slp*⁻, panel B). Thus, in the absence of the repressor Slp, VP16En and En have the same effect on expression of *hh* (and also of *en*; not shown). By contrast, in the presence of *slp*, expression of *hh* is activated by En (Fig. 1D), whereas it is repressed by UAS-VP16En (Fig. 4B). Note that, in *slp*⁻, the effect of VP16En is stronger than that of En.

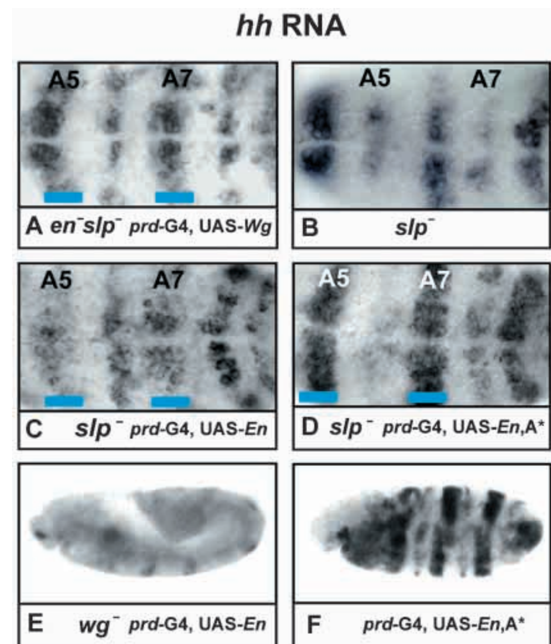

Fig. 7. Wg signaling contributes to the activation of the positive targets of En. (A) Expression of Wg with *paired*-Gal4 in the absence of *en* and *slp* leads to weak, but significant expression of *hh* in odd-numbered segments (where *hh* decays in *slp*⁻). Panels (B) and (C) are repeated from Fig. 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 wg^{CX4} 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 activates Wg expression (Fig. 3D). Therefore, in the absence of *slp*, expression of VP16En leads to expression of both *wg* and *en*, thus causing maximal activation of *hh* expression.

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 a similar effect on *hh* expression (Fig. 6C,D) although VP16En seems to be more potent (an issue to which we will return). According to the logic outlined in Fig. 2, 'same sign' action of En and VP16En suggests that En functions as a bona fide activator. It could either act directly onto its positive targets or it could activate an intermediate activator.

Activation of *hh* expression by En is weaker in *slp* mutants than in *slp*⁺ embryos (compare Fig. 6C with Fig. 1D). Because *slp* mutants lose *wg* expression prematurely (Cadigan et al., 1994), it could be that Wg signaling contributes to the activation of *hh* expression. To address this possibility, we assayed *hh* expression in *en*⁻ *slp*⁻ double mutant in which exogenous Wg was introduced with *paired*-Gal4. Weak but significant activation ensues (Fig. 7A), indicating that Wg signaling does activate *hh* expression even in the complete absence of En activity. Thus, either En or Wg signaling alone has a weak effect. Co-expression experiments show that these effects are additive (possibly synergistic): co-expression of En and activated Armadillo (to activate Wg signaling) in the absence of Slp leads to strong expression 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 *hth*$^{64\text{-}1}$ mutant embryos, (Fig. 8D). Therefore, the *hth*$^{64\text{-}1}$ 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

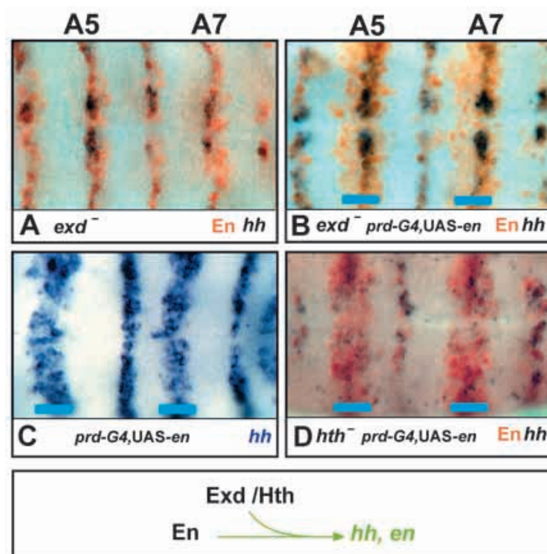

Fig. 8. Exd and Hth are required for En to activate genes in the abdomen. In all panels, abdominal segments A4 to A8 of es11 embryos are shown. (A) Expression of En protein and *hh* RNA in an *exd* deficient embryo. Expression of both En and *hh* has begun to decay. (B) Transcription of *hh* in an *exd*$^-$ embryo expressing exogenous En under the control of the *paired*-Gal4 driver. No ectopic activation is seen (most ochre cells are not black). There may be slightly increased residual expression of *hh* (compare black signal in A and B) but this could be in underlying neuroblasts. (C) In the presence of *exd*$^+$, *paired*-Gal4-driven En activates *hh* expression through the domain of ectopic expression (see the widening of *hh* expression in alternate segment as shown for anterior segments in Fig. 1D). (D) Ectopic En does not activate *hh* expression in a zygotic *hth* mutant embryo (*hth*$^-$ *paired*-Gal4, UAS-*en*). This shows that a mutation in *hth* has the same effect as removal of *exd* (compare with panel B).

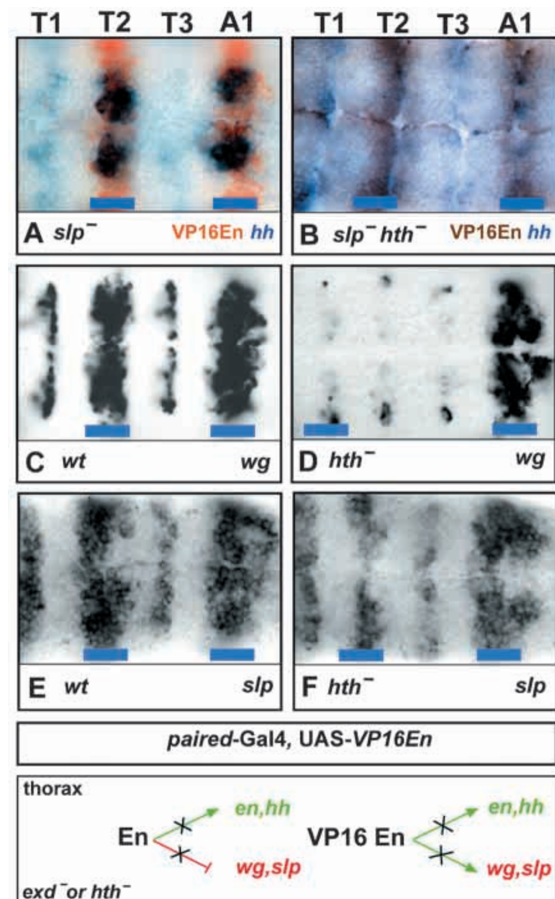

Fig. 9. VP16En requires *hth* throughout the germ band to activate positive targets of En and only in anterior thoracic segments to activate negative targets. All panels show anterior segments (T1, T2, T3, A1, in register) of st11 embryos that express VP16En under the control of the *paired*-Gal4 driver. (A) In the absence of *slp*, VP16En (in ochre, seen with anti-HA) activates expression of *hh* (blue), especially in the midventral region. This point is already made in Fig. 6D for abdominal segments. The same effect is seen on *en* expression (not shown). (B) In the absence of *hth* (and also *slp*), VP16En no longer activates ectopic *hh* expression. This panel shows a *slp hth* double mutant with VP16En in brown and *hh* in blue. Almost all endogenous *hh* has disappeared and no ectopic expression is seen. (C) VP16En activates ectopic expression of *wg* in T2 and A1 (where *paired*-Gal4 is active) in an otherwise wild-type embryo. (D) In a *hth* mutant, ectopic *wg* expression is still activated in A1 but not in T2. (E,F) A similar effect is seen on the expression of *slp*, another negative targets of En.

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 germband. 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 co-expressed Ubx and En (not shown). Co-expression 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 co-expression of Ubx 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)Ubx[109], 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)Ubx[109] 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

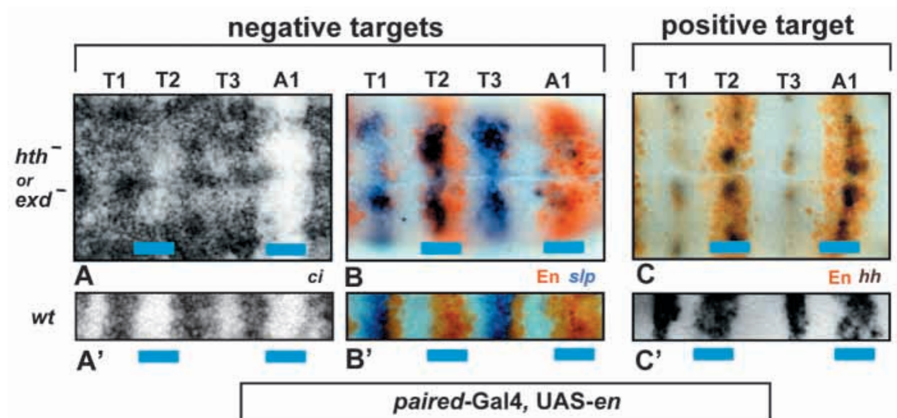

Fig. 10. En requires *exd*/*hth* to repress endogenous targets, but only at the anterior of the germ band. Embryos shown at the top (A, B, C) are all deficient for *exd* (or *hth*) and, in all panels, En is expressed under the control of *paired*-Gal4 (e.g. in T2 and A1). (A) In *hth*–, expression of *ci* is repressed by En in A1 but not in T2. In this embryo, all En originates from the transgene since endogenous expression decays in *hth* mutants. (A′) For comparison, the effect of ectopic En on *ci* expression in *hth*+ is reproduced from Fig. 1B (showing only the left side of the embryo). (B) As with *ci*, expression of *slp* is repressed in A1 but not in T2 in *exd*– embryos. Here ectopic En is shown in ochre (as detected with an antibody) and *slp* expression is in blue. Note that in A1, En is present but not *slp*. (B′) Again, for comparison, we show the effect of En expression on *slp* expression in an otherwise wild type embryo (as in Fig. 5B). (C) In *exd* mutants, activation of *hh* expression by En is abolished in both T2 and A1. Here, En protein is shown in ochre and expression of *hh* is shown in brown. Residual expression (a probable remnant of previous activation by pair-rule gene products) is seen in the normal domain, but not ectopically as happens if *exd*+ is present (shown in C′). (C′) Expression of *hh* is activated in T2 and A1 of otherwise wild type embryos (as shown previously in Fig. 1D).

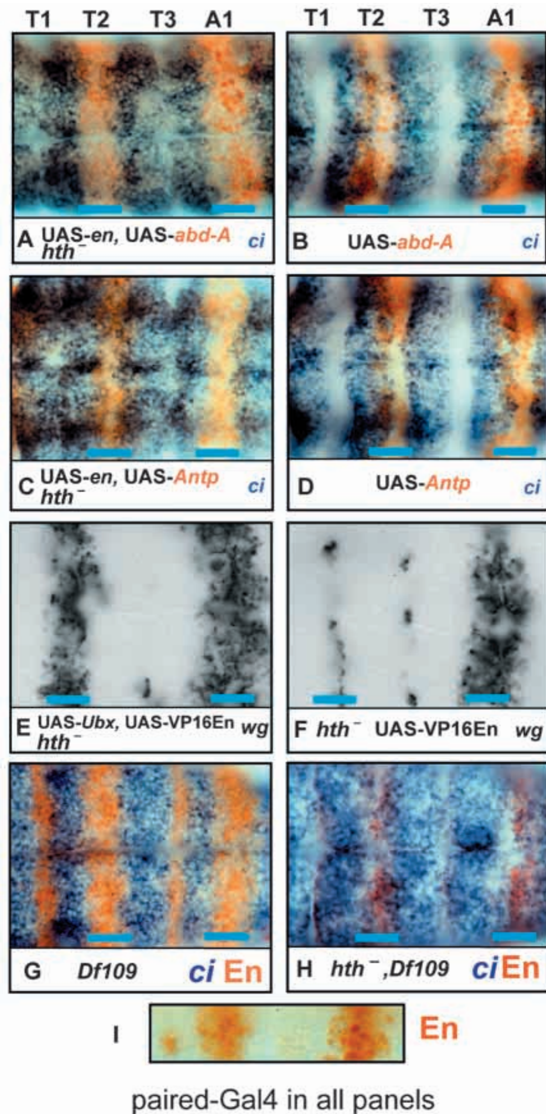

Fig. 11. Role of Ubx or Abd-A in repression by En. (A) Coexpression of En and Abd-A (ochre) repress *ci* expression at the anterior of the germ band (as well as in the abdomen) of a *hth* mutant embryo. This includes T2 and also head segments (not shown). Remember that expression of En alone in a *hth* mutant does not lead to repression there (Fig. 10A). (B) Ectopic expression of Abd-A alone in a wild-type embryo (or in a *hth* mutant; not shown) has no effect on *ci* expression. Normal repression in the domain of *en* expression is seen but no ectopic repression is seen in the *paired* domain (marked with an anti-Abd-A antibody in ochre). (C) Co-expression of En and Antp does not lead to *ci* repression in T2 of a *hth* mutant. Expression of *ci* is the same as if En alone were expressed (as in Fig. 10A). In this picture, expression of Antp (ochre) is partially masked by the signal coming from *ci* transcripts (blue). (D) As expected, *paired*-Gal4 driven Antp alone has no effect on *ci* expression whether in a wild type or a *hth* mutant (not shown). (E-F) Co-expression of Ubx allows VP16En to activate *wg* expression in T2 of a *hth* mutant embryo (E), whereas VP16En is unable to activate there on its own (F; also shown in Fig. 9D). (G) Distribution of *ci* transcripts (blue) and En protein (ochre) in a *Ubx, abd-A* double mutant [Df(3R)Ubx^{109}] overexpressing En with *paired*-Gal4. In such embryos, repression of *ci* occurs throughout the domain of ectopic En expression. There is no overlap between En (ochre) and *ci* (blue). Thus, removal of Ubx and Abd-A does not prevent En from repressing in the abdomen (compare to Fig. 1B). (H) Expression of *ci* (blue) and En (ochre) in a *Ubx, abd-A, hth$^{64-1}$* triple mutant. Here, no repression of *ci* expression is seen despite the strong ectopic expression of En (see panel I). Thus in the absence of Ubx, Abd-A and Hth, the repressive activity of En is abolished. (I) Embryos of the same genotype as in H stained for En alone. This panel shows that the *paired*-Gal4 driver is not affected in the triple mutant.

this possibility, we assayed En's activity in embryos lacking Ubx, abd-A and *hth* (Df(3R)Ubx^{109} hth$^{64-1}$). 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 *slp1* and *slp2*. These two genes are repressed by En and their products repress *en* expression (see also Kobayashi et al., 2003). Importantly, Slp1 and Slp2 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 positive 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 Maschat, 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 Maschat, 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*/*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.

Homeotic 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 homeotic genes do participate in the segmentation cascade and link two regulatory networks previously thought to be independent.

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