

## Engrailed cooperates with *extradenticle* and *homothorax* to repress target genes in *Drosophila*

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### SUMMARY

Engrailed is a key transcriptional regulator in the nervous system and in the maintenance of developmental boundaries in *Drosophila*, and its vertebrate homologs regulate brain and limb development. Here, we show that the functions of both of the Hox cofactors Extradenticle and Homothorax play essential roles in repression by Engrailed. Mutations that remove either of them abrogate the ability of Engrailed to repress its target genes in embryos, both cofactors interact directly with Engrailed, and both stimulate repression by Engrailed in cultured

cells. We suggest a model in which Engrailed, Extradenticle and Homothorax function as a complex to repress Engrailed target genes. These studies expand the functional requirements for *extradenticle* and *homothorax* beyond the Hox proteins to a larger family of non-Hox homeodomain proteins.

Key words: *Drosophila*, Embryogenesis, Homeodomain, Transcription, Repressor, Cofactor, Pbx, Meis

### INTRODUCTION

The *engrailed* (*en*) gene is a homeobox-containing gene crucial for the establishment and maintenance of compartment boundaries during *Drosophila* development (Akam, 1987; Blair, 1995). Its homologs in mammals are important in brain and limb development, where they might provide a similar function (Augustine et al., 1995; Joyner, 1996; Wurst et al., 1994). The En gene product regulates several key target genes. It helps to maintain the activities of the *hedgehog* gene and of its own gene (Heemskerk et al., 1991), and it represses the genes *wingless* (*wg*) (Heemskerk et al., 1991) and *cubitus interruptus* (*ci*) (Eaton and Kornberg, 1990), all of which are involved in the functions of compartment boundaries and the signals they provide to developing tissues (DiNardo et al., 1994).

En carries out these functions, at least in part, by repressing downstream target genes (Smith and Jaynes, 1996; Tolkunova et al., 1998). It has two well-characterized repression domains, one of which binds the Groucho co-repressor complex (Han and Manley, 1993; Jaynes and O'Farrell, 1988; Jaynes and O'Farrell, 1991; Jimenez et al., 1997; Tolkunova et al., 1998). Like many eukaryotic DNA-binding proteins, the specificity of DNA binding by En alone appears to be lower than that required for highly selective interaction with specific target genes. This suggests that, like many other DNA binding proteins, En interacts with cofactors that increase its binding specificity. En has been shown to be capable in vitro of binding

cooperatively to DNA with the Extradenticle protein (Peltenburg and Murre, 1996), although a role for this interaction in vivo has not been demonstrated.

The *extradenticle* (*exd*) gene is also a homeobox-containing gene that was initially characterized as a mutation causing multiple homeotic transformations in *Drosophila*, without affecting the expression patterns of the homeotic genes. These observations suggested that it might serve as a cofactor for Hox gene products (Peifer and Wieschaus, 1990). Homeotic transformations are seen in zygotic mutants but, when the maternal contribution to *exd* function is also removed, embryos show alterations that suggest a loss of *en* function, including a loss of *en* gene expression, at later embryonic stages (Peifer and Wieschaus, 1990; Rieckhof et al., 1997). Molecular studies have confirmed the role of Exd as a cofactor for Hox proteins, including a function in altering their specificity of binding to DNA (reviewed in Mann and Chan, 1996). This function appears to be conserved in the mammalian homologs of Exd, the Pbx proteins (Peltenburg and Murre, 1997; Phelan et al., 1995).

A third homeobox gene, *Meis1*, was discovered as an ecotropic retroviral insertion site in mice (Moskow et al., 1995), where its overexpression in conjunction with a subset of Hox proteins leads to leukemic transformation of hematopoietic stem cells (Nakamura et al., 1996). *Meis1* is a mammalian homolog of the *Drosophila homothorax* gene (*hth*), which has been shown to regulate the nuclear localization of Exd (Rieckhof et al., 1997) and to form

complexes with Hox proteins, including co-complexes with Exd (Ryoo et al., 1999). As with the cofactor functions of Exd, the interactions and functions of the Meis1 family appear to be largely conserved between *Drosophila* and mammals (Jacobs et al., 1999; Liu et al., 2001; Saleh et al., 2000; Shanmugam et al., 1999; Shen et al., 1999). In mammals, a Hox-class (Antp-class) protein that is part of an endodermally expressed ParaHox cluster (Brooke et al., 1998; Leonard et al., 1993) has also been shown to interact functionally with mammalian homologs of Exd and Hth (Swift et al., 1998).

In this work, we show that En interacts specifically with Exd and Hth in vitro and in cultured cells, that Exd and En mediate co-operative repression in cultured cells, and that the genetic functions of both *exd* and *hth* are crucial to the repression activity of En in embryos. We identify *sloppy paired* (*slp*) as a direct target gene of En, and show that *exd* and *hth* are required for En effectively to repress both *slp* and a second key target gene, *wg*, in developing embryos. The involvement of *exd* and *hth* function in direct repression by En, in conjunction with the observed molecular interactions, suggest that they are likely to participate in En repression complexes in vivo.

## MATERIALS AND METHODS

### Yeast two-hybrid and in vitro binding assays

Yeast two-hybrid assays were performed as previously described (Tolkunova et al., 1998), using a *lacZ* reporter gene. Briefly, strain Y190 was co-transformed with a pAS2 and a pACT2 derivative, and *lacZ* activity assays were performed on colonies streaked and grown on filters. The experiments shown in Fig. 5A used pACT2-Exd, in which the complete *Exd* open reading frame (ORF) was inserted, pACT2-Hth, containing the complete *Hth* ORF, pACT2-Meis1b and pAS2-Meis1b (Steelman et al., 1997), and pAS2-En (Tolkunova et al., 1998). The p53 controls used pAS2-p53, which contains the mouse p53 ORF. Details of plasmid construction are available on request.

Glutathione-S-transferase (GST) pull-down assays were performed as described previously (Tolkunova et al., 1998). GST-En contains En amino acids 1-544 in pGEX-5X-1; full-length *Exd* was transcribed from pET15b-Exd, which contains the complete *Exd* ORF. Full-length *Meis1b* was expressed from pET28b-Meis1b (Steelman et al., 1997).

### Cell culture assays

Transfections of Schneider line 2 (S2) cells were performed as described previously (Jaynes and O'Farrell, 1991). Plasmids used for Fig. 5C were pRM-HA3-hth (encoding full-length Hth, 3 µg per 60 mm plate), pAc-en (Jaynes and O'Farrell, 1988) (8 µg per plate), pP<sub>Ac</sub>-His<sub>6</sub>Exd (encoding full-length Exd tagged at its N-terminus, driven by the *actin5C* promoter, 8 µg per plate) and pCaSpeR-hs as filler (to 19 µg total DNA per plate). Nuclear extracts for co-immunoprecipitation (coIP) experiments were performed as described by Han and Manley (1993). For coIPs, antibodies were immobilized on either Protein-A/agarose or Protein-G/sepharose in Buffer A (20 mM HEPES pH 7.9, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10% glycerol), blocked with non-transfected nuclear extract for 4 hours, incubated with transfected nuclear extract overnight, washed extensively at 23°C with Buffer A plus 0.2% Triton X-100, eluted in Laemmli buffer, and analyzed by western blotting essentially as described by Han and Manley (Han and Manley, 1993), except that specific proteins were visualized using the ECL detection system (Amersham). All incubations were at 4°C unless otherwise indicated.

Plasmids used for Fig. 6 were: (1) pT<sub>3</sub>CM<sub>6</sub>-CAT, a chloramphenicol acetyltransferase (CAT)-expressing plasmid that can be activated by glucocorticoid receptor (GR) and is derived from

pT3N6D-33CAT (Jaynes and O'Farrell, 1991) by replacing the N6 homeodomain binding sites with a tandem array of six composite En-Exd binding sites (Peltenburg and Murre, 1996) or by mutated versions of this site (as indicated in the text and in the legend for Fig. 6); (2) pTAT<sub>3</sub>-CAT (Jaynes and O'Farrell, 1991), the equivalent plasmid without En-Exd sites; (3) pRK232 (Heemskerk et al., 1991), an En expression construct; (4) pRM-HA3-hth, a metallothionein-promoter-driven Hth expression construct; and (5) pLac82SU (Dorsett et al., 1989) as a reference. The metallothionein promoter was induced by adding CuCl<sub>2</sub> to 0.7 mM 24 h after transfection; the GR was activated by adding triamcinolone acetonide to 10<sup>-7</sup> M 48 hours after transfection. Fig. 6A used 1 µg each of pRK232 and either pTAT<sub>3</sub>-CAT or pT<sub>3</sub>CM<sub>6</sub>-CAT, as indicated (without or with En-Exd sites, or with mutated sites), 0.5 µg pRM-HA3-hth, and 0.5 ng pLac82SU per 60 mm culture dish. Fig. 6B used 1 µg each of pT<sub>3</sub>CM<sub>6</sub>-CAT and pRK232, 0.04 µg pP<sub>Ac</sub>-GR (Jaynes and O'Farrell, 1991), and 0.5 ng pLac82SU per 60mm culture dish, and either 0.5 ('low') or 1 µg ('hi') of pRM-HA3-hth where indicated in the figure. Total DNA was normalized to 6 µg per dish in both Fig. 6A and Fig. 6B. Fig. 6C used 0.36 µg pT<sub>3</sub>CM<sub>6</sub>-CAT, 0.5 µg pRM-HA3-hth, 0.014 µg pP<sub>Ac</sub>-GR and 0.18 ng pLac82SU per 35 mm culture dish, and either 0.108 ('low') or 0.36 µg ('high') pRK232, as indicated in the figure. Total DNA was normalized to 2.16 µg per dish. Three hours prior to transfection, each dish was treated as described by Clemens et al. (Clemens et al., 2000) with 24 µg of double-stranded RNA (dsRNA) encoding either Rad9 or Exd.

### *Drosophila* stocks and assays

In situ hybridization was performed as described (Tautz and Pfeifle, 1989). Antibody staining with anti-*lacZ*, anti-En, anti-Exd (Rieckhof et al., 1997) and female-specific anti-Sex-lethal antibodies (Bopp et al., 1991) was performed as described previously (Kobayashi et al., 2001).

To generate *hth* mutants with *hs-En* (Heemskerk et al., 1991), *P[hs-En E3D]*; *hth<sup>P2</sup>/TM3 Sb ftz-lacZ* strains were self-crossed; as controls, *P[hs-En E3D]*; *TM3 Sb ftz-lacZ/+* strains were self-crossed.

To generate *exd* maternal mutant embryos carrying *hs-En*, *exd<sup>1</sup> FRT 18D/FM7-B<sup>1</sup>* females were crossed with *OVO<sup>D2</sup> FRT 18D*; *hs-Flp1* males to generate *exd<sup>1</sup> FRT 18D/OVO<sup>D2</sup> FRT 18D*; *hs-Flp1/+* females (non-B), which were heat shocked during the wandering larval stage (1 hour at 37°C every 12 hours) to induce mitotic recombination. These females with homozygous mutant germ-line clones were then crossed with either *P[hs-En E5]* (on the third chromosome) or wild-type (control) males. The *exd<sup>1</sup>* allele (a.k.a. *exd<sup>XP11</sup>*) is amorphic (Rauskolb et al., 1995).

To generate *exd* maternal mutant embryos carrying *UAS-en* (Tabata et al., 1995), *exd<sup>1</sup> FRT 18D/FM7-B<sup>1</sup>*; *UAS-en* females were crossed with *OVO<sup>D2</sup> FRT 18D*; *hs-Flp1*; *UAS-en* males to generate *exd<sup>1</sup> FRT 18D/OVO<sup>D2</sup> FRT 18D*; *hs-Flp1/+*; *UAS-en* females (non-B), which were heat shocked as above to induce mitotic recombination. These females were then crossed with *prd-Gal4/TM3 Sb hb-lacZ* males.

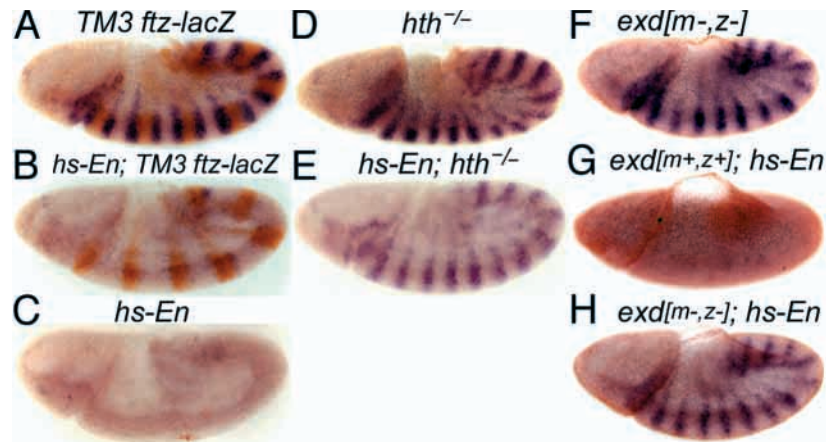
Cuticle preparations were performed as described previously (Fujioka et al., 1999). For Fig. 4, embryos were collected and aged for 22 hours at 25°C. Cuticles were prepared and categorized by severity of defects as follows. The number of abdominal denticle bands fused was determined in at least 120 embryos (for each data point) of the genotypes indicated in Fig. 4 and its legend. The categories, from least to most severe, had the following number of denticle bands fused (two bands completely fused together were counted as two bands fused, partial fusions were counted as one band fused): 1-3, 4, 5, 6, 7-8.

## RESULTS

### *exd* and *hth* cooperate with En to repress the direct target gene *slp*

In the absence of *exd* or *hth* function, expression of the

**Fig. 1.** Ubiquitously expressed Engrailed requires *exd* and *hth* to repress *slp* in vivo. Repression of *slp* by En is abrogated by loss of either *hth* or *exd* function. Embryos were collected for 1 hour, aged for 3.5 hours and heat shocked at 37°C for 10 minutes to induce En expression in *hs-En* embryos, which carry a hs-inducible transgene expressing full-length En. They were then aged for an additional 15 minutes, fixed and stained for *slp* RNA (dark blue, by in situ hybridization) and for β-galactosidase protein (A,B,D,E) (orange, anti-horseradish-peroxidase stain). *lacZ* is expressed from a *ftz-lacZ* transgene on the *TM3* balancer chromosome. (A) Wild type (no *hs-En*, genotype *TM3/hth<sup>P2</sup>* or *TM3/TM3*, which are indistinguishable). (B) *hs-En*, *TM3* containing (*TM3* carries a wild-type *hth* allele); notice the almost complete repression of *slp* by *hs-En*. (C) Control to show that *TM3* itself does not affect repression of *slp* by *hs-En* (genotype *hs-En*; +/+, stained in parallel with the others). (D) An *hth* mutant (genotype *hth<sup>P2</sup>/hth<sup>P2</sup>*, distinguishable in the same population as in A by the absence of β-galactosidase staining). (E) An *hth* mutant with *hs-En* (genotype *hs-En*; *hth<sup>P2</sup>/hth<sup>P2</sup>*, inferred from the absence of β-galactosidase staining; this and the embryo in B were stained together in the same tube). Notice the weak repression of *slp* relative to B. (F-H) Embryos in F and H, designated '*mat.exd<sup>-</sup>*' below ('*m-*' in the figure), were derived from germline clones in the mother that were homozygous for the null *exd* allele *exd<sup>1</sup>*; therefore, no maternally derived Exd was present. These females with *exd<sup>-</sup>* germlines were crossed either to wild-type males, to generate the embryo in F, or to males homozygous for a *hs-En* transgene, to generate the embryo in H. Wild-type females were crossed to the *hs-En* males to generate the embryo in G. (F) *mat.exd<sup>-</sup>*, without *hs-En*. (G) *exd<sup>+</sup>*, with *hs-En*. (H) *mat.exd<sup>-</sup>*, with *hs-En*. Notice the increase in *slp* RNA when Exd is missing (H versus G), indicating a requirement for Exd in order for En to efficiently repress *slp*. These embryos were also stained for the female-specific protein Sex-lethal, to indicate whether they were male or female. Because *exd* is on the X chromosome, females received a wild-type copy of the *exd* locus from their fathers, whereas males did not. The embryos shown were male, and so they received their only *exd* allele from their mother ('*z-*' or '*z+*'). A paternal-only contribution to *exd* function (that is, in females derived from females with *exd<sup>-</sup>* germlines) significantly increased the ability of *hs-En* to repress *slp* (data not shown).



endogenous *en* gene in embryos is unstable and is progressively lost during the middle and later stages of development (Peifer and Wieschaus, 1990; Rieckhof et al., 1997). Therefore, although there is loss of *en* activity in the absence of *exd*, this might be secondary to the loss of *en* expression. To circumvent this ambiguity and to test whether *exd* and *hth* are required for the function of En in vivo, we expressed En from transgenes that are independent of *exd* function. We assayed *en* activity by examining expression of its target genes, and by examining the consequences for the pattern of embryonic cuticular structures, which is a sensitive function of *en* activity.

First, we expressed En ubiquitously using a heat shock promoter-driven transgene. This allowed us to focus on a direct target gene of En, based on the immediate response to ectopic En expression. We examined the responses of *slp* and *wg*, which are repressed by *en*, and of the *en* gene, which is ectopically activated by ubiquitously expressed En (Heemskerk et al., 1991). The gene that responded most rapidly to En was *slp*, which was noticeably repressed relative to control heat-shocked embryos within 7 minutes of a 5 minute heat pulse (data not shown). Such a rapid response makes it very likely that the response is direct (Manoukian and Krause, 1992; Saulier-Le Drean et al., 1998), and strongly suggests that *slp* is a direct target of repression by En.

To determine whether repression of *slp* by En was affected by a loss of *hth* or *exd* function, we examined the effect of the strong *hth* allele *hth<sup>P2</sup>* (Kurant et al., 1998) in combination with a *hs-En* transgene. As shown in Fig. 1A-E, the loss of *hth* function clearly reduced the repression of *slp* by En. This repression of *slp* expression persisted to later stages of

embryogenesis (data not shown), although the degree of persistence varied from embryo to embryo because of the relatively mild induction of ectopic En used here. This mild induction resulted in a level of expression comparable to or less than that in the endogenous En stripes. We also tested whether loss of *hth* function affected the expression of En from the *hs-En* transgene by staining embryos with En-specific antiserum. We detected no difference in the strength or persistence of the ectopic En signal between wild-type and *hth* mutant embryos (data not shown), showing that the repression activity of En is specifically affected in the *hth* mutants.

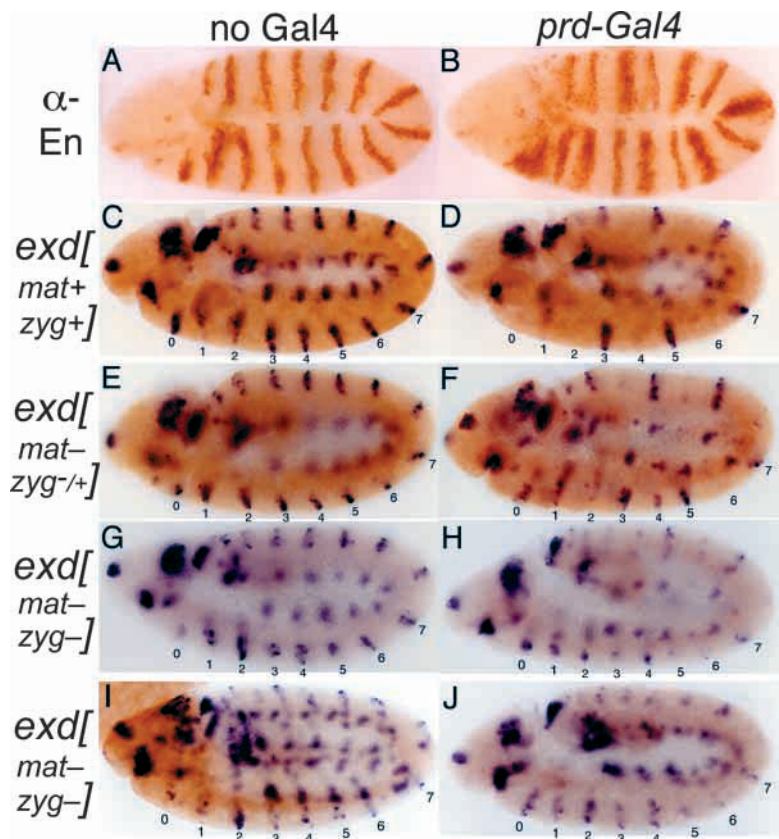
Although repression of *slp* was strongly reduced in *hth* mutants, it was not abolished. With longer inductions of *hs-En* expression, *slp* was more effectively repressed, again consistent with a residual activity in *hth* mutants (data not shown). A possible explanation for this residual activity is that the *hth<sup>P2</sup>* allele is not a complete null. Alternatively, En might retain some repression activity even in the absence of Hth.

We used the same approach to test whether En activity in embryos requires Exd, using a null allele of *exd*. As shown in Fig. 1F-H, complete removal of both maternal and zygotic *exd* function caused a dramatic drop in En repression activity on the *slp* gene. However, as with *hth*, there appeared to be residual activity even in the absence of *exd* function. Thus, although En retains a residual repression activity in vivo without Hth and Exd, its effectiveness is severely reduced in the absence of either.

### Repression of both *wingless* and *slp* by En is facilitated by *exd*

To examine further the requirements for *exd* and *hth* by En, we

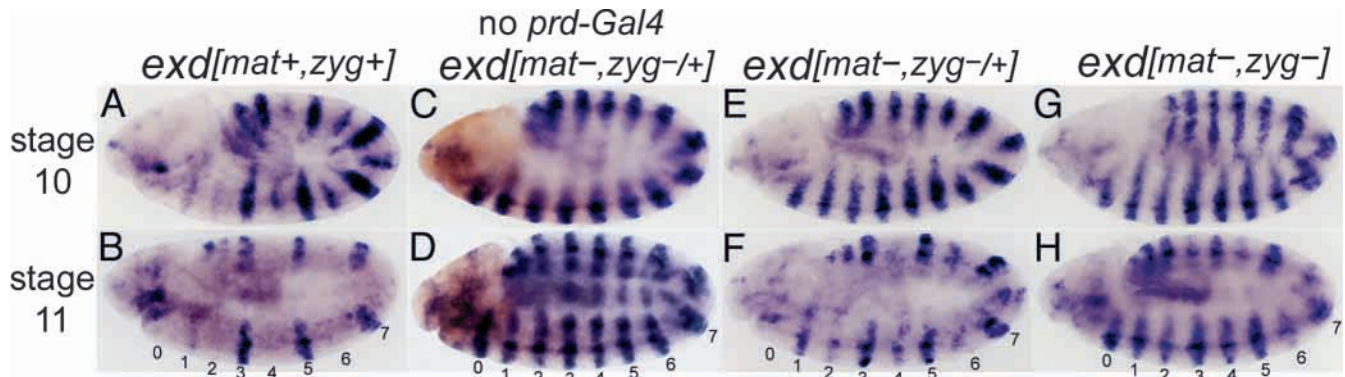
**Fig. 2.** Ectopically patterned En uses *exd* to repress *wg* in vivo. All embryos carry a *UAS-en* transgene. (A) Wild-type embryo stained for En. (B) Embryo carrying a *prd-Gal4* driver transgene, in addition to *UAS-en*. Notice the anteriorly expanded alternate En stripes, which overlap alternate *wg* and *slp* stripes (Fig. 3). (C–J) Embryos were derived from either *exd*<sup>+/+</sup> females (C,D) or from females with *exd* mutant germlines (E–J), and received either a wild-type *exd* allele (E,F) or no *exd* allele (G–J; *exd* is on the X chromosome) from their father. Thus *exd*[*mat*<sup>+</sup> *zyg*<sup>+</sup>] refers to either a homozygous or hemizygous wild-type *exd* genotype; *exd*[*mat*<sup>-</sup> *zyg*<sup>+/+</sup>] refers to heterozygous females that lack a maternal contribution; and *exd*[*mat*<sup>-</sup> *zyg*<sup>-</sup>] refers to hemizygous mutants that also lack a maternal contribution. These embryos were double-stained for *wg* RNA by in situ hybridization and either for Exd (C–H), indicating whether they do or do not have a wild-type *exd* allele, or for a balancer marker (I,J; *hb-lacZ*, in brown, indicating absence of the *prd-Gal4* driver transgene; *lacZ*-negative indicates the presence of the driver). Notice that, in *exd* wild-type embryos, *wg* is completely repressed by the ectopic En expression within alternate (even-numbered) stripes induced by *prd-Gal4*, whereas, in heterozygous *exd* embryos (which lack a maternal *exd* contribution), this repression is reduced. This effect was greater in embryos lacking all *exd* function (H,J). J is inferred to be *exd* mutant because of the weak and incomplete odd-numbered *wg* stripes that characterize them, as seen in H; H is inferred to contain *prd-Gal4* because of the repression of even-numbered *wg* stripes in the abdomen, as seen in J. Notice the lack of repression of even-numbered stripes (particularly stripes 0, 2 and 4) in H and J, and, to a lesser extent, in F. Staining for En showed no difference in either the pattern or extent of ectopic expression between the wild-type and *exd* mutant populations (not shown).



expressed En ectopically using the Gal4-UAS system (Brand and Perrimon, 1993), in a pattern that partially overlaps with the expression of two En target genes, *wg* and *slp*. This patterned ectopic expression provides an internal control within individual embryos of unrepressed target gene expression, as well as avoiding the potential complicating effects of heat shock. In this system, a transgene (the driver) expresses the Gal4 activator protein in a pattern, and this drives the expression of a responding transgene, in this case *UAS-en*. Using the *prd-Gal4* driver [made by L. Fasano and C. Desplan (see Yoffe et al., 1995)], *UAS-en* is activated in a striped pattern (Fig. 2B) that covers every other stripe of both *wg* and *slp* in the segmented portion of the embryo (*wg* is expressed within the *slp* domain, just anterior to each normal *en* stripe). The effects of this ectopic En expression were assayed in wild-type and *exd* mutant embryos. The effects on *wg* expression are shown in Fig. 2. Alternate *wg* stripes (in even-numbered parasegments) were repressed completely in wild-type embryos (Fig. 2D), whereas repression of these stripes was less complete in embryos that lacked a maternal supply of Exd protein and also had a reduced zygotic gene dose, and appeared to be delayed relative to the wild-type controls (Fig. 2F, and data not shown; notice that some expression remains, particularly in parasegments 0, 2 and 4). In embryos that completely lack *exd* function (both maternally and zygotically), the situation is complicated by the fact that continued *wg* expression requires *exd*. The weakening of *wg*

stripes in *exd* null embryos might be secondary to the loss of *en* expression, because *en* is necessary to maintain *hedgehog* and *wg* transcription (Heemskerk et al., 1991; Ingham, 1993). Thus, even without ectopic En expression, *wg* expression is weaker, particularly in odd-numbered parasegments such as 3 and 5 (Fig. 2G,I). Nevertheless, against this ‘background’ of weakened odd-numbered *wg* stripes, the ability of *prd-Gal4*-driven En (expressed only in even-numbered parasegments) to repress *wg* was significantly reduced. Although this effect was also seen in more-posterior parasegments, the effect was noticeably stronger anterior to the abdomen (particularly in parasegments 0, 2 and 4), where there appeared to be very little repression of *wg* compared with *exd* mutant embryos in which En was not ectopically expressed (Fig. 2G–J).

In similar collections of embryos, we examined the consequences of loss of *exd* function on the ability of En to repress *slp*. As with *wg*, alternate *slp* stripes were strongly repressed by *prd-Gal4*-driven En (Fig. 3A,B). Simultaneously eliminating the maternal contribution and reducing the zygotic dose of *exd*<sup>+</sup> caused a delay in repression and an ultimately less-complete reduction of *slp* expression (Fig. 3E,F). In embryos completely lacking both maternal and zygotic *exd* function, repression activity was strongly reduced (Fig. 3G,H), consistent with the results involving ubiquitously expressed En (Fig. 1). As with repression of *wg*, the repression of *slp* by En was more dependent on *exd* function in the gnathal-thoracic region than in the abdomen (Fig. 3H, parasegments 0, 2 and 4



**Fig. 3.** Ectopically patterned Engrailed uses *exd* to repress *slp* in vivo. Embryos collected as in Fig. 2 were double stained for a balancer marker (*hb-lacZ*, in brown in C,D, indicating absence of the *prd-Gal4* driver transgene; all others are *lacZ* negative, indicating that they carry the driver) and for *slp* RNA by in situ hybridization. Notice that, in *exd* wild-type embryos (A,B), *slp* is progressively repressed by the ectopic En expression within alternate stripes; in heterozygous *exd* embryos, which lack a maternal *exd* contribution (E,F), the repression is delayed and reduced; in embryos that lack all *exd* function both maternally and zygotically (G,H), this effect is stronger. Because repression is progressive, mutant embryos are shown that are at least as old as the corresponding wild-type controls.

vs 6, 8, 10 and 12). However, even in the abdomen, repression by En was significantly reduced in the absence of *exd* (Fig. 3G,H vs A,B).

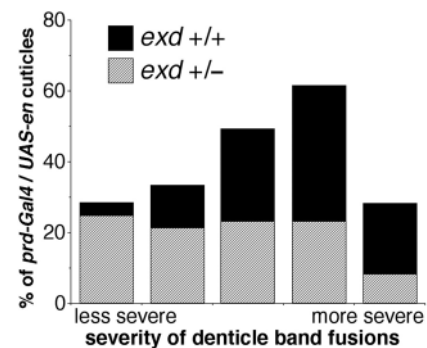
**Activity of En in pattern formation is dependent on both *exd* and *hth***

To test the significance of the changes in the specific En target genes described above for the overall ability of En to regulate its target genes, we examined the extent to which reduced *hth* and *exd* function affected the ability of ectopically expressed En to alter the pattern of structures produced at the end of embryogenesis in the external larval cuticle. First, in *hth* mutant embryos, we examined the severity of cuticle defects produced by *hs-En*. As Table 1 shows, the ability of *hs-En* to cause severe cuticle defects was reduced in *hth* heterozygotes and homozygotes, suggesting that the observed reductions in En repression activity on the specific target genes *wg* and *slp* accurately reflects its overall ability to regulate the target genes responsible for cuticle pattern.

We also quantified the severity of cuticle defects caused by ectopically patterned En expression and compared the results between the *exd*<sup>+/+</sup> and *exd*<sup>+/-</sup> populations, which are both wild type in the absence of *hs-En*. The analysis (Fig. 4) showed that, in the embryo, the reduction in *exd* activity caused by simultaneously removing maternal *exd* function and reducing the zygotic gene dose had a significant effect on the ability of *prd-Gal4*-driven En expression to disrupt the proper development of abdominal cuticular structures. This confirms the developmental impact of the changes in target gene expression characterized above. These results also confirm that En has a significant requirement for *exd* in the abdomen, as well as in more anterior regions.

**En interacts directly with both Exd and Hth**

As a first step in determining the mechanisms whereby *exd* and *hth* contribute to repression by En in vivo, we examined the possibility of direct interaction. Previous studies had shown that En can bind co-operatively with Exd in vitro to artificial DNA sites (Peltenburg and Murre, 1996). We tested whether a



**Fig. 4.** Flies with *exd* mutations show dose-dependent interactions with ectopically patterned En in embryos. Embryos collected as in Figs 2 and 3 were allowed to develop to the end of embryogenesis, and cuticles were prepared and analyzed. Defects in abdominal denticle bands (mostly pairwise fusions of varying severity) were categorized and tabulated, and the results are shown in a stacked bar graph. As indicated by the percentage of defective cuticles in both the *exd*<sup>+/+</sup> population and the population derived from *exd* mutant germline clones (data not shown), those embryos that did not receive a copy of the *prd-Gal4* driver showed no defects other than those expected from the complete absence of *exd* function (embryonic cuticles from *exd* germline clones are completely rescued by one wild-type gene from the father). We did not attempt to analyze *exd*-null cuticles (which were clearly distinguishable from the *exd* heterozygotes) for effects of En ectopic expression induced by the driver. Rather, the denticle band fusions caused by ectopic En expression in the population that received a wild-type *exd* allele from their father were analyzed, and the graph shows the percentage of defects in each category among this population (which are *exd*<sup>+/-</sup>, as indicated in the key). Thus, the percentages add to 100% in each case because they include only those cuticles that showed defects caused by ectopic En expression that were of the indicated *exd* genotype (in each case, these represented the expected overall percentage of cuticles). Notice that when the maternal contribution of *exd* is removed and the zygotic contribution is simultaneously reduced by half (*exd*<sup>+/-</sup>), the overall severity of abdominal cuticle defects in the population caused by ectopic En expression is significantly reduced, indicating a substantial requirement for Exd in En function in the developing abdomen.

**Table 1. *hth* mutants show dose-dependent interactions with ubiquitous En in embryos\***

| Genotype of parent stock    | Defective cuticles (%) | <i>hth</i> -like defects (%) | Severely defective (%) |
|-----------------------------|------------------------|------------------------------|------------------------|
| Wild type (300)             | 1                      | 0                            | 0                      |
| <i>hth/TM3</i> (440)        | 22                     | 22 (3)                       | 0                      |
| <i>hs-En; +/TM3</i> (254)   | 36                     | 0                            | 23                     |
| <i>hs-En; hth/TM3</i> (392) | 37                     | 34 (16)                      | 7                      |

\*Cuticle defects caused by ectopically expressed En are abrogated by loss of *hth* function. Cuticles were prepared after a mild heat shock (7 minutes at 37°C) of embryos from crosses of stocks of the indicated genotype. Those showing defects were categorized as follows: 'defective' means having clear-cut fusions or deletions in ventral denticle bands (shortened dorsal cuticles resulting in an abnormally curved overall shape, characteristic of most of the *hs-En* embryos, were not included); '*hth*-like' refers to characteristic denticle-band defects caused by loss of *hth* function, which include posteriorward transformations and loss of denticle diversity, particularly loss of row-1 denticles; 'severely defective' means multiple fusions or deletions of ventral denticle bands. Numbers in parentheses under 'genotype' indicate the number of embryos examined for each. Numbers in parentheses under '*hth*-like defects' refer to the percentage showing denticle-band fusions in addition to the '*hth*-like' defects described above.

The 22% defective cuticles with *hth* alone is in line with the 1/4 expected to be *hth/hth*, lacking *hth* function. Comparing *hth* alone with *hth* in combination with *hs-En*, the defective population increased by only 15%, from 22% to 37% (compared with an increase of 35% for *hs-En* relative to wild type), and half of this 37% (34%–16%=18%) showed only *hth*-like defects. Strikingly, the percentage of severely defective cuticles was strongly reduced in the *hs-En* lines by the removal of *hth* function. Only 7% of *hs-En; hth/TM3* progeny showed severe defects, compared with 23% when *hth* function was wild type. Overall, it appears that *hs-En* defects are reduced in severity not only in *hth* homozygotes but in heterozygotes as well, because a reduction in homozygotes would be expected to reduce the percentage of defects by only 25% (in this case 1/4 of 23%, or ~6%), whereas the reduction of severe defects is from 23% to 7%, a reduction of about 3/4, consistent with a significant reduction in both homozygotes and heterozygotes. The increase in '*hth*-like' defects is probably due to the partial overlap in the consequences of low-level, ubiquitous En induction and reduced *hth* or *exd* function. For example, as seen in Fig. 2, both loss of *exd* and ectopic En induction result in decreased *wg* expression. In spite of this, the combination of the two produced fewer severely defective cuticles than *hs-En* alone, consistent with the conclusion that *hth* function is required for *hs-En* to be fully effective. (This experiment was done using a mild heat shock, to test the effect of *hth* under conditions expected to be sensitive to modification. Stronger heat shocks produce a much higher percentage of ventral cuticle defects in *hs-En* embryos, without producing defects in wild-type embryos, data not shown.)

direct En-Exd interaction could also occur in other contexts, using yeast two-hybrid and in vitro assays. We also tested whether En could interact similarly with Hth. Fig. 5A shows the results of two-hybrid assays in yeast. En appeared to interact robustly with Exd in this system, because the signal strength observed with both isolated colonies (data not shown) and colony streaks (Fig. 5A) was consistently higher than that seen with some of our positive controls, including the functionally important interaction between En and Groucho (data not shown) (Tolkunova et al., 1998). This signal was also comparable to that seen with Exd and the mouse homolog of Hth, Meis1 (Fig. 5A; Hth fused with the Gal4 DNA-binding domain gave a high background signal, so that parallel results using Hth with Exd were uninformative). En also gave a somewhat weaker, but apparently specific, signal in combination with either Hth or Meis1 (Fig. 5A).

In vitro, En also interacts specifically with both Exd and Meis1 (Fig. 5B). Here, En fused with GST, but not GST alone,

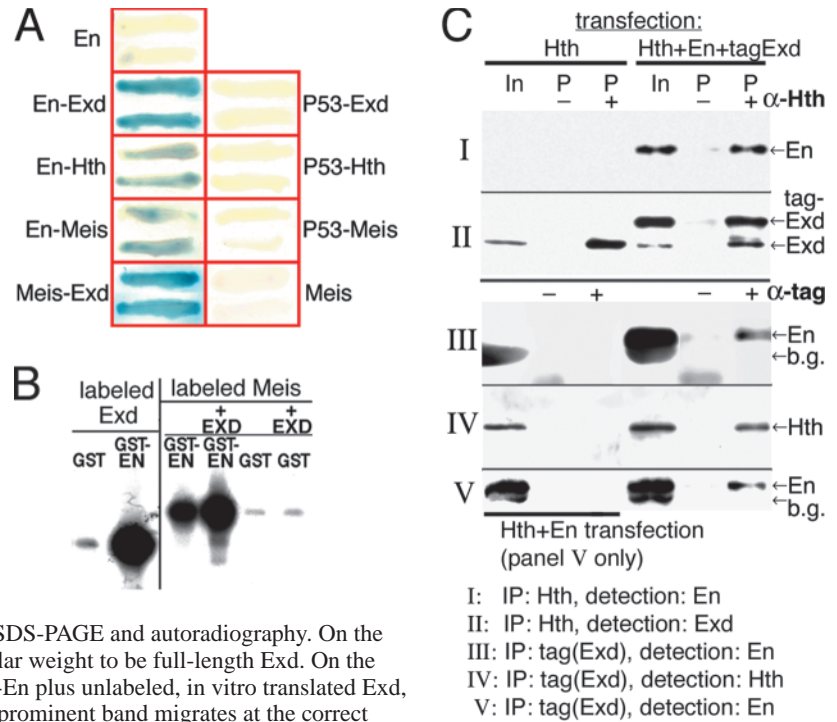
effectively pulls down either Exd or Meis1. Meis1 was used in these studies because of the high level of non-specific interaction observed with in-vitro-translated Hth, perhaps owing to the heterologous nature of the translation system. In this system, it is unlikely that the interactions are due to co-operative binding to DNA, and we interpret these results to mean that these interactions can occur in solution. Furthermore, Meis1 appears to interact more strongly with En in the presence of Exd, suggesting that the three proteins form a co-complex.

We tested whether these molecular interactions can also occur in cultured *Drosophila* cells (S2 cells). We transfected these cells with a Hth expression plasmid, which induces nuclear localization of endogenously expressed Exd (Rieckhof et al., 1997), either alone or with expression plasmids for En and for a tagged form of Exd (His<sub>6</sub>Exd, see Materials and Methods). In nuclear extracts from these cultures, anti-Hth antiserum specifically precipitated both En and endogenous Exd, as well as the tagged form of Exd (Fig. 5C). Conversely, anti-His<sub>6</sub> antibodies specifically precipitated both En and Hth (Fig. 5C). This ability of either Hth or His<sub>6</sub>Exd to mediate precipitation of En occurs at a salt concentration of 150 mM and with 0.2% Triton (see Materials and Methods), suggesting that the complexes involved are reasonably stable. However, the interaction of Hth with Exd, which is known to be functionally important, might be somewhat more stable, because the background seen under conditions needed to generate a readily detectable signal is somewhat lower between Hth and endogenous Exd, and between His<sub>6</sub>Exd and transfected Hth, than that seen with En (notice the slight background visible in the '- antibody' lane at the positions of En and tag-Exd in Fig. 5C, and the lack of any background at the positions of endogenous Exd and Hth). Although the interactions seen with En might be weaker than those between Exd and Hth, they are nonetheless strong enough to suggest the direct involvement of Exd and Hth in repression by En in vivo.

### Hth and Exd augment repression by En in cultured cells

As a first step in assessing the influence of the physical interactions identified above on the transcriptional activity of En, we used a previously constructed co-operative binding site for En and Exd (Peltenburg and Murre, 1996) as a target site in transfection assays. Repression by En has been extensively studied in *Drosophila* S2 cells, which have been shown to express Exd, but not Hth, constitutively (Rieckhof et al., 1997). Co-operative binding sites were inserted into a reporter vector previously shown to be unresponsive to En in the absence of inserted sites (Jaynes and O'Farrell, 1991) and their responses to En and to En in combination with Hth were measured. Consistent with previous results, En was able to repress this artificial target gene only when it contained the inserted sites and, without these sites, neither Hth alone nor En plus Hth caused any repression (Fig. 6A). Importantly, with the binding-site-containing reporter, the presence of Hth significantly increased the effectiveness of repression by En, either on the basal expression level of the target gene (Fig. 6A) or, in an established assay for active repression, when this gene was activated from separate activator binding sites (Fig. 6B). Because Hth has been shown to induce the nuclear localization of endogenously expressed Exd in these cells (Abu-Shaar et

**Fig. 5.** Engrailed interacts with Extradenticle and Homothorax in yeast, in vitro and in cultured cells. (A) En can interact with both Exd and Hth. In the yeast two-hybrid system, En was tested for interaction with full-length Exd, Hth and mouse Meis1. In each case, the protein listed first (or by itself) was expressed as a fusion with the Gal4 DNA-binding domain (DBD, in pAS2), whereas that listed second was fused with the Gal4 activation domain (in pACT2). En shows a strong signal with Exd and a weaker, but still specific, signal with both Hth and Meis1 (relative to these proteins either alone or in combination with the negative control P53, as indicated). Exd also generates a consistently strong signal with Meis1. (Hth produces a strong signal by itself when fused with the Gal4 DBD, so that similar experiments using it were uninformative.) (B) En interacts directly with both Exd and Meis1 in vitro and the three appear to form a co-complex. Glutathione-*S*-transferase (GST) fused to full-length En was produced and affinity purified from bacteria, then mixed with in vitro translated Exd (either labeled or unlabeled) and/or Meis1 (labeled), as indicated, and extracted from the mixture using glutathione agarose beads. Proteins captured by the beads were examined by SDS-PAGE and autoradiography. On the left, the single strong band migrates at the correct molecular weight to be full-length Exd. On the right, labeled Meis1 was mixed with either GST-En, GST-En plus unlabeled, in vitro translated Exd, GST alone or GST plus unlabeled Exd, as indicated. The prominent band migrates at the correct molecular weight to be authentic Meis1. (C) En interacts with Hth and Exd in cultured cells.



*Drosophila* S2 cells were transfected with plasmids expressing Hth (panels I-IV, lanes 1-3), Hth plus En (panel V, lanes 1-3) or Hth plus En and His<sub>6</sub>Exd ('tagExd', lanes 4-6, all panels), and nuclear extracts were prepared (see Materials and Methods). Hth-specific antiserum (+, panels I and II) or preimmune serum control (-) was incubated with Protein-A/agarose beads and then with the nuclear extracts. His<sub>6</sub>-specific monoclonal antibodies (+, panels III-V) or nonspecific IgG control (-) were incubated with Protein-G/Sepharose beads and then with the nuclear extracts. 'In' indicates one-fifth of input extract (except panel III, where lanes 1 and 4 are shown at a shorter exposure to allow the En band to be clearly distinguished from a background band that is detected by the anti-En antiserum); P indicates a pellet (bead) fraction. Lanes 1-3 contain extract from the Hth-only (or Hth plus En, panel V only) transfection. Lanes 4-6 contain extract from Hth plus En and His<sub>6</sub>Exd transfection, analyzed by SDS-PAGE and western blotting, after incubation with control beads (-), anti-Hth ('α-Hth') beads (+, panels I and II) or anti-His<sub>6</sub> ('α-tag') beads (+, panels III-V), followed by extensive washing, followed by detection of either En (panels I, III and V), Exd (panel II) or Hth (panel IV) with specific antisera. Notice that the background band ('b.g.') in panels III and V, which migrates faster than En and is present in both extracts, is not precipitated. This band does not appear in panel I because of the use of monoclonal anti-En antibody, whereas polyclonal anti-En antibody was used in panels III and V.

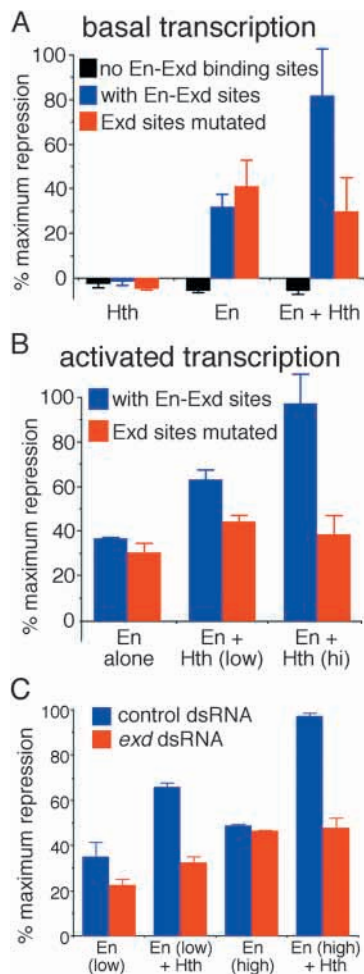
al., 1999), this might be due to an increased nuclear concentration of En-Exd complexes, to a Hth-En interaction or to a combination of the two.

In order to test whether the increase in repression by En caused by expression of Hth was mediated by cooperative binding between En and Exd, we tested mutated versions of the cooperative binding site. A two-nucleotide change in an Exd core consensus binding sequence (TGAT) within the 30-nucleotide oligonucleotide has previously been shown to abolish co-operative binding by En and Exd (van Dijk, 1994). As shown in Fig. 6, this Exd-site-mutated target gene still responded to En, but the increased repression caused by Hth was largely absent. Although there was no apparent increase in repression of basal transcription caused by Hth with this mutated target gene, some residual increase in repression of activated transcription appeared to persist. This was true even when the other core Exd consensus binding sequences (ATCA) within the oligonucleotide were also mutated (data not shown). Thus, eliminating co-operative binding between Exd and En significantly reduced the cooperative repression caused by Hth expression, suggesting that this effect of Hth is mediated at least in part by co-operative binding between Exd and En, whereas a secondary effect of Hth expression on repression of

activated transcription might be independent of such co-operative binding. Consistent with previous results (Jaynes and O'Farrell, 1991), when the En core consensus sequence ATTA was mutated to AGGA, repression by En, or by En plus Hth, was eliminated (data not shown).

To test the influence of Exd more directly, we turned to RNA interference (Clemens et al., 2000) to reduce endogenous Exd levels in S2 cells. As shown in Fig. 6C, treatment of S2 cells with Exd dsRNA reduced En-mediated repression from the co-operative binding sites significantly, relative to non-specific dsRNA. This effect was not seen at higher concentrations of En, possibly owing to saturating levels of En. However, the increase in repression by Hth was clearly reduced by Exd dsRNA at both high and low En concentrations, suggesting that Hth and Exd cooperate to increase the repression activity of En.

Although these results might be accounted for by a combination of Hth-induced nuclear localization of Exd and co-operative binding by Exd and En to the target gene, the observed ability of En to form complexes with both Hth and Exd suggest the simple model that repression by En alone is less effective than repression by a trimolecular complex of En, Exd and Hth.



**Fig. 6.** Hth and Exd enhance repression by En in cultured cells. *Drosophila* S2 cells were transfected with a target gene containing a CAT reporter, binding sites that are bound co-operatively by En and Exd in vitro (oligonucleotide sequence TCGAGTCAATTTAAATGATCAATCAATTTCG); or, as indicated in the key, the same plasmid without these sites, or with a two-nucleotide change in an Exd core binding site (oligonucleotide sequence: TCGAGTCAATTTAAAGCATCAATCAATTTCG) and activator binding sites. Cells were harvested after 66 hours and assayed for CAT and a co-transfected reference gene. The vertical axis shows results normalized to reference gene activity, as a percentage of the maximum repression observed in each experiment. Maximum repression was eightfold for basal transcription (A) and at least tenfold for activated transcription (B,C). Error bars show the range of values from two parallel transfections. (A) The reporter (either with or without En-Exd sites, or with mutated sites) and reference plasmids were co-transfected with either a Hth expression construct, an En expression construct, or both together. Bars below the baseline indicate the slight activation seen without binding sites. (B) The reporter (with or without Exd sites mutated as indicated in the key), activator and reference plasmids were co-transfected with an En expression construct either alone or in combination with a Hth expression construct at either a low or a higher concentration as indicated (see Materials and Methods for plasmid amounts and other transfection details). There was no effect of Hth alone on activated transcription. (C) The reporter, activator and reference plasmids were co-transfected with the En expression construct (at either a low or higher concentration) either alone or with the Hth expression construct, after a prior treatment of the cells with either control or Exd dsRNA, as indicated (see key).

Therefore, En might interact with these cofactors similarly to Hox proteins, which have been shown to form such three-way complexes (Ferretti et al., 2000; Jacobs et al., 1999; Ryoo et al., 1999).

In cultured *Drosophila* cells, Exd and Hth cooperate with En to repress transcription. Using a co-operative binding site for Exd and En (Peltenburg and Murre, 1996) to construct an En-responsive target gene, we found that both Exd and Hth are required for full repression activity (Fig. 6). When a mutation was introduced into an Exd consensus binding sequence that eliminates co-operative binding, co-operative repression was largely eliminated, whereas mutating the En consensus binding sequence eliminated repression. This, along with the fact that RNA interference directed against Exd mRNA also largely eliminated co-operative repression (Fig. 6C), suggests that a complex containing Exd and En is responsible for the co-operative repression caused by coexpression of Hth and En (Exd is constitutively expressed in these cells). Because Hth regulates the nuclear localization of Exd, it can allow Exd-En repression complexes to form in the nucleus. In addition, the observed molecular interactions suggest that the fully active repression complex might include all three proteins.

#### Exd cooperate s with En to repress target genes and to pattern embryos

Loss of *exd* function has been shown to result in a loss of *en* expression at later embryonic stages (Rieckhof et al., 1997). Because *en* function is required to maintain its own expression (Heemskerk et al., 1991), the loss of *en* expression could be a downstream effect of a loss of *exd* function, or it could be due to some other consequence of the lack of *exd*. This ambiguity concerning the role of *exd* in *en* function led us to investigate whether the activities of ectopically expressed En (Heemskerk

## DISCUSSION

We show here that En, a non-Hox homeodomain protein with multiple roles in development, is dependent on the functions of the Hox cofactors *exd* and *hth* to repress target genes in vivo. Molecular studies demonstrate the ability of En to form complexes with Hth and Exd in vitro, in yeast and in *Drosophila* cells, leading to the model that complexes among these genetic partners carry out repression in vivo. This model is supported by the ability of Exd and Hth to facilitate repression by En from co-operative binding sites in co-transfection assays. Previously, Hth and Exd were known to act as cofactors only for Hox proteins. This work therefore sheds new light on how En might achieve target specificity in vivo, and expands the diversity of transcriptional regulators that require *hth* and *exd* function. Below, we discuss the evidence supporting this model, as well as its implications.

#### Exd and Hth form complexes with Engrailed

En can form complexes with both Exd and Hth (as well as its mammalian homolog Meis1) in vitro, in yeast and in cultured cells (Fig. 5). Interestingly, rather than the two-way interactions being mutually exclusive, En appears to be able to interact simultaneously with Exd and Meis1 in vitro (Fig. 5B). In extracts from transfected cultures, En is specifically precipitated by antisera against both Exd and Hth (Fig. 5C).



et al., 1991) are dependent on *exd* function. We expressed En ectopically in two ways: from a heat-shock promoter and using a patterned Gal4 'driver' transgene. An advantage of the former approach is that one can often distinguish between immediate and secondary downstream effects based on how rapidly they occur following heat induction. Advantages of the second approach include having normal and altered expression in parts of the same embryo, providing a rigorous internal control. Both of these approaches led to similar conclusions (Figs 1-4), that *exd* function is important for the repression by En of its direct target gene *slp*, that *wg* also shows a strong dependence on *exd* function for its repression by En and that the ability of En to alter the pattern of embryonic cuticles is sensitive to the gene dosage of *exd*. Further, in each set of experiments, the observed dependence of repression on *exd* was accompanied by a residual repression activity when *exd* function was removed both maternally and zygotically. This residual *exd*-independent repression activity might be due to the ability of En to bind to target sites independently of *exd* but with a reduced affinity, or it could be accounted for by the existence of two classes of binding sites, one *exd* dependent and the other *exd* independent. This possibility is paralleled by the relationship of Exd with Ubx, which has been shown to function either co-operatively with Exd or alone on multiple binding sites in target genes (Galant et al., 2002). Alternatively, *exd* might be exerting an indirect effect on repression by En. However, because Exd forms complexes with En in yeast and in vitro, and because it appears to facilitate repression by En directly in cultured cells, it seems likely that the dependence of En on *exd* function in vivo is due at least in part to the direct action of En-Exd complexes. Confirmation of this model will require the analysis of specific regulatory sites, which have not yet been identified, in target genes such as *slp*. If this model is correct then our results suggest that the repression activity of Exd-En complexes might come exclusively from En repression domains (Han and Manley, 1993; Jaynes and O'Farrell, 1991; Jimenez et al., 1997; Smith and Jaynes, 1996; Tolkunova et al., 1998), because Exd has been shown to act as a cofactor in the activation of target genes in vivo in conjunction with Hox proteins (reviewed in Mann and Chan, 1996; Grieder et al., 1997; Inbal et al., 2001; Liu et al., 2001; Pinsonneault et al., 1997; Ryoo et al., 1999).

The effects of eliminating *exd* function on repression by En appear to be different in the abdomen and the more-anterior regions (Figs 2, 3), in that En is less dependent on *exd* in the abdomen (parasegments 6-12). Similar results have recently been obtained by Alexandre and Vincent (Alexandre and Vincent, 2003), as described in their accompanying paper. One possible explanation is that *hth* can provide the observed *exd*-independent activity. However, in *exd* mutants, Hth levels are reduced, probably because Hth protein is less stable without Exd (Abu-Shaar et al., 1999). Nevertheless, our data are consistent with the possibility that, on their own, either Hth or Exd might provide partial cofactor activity, whereas both together might be required for full activity. The latter possibility is suggested by our observation that maximal repression activity in S2 cells requires all three gene products.

An additional possibility to account for the residual *exd*- and *hth*-independent repression activity of En in the abdomen is that other cofactors assist En in binding to its target genes in the abdomen. If there are other cofactors at work, it is likely

that their activity (or expression) is dependent, either directly or indirectly, on the Hox genes *Ubx* and *abd-A*, because these genes are responsible for all known aspects of differential segment identity in this region of the embryo. This expectation has been directly confirmed by Alexandre and Vincent (Alexandre and Vincent, 2003).

It is noteworthy that the difference in the dependence of En on *exd* in the abdomen versus the thorax is seen only after stage 9 (for example, it is not seen in Fig. 1), when the levels of Hth, and the consequent nuclear concentration of Exd, have declined in the abdomen (Rieckhof et al., 1997). Thus, the dependence of En on *exd* parallels the nuclear concentration of Exd, and might reflect an evolutionary adaptation to the changing levels of Exd in different regions of the embryo.

### Requirements for *hth* and *exd* in En activity are similar in vivo

Hth has been shown to act in part through its facilitation of the nuclear localization of Exd, and strong *hth* and *exd* mutants have very similar phenotypes (Rieckhof et al., 1997). Although Hth can also interact with En independently of Exd (Fig. 5), transfection assays in cultured cells suggest that Hth might depend entirely on Exd for its ability to increase repression by En, at least from artificial En-Exd co-operative binding sites (Fig. 6C). Because Hth forms complexes with En in these cells (Fig. 5C), in addition to increasing its repression activity (Fig. 6A,B), a simple model is that maximal repression activity is due to complexes containing En, Exd and Hth. However, we cannot rule out the possibility that Hth acts solely by making Exd available to interact with En on target sites, through its ability to bring Exd into the nucleus.

We tested whether the repression activity of ectopically expressed En in vivo is dependent on *hth* function, using assays similar to those used for *exd* (Fig. 1, Table 1). In each case, we observed a close similarity to results with *exd* mutants. En activity showed a strong dependence on *hth* function, although residual activity remained in *hth* mutants. In addition, En activity showed a sensitivity to the *hth* gene dose. All of these results are consistent with the effects of Hth being exerted through its effect on Exd nuclear localization, provided that the nuclear targeting of Exd is necessary for its ability to function with En. However, as noted above, Hth might also increase the effectiveness of En repression directly, by forming complexes with En and/or as part of En-Exd complexes. A detailed analysis of a number of in vivo target sites will be necessary to distinguish among these possibilities.

### New *exd* and *hth* functions

Exd and Hth are essential to the correct regulation of target genes by the homeodomain proteins of the Hox clusters (reviewed in Mann and Affolter, 1998). However, their functional interactions have not previously been shown to extend beyond the highly restricted subset of homeodomain proteins that are found within the Hox clusters (the Antp, Abd-B and Labial classes). The identification of functional interactions with En suggests that *exd* and *hth* might provide functional specificity in conjunction with other non-Hox-class homeodomain proteins.

The identification of *slp* as a direct target gene of En has implications for the mechanism by which En helps to maintain the activity of its own and other genes, including *hedgohog*,

within its domains of expression, the posterior compartments. The *slp* locus produces two closely related, coordinately regulated gene products (Slp1 and Slp2), which have essentially indistinguishable functions (Cadigan et al., 1994; Grossniklaus et al., 1992). They are forkhead-domain transcription factors (Grossniklaus et al., 1992) that repress *en* expression (Cadigan et al., 1994), and both contain a conserved motif (homology region II) (Grossniklaus et al., 1992) that is similar to the Groucho-binding domain of En (Tolkunova et al., 1998). Slp1 has also been shown to bind the Groucho co-repressor in vitro (Kobayashi et al., 2001), suggesting that it is a repressor and therefore that its action on the *en* gene is likely to be direct. Thus, the mechanism of *en* autoregulation, as well as the ability of En to activate other target genes, is likely to be due, at least in part, to an indirect effect of repression of *slp* expression. Similar conclusions have been reached by Alexandre and Vincent (Alexandre and Vincent, 2003), as described in the accompanying paper. In addition, En might activate target genes indirectly by repressing other repressors that are also normally excluded from its expression domain, such as Odd-skipped (Mullen and DiNardo, 1995; Saulier-Le Dreaan et al., 1998) and the repressor form of Cubitus interruptus (Eaton and Kornberg, 1990).

Although there have been previous suggestions that Exd and Hth might participate in active repression as well as activation complexes (Abu-Shaar and Mann, 1998; Abzhanov et al., 2001; Manak et al., 1994; White et al., 2000), most of the well-characterized direct Exd-Hth-Hox target genes are activated in an *exd*- or *hth*-dependent fashion (Manak et al., 1994; Pinsonneault et al., 1997; Ryoo and Mann, 1999; Ryoo et al., 1999). In fact, these observations raised the question of whether Exd and Hth might be dedicated to gene activation (Pinsonneault et al., 1997). Recently, Hth and Exd have been shown to act directly with Ubx to repress the Hox target gene *Distalless* in the *Drosophila* abdomen (Gebelein et al., 2002). The partnership with En in repression further argues that these cofactors can increase the target site discrimination of homeodomain proteins without restricting the resulting transcriptional activity to activation alone. Based on these results, we suggest that Hth and Exd increase the target-site discrimination of several classes of homeodomain proteins and that they do so without defining the transcriptional activity of the resulting protein complex.

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