The EMBO Journal Vol.17 No.13 pp.3704–3713, 1998

Molecular mechanism of *polyhomeotic* activation by Engrailed

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The *Drosophila* Engrailed homeoprotein has been shown to activate directly a *Polycomb*-group gene, *polyhomeotic*, during embryogenesis. The molecular mechanism involved in this activation has been studied. Two different types of Engrailed-binding fragments have been detected within the *polyhomeotic* locus. The P1 and D1 fragments contain several 'TTAATTGCAT' motifs, whereas the D2 fragment contains a long 'TAAT' stretch to which multiple copies of Engrailed bind cooperatively. Another homeodomain-containing protein, Extradenticle, establishes protein–protein interactions with Engrailed on the D2 fragment. We have shown by CAT assays that both types of Engrailed-binding sites (P1 or D1 and D2), as well as Extradenticle, are necessary to obtain activation by Engrailed. *In vivo*, we have also shown that normal *polyhomeotic* expression depends on *extradenticle* expression. Moreover, in the absence of Extradenticle, overexpression of Engrailed protein represses *polyhomeotic* expression.

Keywords: *Drosophila*/Engrailed/Extradenticle/*polyhomeotic*/transcriptional activation

Introduction

During *Drosophila* embryogenesis, anterior–posterior organization is generated by a transcriptional cascade involving several classes of maternal and zygotic genes. This cascade sequentially subdivides the embryo into smaller domains along the anterior–posterior axis and leads to the establishment of segments and compartments within these segments (reviewed by Ingham, 1988). The last genes in this cascade, the 'segment polarity' genes, are expressed in segmentally reiterated stripes and encode products necessary for the establishment of compartments within segments. The *engrailed* (*en*) gene belongs to this last class of genes and plays an essential role during *Drosophila* development.

Genetic and molecular studies have shown that the Engrailed (En) protein is necessary to direct the posterior compartment developmental pathway and to specify posterior identity (Lawrence and Morata, 1976; Kornberg, 1981b). The *en* gene also plays an essential role in the establishment of compartment boundaries within each segment (Lawrence and Morata, 1976; Kornberg, 1981a).

En is a nuclear protein of 552 amino acids that contains a homeodomain (Fjose *et al.*, 1985; Poole *et al.*, 1985). En is able to bind DNA with high affinity through its homeodomain (Desplan *et al.*, 1985, 1988; Kissinger *et al.*, 1990). Transfection experiments into cultured cells have demonstrated that En can act as a passive repressor, by competing with specific activators for binding sites located upstream of a basal promoter (Jaynes and O'Farrell, 1988; Han *et al.*, 1989). Repression by En can also occur by competition with the general transcription factor, TFIID, for binding to the TATA box (Ohkuma *et al.*, 1990). En has also been found to act as an active repressor of transcription by binding to sites distinct from those bound by activators (Jaynes and O'Farrell, 1991). The repressor domain of the En protein has been mapped and is located outside of the homeodomain (Jaynes and O'Farrell, 1991; Han and Manley, 1993). This repressor domain is able to confer the capacity to repress transcription in transfected cultured cells when transferred to a heterologous DNA-binding domain (Jaynes and O'Farrell, 1991; Han and Manley, 1993), as well as *in vivo* (John *et al.*, 1995). These results suggest that En could regulate the expression of particular genes directly at the transcriptional level, as has been shown for other homeodomain-containing proteins (Krasnow *et al.*, 1989; Winslow *et al.*, 1989).

Although genetic experiments have shown that En is able to activate the expression of several genes, for example *en* itself (Heemskerk *et al.*, 1991), *hedgehog* (Tabata *et al.*, 1992) and *polyhomeotic* (Serrano *et al.*, 1995), no activation has been described after transfection into cultured cells. This has suggested that activation by En is indirect, that the En-binding sites involved in activation have not been identified and are different from those mediating repression, or that En is unable to act as a transcriptional activator on its own.

Recently, the homeodomain-containing protein, Extradenticle (Exd), has been shown to interact *in vitro* with two homeoproteins, Ultrabithorax (Ubx) and AbdominalA (AbdA), and to raise their DNA-binding specificity (Chan *et al.*, 1994; van Dijk and Murre, 1994). Exd also binds to DNA cooperatively with the En protein, although to a different sequence from that bound by the Exd–Ubx and Exd–AbdA heterodimers (van Dijk and Murre, 1994). Genetic studies have suggested that Exd might modulate the *in vivo* functions of the homeotic gene products (Peifer and Wieschaus, 1990; Rauskolb and Wieschaus, 1994). Strikingly, complete removal of both the maternal and zygotic Exd product leads to segmentation defects that result from failure to maintain *en* expression (Peifer and Wieschaus, 1990). This observation suggests that Exd may be required for the autoactivation of *en* during embryogenesis (Heemskerk *et al.*, 1991). Interestingly,

autoactivation of *en* has never been reproduced in cultured cells.

We identified a *Polycomb* group gene, *polyhomeotic* (*ph*) as a potential En target gene on the basis of Engrailed binding to the cytological location of *ph* on polytene chromosomes (Serrano *et al.*, 1995). En was indeed shown to activate *ph* expression in posterior compartment cells during embryogenesis. Three En-binding fragments were identified *in vitro* within the 49 kb covering the *ph* locus. These three fragments were also shown to be bound by En *in vivo* (Serrano *et al.*, 1995). These results suggested that these En-binding fragments were likely to mediate *ph* activation by En, and offered a unique opportunity to analyse the molecular mechanism of activation by En.

To understand better how En mediates activation of its target genes, we have analysed further the interactions between En and *ph* at the molecular level. We have characterized two different types of En-binding sites that differ by their sequences, their affinity for En, their footprint profiles and their effect on transcription after transfection into cultured cells. While two of the three En-binding fragments (named P1 and D1) behave like the classical 'NP' binding site (Desplan *et al.*, 1988), one of the fragments (named D2) has a 5-fold higher affinity for En. In the presence of En protein, 150 bp within this D2 fragment are protected against DNase I, creating an unusually large footprint. We have also shown that En binds to D2 in a cooperative manner. In addition, while P1 and D1 are able to mediate repression after transfection into cultured cells, D2 mediates a 2-fold activation. Exd raises En affinity for the D2 fragment, through the formation of an Exd–En–D2 complex, involving protein–protein interactions between En and Exd. Functional analysis by CAT assays has shown that activation of *ph* expression by En requires the presence of Exd and D2 as well as either P1 or D1 En-binding fragments. We also verified that in germband-extended embryos, *ph* expression and its activation by *en* are dependent on *exd* expression.

Results

Identification of the Engrailed-binding sites within the polyhomeotic locus

The *ph* genomic region has been cloned (Dura *et al.*, 1987) and the *ph* transcription units have been sequenced (Deatrick *et al.*, 1991). *ph* is a complex locus containing two duplicated transcription units largely redundant in terms of function.

Three En-binding fragments were identified within the *ph* locus by immunoprecipitation and gel shift experiments (Serrano *et al.*, 1995). One of these, a 441 bp fragment named P1, lies upstream of the proximal transcription unit, and the other two fragments (a 300 bp fragment, named D1, and a 200 bp fragment, named D2) are localized upstream of the distal transcription unit (Figure 1; Serrano *et al.*, 1995).

En can bind with high affinity to sequences either related to the 'TTAATTGCAT' consensus or containing repeated 'TAAT' motifs, as can many homeodomain-containing proteins (Desplan *et al.*, 1988). Sequence analysis of the En-binding fragments identified within the *ph* locus revealed that, whereas P1 and D1 contain six sequences closely related to the 'TTAATTGCAT' En consensus binding sequence, D2 contains a long stretch of 'TAAT' motifs (Figure 1). Footprinting analysis determines that four regions within the P1 or D1 fragments are protected against DNase I treatment in the presence of a purified bacterial T7 En protein (Figure 1). These short footprints, each ~30 bp, cover the consensus binding sequences (Figure 1, in bold and underlined). Within the D2 fragment, a large sequence (~150 bp) is protected against DNase I in the presence of En protein. This long footprinted region covers the 'TAAT' motifs (Figure 1).

Analysis of Engrailed affinities for P1, D1 and D2

En affinity for the P1, D1 and D2 fragments was estimated by gel shift assays in which the concentration of En protein was varied relative to a constant amount of DNA.

Binding sites were titrated with HS-EN protein, which has been shown to bind DNA with a 10-fold higher affinity than the T7 En protein (Serrano *et al.*, 1995). Binding specificity was verified both by titrating P1, D1 and D2 fragments with a purified En bacterial protein (Serrano *et al.*, 1995) and by supershift experiments in the presence of anti-En antibody (data not shown).

Six complexes are formed within the P1 (Figure 2A) and D1 fragments (data not shown), whereas only one major complex is formed with the D2 fragment (Figure 2B). Note that a minor complex with a lower affinity is also detectable. Considering all the complexes, the estimated K_D with the HS-EN protein for P1 (Figure 2A) and D1 (not shown) is 10^{-9} M. This K_D corresponds to the estimated affinity of the HS-EN protein for one 'TTAATTGCAT' consensus binding sequence. For D2, the En affinity was estimated to be 2×10^{-10} M, which is 5-fold higher than for the P1 or D1 fragments (Figure 2B).

There is a correlation between the number of consensus sequences within P1 and D1 and the number of complexes observed by gel shift experiments (Figures 1 and 2).

The formation of only one major complex with D2 suggests that the large footprint observed on D2 results from the binding of more than one En protein to the 'TAAT' motifs. It has been shown that a $(TAA)_4$ sequence is able to bind En protein with the same affinity as one consensus binding site (Desplan *et al.*, 1988). Therefore, the observed complex probably results from the binding of several En proteins to different 'TAAT' motifs within D2. The binding of multiple proteins is likely to be highly cooperative, since reducing the protein concentration does not result in the appearance of slower migrating complexes or shorter footprints (Figure 2B).

To test this hypothesis, the D2 fragment was titrated with the isolated homeodomain of the En protein (HD-EN). In contrast to what is observed with the full-length En protein, several complexes were formed in the presence of increasing amounts of HD-EN, creating a 'ladder' gel shift profile (Figure 2C). By counting the number of complexes, we estimated the number of En homeodomains able to bind the D2 fragment to be 10 (Figure 2C).

This experiment, together with footprint and gel shift assays with HS-EN protein, strongly suggests that several En proteins (up to 10) bind to D2 in a cooperative manner. Since HD-EN binds to D2 in a non-cooperative manner (Figure 2C), the protein–protein interactions observed with HS-EN must involve a region of the protein outside of the homeodomain. In contrast, En protein binds to the P1

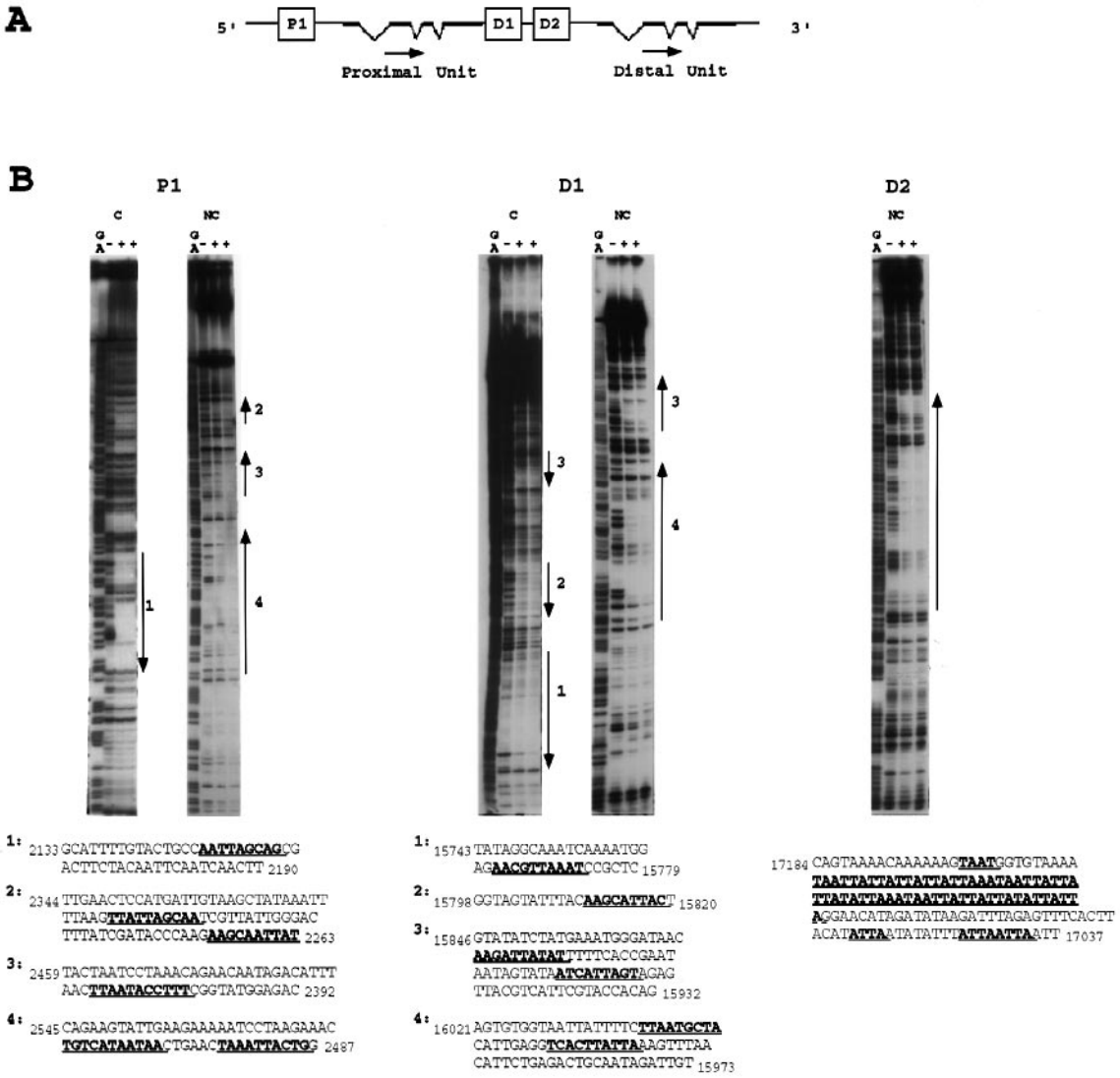

Fig. 1. Localization of En-binding fragments within the *ph* locus. (**A**) Diagram of 49 kb of the *ph* region (Dura *et al.*, 1987). Thick lines correspond to repeated sequences in the two transcription units. The intron/exon structure of the proximal and distal transcripts is shown. P1, D1 and D2 boxes correspond to fragments that were shown specifically to bind En (Serrano *et al.*, 1995). (**B**) DNase I footprints in the presence of purified T7-En protein on fragments P1, D1 and D2 are shown. G/A represents the Maxam–Gilbert sequencing. (–) corresponds to the DNase I profile without any protein; (+) corresponds, from left to right, to two different amounts of T7-En protein, which are 0.6×10^{-8} and 1.25×10^{-8} M. Both DNA strands have been tested except in the case of the D2 fragment. DNase I footprinted sequences are shown below and numbered according to Deatrick *et al.* (1991). Sequences related to the En consensus binding site are in bold and underlined.

and D1 fragments in a non-cooperative manner, at the ratio of one En protein per consensus binding sequence.

Differential behaviour of P1, D1 and D2 for Engrailed binding

In order to analyse further the differences in En binding to sites P1, D1 and D2, we carried out cross-competition experiments between these fragments. Competition experiments were carried out using the HS-EN protein as a source of En protein.

No significant competition was observed for any of the fragments in the presence of herring sperm DNA (HSDNA), used as non-specific competitor DNA (Figure 3A–C). In contrast, binding of En to fragments P1 and D1 can be competed with either P1, D1 or D2 fragments. A 100-fold excess of P1 or D1 strongly reduces complex formation on the labelled site and a 1000-fold excess nearly abolishes binding (Figure 3A and B). Consistent with its 5-fold higher affinity for En, D2 can compete for En binding to P1 or D1 more efficiently than P1 or D1 can themselves. Actually, a 100-fold excess of fragment D2 competes as well as a 1000-fold excess of P1 or D1 (Figure 3A and B).

Unexpectedly, P1 and D1 are completely unable to compete En binding to D2 (Figure 3C). Competing with P1 and D1 is as ineffective as competing with HSDNA or with 3.1 (Figure 3C), a *ph* fragment that is unable to bind En *in vitro* (data not shown). Fragment D2 was able to compete for its own binding very efficiently: a 100-fold excess of D2 fragment totally abolished complex formation. These results are consistent with the idea of cooperative binding of several En proteins to D2. Strong protein–protein interactions might occur between En proteins bound to D2, resulting in the formation of a tight

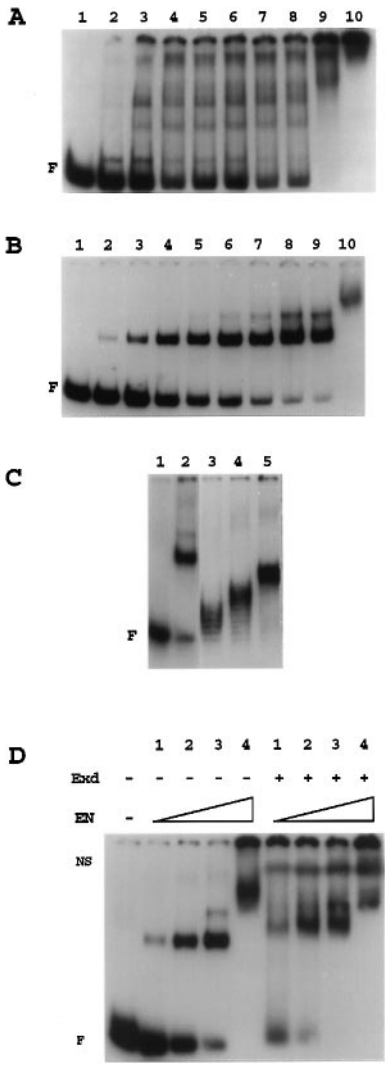

Fig. 2. En binding to P1 (A) and D2 (B, C and D) fragments. (A–C) Mobility shift analysis of En protein-binding sites within the *ph* locus. Relative affinities of En-binding sites were measured by maintaining a constant amount of DNA (0.5 ng/lane) and varying the concentration of En protein. (**A**) P1 fragment titrated with increasing amounts of HS-EN protein. Protein concentrations were calculated to be: lane 1, 0; lane 2, 10^{-10} M; lane 3, 5×10^{-10} M; lane 4, 6×10^{-10} M; lane 5, 7×10^{-10} M; lane 6, 8×10^{-10} M; lane 7, 9×10^{-10} M; lane 8, 10^{-9} M; lane 9, 2.5×10^{-9} M; lane 10, 5×10^{-9} M. The estimated K_D is 10^{-9} M. (**B**) D2 fragment titrated with increasing amounts of HS-EN protein. Protein concentrations were calculated to be: lane 1, 0; lane 2, 5×10^{-11} M; lane 3, 10^{-10} M; lane 4, 2.5×10^{-10} M; lane 5, 4×10^{-10} M; lane 6, 5×10^{-10} M; lane 7, 6×10^{-10} M; lane 8, 8×10^{-10} M; lane 9, 10^{-9} M; lane 10, 5×10^{-9} M. The estimated K_D is 2×10^{-10} M. (**C**) D2 fragment with no protein (lane 1) titrated with 5×10^{-10} M HS-EN protein (lane 2) or increasing amounts of HD-EN protein (lane 3, 7×10^{-8} M; lane 4, 3.6×10^{-7} M; lane 5, 7×10^{-7} M). (**D**) Analysis of En binding on D2 in the presence of Exd. Fragment D2 (0.5 ng) was incubated with increasing amounts of HS-EN protein in the absence (–) or the presence (+) of 3 ml of *in vitro* translated Exd protein, as indicated. HS-EN concentrations are: lane 1, 10^{-10} M; lane 2, 5×10^{-10} M; lane 3×10^{-9} M; lane 4, 5×10^{-9} M. NS indicates a non-specific band due to the presence of labelled Exd translation product. F indicates free DNA.

complex on this fragment that prevents P1 and D1 from interfering with En binding to D2.

To test this hypothesis, we carried out competition experiments for binding to the D2 fragment using HD-EN as a source of protein. If P1 and D1 are unable to compete because of cooperative binding of the entire En protein on D2, using HD-EN as a source of protein should allow competition. When the P1 and D1 fragments are used as competitors and HD-EN is the source of protein, a downshift of the complex formed with D2 is observed. This downshift corresponds to a decrease in the number of complexes formed on D2 in the presence of competitors. This result confirms that P1 and D1 are indeed perfectly able to compete En homeodomain binding to D2. A 1000-fold excess of either P1, D1 or D2 totally abolishes complex formation (Figure 3D).

These results demonstrate the presence of two different types of En-binding sites within the *ph* locus. The P1 (or D1) and D2 fragments differ by their binding recognition sequences, their affinities for En protein and the length of the footprinted regions in the presence of En. These characteristics seem to result from different mechanisms of En protein binding to these sites. On one hand, En binds as a monomer to each consensus binding site within P1 and D1. On the other hand, binding to D2 seems to result from cooperative binding of several En proteins to repeated 'TAAT' motifs covering 150 bp. In agreement with this hypothesis, En has a 5-fold higher affinity for D2 than for P1, D1 or for an isolated consensus En-binding site.

Functional role of the two different types of Engrailed-binding sites

To address the *in vivo* significance of the observed differences between the P1, D1 and D2 En-binding sites, we carried out a functional analysis by transfection into cultured cells and CAT assays. En-binding fragments from the *ph* locus were cloned upstream of the *Adh-33* minimal promoter, which had been fused to the *CAT* reporter gene in the pD-33CAT vector (Jaynes and O'Farrell, 1988; Figure 4A). This vector was transfected into S2 *Drosophila* cells in the absence or the presence of the pAc-En En-producing vector (Jaynes and O'Farrell, 1988).

When placed upstream of the *Adh-33–CAT* fusion gene, P1 and D1 fragments mediate a 2-fold repression of CAT activity in the presence of En protein (Figure 4B). A multimere containing six repeated 'TTAATTGCAT' consensus binding sequences mediated a 300-fold activation in the presence of a Fushi tarazu (Ftz)-producing vector. However, in the presence of both Ftz and En, activation was reduced 100-fold (data not shown). Thus, the consensus sequences present within P1 and D1 can mediate a strong repression under certain conditions (data not shown). This result is similar to the one previously obtained with the NP consensus sequence (Jaynes and O'Farrell, 1988).

In contrast, no repression of CAT activity was observed with D2 in the presence of En protein (Figure 4B). Rather, this site mediated a 2-fold activation of CAT activity in the presence of En. Multimerization of this 'TAAT' stretch did not increase the observed activation (Figure 4B; data not shown).

Thus, unlike P1 and D1, the D2 site does not behave as a repressor site. En is, however, unable to act on its own as a strong activator through binding to either the P1, D1 or D2 fragments.

Extradenticle interacts with Engrailed on the D2 fragment

Since none of the En-binding fragments identified within the *ph* locus allowed significant activation by En, we

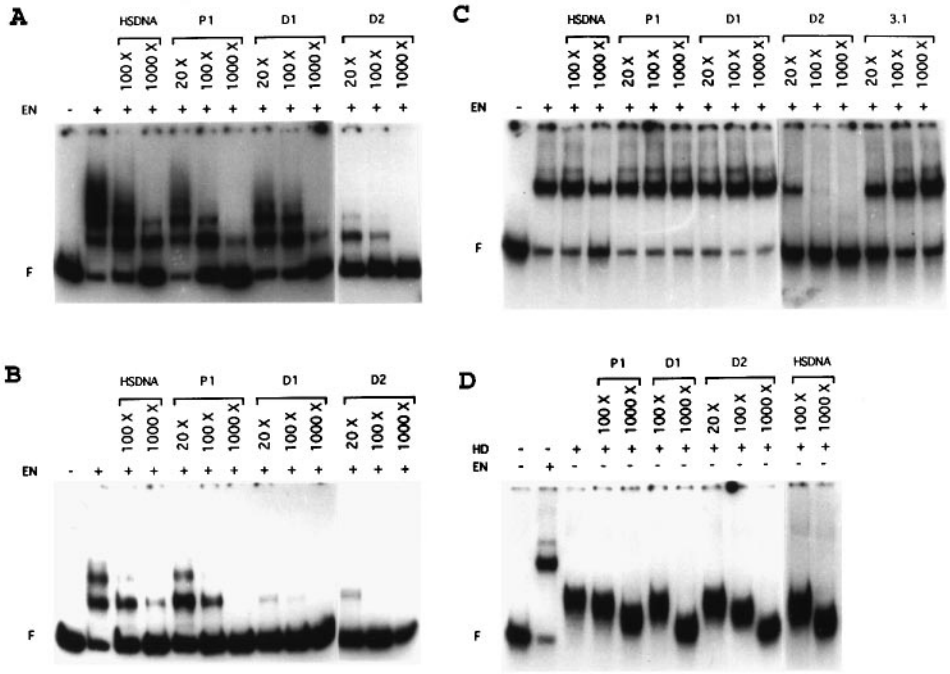

Fig. 3. Competition for En binding between fragments P1, D1 and D2. The competitor and probe are mixed in proportions indicated above each lane. Labelled P1 (**A**) or D1 (**B**) fragments (0.5 ng) used as probe were titrated by 2×10^{-9} M of HS-EN protein in the absence or presence of specific or non-specific competitor DNA, as indicated. (**C**) Labelled D2 fragment (0.5 ng) was titrated by 5×10^{-10} M of HS-EN protein in the presence or absence of specific or non-specific competitor DNA as indicated. (**D**) Labelled D2 fragment (0.5 ng) was titrated by 5×10^{-10} M of HS-EN protein or 4×10^{-7} M HD-EN protein in the presence or absence of specific or non-specific competitor, as indicated. F indicates free DNA.

tested the possibility that En might need the presence of a co-factor to drive activation. Previous results have shown that Exd raises the DNA-binding specificity of En through the formation of a heterodimeric En–Exd DNA-binding complex (van Dijk and Murre, 1994). En and Exd were also shown to bind cooperatively to DNA (van Dijk and Murre, 1994).

We tested whether Exd could interact with En upon binding to the P1, D1 or D2 fragments. We analysed by gel shift experiments the complexes formed on each of these fragments in the presence of either En or Exd protein alone or of both proteins in combination.

First, we analysed possible interactions between En and Exd on P1 and D1. As previously shown, increasing amounts of En protein generate several complexes on P1 and D1 (Figure 2A). In the presence of *in vitro* translated Exd protein, no difference in the complexes formed with En was observed (data not shown). This indicates that En and Exd do not interact at P1 and D1.

The same analysis was carried out with the D2 fragment. In the presence of increasing amounts of HS-EN protein, one major and one minor complex were formed (Figure 2B and D). In the presence of *in vitro* translated Exd protein, a slower mobility complex replaced the En–D2 major complex (Figure 2D). Titration of the D2 fragment with increasing amounts of HS-EN protein in the presence of *in vitro* translated Exd protein showed that Exd raises the affinity of En for D2 2-fold (to an estimated K_D of 10^{-10} M).

Supershift experiments using specific antibodies, directed against either En or a tag present in the Exd protein, allowed us to confirm that the slower mobility complex observed in the presence of Exd is due specifically to binding of both En and Exd to D2.

Interestingly, when En homeodomain was used as a source of protein in the same experiments, no supershift was observed (data not shown). This suggests that protein–protein interactions between En and Exd are necessary to allow cooperation between the two proteins for binding to DNA and that the region of the En protein involved in this interaction must be located outside the homeodomain. A supershift was observed using a truncated version of the En protein lacking the first 297 N-terminal amino acids (data not shown), suggesting that the first 297 N-terminal amino acids of the En protein are not required for the protein–protein interaction between En and Exd.

These results demonstrate that En and Exd are able to cooperate to increase the affinity of En for fragment D2, but not for fragments P1 and D1. This binding may involve a highly conserved Exd-binding site located downstream of the 'TAAT' motifs within D2 (Deatrick *et al.*, 1991).

Extradenticle cooperates with Engrailed to activate transcription

Since Exd is able to cooperate with En for binding on the D2 fragment, we tested whether the interaction between the two proteins at D2 could account for the observed activation of transcription.

Preliminary data have shown that in 0–16 h embryos En binds to the P1, D1 and D2 fragments (Serrano *et al.*, 1995). We therefore tested the possibility that En binding to both types of sites is necessary for *ph* activation.

We carried out transfection experiments into cultured cells and performed CAT assays. D2, P1–D2 or D1–D2 fusions were cloned upstream of the *Adh-33–CAT* reporter gene and analysed in the presence of En- and Exd-producing vectors (Figure 4A). Alternatively, we used S2-EN cells that constitutively express En (J.Knight and

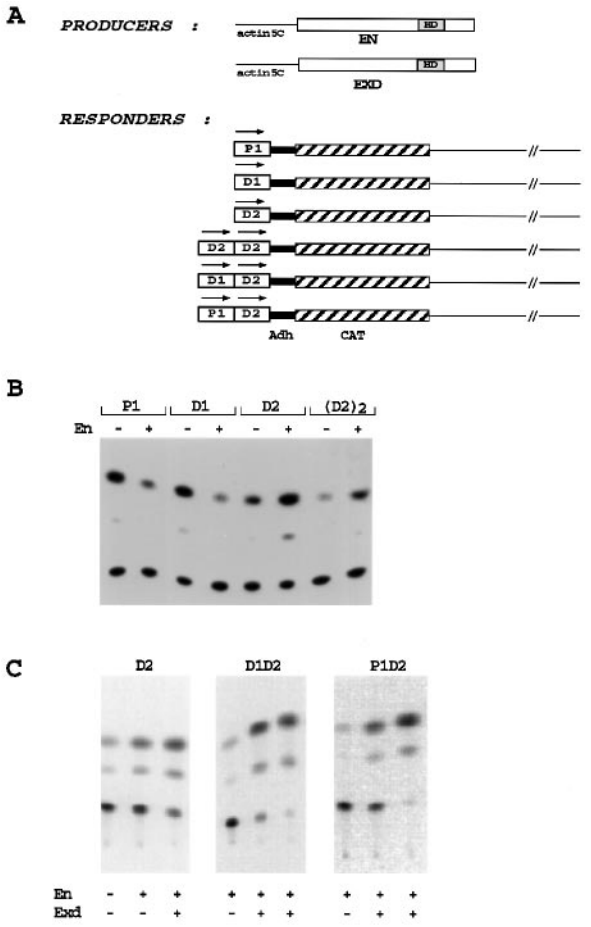

Fig. 4. CAT activity driven by different fragments in the absence or presence of En and Exd. (**A**) Plasmids used to transfect cultured cells. pPAc-en was used as the En protein producer plasmid (Jaynes and O'Farrell, 1988). The producer plasmid for Exd contains the Exd-coding sequence cloned downstream of the actin promoter, in the pPAc vector. En-binding fragments P1, D1, D2, a D2 dimer, and the P1–D2 and D1–D2 fusions were cloned upsteam of the *Adh-33–CAT* reporter gene. (**B**) Chromatography autoradiogram of the CAT assay measuring the CAT activity of the different constructs in the absence (–) or the presence (+) of the En-producing vector, as indicated. (**C**) Chromatography autoradiogram of the CAT assays measuring the CAT activity of different constructs in the presence of Exd in S2-EN cells that constitutively express En (J.Knight and T.Kornberg, personal communication). Experiments with only En have been carried out in S2 cells, whereas experiments with Exd result from transfection of S2-EN cells. CAT activity of the different constructs was tested in the absence (–) or the presence (+) of En- or Exd-producing vector, as indicated below.

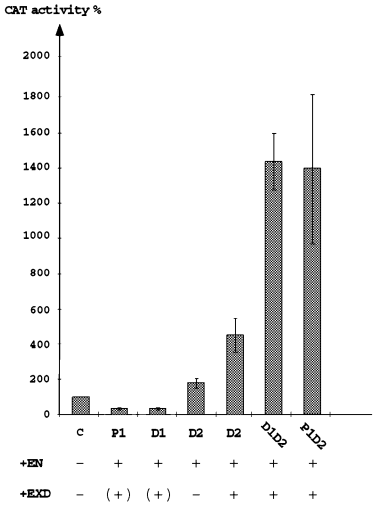

Fig. 5. Quantification of chromatography autoradiograms from Figures 4 and 6. The responders have been tested '–' in the absence or '+' in the presence of En or Exd. (+) indicates that the same result was found in the presence or absence of Exd. The CAT activity is estimated from at least six independent experiments. Error bars are indicated.

T.Kornberg, personal communication) in the absence or in the presence of Exd. The obtained results were similar, although S2-EN cells gave more reproducible results, probably because of the use of only two plasmids for transfection.

When placed upstream of the *Adh-33–CAT* reporter gene, the D2, P1–D2 and D1–D2 fusions mediate a 2-fold activation of CAT activity in the presence of En protein, suggesting that the presence of both sites is not sufficient to mediate strong activation by En. In the presence of the Exd-producing vector alone, no effect on CAT activity was detected with any of the reporter gene constructs (data not shown).

When cells were co-transfected with the D2–*Adh-33–CAT* responder and both the En- and Exd-producing vectors, a 6-fold activation was detected (Figure 4C). Interestingly, with the P1–D2 or D1–D2 responders, a 16-fold activation was observed in the presence of both En and Exd proteins (Figure 4C).

All the CAT assay results are summarized on the histogram on Figure 5. They suggest that activation of *ph* expression by En requires the presence of D2 together with either P1 or D1 as well as the cooperation of the Exd protein. Thus, Exd not only acts as a co-factor for En binding to specific DNA sequences, but is also able to modulate En function as a transcription factor.

Engrailed is able to activate polyhomeotic only in the presence of Extradenticle in vivo

Since Exd seems to be the co-factor necessary for En to act as an activator in cultured cells, we analysed *ph* expression in an *extradenticle* mutant background. Because *exd* maternal product persists until germband extension (Peifer and Wieshaus, 1990), the stage at which *ph* is transiently activated by En (Serrano *et al.*, 1995), we analysed *ph* expression in embryos lacking the maternal *exd* product. We monitored *ph* expression using the ph^{lac} enhancer trap which presents an *engrailed*-dependent striped pattern at germband extension (Serrano *et al.*, 1995). In fully extended *exd* mutant embryos, *ph* expression shows an abnormal striped pattern (Figure 6A1 and B1). This result shows that in the absence of Exd, *ph* is not normally activated.

Because *en* maintenance is affected similarly in these mutants (Figure 6A2 and B2; Peifer and Wieshaus, 1990), we could not determine whether the observed misregulation of *ph* resulted from the misregulation of *en* or from the absence of Exd. To address this issue, we tested whether ectopic expression of *en* induces *ph* expression in the absence of *exd*. Using a *hs-en* transgenic line, *en* can be ectopically expressed in all the cells of germband-extended embryos. These embryos also ectopically express *ph* at high levels (Figure 6A3). In contrast, heat-shock induction of *en* in embryos lacking maternal Exd does not

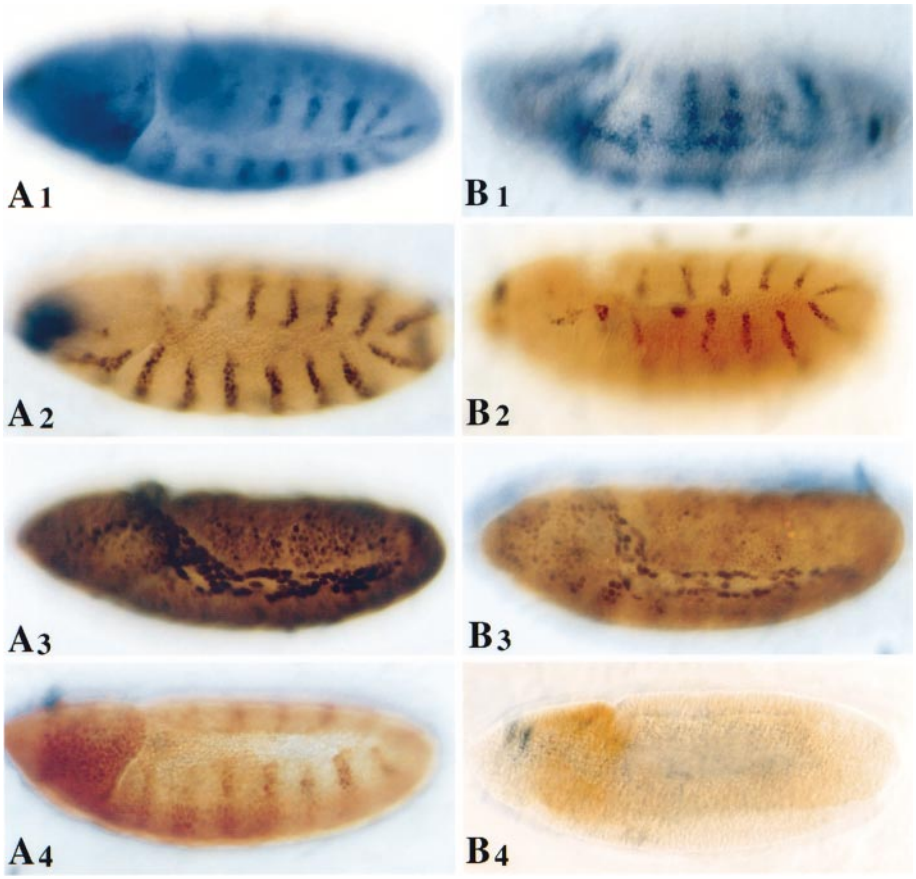

Fig. 6. Analysis of *ph* expression in *exd*$^{-}$ germline clones. All the embryos are at germband extension and are oriented anterior left and dorsal up. *ph* expression is followed by *LacZ* expression in the *ph*^{lac} enhancer trap transgenic line (Serrano *et al.*, 1995). (A) Wild-type background and (B) *exd* maternal effect mutants. (**A1**) and (**B1**) *ph*^{lac} expression monitored using an anti-β-galactosidase antibody and detected by an alkaline phosphatase-conjugated secondary antibody. (**A2**) and (**B2**) Double stainings of *ph* expression [detected as in (A1) and (B1), in blue] and endogenous *en* expression, detected by a polyclonal anti-En antibody monitored using a biotinylated secondary antibody (in brown). (**A3**) and (**B3**) Double stainings as in (A2) and (B2) showing *ph* (in blue) and *en* (in brown) expression in a *hs-en* background under heat shock conditions. (**A4**) The normal *ph* striped pattern monitored using an anti-β-galactosidase antibody detected by a biotinylated secondary (in brown). (**B4**) An embryo under the same conditions as that in (B3), but only stained for *ph* expression [detected as in (A4)].

result in the induction of *ph* expression in the epidermis (Figure 6B3). This result indicates that En is unable to activate *ph* in the epidermis in the absence of Exd (Figure 6B3 and B4). It is noticeable that even the normal *ph* stripe pattern disappears in these embryos, suggesting that in the absence of Exd, high levels of En repress *ph* (Figure 6A4 and B4).

Discussion

The En homeodomain-containing protein binds DNA with high affinity and acts as a transcriptional repressor after transfection into cultured cells (Jaynes and O'Farrell, 1988, 1991; Han *et al.*, 1989; Saenz-Robles *et al.*, 1995). *In vivo*, genetic experiments have shown that En is able not only to repress (Eaton and Kornberg, 1990; Serrano *et al.*, 1997) but also to activate the expression of several target genes (Heemskerk *et al.*, 1991; Tabata *et al.*, 1992). In these examples of activation, no direct transcriptional activation by En has been described. This suggested the possibility that either activation by En is indirect or that it requires a co-factor. Because *ph* was identified as a direct target of En (Serrano *et al.*, 1995), it provided a unique opportunity to analyse the molecular mechanisms of En-mediated activation.

ph expression is activated by En in posterior compartment cells during germband elongation (Serrano *et al.*, 1995) and during wing morphogenesis (F.Maschat, in preparation). En-binding sites have been identified within the *ph* locus and shown to be bound by En, *in vivo*, under physiological conditions during embryogenesis (Serrano *et al.*, 1995). Further analysis of En binding within the *ph* locus has allowed the identification of two different types of En-binding sites. In addition to two fragments, P1 and D1, that contain six typical 'TTAATTGCAT' motifs (NP site), we have identified an additional fragment that contains a new type of high affinity En-binding site. This fragment, named D2, does not contain any NP consensus binding sequence, but instead a long 'TAAT' stretch. Up to 10 En proteins can bind cooperatively to this fragment, resulting in a large footprint and a 5-fold higher affinity for this fragment than for the P1 and D1 fragments. Furthermore, whereas P1 and D1 mediate a 2-fold repression after transfection into cultured cells, D2 mediates a 2-fold activation in the presence of En.

Because none of the En-binding sites alone or in combination mediated a strong transcriptional activation in the presence of En, we tested the possibility that En requires a co-factor to act as a transcriptional activator. Recent results had shown that Exd was able to cooperate

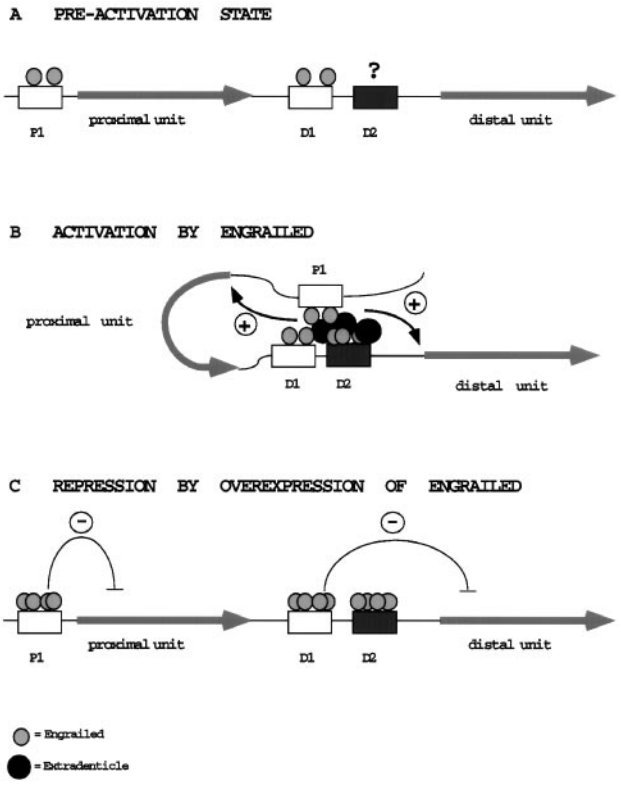

Fig. 7. Molecular model of *ph* regulation by En. (**A**) Pre-activation state. In the absence of Exd, no activation of *ph* occurs. Sites P1 and D1 are bound by En, while site D2 may be occupied either by En or different homeodomain-containing proteins, indicated by a question mark. (**B**) Activation of *ph* by En. This activation involves interactions between En, bound to sites P1, D1 and D2, and Exd. En interacts with Exd at site D2, allowing Exd binding on this site. DNA bending might be necessary to allow interactions between the En protein bound at the three sites and the Exd co-factor bound to D2, leading to activation of both transcription units. (**C**) *ph* repression in the presence of high amounts of En. In the absence of Exd, non-physiological amounts of En are able to drive repression. This repression may be mediated by sites P1 and D1, since CAT assays have shown that these sites can act as repressor sites. It may also occur by displacing other homeodomain-containing proteins acting as activators of *ph* and bound to different sites.

with En for binding to DNA (van Dijk and Murre, 1994). Similar interactions had also been seen between Exd and two other homeodomain-containing proteins, Ubx and AbdA (Chan *et al.*, 1994; van Dijk and Murre, 1994). Exd was, therefore, a good candidate to act as a co-factor for En. We have found that Exd interacts with En at D2, but not P1 or D1, and increases the affinity of En for this fragment. En binding on D2 is necessary to bring Exd to its consensus sequence located dowstream of the 'TAAT' stretch. Cooperation between En and Exd requires protein–protein interactions that involve a domain of the En protein located outside the homeodomain. More strikingly, the presence of both En and Exd mediates transcriptional activation after transfection into cultured cells in the presence of both D2 and P1 (or D2 and D1) En-binding sites. Thus, En is able to act as a transcriptional activator in the presence of Exd protein and, in the case of *ph*, this activation requires the presence of both a typical NP site and a 'TAAT' stretch.

On the basis of these results, we can propose a model to explain *ph* regulation by En (Figure 7). In the absence of Exd, En protein is probably bound to P1 and D1, while D2 might be bound either by En or by other homeodomain-containing proteins. Under such conditions, no activation of *ph* is observed (Figure 7A). This situation might represent what happens in the embryo prior to full germband extension.

When Exd is present, activation of *ph* occurs (Figure 7B). Although Exd is expressed prior to the time at which En activates *ph*, Exd might not be active at those earlier stages: Exd activity has been shown to be regulated by nuclear import and was described to be exclusively cytoplasmic in the embryo before germband extension (Mann and Abu-Shaar, 1996).

Because the En-binding sites are so far apart within the *ph* locus and because D2 is required for activation, acting as an enhancer on both *ph* transcription units, we further suggest that DNA bending might be involved in activation. In particular, En binding to P1 and D1 could be involved in bringing the proximal and distal *ph* promoters close to D2 in the presence of Exd, allowing *ph* activation from both transcription units (Figure 7B). En binding to P1 and D1 prior to germband extension might thus prepare the chromatin for rapid activation. At least two lines of evidence support the idea that D2 interacts with P1 as well as D1, to mediate activation of both the proximal and the distal transcription units. First, the P1–D2 and D1–D2 fusions behave similarly in CAT assays. Second, the *in vivo* activation was observed with the ph^{lac} enhancer trap, which corresponds to an insertion in the proximal transcription unit of *ph* (Fauvarque *et al.*, 1995).

Interestingly, our *in vivo* experiments show that in the absence of Exd and in the presence of high, non-physiological amounts of En, repression of *ph* is observed (Figures 6B3, B4 and 7C). This repression could be mediated by a more complete binding of En on the P1 and D1 sites. Alternatively, En could displace other homeodomain-containing regulators of *ph* from the *ph* promoter.

Because no transcriptional activation by En has been described previously, we further propose that interactions between En and Exd, or other co-factors, might be a general prerequisite for direct activation by En.

Materials and methods

Proteins

Purified full-length T7-En and En homeodomain proteins were kindly provided by Henri Bourbon and Enrique Martin-Blanco and purified in conditions described in Bourbon *et al.* (1995). A soluble nuclear extract-containing En protein was prepared from a Schneider 2 cell line (termed HS-EN) transformed with a gene fusion composed of the *hsp70* promoter–*en* cDNA–*en* polyadenylation site (Gay *et al.*, 1988), in conditions described in Serrano *et al. (*1995). Exd protein and a tagged version of Exd were prepared by *in vitro* combined transcription–translation. *Drosophila exd* coding region (van Dijk and Murre, 1994) as well as a version fused to the influenza tag were cloned into the pSP64-ATG vector (van Dijk and Murre, 1994), downstream of the SP6 promoter. Combined transcription–translation was performed using the 'TNT Reticulocyte Lysate system' (Promega). Exd DNA (0.5 mg) was incubated for 30 min at 30°C in the presence of 25 ml of rabbit reticulocyte lysate, 1 pmol of amino acids mixture minus methionine, 2 ml of TNT buffer, 40 U of RNase block I, 40 mCi of [^{35}S]methionine (1000 Ci/mmol) and 50 U of SP6 polymerase. The reaction was stopped by transfer into ice. Proteins were stored in 5 ml aliquotes at –80°C.

Antibodies

The anti-Engrailed antibody is a polyclonal rabbit antibody, provided by F.Payre and A.Vincent (Serrano *et al.*, 1997). Monoclonal anti-β-galactosidase antibody was provided by Promega.

Gel shift assays

Binding assays contained ~10^{-10} M DNA. En protein (diluted in 25 mM HEPES pH 7.6, 10% glycerol, 100 mM KCl) was incubated with end-labelled DNA (0.5 ng) for 30 min at 4°C in 10 ml of 25 mM HEPES pH 7.6, 10% glycerol, 100 mM KCl, 1 mM dithiothreitol (DTT), 1% polyvinyl alcohol (PVA), 1% NP-40, 0.1% bovine serum albumin (BSA) and 200 ng of poly(dI–dC). DNA–protein complexes were resolved on 6% native polyacrylamide minigels in 0.5× TBE (pH 8.3) buffer. Gels were pre-run at 4°C for 1 h at 100 V and run at 4°C for 2 h at 120 V.

For competition experiments, unlabelled competitor DNA was added to the mix and incubated with the protein and labelled DNA for 30 min at 4°C, before loading on the gel. For supershift experiments, *in vitro* translated Exd protein or antibodies were incubated together with En protein and the labelled DNA for 30 min at 4°C, before loading on the gel.

Footprints

DNA fragments, cloned in the Bluescript vector, were labelled on one end by PCR amplification using either kinased T3 or kinased T7 primers. DNA (10^{-10} M) was diluted in 25 ml of buffer, containing 0.4% PVA, 40 mM HEPES pH 8.0 and 2 mg/ml poly(dI:dC), in the presence of 0, 12.5 or 25 ml of a 5×10^{-8} M solution of purified T7-En bacterial protein. The volume was adjusted to 50 ml with HEMG buffer (25 mM HEPES pH 7.6, 0.1 mM EDTA pH 8.0, 12.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol and 100 mM KCl). After 15 min at 4°C, 50 ml of DNase buffer (20 mM $MgCl_2$, 10 mM $CaCl_2$) and 1 ml of 50 mg/ml DNase I (dilution buffer: 10 mM HEPES pH 7.6, 5 mM $MgCl_2$ and 1 mM $CaCl_2$) were added. After 1 min incubation at room temperature, the reaction was stopped by adding 100 ml of stop solution (0.1% SDS, 200 mg/ml tRNA carrier, 20 mM EDTA, 0.2 M NaCl) and then 100 ml of phenol/chloroform mixture. After a second extraction with chloroform, the DNA was ethanol precipitated. The pellet was resuspended in 90% formamide, 10 mM HEPES pH 7.6, 1 mM EDTA and a bromophenol–xylene cyanol mixture, and loaded on an 8% sequencing gel. Similar DNA treated with the chemical sequencing G+A reactions of Maxam and Gilbert (1980) was also run.

Plasmids

En protein producer plasmid was kindly provided by J.Jaynes. It contained the complete *en* cDNA sequence inserted into the actin 5C promoter/polyadenylation signal vector, pPAc (Jaynes and O'Farrell, 1988). Exd producer plasmid was obtained by cloning the complete *exd* cDNA sequence upstream of the actin 5C promoter, into the pPac vector. En-binding fragments to be tested have been cloned upstream of the *Adh* minimal promoter contained in the reporter vector pD-33CAT (Jaynes and O'Farrell, 1988). Fragments were produced by PCR amplification, using specific primers flanked by either *Bam*HI or *Bgl*II overhangs. The obtained fragments were then ligated into the *Bam*HI site of the pD-33CAT vector. Multimers were purified after ligation of the PCR-amplified fragments, creating non-digestible *Bam*HI–*Bgl*II junctions, followed by a double *Bam*HI–*Bgl*II digestion and size selection (Dobens *et al.*, 1991).

Cell culture and transfection

The S2-EN cell line was kindly provided by J.Knight and T.Kornberg. This line was obtained by transfecting S2 cells with equal quantities (5 μg each) of three plasmids by calcium phosphate. The plasmids were pUAS-en, pUChsneo-act and pA5cGal4. *en* expression in this S2-EN cell line has been verified by Western blot (J.Knight and T.Kornberg, personal communication). *Drosophila* Schneider cell lines (S2 and S2-EN) were cultured at 25°C in Schneider's medium (Gibco) supplemented with 5% fetal bovine serum (Gibco), in the presence of G418 (1 μg/ml) to maintain the S2-EN cell line.

Transfection was performed by calcium precipitation. For this purpose, cells were seeded at 4×10^5–10^6 cells/ml into 5 ml of medium in a 60 mm Petri dish 24 h before transfection. DNAs were prepared with the maxiprep kit (Qiagen). Twenty μg of DNAs to be transfected were added to 31 μl of 2 M $CaCl_2$ and 219 μl of H_2O. The DNA mixture was added to 245 μl of HBS2X (280 mM NaCl, 1.5 mM $Na_2HPO_4{\cdot}2H_2O$, 50 mM HEPES pH 7.1) supplemented with 5 μl of phosphate-buffered saline (PBS) pH 6.8 (1.3 M NaCl, 0.07 M $Na_2HPO_4{\cdot}2H_2O$, 0.03 M NaH_2PO_4). The transfection mixture, mixed carefully, was incubated for 30–60 min at room temperature before adding it to cells. Cells were cultured for 48 h, before preparing the cellular extract.

CAT assay

Cells were harvested by centrifugation and rinsed twice in 4 ml of PBS pH 6.8 (1.3 M NaCl, 0.07 M Na_2HPO_4, 0.03 M NaH_2PO_4). Cells were suspended in TEN (40 mM Tris–HCl pH 7.5, 1 mM EDTA, 150 mM NaCl), gently centrifuged for 5 min and resuspended in 0.25 M Tris pH 7.8. Cells were then broken by freezing in dry ice and thawing at 37°C three times, and debris was removed by centrifugation for 5 min at 4°C. The supernatant was assayed for CAT activity following the protocol of Gorman *et al.* (1982). The assay mixture contained 40 mg of protein extract, 70 mg of acetyl-CoA, 0.1 mCi of [^{14}C]chloramphenicol (50 mCi/mmol), adjusted to 150 ml with 0.25 M Tris–HCl pH 8.0. After 30 min incubation at 37°C, the reaction was stopped with 500 ml of ethyl acetate, also used to extract chloramphenicol. The organic layer was dried and taken up in 10 ml of ethyl acetate, spotted on silica gel thin-layer plates and run with 95% chloroform/5% methanol. Quantification was carried out with ImageQuant Phosphorimager (Molecular Dynamics).

Fly stocks and immunohistochemistry

Embryos without maternal and zygotic *exd* functions were generated in conditions described in Chan *et al.* (1994), except that the *exd* stock is also carrying a ph^{lac} enhancer trap transgene. *w* ph^{lac} exd^{XP11} f^{36A} *FRT18D/y w FM7* females have been crossed with Ovo^D *FRT18D/Y; hsFLP38* males, and larvae obtained from this cross have been shocked for 1 h at 37°C at L1 and L2, in order to activate the *flip-recombinase*. *w phlac* exd^{XP11} f^{36A} *FRT18D/ovoD FRT18D; hsFLP38/+* females were crossed with different males. In order to check *phlac* expression in *exd* germinal clones, these females have been crossed with *simlac* (X) transgenic males, in order to descriminate embryos affected both in maternal and zygotic *exd* functions. To test ph^{lac} activation by En in the *exd* mutant background, *w* ph^{lac} exd^{XP11} f^{36A} *FRT18D/ovoD FRT18D; hsFLP38/+* heat-shocked females were crossed to *hsen* males (Serrano *et al.*, 1995). Three-hour collections of embryos were made at 25°C and transferred for 1 h at 37°C in order to activate En ectopically.

For antibody double stainings, embryos were fixed as described by Sullivan *et al.* (1993). Fixed embryos were stained with different antibodies diluted to the desired concentration. Primary antibodies were detected by biotinylated secondary antibody, visualized by use of the Vectastain ABC kit (Vector) or by secondary antibodies directly conjugated to alkaline phosphatase (Biosys). Double stainings with primary antisera from rabbit and mouse were carried out for both antisera simultaneously.

Acknowledgements

We wish to acknowledge Jean-Antoine Lepesant for welcoming us into his laboratory and for providing accommodation. We thank Peter Follette for useful comments on the manuscript; Jonathan Knight and Thomas Kornberg for the S2-EN cuture cell line; Henri-Marc Bourbon, Enrique Martin-Blanco and Thomas Kornberg for purified T7-En proteins; Cornelis Murre and Lucy Peltenburg for Exd and Exd-tag cDNAs cloned into the pSP64-ATG vector; and Jim Jaynes for the pPAc-En and pD-33CAT vectors. Conversations with Jean-Antoine Lepesant were valuable during the course of this work. N.S. was supported by the Ministère de l'Education Superieure et de la Recherche and by a fellowship from the Association pour la Recherche sur le Cancer. Funding of the research is supported by grants from the CNRS and from INSERM (grant CRE 93107), ACC SV4 No. 9504211 and ARC 1370 to F.M.

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Received October 6, 1997; revised May 7, 1998; accepted May 8, 1998