The EMBO Journal vol.10 no.6 pp.1427 – 1433, 1991

Active repression of transcription by the Engrailed homeodomain protein

James B.Jaynes and Patrick H.O'Farrell

Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143, USA

Communicated by M.Yaniv

The *Drosophila engrailed* gene product (En) is a homeodomain-containing protein that contributes to segmental patterning. In transfection assays it acts as a transcriptional repressor. We show that En is an active repressor, blocking activation by mammalian and yeast activators that bind to sites some distance away from those bound by En. Active repression is distinct from the effects of passive homeodomain-containing proteins, which repress when competing with activators for binding sites and activate when competing with En. Active repression activity maps outside the En homeodomain, and this activity can be transferred to a heterologous DNA binding domain.

Key words: Drosophila development/*engrailed*/homeodomain/repression/transcriptional control

Introduction

Pattern formation in *Drosophila* embryos appears to be guided mainly by regulators of transcription. For example, one class of transcriptional regulators, the homeodomain-containing proteins (homeoproteins), make up ~50% of the gene products that genetics has implicated in patterning (Scott and O'Farrell, 1986; Scott and Carroll, 1987; Levine and Hoey, 1988). A number of homeoproteins bind to DNA with similar sequence specificity (Desplan *et al.*, 1988; Hoey and Levine, 1988; Levine and Hoey, 1988; Treisman *et al.*, 1989; Hanes and Brent, 1989), suggesting that they might compete for binding sites in target genes. In fact, functional analyses in cultured cells have demonstrated that multiple homeoproteins can act on a single target gene. For example, the *fushi tarazu* gene product (Ftz) activates expression of target genes that contain homeodomain binding sites (HDBS), while the *engrailed* gene product (En), which can bind to the same HDBS, effectively represses Ftz-activated gene expression (Jaynes and O'Farrell, 1988; Han *et al.*, 1989; Jaynes *et al.*, 1990; Ohkuma *et al.*, 1990).

Transcriptional repression involving specific *cis*-acting sequences has been observed in a number of systems (reviewed in Levine and Manley, 1989; Doyle *et al.*, 1989; Yoo *et al.*, 1989; Baniahmad *et al.*, 1990; Diamond *et al.*, 1990; Finley *et al.*, 1990; Gottschling *et al.*, 1990; Hudson *et al.*, 1990; Licht *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990). Two distinct classes of repressors are envisaged. Passive repressors would directly interfere with the binding of activators, while active repressors could counteract activators that bind to independent sites. Active repressors are also expected to include a repression domain more or less distinct from the DNA binding domain.

Here we show that homeoproteins can function as active repressors. Specifically, we find that in addition to its previously characterized activities, En can repress target genes activated by known mammalian and yeast proteins that function at unrelated binding sites. This behavior is contrasted to that of passive homeoprotein repressors, which can only repress by direct competition. En is shown also to include a repression function independent of its DNA binding domain. As an active repressor, En is capable of playing a number of roles in the network of regulatory interactions that govern early development.

Results

En counteracts activators that bind to distinct sites

To test whether En represses transcription only by passive displacement of activators, we investigated the effects of En on promoters activated by mammalian and yeast proteins that bind to sites on the DNA distinct from those bound by En. Firstly, in cultured *Drosophila* cells, the rat glucocorticoid receptor (GR) and the yeast Gal4 protein activated promoters that contain their binding sites (GREs and Gal4 binding sites, respectively; see also Fischer *et al.*, 1988; S.K.Yoshinaga and K.R.Yamamoto, submitted). Increasing amounts of these activators caused increasing levels of target gene expression (Figure 1). When En was coproduced, it effectively suppressed this activation when HDBS were present in the target genes (Figure 1a and c). On the other hand, En did not repress GR activation when the target gene lacked HDBS (Figure 1b). Therefore, En can repress an activated promoter by acting at distinct sites upstream of the activator binding sites.

To occlude an activator binding site, a passive repressor must bind to an overlapping or adjacent site. In contrast, repression of GR activation by En occurred when HDBS were either 14 or 25 nucleotides (nt) upstream of the activator binding sites (Figures 1a and 2b), or when 40 or 110 nt downstream of the activator sites (Figure 3b and data not shown). Gal4 activation was repressed by En from 60 nt upstream (Figure 1c). Repression was not detected when the En binding sites were placed very far away (3000 nt, Figure 1d). In addition to repressing activated promoters at a distance, En represses the basal activity of a number of promoters, with HDBS as far as 200 nt away from the transcription start site (Jaynes and O'Farrell, 1988). Therefore, En is likely to possess an active repression function in addition to its DNA binding activity.

DNA binding is not sufficient for active repression

To test whether site occupancy is sufficient for repression, we used homeodomain-containing proteins from which activation domains had been deleted. Such crippled activators

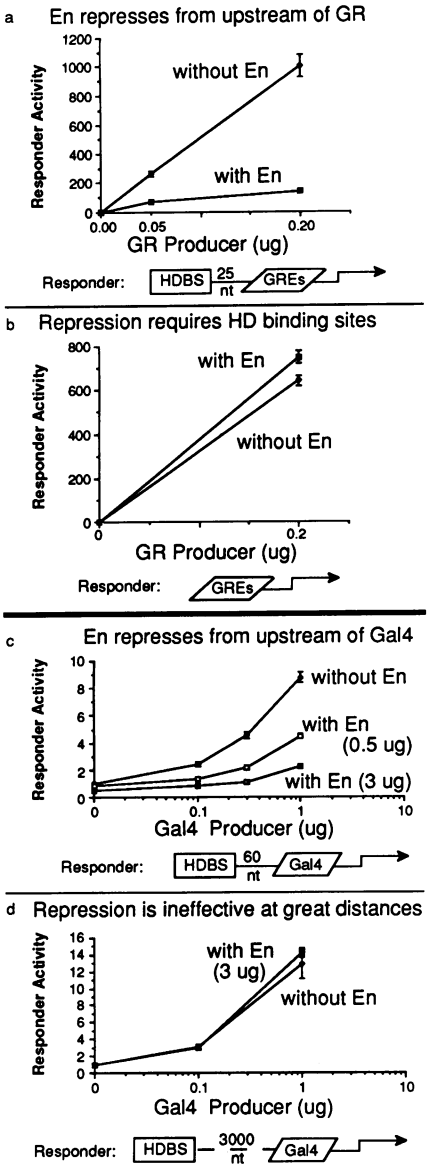

Fig. 1. En represses target genes ('responders') activated by the rat glucocorticoid receptor (GR) or by the yeast Gal4 protein. A constant amount of a responder plasmid containing the target gene shown below each graph (HDBS = homeodomain binding sites, GREs = GR binding sites, Gal4 = Gal4 binding sites) was transfected into cultured *Drosophila* cells. Responder gene activities were determined by measuring the levels of reporter enzyme activity (relative to the activity of a co-transfected reference gene), normalized to basal expression (activity in the presence of control producer plasmid). Induction by GR or Gal4 was assessed by co-transfecting the indicated amounts of activator 'producer' plasmids. The effect of En on expression was determined by including in the co-transfection either En producer ('with En') or control producer ('without En'). Error bars indicate the range of values of two separate transfections within one experiment. All results are representative of those obtained in at least two separate experiments. See Materials and methods for details and plasmid constructions. (**a**) GR producer [$pP_{Ac}GR$, (S.K.Yoshinaga and K.R.Yamamoto, submitted)] was co-transfected with 1 μg of HDBS-containing responder (pN6T3D-33CAT$_B$) and with 5 μg of either En producer [pAc-en (Jaynes and O'Farrell, 1988)] or control producer [pAc-en^{STOP}, En producer with STOP codons inserted into the coding region (Jaynes and O'Farrell, 1988)]. (**b**) As in (a), except that no HDBS were present in the responder (pT3D-33CAT). (**c**) Gal4 producer (pRK245) was co-transfected with 0.2 μg of a responder (pN6G2hZ) that contains HDBS 60 nt upstream of the Gal4 binding sites and with either the indicated amount of En producer or an equal amount of control producer ('without En'). (**d**) As in (c), except that the responder (pG2hZN6) contained HDBS 3000 nt away from the Gal4 binding sites.

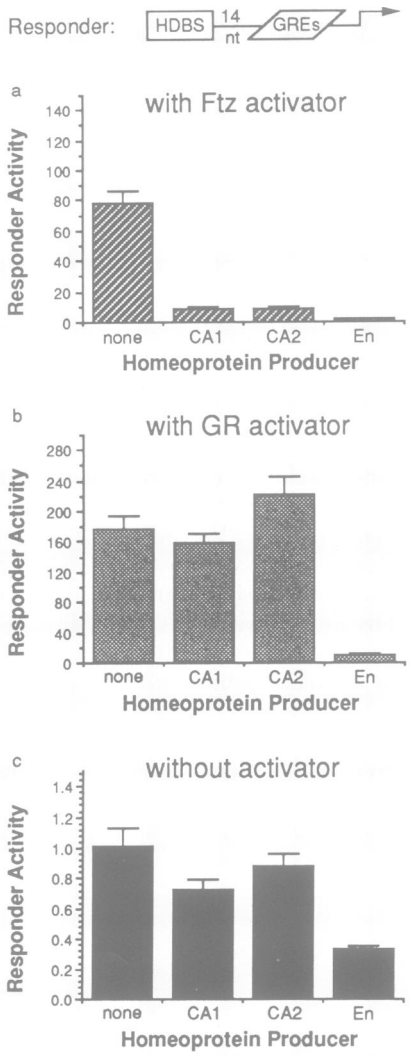

Fig. 2. Homeodomain binding activity is sufficient to suppress activation by a homeoprotein (Ftz), but not to suppress activation by GR. A responder capable of being activated by either Ftz or GR (pN6T3D-33CAT$_A$, diagrammed at top) was co-transfected with either Ftz producer (**a**), GR producer (**b**) or control plasmid (**c**). The influence of the indicated homeoproteins was assessed by co-transfecting with each of the corresponding producers. CA1 and CA2 are truncated homeoproteins with the homeodomain (DNA binding domain) intact but with little or no transcriptional regulatory activity (see c). Responder activity was determined as in Figure 1. The following plasmid amounts were used: 3 μg of one of the homeoprotein producers just described [CA1: pAct5C-zen-Δ2n; CA2 pAct5C-z2-Δ2 (Han *et al.*, 1989); En: pAc-en; 'none' indicates co-transfection with control plasmid, pP_{Ac}, containing the same promoter, but lacking the homeoprotein coding sequences], with 1 μg of responder, and, in (a) 0.1 μg of Ftz producer [pP_{Ac}ftz (Winslow *et al.*, 1989)]; in (b) 0.05 μg of GR producer; in (c) control producer. See Materials and methods for details and plasmid constructions.

were constructed by Han *et al.* (1989) using the *zerknüllt* (zen) and *z2* coding regions. These altered proteins should have no intrinsic transcriptional regulatory function, yet like their parent proteins, they should be able to bind to the HDBS in our target genes.

As expected for passive repressors, the crippled activators can cause substantial repression of Ftz activation (Figure 2a, CA1 and CA2), presumably by competing with Ftz for binding to the HDBS. However, they do not repress GR activation (Figure 2b). On the other hand, En is an effective repressor in both cases (Figure 2a,b). Thus repression in

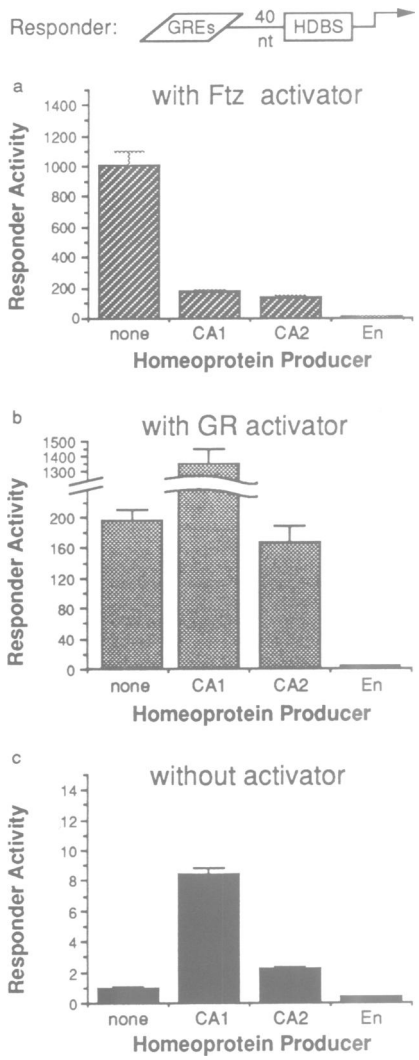

Fig. 3. An active function is required for repression even when repressor binds between activator and basal promoter. A responder capable of being activated by either Ftz or GR, but with the order of activator binding sites opposite to that in Figure 2 (diagrammed at top), was co-transfected with either Ftz producer (**a**), GR producer (**b**) or control plasmid (**c**). The effect of each of the indicated homeoproteins on responder expression was assessed as in Figure 2. The amounts of transfected plasmid DNA were as described in Figure 2, except that pT3N6D-33CAT$_B$ was substituted for pN6T3D-33CAT$_A$.

the two assays has different requirements, and En has an active repression function that the crippled activators lack.

The target gene used in the previous set of experiments contained HDBS upstream of the activator binding sites. It has been suggested that binding of a protein between an activator binding site and the TATA box can passively interfere with activation (Brent, 1985). To test this possibility, we reversed the order of the sites. Figure 3 shows that even in a proximal position relative to the promoter, only En is able to interfere with GR activation. The failure of the crippled activators to repress activation of this target gene by GR does not appear to result from a failure to bind to the HDBS, since, as before, they are effective repressors of activation by the Ftz homeoprotein. In fact, one of the crippled activators increased the effect of GR in activating transcription (CA1, Figure 3b). This is consistent with the fact that it exhibits a weak activation function when tested alone (Figure 3c). Thus, we conclude that binding of a protein between an enhancer and a promoter is not sufficient to interfere with activation, so that repression by En, even when it binds in this position, requires a specific repression function.

The *even-skipped* gene product (Eve) also behaves as an active repressor in these assays. In fact, it is even more effective than En, in both the proximal and distal positions relative to GR (data not shown, and Figure 6; see also Biggin and Tjian, 1989). This is true even though Eve is considerably smaller than En (376 versus 552 amino acids).

Transcriptional activities map outside of the homeodomain

Although En and Ftz bind the same sequence, their homeodomains are substantially different. Since the regulatory distinctions between homeotic gene products appear to be determined, at least in part, by their homeodomains (Kuziora and McGinnis, 1989; Stern *et al.*, 1989; González-Reyes *et al.*, 1990; Mann and Hogness, 1990) we asked whether the distinct transcriptional regulatory properties of En (an active repressor) and Ftz (a potent activator) were functions of their homeodomains. To address this question, we replaced precisely the En homeodomain with that of Ftz (see Materials and methods). The resulting chimeric protein retains the transcriptional regulatory properties of the active repressor. It is able to strongly repress a GR activated target gene (active repression, Figure 4a), as well as a Ftz activated target gene (Figure 4b). The chimeric protein also represses basal level activity when tested alone (Figure 4c), as does the normal En. These results suggest that the transcriptional activities of both En and Ftz are due to regions other than the homeodomain.

The En repression domain is portable

To localize the transcriptional regulatory activity of En, and to determine whether active repression function could be transferred to a heterologous DNA binding domain, fusions were made between En coding sequences and the GR DNA binding domain. These fusion proteins (Figure 5a) contained either 81 or 298 amino acids (aa) from the N terminus of En and a minimal (85 aa) GR DNA binding domain (Diamond *et al.*, 1990). We compared the ability of these fusion proteins to repress transcriptional activation by GR itself, or by Ftz when Ftz binds to downstream sites. While the longer fusion (En298−GR) repressed in both cases (with the activator binding to either the GREs or the HDBS), the shorter fusion exhibited the characteristics of a passive repressor, inhibiting only by direct competition at the GREs (Figure 5b,c). Upstream repression by En298−GR is effective with either 10 or 39 nt of DNA between the GREs (where En298−GR binds) and the HDBS, which are bound by Ftz (Figure 5b and data not shown). As with full-length En, active repression by En298−GR requires binding to DNA, since a Ftz activated target gene containing HDBS but no GREs was essentially unaffected by the presence of En298−GR (<2-fold repression, data not shown). Thus, an active repression activity can be transferred from En to a non-HD DNA binding domain.

Displacement of an active repressor results in activation

To distinguish clearly between the actions of active repressors and a transcriptionally neutral DNA binding

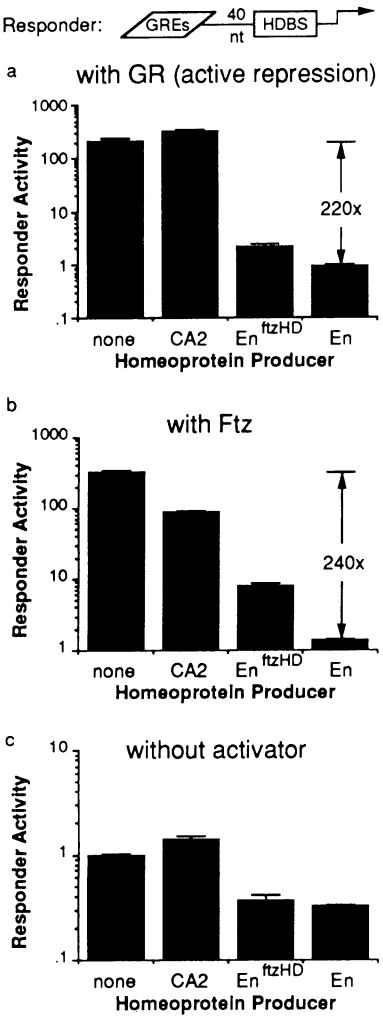

Fig. 4. Replacing the homeodomain of an active repressor (En) with that of an activator (Ftz) produces an active repressor. A responder (same as in Figure 3, diagrammed at top) capable of being activated by either GR or Ftz was used to test the chimeric homeoprotein En^{ftzHD} either for active repression function: by co-transfection with GR producer, with or without En^{ftzHD} producer (**a**); for passive repression in combination with Ftz producer (**b**); or for activity on its own (**c**). CA2 (passive repressor) and En (active repressor) producers were included for comparison. Note the log scale to allow visualization of the smaller (repressed) activity levels. Responder activities were determined as in Figure 1. The amounts of plasmids used were as follows: 3 μg of the indicated homeoprotein producer ('none' = control plasmid pP_{Ac}) with 2 μg of the responder, $pT3N6D\text{-}33CAT_B$ and: in (**a**) 0.05 μg GR producer; in (**b**) 0.3 μg of Ftz producer; and in (**c**) control producer. See Materials and methods for details and plasmid constructions.

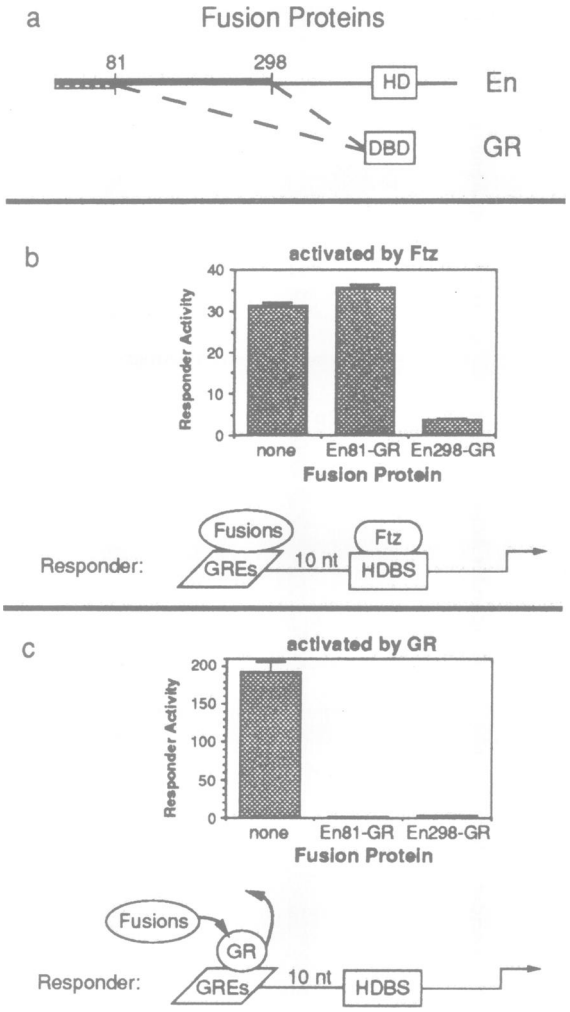

Fig. 5. Active repression activity is contained within a portion of En that does not include the HD. Two fusion proteins (diagrammed in **a**) containing N-terminal En sequences (either 81 or 298 aa) and a minimal GR binding domain (85 aa) were produced in cultured *Drosophila* cells. The ability of these proteins to repress Ftz-activated transcription from an upstream position was tested using a responder ($pT6N6D\text{-}33CAT_A$, diagrammed in **b** and **c**) capable of binding both the minimal GR binding domain of the fusion proteins (at the GREs) as well as Ftz (at the HDBS). In (b), 0.1 μg of Ftz producer was co-transfected with 2 μg of the responder, and with 3 μg of either control producer pP_{Ac} ('none'), or pAc-en81GR ('En81-GR') or pAc-en298GR ('En298-GR'). In (c), 0.05 μg of GR producer was co-transfected with 2 μg of responder and either control producer pP_{Ac}, pAc-en81GR or pAc-en298GR (3 μg each). Responder activities were determined as in Figure 1. See Materials and methods for details and plasmid constructions.

protein, we asked whether such a neutral protein could activate by displacing an active repressor. Figure 6 shows the effects of the crippled activator CA2 on a target gene that is initially in an actively repressed state (bound by both GR and active repressor). CA2 was able to activate by displacing either Eve, En or the chimeric En^{ftzHD}. (Recall that CA2 repressed transcription when in competition with Ftz for binding; Figures 2a and 3a.) In the absence of active repressor, CA2 had no significant effect, either in combination with GR (Figure 6) or by itself (data not shown). These results clearly show that active repression requires an activity in addition to DNA binding, since the transcriptional effect of active repressors is qualitatively distinct from that of a neutral DNA binding protein.

Discussion

In *Drosophila*, the Engrailed homeoprotein (En) is required for proper segmentation and for maintaining the identity of posterior compartment cells throughout development. *In vitro* En is a sequence specific DNA binding protein (Desplan *et al.*, 1985, 1988; Hoey and Levine, 1988), and in transiently transfected tissue culture cells it acts as a repressor of transcription (Jaynes and O'Farrell, 1988; Han *et al.*, 1989). Here, we show that En can function as an active repressor, an activity for which occupancy of a DNA site is not sufficient.

We have argued previously that direct competition contributed to repression of Ftz induction by En and the even-skipped protein, Eve (Jaynes and O'Farrell, 1988). In

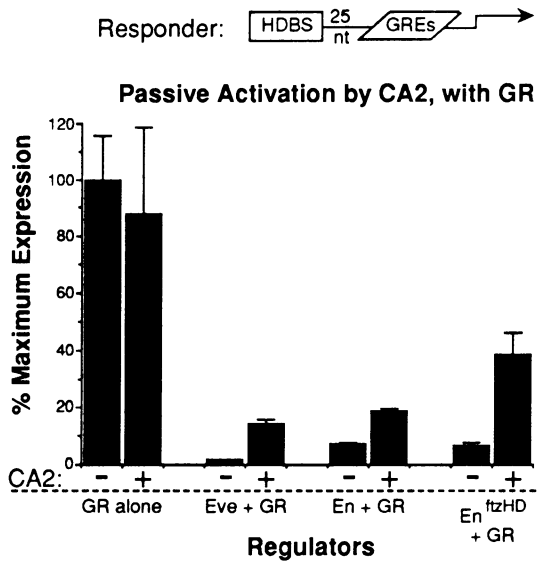

Fig. 6. A passive binding protein activates transcription by displacing active repressor. A responder (same as in Figure 1a, diagrammed at top) capable of binding both GR (activator) and homeoproteins was used to study the effects of competition for binding sites between active repressors and a passive binding protein. A constant amount of GR producer was co-transfected with producers for either passive binding protein (CA2), active repressor (Eve, En or En^{ftzHD}), or a combination of active and passive proteins. Passive activation refers to the increases in activity seen when CA2 competes with an active repressor. Responder activities were determined as in Figure 1. Plasmid amounts used were as follows: 0.05 μg GR producer and 2 μg responder (pN6T3D-33CAT$_B$) in all transfections, with either 5 μg CA2 producer ('+' CA2) or control plasmid pP_{Ac} ('−' CA2), and with either 1 μg Eve producer (pAE1315, gift of A.Ephrussi), 3 μg En producer or 3 μg En^{ftzHD} producer. Total amounts of vector (with actin promoter) was kept constant by adding control producer pP_{Ac}. See Materials and methods for details and plasmid constructions.

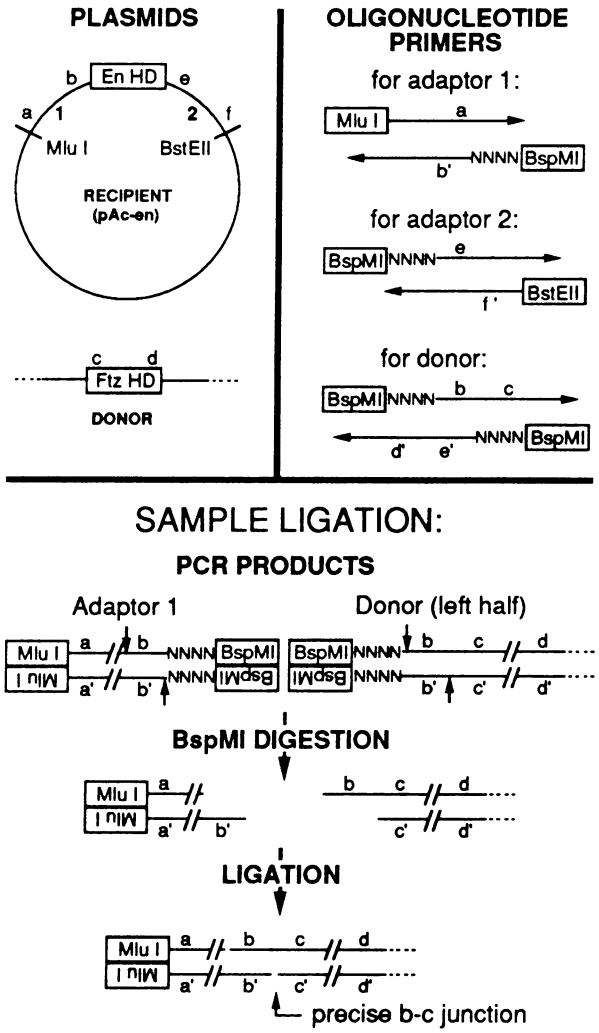

Fig. 7. Exact transplacement of one plasmid sequence for another using PCR. Oligonucleotides (upper right panel) were designed to amplify three plasmid sequences (upper left panel): the donor sequence to be inserted into the recipient, and two adaptor sequences flanking the region to be replaced in the recipient. Lower case letters refer to DNA sequences in the clockwise or left-to-right direction, while primed lower case letters refer to the reverse complement of those sequences (e.g. for priming in the opposite direction). The donor oligonucleotides include 4 nt sequences from the recipient (b and e') that will serve as 'sticky ends' for ligation following digestion with an enzyme (*Bsp*MI) that cuts outside its recognition sequence (lower panel). Since the sticky ends are different for each junction, efficient ligation will occur only between the desired fragment ends. See Materials and methods for more detail.

support of this suggestion, we show that a homeoprotein (CA) deprived of its activation domain but retaining its DNA binding domain is capable of suppressing Ftz induction. This suggests that a protein need only be able to compete for site occupancy to function as a repressor in this assay. However, as we show here by other assays, En can also be an active repressor.

We provide three lines of evidence that En is an active repressor. Firstly, using characterized transcriptional activators we show that En can act at HDBS to inhibit activation from distinct sites. Second, repression occurs using a variety of spacings between activator and repressor binding sites. Third, a repression domain distinct from the DNA binding homeodomain can be transferred to the zinc finger DNA binding domain of GR to create a novel repressor. Studies with the Krüppel protein, a segmentation gene product containing a zinc finger DNA binding motif, have defined a similar repression activity, showing it to be functionally separable from the Krüppel DNA binding domain (Licht *et al.*, 1990).

Active repression probably involves protein−protein interactions that result in either displacement of activators or interference with their functioning. There are two general ways that this might work. First, the inhibitor might act by directly contacting the basal transcriptional machinery. Second, active repression might be due to anti-enhancement occurring via an interaction with activators themselves, either directly or through a third component. Such an interaction would have no direct effect on the basal machinery. These models, direct inhibition and anti-enhancement, make different predictions about the behavior of active repressors that bind to complex *cis*-acting regulatory regions. If active repressors interact with the basal machinery, they would be expected to inhibit activation by any or all activators, more or less indiscriminately. However, interaction with activators or their cofactors would leave the promoter open for stimulation by additional activators. This latter model is favored by the regulatory behavior of a number of developmental genes with complex control regions. Molecular and genetic dissection of such complex control regions suggests a modular organization of enhancers where discrete segments of the regulatory sequence can direct distinct components of pattern (DiNardo *et al.*, 1988; Howard *et al.*, 1988; Goto *et al.*, 1989; Harding *et al.*, 1989; Stanojevic *et al.*, 1989;

Carroll, 1990). Although each module of control is simpler than the whole, multiple regulators, both positive and negative, appear to interact with each segment of regulatory sequence to direct a component of pattern (Goto *et al.*, 1989; Harding *et al.*, 1989; Stanojevic *et al.*, 1989). In cases where such modular control regions act autonomously, repression of one module is not dominant over activation by other modules. That is, active repressors appear to act only within a module. Consequently, in such cases it is unlikely that the repressors interact directly with the basal transcriptional machinery. If the action of repressors is short range relative to that of activators, short stretches of DNA could functionally isolate such control regions. This organization might facilitate the evolution of complex spatial and temporal patterns of gene expression by allowing independent refinement and recombinational assortment of control regions that direct components of pattern.

One of the more interesting puzzles surrounding the *Drosophila* homeoproteins remains to be resolved. It is not yet clear what activities or interactions at the molecular level are responsible for their functional differences. A part of the functional specificity of homeoproteins might be due to differences in their potency as transcriptional activators or repressors. However, this is not likely to be the only distinction. In fact, exchanges of homeodomain sequences suggest that the regulatory specificity of homeotic genes is substantially determined by homeodomain sequences, while we find that the homeodomain sequence has little effect on the activator/repressor function. Perhaps the homeodomain contributes to target gene specificity in ways that have not been detected in current DNA binding studies. Interactions with other DNA binding proteins could provide additional specificity. Alternatively, protein−protein interactions involving the homeodomain might allosterically alter the specificity of binding by the helix−turn−helix region, either increasing the degree of specificity, or changing the preferred binding site. Further study of interactions between regulators in simplified systems should contribute to our understanding of the more complex combinatorial mechanisms at work in the control of embryonic pattern formation.

Materials and methods

Transfections and assays

Drosophila cultured cells [Schneider line 2 (Schneider, 1972)] were grown and transfected as described previously (Jaynes and O'Farrell, 1988). Each 60 mm culture dish was transfected with a total of 10 μg plasmid DNA, consisting of the amounts of specific plasmids specified in the figure legends, plus a reference gene [either 0.3 ng pLac82SU (Dorsett *et al.*, 1989), or 10 ng pCOPiCAT, (Jaynes and O'Farrell, 1988)], plus filler DNA to 10 μg [either the actin-5C promoter vector pP_{Ac} (Krasnow *et al.*, 1989), or appropriate control plasmids identified in the figure legends]. Cultures were harvested 48−60 h after transfection and assayed for responder (target gene) and reference gene activities as described previously (Jaynes and O'Farrell, 1988). Dexamethasone was added 14−18 h prior to harvesting (to 100 nM) to the growth medium of cultures transfected with GR producer (as well as control cultures in the same experiments).

Constructions

Gal4 responders were constructed by inserting a *Bgl*II−*Bam*HI fragment from pEG72 (see Fischer *et al.*, 1988), containing two tandem Gal4 17mer binding sites upstream of a −43 hsp70 promoter/LacZ fusion gene, into the *Bam*HI site of pNP6bs. One orientation yielded pN6G2hZ, with the NP6 HDBS [six tandem repeats of the 'NP' consensus site (Jaynes and O'Farrell, 1988)] separated from the Gal4 site by 60 bp of intervening DNA. The other orientation gave pG2hZN6, with the HDBS 3 kb away. pNP6bs was made by inserting the *Pst*I−*Eco*RI fragment from an M13mp10 clone containing NP6 in the *Bam*HI site into *Pst*I−*Eco*RI cut Bluescript$^+$ (Stratagene).

GR responders were constructed by inserting NP6 HDBS from modified versions of pNP6bs (see below) into a plasmid [$pG_6tA_{-33}CO$ (S.K.Yoshinaga and K.R.Yamamoto, submitted)] containing tandem copies of a GR binding site upstream of a −33dADH promoter/*CAT* fusion gene (in pD-33CAT, England *et al.*, 1990). Responders with HDBS downstream of GREs ($pT6N6D\text{-}33CAT_A$, $pT3N6D\text{-}33CAT_B$) were constructed as follows: pNP6bs-Nhe was made from pNP6bs by inserting a single *Nhe*I linker (New England Biolabs, NEB) into the *Eco*RV site. The *Xba*I−NheI fragment from pNP6bs-*Nhe* was cloned into *Xba*I cut $pG_6tA_{-33}CO$. One orientation regenerated the *Xba*I site on the upstream side (relative to *CAT* gene transcription), yielding $pT6N6D\text{-}33CAT_A$. The other orientation regenerated the *Xba*I site on the downstream side; three of the six original GREs were then removed by cutting with *Pst*I, adding *Xho*I linkers (NEB), cutting with XhoI, and ligating, to yield $pT3N6D\text{-}33CAT_B$. The responders with HDBS upstream of GREs ($pN6T3D\text{-}33CAT_{A,B}$) were constructed as follows. pNP6bs-*Nsi* was made from pNP6bs by inserting a single *Nsi*I linker (NEB) into the *Eco*RV site. The *Pst*I−*Nsi*I fragment from pNP6bs-*Nsi* was cloned into *Pst*I cut $pG_6tA_{-33}CO$. One orientation regenerated a *Pst*I site on the upstream side, and following removal of three of six GREs by cutting with *Sma*I, adding *Xho*I linkers, cutting with *Xho*I and ligating, yielded $pN6T3D\text{-}33CAT_A$. The other orientation regenerated a *Pst*I site on the downstream side, and following removal of three of six GREs by cutting with *Pst*I, adding *Xho*I linkers, cutting with *Xho*I and ligating, yielded $pN6T3D\text{-}33CAT_B$. The control GR responder T3D-33CAT was made from $pG_6tA_{-33}CO$ by cutting with *Pst*I, adding *Xho*I linkers, cutting with *Xho*I (to remove three of six GREs) and ligating.

CA1 producer (pAct5C-zen-△2n, kindly provided by K.Han and J.Manley) contains the N-terminal 235 amino acid (out of 354) coding region of the *zerknüllt* gene fused to an open reading frame of one additional amino acid (K.Han, personal communication). CA2 producer (pAct5C-z2-△2, Han *et al.*, 1989) contains the N-terminal 124 amino acid (out of 252) coding region of the *z2* gene fused to a 136 foreign amino acid open reading frame (K.Han, personal communication). Gal4 producer (pRK245, gift of R.Kostriken) contains the entire Gal4 protein coding region [from pEG46 (E.Giniger, unpublished)] downstream of the Act5C promoter from pP_{Ac} (Krasnow *et al.*, 1989) in a pCaSpeR vector (Thummel *et al.*, 1988).

En−GR fusion protein producers (pAc-en81GR, pAc-en298GR) were constructed as follows. pAc-en (Jaynes and O'Farrell, 1988) was cleaved at a unique *Bst*EII site 13 bp downstream of the translation stop codon. After adding *Xba*I linkers and digesting with *Xba*I, the plasmid was cut within the En coding sequence with either *Not*I (for pAc-en81GR) or *Bam*HI (for pAc-en298GR). The larger, vector containing fragment was isolated and ligated with a fragment from pT7-440-525 (L.P.Freedman, unpublished) that contained the coding sequence of the GR DNA binding domain. Appropriate linkers were added to maintain the open reading frame in each case. pAc-en81GR encodes the first 81 aa of En, followed by the rat GR DNA binding domain (DBD). pAc-en298GR encodes the first 298 aa of En followed by Arg, followed by the rat GR DBD. [The rat DBD includes a Met, followed by coding sequences described in Hard, *et al.* (1990).]

Exact transplacement: cloning of pAc-en^{ftzHD}

In order to replace the En HD coding sequences exactly with those of the Ftz HD, a method was used with general applicability for exact transplacement of any donor plasmid sequence with any sequence in a recipient. Three fragments are amplified using the polymerase chain reaction (PCR): one is the donor sequence to be transferred and the other two are adaptor fragments amplified from the recipient. In this case the donor fragment was the Ftz HD coding sequence, and the adaptor sequences were those immediately flanking the En HD coding region on either side, extending to restriction sites unique in pAc-en (*Mlu*I and *Bst*EII). The resulting PCR products were ligated together and used to replace the corresponding pAc-en fragment between the *Mlu*I and *Bst*EII sites. A new method was used to produce PCR products which would ligate efficiently in the desired orientation, as follows. The oligonucleotides used to prime synthesis of what were to be the four internal ends of the 3-fragment ligation were designed with a recognition site for a restriction enzyme that cuts outside of its recognition sequence, and leaves protruding single-stranded (sticky) ends. Restriction digestion therefore removes the recognition site, leaving an overhang with an arbitrary sequence, i.e. one which by design consists of sequence at the junction of the fragments to be joined (see Figure 7). Since the junctions are different from each other, as well as from the sticky ends generated by the enzymes cutting within pAc-en, the desired ligation product will be greatly favored over all other possible combinations. In practice, the enzyme cutting outside of its recognition sequence may not digest the DNA efficiently. In our hands, $<10\%$ of the PCR products containing these

sites near their ends were digested (we used *Bsp*MI, and our oligonucleotides had a CG dinucleotide at their 5′ ends, followed by the recognition sequence). We used therefore a second PCR amplification to obtain sufficient quantities of the replacement insert: following gel isolation of the *Bsp*MI-cut fragments, and ligation of all three together, we amplified using the external oligonucleotides. The major product was of the correct size to contain all three sequences, and this was gel purified, digested with *Mlu*I and *Bst*EII, and cloned into pAc-en to replace the corresponding En coding region. The second PCR amplification was done in duplicate, and inserts from each were cloned separately into pAc-en. Two independent isolates from each cloning were tested in the transfection assay, and all four gave virtually identical results, suggesting that they contain identical protein coding sequences.

Acknowledgements

We thank K.Han, J.Manley, R.Kostriken, L.Freedman, A.Ephrussi and E.Giniger for sharing plasmids and information prior to publication, and C.Lehner, J.Vincent, J.Heemskerk and B.Kalionis for critical comments on the manuscript. Support for this work was provided by an NIH grant to P.H.O. and by an American Cancer Society postdoctoral fellowship to J.B.J.

References

Baniahmad,A., Steiner,C., Köhne,A.C. and Renkawitz,R. (1990) *Cell*, **61**, 505–514.
Biggin,M.D. and Tjian,R. (1989) *Cell*, **58**, 433–440.
Brent,R. (1985) *Cell*, **42**, 3–4.
Carroll,S.B. (1990) *Cell*, **60**, 9–16.
Desplan,C., Theis,J. and O'Farrell,P.H. (1985) *Nature*, **318**, 630–635.
Desplan,C., Theis,J. and O'Farrell,P.H. (1988) *Cell*, **54**, 1081–1090.
Diamond,M.I., Miner,J.N., Yoshinaga,S.K. and Yamamoto,K.R. (1990) *Science*, **249**, 1266–1272.
DiNardo,S., Sher,E., Heemskerk-Jongens,J., Kassis,J.A. and O'Farrell, P.H. (1988) *Nature*, **332**, 604–609.
Dorsett,D., Viglianti,G.A., Rutledge,B.J. and Meselson,M. (1989) *Genes Dev.*, **3**, 454–468.
Doyle,H.J., Kraut,R. and Levine,M. (1989) *Genes Dev.*, **3**, 1518–1533.
England,B.P., Heberlein,U. and Tjian,R. (1990) *J. Biol. Chem.*, **265**, 5086–5094.
Finley,R.L., Jr, Chen,S., Ma,J., Byrne,P. and West,R.W., Jr (1990) *Mol. Cell. Biol.*, **10**, 5663–5670.
Fischer,J.A., Giniger,E., Maniatis,T. and Ptashne,M. (1988) *Nature*, **332**, 853–856.
González-Reyes,A., Urquia,N., Gehring,W.J., Struhl,G. and Morata,G. (1990) *Nature*, **344**, 78–80.
Goto,T., Macdonald,P. and Maniatis,T. (1989) *Cell*, **57**, 413–422.
Gottschling,D.E., Aparicio,O.M., Billington,B.L. and Zakian,V.A. (1990) *Cell*, **63**, 751–762.
Han,K., Levine,M.S. and Manley,J.L. (1989) *Cell*, **56**, 573–583.
Hanes,S.D. and Brent,R. (1989) *Cell*, **57**, 1275–1283.
Hard,T., Kellenbach,E., Boelens,R., Kaptein,R., Dahlman,K., Carlstedt-Duke,J., Freedman,L.P., Maler,B.A., Hyde,E.I., Gustafsson,J.-A. and Yamamoto,K.R. (1990) *Biochemistry*, **29**, 9015–9023.
Harding,K., Hoey,T., Warrior,R. and Levine.M. (1989) *EMBO J.*, **8**, 1205–1212.
Hoey,T. and Levine,M. (1988) *Nature*, **332**, 858–861.
Howard,K., Ingham,P. and Rushlow,C. (1988) *Genes Dev.*, **2**, 1037–1046.
Hudson,L.G., Santon,J.B., Glass,C.K. and Gill,G.N. (1990) *Cell*, **62**, 1165–1175.
Jaynes,J.B. and O'Farrell,P.H. (1988) *Nature*, **336**, 744–749.
Jaynes,J.B., Vincent,J. and O'Farrell,P.H. (1990) In Mahowald,A. (ed.), *Genetics of Pattern Formation and Growth Control, 48th Symposium of the Society for Developmental Biology*. John Wiley and Sons, New York, pp. 47–64.
Krasnow,M.A., Saffman,E.E., Kornfeld,K. and Hogness,D.S. (1989) *Cell*, **57**, 1031–1043.
Kuziora,M.A. and McGinnis,W. (1989) *Cell*, **59**, 563–571.
Levine,M. and Hoey,T. (1988) *Cell*, **55**, 537–540.
Levine,M. and Manley,J.L. (1989) *Cell*, **59**, 405–408.
Licht,J.D., Grossel,M.J., Figge,J. and Hansen,U.M. (1990) *Nature*, **346**, 76–79.
Mann,R.S. and Hogness,D.S. (1990) *Cell*, **60**, 597–610.
Ohkuma,Y., Horikoshi,M., Roeder,R.G. and Desplan,C. (1990) *Cell*, **61**, 475–484.
Schneider,I. (1972) *J. Embryol. Exp. Morphol.*, **27**, 353–365.
Schüle,R., Rangarajan,P., Kliewer,S., Ransone,L.J., Bolado,J., Yang,N., Verma,I.M. and Evans,R.M. (1990) *Cell*, **62**, 1217–1226.
Scott,M.P. and Carroll,S.B. (1987) *Cell*, **51**, 689–698.
Scott,M.P. and O'Farrell,P.H. (1986) *Annu. Rev. Cell Biol.*, **2**, 49–80.
Stanojevic,D., Hoey,T. and Levine,M. (1989) *Nature*, **341**, 331–335.
Stern,S., Tanaka,M. and Herr,W. (1989) *Nature*, **341**, 624–630.
Thummel,C.S., Boulet,A.M. and Lipshitz,H.D. (1988) *Gene*, **74**, 445–456.
Treisman,J., Gönczy,P., Vashishtha,M., Harris,E. and Desplan,C. (1989) *Cell*, **59**, 553–562.
Winslow,G.M., Hayashi,S., Krasnow,M., Hogness,D.S. and Scott,M.P. (1989) *Cell*, **57**, 1017–1030.
Yang-Yen,H.-F., Chambard,J.-C., Sun,Y.-L., Smeal,T., Schmidt,T.J., Drouin,J. and Karin,M. (1990) *Cell*, **62**, 1205–1215.
Yoo,H.S. and Cooper,T.G. (1989) *Mol. Cell. Biol.*, **9**, 3231–3243.

Received on January 25, 1991