Transcriptional repression by the *Drosophila* Even-skipped protein: definition of a minimal repression domain

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We have used a transient expression assay employing *Drosophila* tissue culture cells to study the transcriptional repression activity of the homeo domain protein Even-skipped (Eve). Eve was found to repress all promoters that contained Eve-binding sites, including both TATA-containing and TATA-lacking minimal promoters, as well as promoters activated by several different classes of activator proteins. These findings suggest that the general transcription machinery can be a target of Eve. By analyzing properties of a variety of Eve mutants and chimeric fusion proteins, we have identified several features important for efficient repression. In addition to the DNA-binding domain, a potent repressor requires a repression domain, which can be as small as 27 residues. The minimal 57-residue Eve repression domain, as well as several others studied here, were all found to be proline rich and to contain a high percentage of hydrophobic residues. An intriguing feature of the strong repressors was that their DNA-binding activities, measured by gel retention assays with nuclear extracts, were significantly less than those of derivatives inactive in repression.

[Key Words: *Drosophila*, homeo domain protein, transcriptional repression]

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Transcription initiation is regulated by sequence-specific DNA-binding proteins that appear to interact, directly or indirectly, with the general transcriptional machinery. A great number of eukaryotic transcriptional activators have been identified [for review, see Mitchell and Tjian 1989; Johnson and McKnight 1990]. These proteins stimulate transcription of specific genes by binding to *cis*-acting sequence elements within the target gene boundaries. In general, transcriptional activators are composed of at least two distinct domains: One is involved in contacting specific DNA sequence elements, and the other, the so-called activation domain, interacts directly or indirectly with one or more components of the general transcription machinery [e.g., Ptashne and Gann 1990; Stringer et al. 1990; Dynlacht et al. 1991; Lin and Green 1991; Lin et al. 1991]. There are at least three relatively well-defined types of activation domains [for review, see Mitchell and Tjian 1989], which are characterized by the identity of the amino acid residues that they contain: acidic [Hope and Struhl 1986; Ma and Ptashne 1987a,b; Cress and Triezenberg 1991], glutamine rich [Courey and Tjian 1988; Tanaka and Herr 1990], and proline rich [Mermod et al. 1989].

It is likely that repressors as well as activators play essential roles in controlling transcription. Sequence-specific DNA-binding proteins that repress transcription can in principle participate both in inhibiting already active promoters and in keeping inactive promoters turned off. However, compared with activators, the number of transcriptional repressors that have been identified and characterized is small. Only one repression "domain", a region from the *Drosophila* Krüppel protein that is rich in alanine residues, has been described in any detail [Licht et al. 1990]. Several models have been proposed for mechanisms of transcriptional repression by sequence-specific DNA-binding proteins in eukaryotes [see Levine and Manley 1989]. First, some repressors might function by competing with specific transcriptional activators for binding to overlapping or closely linked target DNA sites [competition; recent examples: Ohkuma et al. 1990a; Descombes and Schibler 1991; Foulkes et al. 1991]. It has also been suggested that sequence-specific repression might occur by competition with a general transcription factor, such as, TFIIID [Ohkuma et al. 1990b; Kato et al. 1991; Kaufman and Rio 1991]. Because this type of repression results from displacement of transcription factors from the DNA, such repressors might not necessarily need to possess an active repression function. Second, certain repressors, the best example being the yeast α2 protein, appear to complex with sequence-specific activators without displacing them from the DNA, thereby preventing the activator from making proper contacts required for its function [quenching; Keleher et al. 1988, 1992]. Repression in this case depends on the presence of...
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a target activator, and such repressors would therefore inhibit only promoters that contain appropriate activator-binding sites. A third class of repressors might interfere directly with the formation or activity of the general transcription machinery by binding to nonoverlapping DNA sequences and contacting basal factors [direct repression] for possible examples, see Biggin and Tjian 1989; Damm et al. 1989; Banaihmad et al. 1990], such repression would presumably involve protein–protein interactions analogous to those of certain activators, except that their effect would be negative instead of positive [Johnson and Krasnow 1992].

The Drosophila even-skipped gene product [Eve] is a homeo domain protein that participates in controlling segmentation during early embryonic development [for review, see Scott et al. 1989, Hayashi and Scott 1990]. A number of different homeo domain proteins have been shown to be transcriptional regulators in transfection assays [Jaynes and O’Farrell 1988; Driever and Nüsslein-Volhard, 1989; Han et al. 1989; Krasnow et al. 1989; Winslow et al. 1989] and in in vitro assays [Biggin and Tjian 1989; Johnson and Krasnow 1990, 1992, Ohkuma et al. 1990a]. We and others have reported previously that Eve acts as a transcriptional repressor [Jaynes and O’Farrell 1988; Biggin and Tjian 1989; Han et al. 1989]. Here, we examine further some of the requirements for Eve repression, define features of the protein that allow it to function as a repressor, and suggest how these properties may reflect general mechanisms of transcriptional repression.

**Results**

The goal of these experiments was to analyze in detail some of the properties of the transcriptional regulator Eve, with the specific aim of learning more about how a repressor protein can function. Several properties of the cotransfection assay were initially considered to optimize its sensitivity and reliability. First, to reduce the possibility of having fortuitous binding sites for endogenous transcriptional regulators in the chloramphenicol acetyltransferase (CAT) reporter plasmid [see Han et al. 1989], we reduced its size, by deleting nonessential sequences, to 2.9 kb [see Materials and methods]. Second, we designed minimal synthetic promoters in the reporter constructs, both for basal and activated transcription, to reduce further any possible interference from extraneous binding sites. The 17-bp mammalian terminal deoxynucleotidyltransferase [TdT] initiator element [Smale and Baltimore 1989], rather than a TATA box, was used as the minimal basal promoter in most experiments. This sequence has no homology with binding sites for Eve and the activators used in this study [see below], and was shown previously to function in Drosophila Schneider cells [Colgan and Manley 1992]. Third, to study repression of activated transcription, we used Sp1 and several GAL4 fusion proteins, most frequently GAL4–VP16 [Sadowski et al. 1988], because there is no endogenous Sp1 activity in Schneider cells [Courey and Tjian 1988] nor are there endogenous transcriptional regulators that bind to the GAL4-binding sites used here [Fischer et al. 1988; results not shown]. Finally, all Eve proteins were tagged at their amino termini with a 9-amino-acid-long influenza [flu] hemagglutinin epitope [YPYDVPDYA; Wilson et al. 1984; Field et al. 1988], which did not affect their stability or repression activity [data not shown], to monitor relative accumulation of mutant and wild-type derivatives in transfected cells.

**Eve represses basal promoters**

We reported previously that Eve possesses repression activity [Han et al. 1989], although the mechanism by which it functions was not resolved. To determine whether Eve can function as a direct repressor, we first asked whether Eve could repress expression from a plasmid containing only a single promoter element, the 17-bp TdT initiator. For this, reporter plasmids containing the TdT initiator 26 bp upstream of the bacterial CAT gene, plus or minus Eve-binding sites [NP6 = synthetic 6 × 12-mer; Jaynes and O’Farrell 1988; see Fig. 1], were

![Figure 1. Eve represses basal promoters. A minimal promoter–CAT reporter ln = TdT initiator element with and without Eve-binding sites (NP6 = 6 × 12-mer Eve-binding sites), as diagramed above the graph, was cotransfected into Drosophila Schneider cells with increasing amounts of Eve expression vector. Four micrograms of pl–CAT (top), pSGNI–CAT (middle), or pNSI–CAT (bottom) was cotransfected with the indicated amount of pAct5C–eve and 2 µg of an internal control poliovirus LTR–lacZ. Basal CAT activities [without Eve] of pl–CAT, pSGNI–CAT, and pNSI–CAT were 1.9 ± 0.21 [arbitrary unit; mean ± s.d.] 2.45 ± 0.21, and 4.2 ± 0.79, respectively. Relative CAT activities are shown in which the percent activity in the absence of the Eve expression vector was taken as 100%. Error bars indicate standard deviations.](image-url)
Eve also represses activated transcription

The above experiments provided evidence that Eve can function as a direct repressor to block basal transcription. We also wanted to determine whether Eve can block activated transcription. We first used a reporter [pSGNI–CAT] that contains both GAL4-binding sites (synthetic 5 × 17-mer, Fischer et al. 1988) and Eve-binding sites (NP6) separated by 42 bp [Fig. 2A]. Cotransfection of this plasmid with 1 ng of an expression vector capable of synthesizing GAL4–VP16 activated CAT expression significantly (~30-fold). However, when Eve was coproduced, activation was completely blocked [Fig. 2A]. Essentially identical results were obtained when the concentration of GAL4–VP16 expression vector was increased to 10 ng (~250-fold activation; results not shown) or when the distance between the GAL4- and Eve-binding sites was increased to 209 bp [Fig. 2A]. These findings strengthen the conclusion that Eve can repress the strong activator GAL4–VP16 in the absence of competition between the two proteins for DNA-binding sites.

The inhibition of GAL4–VP16-activated expression also does not appear to result from quenching. When the Eve- and GAL4-binding sites in pSGNI–CAT [separated by 42 bp] were moved as a “cassette” an additional 500 bp upstream of the start site, Eve was unable to block GAL4–VP16-activated transcription [Fig. 2A]. This is in keeping with the inability of Eve to repress basal transcription from this distance (see above) and suggests that Eve’s inhibition of GAL4–VP16-activated transcription described above does not reflect an interaction between the two proteins, as such an interaction would not be expected to be influenced by distance from the transcription start site.

We then asked whether Eve could repress expression induced by activators with different types of activation domains. We first examined Sp1, which contains a glutamine-rich activation region. For this, two reporter plasmids that have both Sp1 [GC boxes; Courey and Tjian 1988] and Eve-binding sites were constructed. In one, the Eve sites were situated upstream of the Sp1 sites, whereas the order was reversed in the other. Figure 2B shows that Eve repressed CAT expression effectively from both constructs. Repression appeared more efficient when the Eve-binding sites were situated upstream of the Sp1 sites. Repression was therefore unlikely to have been due simply to a physical block preventing interaction between Sp1 and the basal transcription machinery. To extend these results, we tested the ability of Eve to repress activated transcription brought about by three different GAL4 fusion proteins, each containing a distinct type of activating domain. GAL4–zenST contains a serine- plus threonine-rich region of the Zen protein [Han et al. 1989], GAL4–ftzQ contains a glutamine-rich segment from the Ftz protein [Han et al. 1989], and GAL4–CTF1P contains a proline-rich region of CTF1 [Mermod et al. 1989; Tanese et al. 1991]. Using the pSGNI–CAT reporter, we observed that Eve repressed transcription induced by all three activators [Fig. 2C]. Transcription was reduced to baseline with two of the activators [ftzQ and CTF1P], whereas zenST-activated transcription was reduced somewhat less.

Taken together, the experiments presented in Figures 1 and 2 indicate that Eve can repress transcription from a wide variety of different types of promoters and suggest strongly that Eve can function as a direct repressor.

Alanine- and proline-rich regions are both required for maximal repression

We then wanted to determine which parts of the Eve protein are necessary for transcriptional repression. To map possible repression domains, a number of systematic truncations and internal deletions were made by dividing wild-type Eve into six regions, or domains, and expressing these in various combinations. To assay repression, GAL4–VP16 was used as an activator and pSGNI–CAT as the reporter. First, each of the six domains [A, B, C, D, E, and F] was deleted one at a time from full-length Eve, and the results of cotransfections are shown in Figure 3A. Deleting domain A, E, or F affected repression very little, if at all. However, deleting domain B, C, or D resulted in a significant loss of repression activity. Domain B corresponds to the homeo domain,
Figure 2. Eve represses activated transcription. The indicated CAT reporter plasmids, diagramed above each graph, were cotransfected with a constant amount of the appropriate activator expression vector and varying amounts (0, 0.02, 0.2, 1, or 2 μg) of Eve expression vector. Relative CAT activities, which are 100 × CAT_{activated}/CAT_{basal}/(CAT_{activated}−CAT_{basal}), are shown. CAT_{activated} is the normalized CAT activity in the presence of the activator and in the absence of Eve; CAT_{basal} is the normalized CAT activity in the absence of both activator and Eve. (A) Eve can repress expression activated by GAL4-VP16. Four micrograms of pSGN1-CAT (top), pG209NI-CAT (middle), or pSGN466I-CAT (bottom) was cotransfected with 1 ng of pAct-GAL4-VP16, 0–2 μg of pAct5C-eve, 2 μg of pcopia LTR-lacZ, and 2 μg of pGem1 carrier. CAT_{basal} values of pSGN1-CAT, pG209NI-CAT, and pSGN466I-CAT were 4.7 ± 0.71 (mean ± S.D.) 33 ± 2.33, and 1.95 ± 0.21, respectively. CAT_{activated} values were 148 ± 8.7, 344 ± 73.2, and 34.9 ± 5.4, respectively. (B) Eve represses expression activated by Spl. Four micrograms of pSNI-CAT (top) was cotransfected as in A except that 2 μg of Spl expression vector pAct-Spl was used instead of pAct-GAL4-VP16 and no pGem1 carrier was added. Four micrograms of pNSI-CAT (bottom) was cotransfected as in A except that 10 ng of pAct-Spl was used instead of pAct-GAL4-VP16. CAT_{basal} values were 13.9 ± 1.2 and 7.9 ± 0.4, respectively; CAT_{activated} values were 174 ± 22.5 and 198 ± 33.7, respectively. (C) Eve represses expression activated by different classes of activation domains. One microgram of pSGN1-CAT was cotransfected with 2 ng of pAct-GAL4-ftz0 (top), 5 ng of pAct-GAL4-zenST (middle), or 200 ng of pAct-GAL4-CTF1P (bottom), 0–2 μg of pAct-eve, 2 μg of pcopia LTR-lacZ, and 5 μg of pGem1 carrier. CAT_{basal} value was 1.9 ± 0.5; CAT_{activated} values were 141 ± 6.7, 92.5 ± 10.6, and 43.5 ± 5.0, respectively.

The differences in activities of the mutant proteins described above could have reflected one of several variables, not directly related to repression, such as protein stability, nuclear localization, and/or DNA-binding affinity. We have addressed these possibilities for all of the Eve derivatives described above, and the results are shown in Figures 5 and 6 below.

To measure the relative accumulation and nuclear localization of the proteins, we made use of the 9-residue hemagglutinin epitope [flu] mentioned above, which allowed quantitation of all proteins by immunoblot analysis. To this end, cells were transfected with each of the expression vectors utilized in the experiment shown in Figure 3, nuclear extracts were prepared, aliquots were applied to an SDS–polyacrylamide gel and, following transfer to nitrocellulose, flu-Eve fusion proteins were
Transcriptional repression by Eve

Figure 3. Alanine- and proline-rich regions of Eve are required for maximal repression. The indicated truncation and deletion derivatives of Eve were tested for their ability to repress transcription activated by GAL4-VP16. Full-length Eve, arbitrarily divided into six regions, and the reporter plasmid pSGNI-CAT are diagramed above the graphs. Domain C is rich in alanine (46%); domain D is rich in proline (25%). One microgram of pSGNI-CAT was cotransfected with 1 ng of pAct-GAL4-VP16, 4 μg of each Eve mutant expression vector, 2 μg of pcopia LTR-lacZ, and 3 μg of pGem1 carrier. Relative CAT activities were determined as in Fig. 2. The error bar indicates standard deviations. CAT_{baseline} value was 2.80 ± 0.21; CAT_{activated} value was 97.5 ± 7.7.

detected with mAbl2CA5 [Wilson et al. 1984; Field et al. 1988]. The results, shown in Figure 4, make several points. First, while there were reproducible differences in the levels to which these proteins accumulated in nuclear extracts, there was no correlation between the amount of protein detected and its repression activity. For example, the wild-type protein [lane 2], which was the strongest repressor, appeared to be among the least stable. In contrast, ABF and ABCE accumulated to high levels but were completely inactive as repressors. It is notable that only low levels of the wild-type protein were detected, which may reflect its short half-life in embryos [Frasch et al. 1987]. However, examination of the more stable mutant derivatives did not reveal a specific region of the protein that correlated strictly with apparent instability. A second point is that repression activity was also not correlated with protein size. Thus, while wild-type Eve was the largest protein tested [calculated molecular mass ~41.4 kD], the BCD protein, which was also a very strong repressor, was one of the smallest (~21.6 kD). Finally, wild-type Eve and many of the mutant derivatives gave rise to multiple bands during electrophoresis, suggesting that the protein is extensively modified post-translationally. Much of this heterogeneity appears to involve regions E and F, which do not appear to play a role in repression and contain 26% serine plus threonine residues.

We also performed a Western analysis similar to that shown in Figure 4, except that whole-cell lysates from the transfected cells were used. The results obtained (not shown) were, with one exception, essentially identical to those shown in Figure 5. The exception was mutant AbCDEF, the only derivative with a deletion in the homeo domain, which was found in relatively higher levels in whole-cell lysates as opposed to nuclear extracts. This is in keeping with the fact that there are no sequences that bear any resemblance to a consensus nuclear localization signal [NLS] outside of the homeo domain. Although

Figure 4. Accumulation of Eve proteins in transfected cells. Nuclear extracts were prepared from cells transfected with plasmids encoding the indicated Eve derivatives, and flu-Eve fusion proteins were detected by Western blotting, as described in Materials and methods. The numbers at left indicate the molecular weights of protein markers.
there is also no clear match within the homeo domain, the NLS of the yeast mating-type factor α2 is located at the amino terminus of the homeo domain (Hall et al. 1990). To verify that the lack of repression activity observed with this mutant (Fig. 3A) was not the result of a limiting nuclear concentration, the SV40 NLS (PKK-KRKV, Kalderon et al. 1984) was fused to the amino terminus of the protein. Although this did enhance nuclear localization, the protein was still inactive as a repressor (results not shown).

To estimate the relative DNA-binding affinities of the wild-type and mutant proteins, conditions were developed that allowed us to perform gel retention assays with nuclear extracts prepared from the transfected cells (see Materials and methods). These experiments were initially complicated by the relatively low affinity of homeo domain proteins for DNA (Kd = ~10^-9M, Percival-Smith et al. 1990) and by the fact that only ~10% of the cells expressed the transfected DNA. For these assays, the amount of Eve protein in each nuclear extract was first estimated by Western blotting (Fig. 4), and dilutions were made where appropriate so that all samples had similar amounts of Eve protein. (Dilutions were with nuclear extract prepared from cells transfected with “empty” expression vector so that all samples had equivalent amounts of total protein and transfected DNA.) The DNA probe was an end-labeled 79-bp NP6 fragment, and the specificity of binding was verified with unlabeled competitors (data not shown). The results, with the same mutants analyzed in Figures 3 and 4 above, are shown in Figure 5 and allow two principal conclusions. First, the relative affinities of the wild-type protein (lane 2) and several of the mutants were very low, whereas other mutants, for example, the homeo domain alone (mutant B, lane 9), were significantly higher. The second point is that not only is there no correlation between relatively strong DNA-binding affinity and repression activity, but there is an inverse correlation. Thus, the most active repressors (the full-length protein and the mutants shown in lanes 3, 7, 8, 15, and 16) all gave faint, barely detectable gel shifts, whereas the weak or inactive mutants all gave modest to strong shifts [with the exception of the mutant lacking an intact homeo domain (lane 4), which, as expected, produced no retarded complex]. These results raise the possibility that a relatively low DNA-binding affinity is in some way important for repression (see also below) and, in any event, indicate that the repression-defective mutants were not defective simply because of impaired DNA binding.

Together, the above results suggest that domains B, C, and D (i.e., residues 61–246) of Eve are necessary and sufficient for strong repression. While the role of domain B (the homeo domain) is, at least in part, DNA binding (see below), domains C and D must participate actively in repression. The primary structures of these regions are shown in Figure 6. A prominent feature of C is that it is very rich in alanine residues (18 of 39) and contains 5 prolines and only a single charged residue. Domain D is very rich in proline (15 of 61 residues), as well as, to a lesser extent, methionine and histidine residues (9 each).

Identification of a minimal Eve repression domain
To characterize the Eve repression domain further, we
first constructed and analyzed a series of derivatives of
the highly active ABCD protein (see Fig. 3C) that further
subdivided the CD region. Repression activity was
determined as in Figure 3, and the results are shown in
Figure 7A. With the exception of the D1 region (see Fig.
7A, top), which could be removed without affecting
repression and by itself could increase the activity of the
ABC protein only slightly (cf. Fig. 3B, ABC, and Fig. 7A,
ABCD1), all other derivatives tested, which contained
some combination of regions C1, C2, and D2, gave rise to
20-fold or greater repression. The strongest repression
activity was associated with regions C2 and D2, and a
protein containing only the 57-residue C2D2 segment
fused to AB resulted in 30-fold repression. C2D2 con-
tains the bulk of the alanines and prolines (see Fig. 6),
supporting the view that these residues are important for
strong repression.

We also wanted to learn whether the Eve repression
domain could function when fused to a heterologous
DNA-binding domain or whether the homeo domain
was also necessary for repression. To this end, several
carboxy-terminal fragments of Eve were fused to the
GAL4 DNA-binding domain and tested for their ability
to block expression activated by Spl (Fig. 7B). A GAL4
fusion containing the CDEF region repressed CAT activ-
ity effectively, reducing expression by greater than a fac-
tor of 10, in a GAL4-binding site-dependent manner [re-
results not shown]. Deletion of the EF region, which had
no effect on repression in the context of the Eve DNA-
binding domain, reduced repression, although significant
activity remained. Together, these results indicate that
the Eve repression domain can function when fused to a
heterologous DNA-binding domain. However, there are
qualitative and quantitative differences in the behavior
of these fusion proteins relative to the homologous Eve
derivatives. Possible explanations for these differences
are discussed below.

Proline richness is an important feature
of a strong repression domain

One feature of the Eve repression domain suggested by
the above experiments is that it is enriched in proline
residues. To investigate the possibility that this might be
an important feature of repression domains more gener-
ally, we constructed and analyzed an additional set of
Eve variants. These consisted of the flu epitope, the in-
active AB Eve domain, and several different heterologous
protein segments. One such fusion protein that was par-
ticularly informative was the AB-FS1 derivative. [The FS
proteins were created by ligation of some of the
eve fragments utilized above, but in such a manner that a frame-
shift was introduced following translation of the B [homeo]
domain.] The sequence of the FS1 oligopeptide is
shown in Figure 6, and the results of CAT assays (again
using GAL4–VP16-activated expression as a baseline) are
shown in Figure 8A. The short (27 residue) FS1 peptide
was able to convert the AB protein into a potent repres-
sor, reducing CAT activity by a factor of ~120. AB–FS1
also efficiently repressed expression activated by all of
the other activators tested with Eve and also blocked
expression from the basal TdT initiator plasmid [result
not shown]. In addition, FS1 functioned, although some-
what less efficiently, when fused to the GAL4 DNA-
binding domain (Fig. 7B). The behavior of FS1 was thus
essentially indistinguishable from that of the Eve repres-

Figure 7. Identification of a minimal Eve repression domain.
[A] The minimal repression domain comprises domains C2 and
D2. The indicated deletion derivatives of Eve were tested for
their ability to repress transcription activated by GAL4–VP16.
Experimental conditions were as in Fig. 3. Subdivided domains
C and D are diagramed above the graph. The number of amino
acid residues in each subdomain are denoted. [B] Eve repression
domains function when fused to a heterologous DNA-binding
domain. The reporter plasmid pGSI–CAT is diagramed above
the graph. The indicated GAL4 fusion proteins were tested for
their ability to repress transcription activated by Spl. GAL4-93
without a heterologous peptide fusion served as a control. Four
micrograms of pGSI–CAT was cotransfected with 0.05 μg of
pAct–Spl, 2 μg of GAL4 fusion expression vector, 2 μg of pGem1
LIR–lacZ, and 2 μg of pGem1 carrier. CATbasal value was
7.6 ± 0.85; the CATactivated value was 5730 ± 660.

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Figure 8. Proline richness is an important feature of a strong repression domain. The general structure of the Eve fusion proteins analyzed and of the reporter plasmid (pSGNI-CAT) are shown at the top. CAT assays, Western analysis, and gel retention assays were all performed with extracts from the same transfected cells [see Materials and methods]. (A) CAT assays. Transfections with expression vectors encoding the indicated fusion proteins, together with pSGNI-CAT and ActGAL4-VP16, were exactly as in Fig. 3. The accumulation of molecular weight standards are indicated at left. (C) Gel retention assay. The ability of the indicated Eve derivatives to bind DNA was measured in a gel-shift assay exactly as in Fig. 5. Based on the Western analysis in B, nuclear extracts were adjusted so that the amounts of Eve protein in the samples analyzed in lanes 3, 5, and 6 were essentially identical. Owing to significantly lower accumulation in the transfected cells, the concentration of Eve derivatives in the samples analyzed in lanes 2 and 4 was estimated to be 25–50% that present in the other samples.

such a potent repressor suggests that this composition may reflect a minimal repression domain.

Proline richness is also a characteristic of certain transcriptional activators (for review, see Mitchell and Tjian 1989), and thus cannot be sufficient to define a repression domain. A possible basis for distinguishing between proline-rich activators and repressors is provided by the AB–FS2 protein. AB–FS2 was derived from AB–FS1 by insertion of a 17-residue oligopeptide between the homeo domain and the FS1 repression domain (the first residue of FS1 was changed from tryptophan to alanine; see Fig. 6). Despite its extreme proline richness (9/17), this insertion essentially destroyed the repressor activity of AB–FS1 [Fig. 8A]. We suggest that this was the result of the other notable feature of the 17-mer, which is an abundance of serine and threonine residues (6/17) and that the presence of such residues is incompatible with repression. This is consistent with the fact that proline-rich activation domains (e.g., in the activator CTF; Mermod et al. 1989) are also enriched in these two residues.

Another feature of Eve domain D2 is that it contains a high proportion of histidine residues. This proline–histidine richness is reminiscent of a sequence found in another Drosophila homeo box protein, Paired (Prd; Frigerio et al. 1986, Kilchherr et al. 1986). The so-called prd repeat is found in several other Drosophila segmentation genes, but its function is unknown. Prd functions as a transcriptional activator in cotransfection assays [Han et al. 1989], but the Prd repeat is not essential for this activity [K. Han and J. Manley, unpubl.]. To determine whether the Prd repeat possesses repressor activity, an expression vector (AB–PR) producing the AB domain fused to a 75-residue fragment containing the Prd repeat [see Fig. 6] was constructed. This fragment is highly enriched in proline and histidine residues and, as with the other repression domains analyzed above, contains only a single charged residue (discounting the possible charge of the histidines). AB–PR functioned as a strong repressor, reducing CAT expression by a factor of 10 [Fig. 8A]. This supports the idea that a proline/histidine-rich region can function as a repression domain and raises the possibility that Prd may, in some contexts, function as a repressor.

Finally, we also constructed and analyzed a fusion that does not display any of the features that we have identified as important for repression (AB–FS3; see Fig. 6). The 70-residue FS3 segment is basic and rich in serine residues in its amino-terminal half and contains an acidic region at its carboxyl terminus. Figure 8A shows that not only was AB–FS3 not a repressor, but it was a moderately strong activator, increasing CAT expression ~11-fold.

The accumulation and relative DNA-binding activities of these Eve derivatives were measured as in Figures 4 and 5. The results of a Western analysis using the anti-flu monoclonal antibody are shown in Figure 8B. Although there was again some variation in protein accumulation, there was no correlation between accumulation and repression activity. The strongest repressor, AB–FS1, appeared to be present in the lowest abundance. The ability of the proteins to form protein–DNA complexes, again using adjusted nuclear extracts from the transfected cells, was measured by gel retention, and the results are shown in Figure 8C. Extending the correlation observed in Figure 5, the strongest repressors, AB–FS1 and AB–PR, gave rise to the lowest amounts of DNA–protein complexes (specific shifted bands are more apparent on a longer exposure; results not shown). As above, the AB protein gave a modest gel shift. The most notable result was obtained with the AB–FS3 activator, which was by far the most efficient in complex forma-
tion of all the Eve derivatives tested. Multiple bands of decreasing mobility, which presumably reflect filling of the multiple sites on the NP6 DNA fragment, were detected. Whether the enhanced ability of AB–FS3 to bind DNA reflects the fact that this protein is an activator rather than a repressor is an intriguing possibility. However, the observation that all of the strong repressors displayed significantly reduced DNA-binding activity suggests that this may be an important aspect of Eve-mediated repression.

Discussion

In the Introductory section we described three distinct types of repression: competitive binding, quenching, and direct. Our findings provide strong support for the notion that Eve can function as a direct repressor, that is, it can interfere with the assembly or activity of the basal transcription complex. The fact that Eve can function at distances 100–200 bp from basal promoter elements or activator-binding sites argues strongly against DNA-binding competition as the mechanism of repression in the experiments described here. However, previous studies are consistent with the idea that Eve can also function by this mechanism to block the activity of several homeobox activator proteins [Jaynes and O’Farrell 1988; Han et al. 1989]. That Eve cannot work effectively at distances far removed from the basal promoter does not argue against the direct repression mechanism. It may be, for example, that Eve’s function involves contact with the basal transcription machinery but that the interactions involved are weak and cannot occur stably when the proteins are not in close proximity of each other. This idea has been proposed previously to explain the inability of certain activators to function at a distance [e.g., Carey et al. 1990], a phenomenon that we have also detected with some of the activators used here [K. Han and J. Manley, unpubl.]

The idea that Eve can function as a direct repressor is also supported by our results showing that Eve can repress expression induced by a variety of different transcriptional activators. The fact that Eve thus appears to be a “universal” repressor argues that the mechanism involved is not likely to be quenching [i.e., specific protein–protein interactions involving the activator and the repressor]. This idea is further supported by the fact that Eve represses GAL4–VP16-activated expression very efficiently when binding sites for the two proteins are located near the promoter but not at all when these sites are moved far upstream. The simplest interpretation of these findings is that the inhibition of activated transcription is a consequence of Eve’s ability to block basal transcription. It is possible that Eve can also function by quenching. For example, Eve can weakly repress [two- to fourfold] the activation induced by GAL4–foozQ and Sp1 [but none of the other activators] in the absence of Eve-binding sites [K. Han and J. Manley, unpubl.]. Thus, although the studies described here provide strong evidence that Eve can function as a direct repressor, it is perhaps likely that the protein can work by competitive binding and quenching as well, and all three activities may be important for its activity in the embryo.

Our experiments provide compelling evidence that a transcriptional repressor, like an activator, requires a specific functional domain to facilitate repression. This general conclusion is consistent with other recent studies on transcriptional repressors, for example, the Drosophila Krüppel [Licht et al. 1990] and Engrailed [Jaynes and O’Farrell 1991] proteins, the human Wilms’ tumor protein WT1 [Madden et al. 1991], and the v-erbA oncogene [Baniahmad et al. 1992]. Our studies have extended these by defining with some precision what might be a minimal repression domain. Perhaps the most informative results were obtained with the FS1 fusion protein. This protein was the strongest repressor that we tested, and its repression activity was provided by a very short [27 residue], artificial, proline- plus leucine-rich domain. On the basis of the primary structure of this oligopeptide, we can conclude that an efficient repression domain can be small, unstructured, hydrophobic, and not highly charged. This description fits essentially all of the repression motifs that we have characterized in this paper, including the minimal 57-residue alanine plus proline Eve domain, and is not inconsistent with the much larger domains identified previously. For example, the region of Krüppel necessary for repression is alanine rich, and the segment of WT1 required is enriched in proline plus glutamine residues. Two repression regions that we have characterized [region D2 from Eve and the Prd repeat] also contain high proportions of histidine. Although this could simply reflect a variation on the hydrophobic requirement, it might also suggest that basic residues are compatible with repression activity. Consistent with this, a recent genetic screen for “repression domains” in yeast, analogous to one used previously to identify activation domains [Ma and Ptashne 1987b], has found that such regions are typified by a high proportion of basic residues [M. Ptashne, pers. comm.].

The results obtained with the inactive FS2 derivative suggest that the presence of serine and threonine residues can be sufficient to inactivate a proline-rich repression domain. We suspect that acidic residues, which are a common feature of many activation domains [Ptashne 1988], would similarly prevent repression, as the strong repressors identified here are all characterized by a lack of acidic residues. Thus, although our results strongly suggest that small proline/hydrophobic-rich regions can constitute efficient repression domains, it is possible that this can be generalized to include unstructured regions lacking serine, threonine and acidic residues.

That the Eve repression domain can function when fused to the heterologous GAL4 DNA-binding domain supports the view that this region constitutes a functional repression domain. However, its reduced activity and requirement for the Eve carboxyl terminus [E–F region in this context requires comment, and we offer two possible explanations. First, the conformation of the proteins may be such that the repression domain is not as accessible in the GAL4 fusion protein. This could reflect, for example, the fact that GAL4 binds DNA as a dimer.
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[Marmorstein et al. 1992] while homeo domain proteins, including Eve, apparently function as monomers [Afofylter et al. 1990; K. Han and J. Manley, unpubl.]. The structure of the GAL4–DNA complex [Marmorstein et al. 1992] indicates that the carboxyl termini of the two subunits are in close proximity, and it is possible that the two hydrophobic C–D repression regions in the GAL4–CD fusion protein could interact nonproductively with each other. In this conformation the E–F region might be important to bring about the full activity of the minimal repression domain. The function of the E–F region in the context of native Eve is unclear, although it is not inconceivable that it may function as an activation domain in some circumstances. A second and not mutually exclusive reason for the reduced activity of the GAL4 fusion proteins may reflect the fact that they bind DNA with much higher affinity than the Eve derivatives [K. Han and J. Manley, unpubl.]. As we have detected an inverse correlation between DNA-binding affinity and repression activity, this may contribute to the reduced activity of the GAL4 fusion proteins.

How might a direct repressor function to block transcription? One possibility is that the repression domain may in some way interfere with the formation or activity of the basal transcription complex. Recent in vitro experiments have provided evidence that Eve can interfere with an early step in formation of the initiation complex [Johnson and Krasnow 1992]. By analogy with transcriptional activators, this might involve a specific interaction with one [or more] general transcription factor or perhaps be mediated by a corepressor. Our analysis of the character of repression domains suggests that this putative interaction might involve hydrophobic forces. Although it is clear that numerous specific protein–protein interactions are necessary for transcriptional activation, the chemical nature of these interactions is unknown. However, it is worth noting that the interaction of the acidic activator GAL4–VP16 with the TATA-binding subunit of TFID requires a specific phenylalanine located within the VP16 acidic activation domain [Ingles et al. 1991].

Our finding that Eve derivatives that function as strong repressors have reduced DNA-binding ability in nuclear extracts was unexpected. However, the correlation between strong repression and weak binding was very high, and we therefore believe that it is likely to be significant. But how could this seemingly paradoxical observation reflect the mechanism of repression? One interpretation might be that DNA binding is not necessary for [and perhaps even detrimental to] Eve repression. However, this appears very unlikely. We showed that Eve-binding sites are essential for repression and that several mutations in the homeo domain block DNA binding as well as abolish repression activity [Fig. 4; unpublished data]. It is difficult without additional data to speculate how this property might be linked to efficient repression, but we suggest one possible model, which assumes what we believe to be the most likely mechanism for Eve function, that is, that the protein interferes with the proper assembly of the basal transcription complex. The model suggests that Eve binds to its DNA target and then contacts its protein targets. If this factor is necessary for transcription complex formation or activity, and if the protein–protein interaction is relatively stable, then dissociation of Eve from its DNA-binding site [perhaps only transiently] could help to disrupt the transcription initiation complex. This model is obviously speculative and is only one of several that might be consistent with our data. Nonetheless, it provides an explanation for the DNA-binding results, as well as a framework for future experiments.

Materials and methods

Recombinant plasmids

The plasmid pActPflu (for flu epitope tagging; Wilson et al. 1984; Field et al. 1988) was constructed by inserting the synthetic oligonucleotide

5′-GATCCAAATGTAGCCCTACGAGTCCCGACTACGCTGGTGACATG

3′-GTGATACGATGGCCGCTGCGGCGTTACGGGTGCTG

between the BamHI and SalI sites of pActSCPAA [Han et al. 1989]. These oligonucleotides contain a start codon and encode part [YPYDVPDYA] of the influenza hemagglutinin HA1. The nucleotide sequence flanking the translational start site was chosen according to the Drosophila consensus sequence [Cavener 1987], and the sequence coding for the flu epitope was chosen according to Drosophila codon usage [Streck et al. 1986]. pActPflu-eve was constructed by inserting a 0.18-kb Ndel/Klenow–SalI fragment from pAR-eve [Hoey and Levine 1988] and a 0.4-kb HinClI–NcoI fragment from pActflu into a 8.15-kb SacII–NcoI pActSC–eve fragment [Han et al. 1989] fragment. All other expression vectors were constructed by standard subcloning methods. Portions of DNA around the junctions of all mutations were sequenced to confirm their identities. Detailed strategies for constructing all Eve expression vectors are available on request. Descriptions of all the Eve derivatives used in this study are shown in Table 1 (the single-letter amino acid code is used).

pAct–Sp1 was described by Courcy and Tjian [1988]. pAct–GALE4–VP16 was constructed as ligating a 1.0-kb KpnI-BglII fragment from pSGVP [Sadowski et al. 1988] with a 7.3-kb KpnI–BamHI fragment from pActSCPAA. pAct–GA4 was constructed by ligating a 2.95-kb Xbal–EcoRI fragment from pAct–GA4–VP16 with a 4.9-kb Xbal–EcoRI pActPPA fragment. All expression vectors encoding GAL4 fusion proteins were constructed using pAct–GA4 and plasmids encoding the required activator or Eve. Details of the GAL4 fusion proteins used in this study are shown in Table 2.

To minimize possible complications in our analysis, for example, from endogenous transcription factors binding to cryptic sites in plasmid sequences, we constructed a minimal CAT reporter plasmid, pGAP4–CAT. pGAP4–CAT [2.9 kb] contains a 2.0-kb AatII–PvuII pUC fragment; a polylinker containing HindIII, PstI, SalI, XbaI, BamHI, and Smal sites; a 0.66-kb CAT-coding region; and a 0.24-kb BclI–BamHI SV40 poly[A] signal. Reporter plasmids were constructed by inserting transcription factor-binding sites and one of several minimal promoter elements into the polylinker sequences of pGAP4–CAT. Detailed strategies used for constructing reporter plasmids are available on request.

DNA transfection and transient expression assay

Transient expression assays for Figures 1, 2, 3, and 7 were per-
Table 1. Eve derivatives

| Eve protein | Primary structure | Calculated molecular mass |
|-------------|-------------------|---------------------------|
| ABCDEFG     | M + flu9 + LS + Eve residues 1 to 376 (full length) | 41,376 |
| BCDEFG      | M + flu9 + Eve61 to 376 | 34,574 |
| AbCDEFG     | M + PPPKKKV + LS + YDVPDYA + LS + Eve to 81 + 107 to 376 | 39,145 |
| AB DEF      | M + flu9 + LS + Eve to 139 + S + 186 to 376 | 37,116 |
| ABC EF      | M + flu9 + LS + Eve to 178 + 245 to 376 | 34,169 |
| ABCD F      | M + flu9 + LS + Eve to 245 + 305 to 376 | 35,820 |
| ABCDEF      | M + flu9 + LS + Eve to 305 + RP | 34,250 |
| B           | M + flu9 + LS + Eve61 to 138 + LV | 10,769 |
| AB          | M + flu9 + LS + Eve to 139 | 17,458 |
| ABC         | M + flu9 + LS + Eve to 139 + 305 to 376 | 21,486 |
| ABCD        | M + flu9 + LS + Eve to 139 + 306 to 376 | 24,433 |
| ABCD F      | M + flu9 + LS + Eve to 139 + 308 to 376 | 23,267 |
| BCD         | M + flu9 + LS + Eve to 139 + 306 to 376 | 24,837 |
| ABCD        | M + flu9 + LS + Eve to 139 + 307 to 376 | 26,612 |
| ABC E       | M + flu9 + LS + Eve to 139 + 304 to 376 | 29,989 |
| ABC F       | M + flu9 + LS + Eve to 139 + 305 to 376 | 31,559 |
| AB DE       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,394 |
| AB D F      | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABCD E      | M + flu9 + LS + Eve to 139 + 307 to 376 | 31,559 |
| ABCD F      | M + flu9 + LS + Eve to 139 + 308 to 376 | 30,394 |
| AB C        | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABC D       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABC E       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABC F       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| AB DE       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| AB D F      | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABCD E      | M + flu9 + LS + Eve to 139 + 307 to 376 | 31,559 |
| ABCD F      | M + flu9 + LS + Eve to 139 + 308 to 376 | 30,394 |
| AB C        | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABC D       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABC E       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABC F       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| AB DE       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| AB D F      | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABCD E      | M + flu9 + LS + Eve to 139 + 307 to 376 | 31,559 |
| ABCD F      | M + flu9 + LS + Eve to 139 + 308 to 376 | 30,394 |
| AB C        | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABC D       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABC E       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| ABC F       | M + flu9 + LS + Eve to 139 + 305 to 376 | 30,671 |
| AbCDEFG     | M + flu9 + LS + Eve to 81 + 107 to 376 | 39,145 |
| AbCDEF      | M + PPPKKKV + LS + YDVPDYA + LS + Eve to 81 + 107 to 376 | 39,145 |
| AbCDEF      | M + PPPKKKV + LS + YDVPDYA + LS + Eve to 81 + 107 to 376 | 39,145 |
| AbCDEF      | M + PPPKKKV + LS + YDVPDYA + LS + Eve to 81 + 107 to 376 | 39,145 |
| AbCDEF      | M + PPPKKKV + LS + YDVPDYA + LS + Eve to 81 + 107 to 376 | 39,145 |

Formed in duplicate essentially as described by Han et al. (1989). In the experiments shown in Figures 4, 5, and 8, duplicate 100-mm tissue culture dishes were used for both CAT assay and nuclear extract preparation from the same transfected cells. Cells were plated at 0.6 x 10^7 to 1.2 x 10^7 cells in 15 ml of M3/10% fetal bovine serum medium per 100-mm dish 1 day before transfection. Each transfection contained 3-15 lag of Eve expression vector, 3 ng of pAct-GAL4-VP16, 3 µg of pSGNI-CAT, 6 lag of pcopia LTR-lacZ as an internal control, and 6 µg of pGeml. The total amount of actin promoter plasmid DNA was adjusted to 15 µg by adding, as needed, an empty expression vector pAct5CPPA. The amounts of the different Eve proteins that accumulated in nuclei (determined by Western blot analysis) and the abilities of the proteins to form complexes with the target NP6 DNA (determined by gel retention assay) were the same whether the nuclear extracts were prepared from cells cotransfected with pAct-GAL4-VP16 and pSGNI-CAT DNA or from cells without these two plasmids (K. Han and J. Manley, unpubl.). Two days after transfection, cells were removed from tissue culture dishes by agitation. Cells were washed twice with PBS, and 15% were resuspended in 0.1 ml of 0.25 M Tris-HCl (pH 7.8) and frozen at -70°C. Whole-cell extracts were prepared by thawing and sonicating for 2 min in an ultrasonic sonifier (Branson), followed by centrifugation at full speed for 10 min in a microcentrifuge. Transfection efficiencies were determined by assaying for β-galactosidase activity as described in Han et al. (1989), except that 0.1 M NaPO4 (pH 7.0) was used instead of 0.1 M Tris-HCl (pH 8.0) in buffer Z. CAT assays were performed as described in Han et al. (1989).

Table 2. GAL4 fusion proteins

| GAL4 fusion | Primary structure | |
|-------------|-------------------|---|
| GAL4        | GAL4[1 to 147] + PEFIDI | |
| GAL4-VP16   | GAL4[1 to 147] + PEFPGI + VP16[412 to 490] | |
| GAL4-ftzQ   | GAL4[1 to 147] + PEFPG1 + Ftz[328 to 413] | |
| GAL4-zenST  | GAL4[1 to 147] + PEFPG1 + Zen[185 to 353] | |
| GAL4-CTF1P  | GAL4[1 to 92] + FRRYP + CTF1[399 to 499] | |
| GAL4-93     | GAL4[1 to 93] + IPI | |
| GAL4-CDEF   | GAL4[1 to 92] + F + Eve[140 to 376] | |
| GAL4-CD     | GAL4[1 to 92] + F + Eve[140 to 246] + RP | |
| GAL4-FS1    | GAL4[1 to 93] + FS1 | |
Triton X-100 was added to each cell suspension, cell suspensions were kept on ice for 5 min. Nuclei were harvested by centrifugation at 7000 rpm for 20 sec in a microcentrifuge, re-suspended with two-thirds PCV of salt buffer (20 mM HEPES at pH 7.8, 25% glycerol, 1 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSE), kept on ice for 30 min, and centrifuged at full speed for 15 min in a microcentrifuge in a cold room. The supernatant was saved as nuclear extract.

Western blot analysis
Aliquots were taken from each nuclear extract, with the amounts removed determined by the β-galactosidase activity (to correct for variations in transfection efficiencies), and one volume of 2× Laemmli loading buffer was added. The volumes of the samples were made equal by the addition as needed of 1× Laemmli loading buffer. Proteins were resolved on a 12.5% SDS–polyacrylamide gel, transferred to nitrocellulose, and blotted with the mouse mAB 12CA5 (Berkeley Antibody Company, Niman et al. 1983). A goat anti-mouse IgG antibody, conjugated to alkaline phosphatase (Sigma Chemical Company), was used as the second antibody and was detected essentially as described by Harlow and Lane (1988).

Gel retention assay
The DNA used in the gel retention experiments was a 79-bp XhoI–Smal fragment from pBS–NP6 (Jaynes and O’Farrell 1988) end-labeled with T4 polynucleotide kinase. Binding reactions were performed in a volume of 10 μl in 20 mM HEPES (pH 7.8), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSE, and 15% glycerol and were incubated for 20 min on ice. Reaction mixtures contained 1 μl of nuclear extract, 1 μg of poly[d(C)]-1, 1 μg of poly[d(A-T)]-2, 2 μg of sonicated salmon sperm DNA, and 2 ng of labeled NP6 DNA. Nuclear extracts were diluted before use with extract prepared from cells transfected with empty expression vector to adjust for differences in Eve concentrations as determined by Western blot analysis. Samples were loaded on a prerun 4% polyacrylamide gel (80:1, acrylamide/bis) containing 1× TBE buffer. Electrophoresis was at 200 V at room temperature for 2–3 hr.

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