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Cooperative Binding at a Distance by *even-skipped* Protein Correlates with Repression and Suggests a Mechanism of Silencing

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In this study, we examined how the *Drosophila* developmental control gene *even-skipped* (*eve*) represses transcription. Tissue culture cells were used to show that *eve* contains domains which inhibit transcriptional activators present at the *Ultrabithorax* (*Ubx*) proximal promoter when bound up to 1.5 kb away from these activators. Different portions of *eve* were fused to a heterologous DNA binding domain to show that three adjacent regions of *eve* contribute to silencing. There appear to be two mechanisms by which *eve* protein represses transcription. In this study, we used in vitro transcription and DNA binding experiments to provide evidence for one of these mechanisms. Repression in vitro correlates with binding of *eve* protein to two low-affinity sites in the *Ubx* proximal promoter. Occupancy of these low-affinity sites is dependent upon cooperative binding of other *eve* molecules to a separate high-affinity site. Some of these sites are separated by over 150 bp of DNA, and the data suggest that this intervening DNA is bent to form a looped structure similar to those caused by prokaryotic repressors. One of the low-affinity sites overlaps an activator element bound by the *zeste* transcription factor. Binding of *eve* protein is shown to exclude binding by *zeste* protein. These data suggest a mechanism for silencing whereby a repressor protein would be targeted to DNA by a high-affinity element, which itself does not overlap activator elements. Cooperative binding of further repressor molecules to distant low-affinity sites, and competition with activators bound at these sites lead to repression at a distance.

Eukaryotic transcriptional repressors act by several distinct mechanisms. Some repressor proteins act by directly complexing with sequence-specific activators to inhibit their function (1, 3, 14, 19, 31, 36, 44, 47). Other repressors act by binding directly to DNA sites overlapping those of activator proteins, thereby sterically blocking activator binding (35, 51, 54). In contrast to these mechanisms, some repressors are able to prevent the function of either specific activators or general transcription factors when they are bound anywhere from tens of base pairs to several kilobase pairs away (7, 10, 26, 28, 29, 40, 42, 49, 62). Such repressors have been termed silencer proteins or active repressors, and their mechanisms of action have not been fully characterized.

Many of the genetically defined regulators of *Drosophila* embryogenesis have been shown to act as transcription factors (7, 17, 21, 27, 30, 39, 42, 51, 61, 63, 64; reviewed in references 8 and 22). Studying the mechanisms by which these proteins act should allow a thorough genetic and biochemical understanding of the control of spatial and temporal patterns of transcription. Previously we demonstrated that the homeodomain protein *even-skipped* (*eve*) represses transcription from the *Ultrabithorax* (*Ubx*) promoter in vitro and in tissue culture cells (7). In these experiments, *eve* binding sites were located within 15 bp of basal promoter elements that are important for transcription but did not overlap any activator elements. In vitro, *eve* protein was able to repress when bound upstream or downstream of the RNA start site and when its DNA binding sites were in either orientation. We took these data to indicate that *eve* protein might be able to repress when bound at much greater distances and therefore act as a silencer factor. If this is true, then the mechanism by which *eve* protein represses *Ubx* transcription in vitro might be the same as silencing but occurring over a much shorter distance.

Here, evidence which suggests that *eve* can repress transcription at a distance is presented. Repression by *eve* has been studied by using a combination of tissue culture cell, in vitro transcription, and DNase I footprinting assays. These data suggest that repression involves cooperative binding between *eve* molecules bound at distant sites in a manner similar to that used by some prokaryotic repressors (for examples, see references 18, 23, 43, and 45).

MATERIALS AND METHODS

Plasmid DNA. The construction of plasmids pUbx−185/+45 CAT (also called pUbx Δ3′+45/CAT), pPac (also called pPac U+Nde), and pPac eve is described in reference 7. Plasmids pADHP Δ5′−55, pUbx Δ3′+45, and pUbx Δ3′+114 are described in reference 6. pXS1 and pNS8 contain multiple copies of the Sp1 DNA binding sites TGG GCG GAG TTA GGG GCG GGAT (also used in the study reported in reference 12) inserted at the *Xba*I and *Nae*I sites of pUbx −185/+45 CAT, respectively. pXB5 and pNB2 contain multiple copies of the sequence GGA CGG CAT TAT TGT TAT TAT TGG CC inserted at the *Xba*I and *Nae*I sites of pUbx −185/+45 CAT, respectively. p−185/+45 HN is a recircularized *Hin*dIII-*Nde*I fragment of pUbx −185/+45 CAT lacking sequences between −0.2 and −3.5 kb. The expression plasmid pPac Sp1/C167 contains DNA sequences derived from pAR3040 (59) and a *Not*I linker, which together code for 17 amino acids, followed by DNA sequences coding for the C-terminal 167 amino acids of Sp1 (Fig. 2 in reference 32), all cloned as an *Nde*I-*Xho*I fragment into pPac. pPac eveff contains an *Nde*I-*Xho*I fragment which codes for all

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but the C-terminal amino acid of *eve*, followed by 4 extraneous amino acids due to the presence of a *Not*I linker, followed by the C-terminal 167 amino acids of Sp1. Deletion mutations of eveff (*eve* finger fusion) were constructed by using convenient restriction sites except that BAL 31 digestion, followed by addition of an *Nde*I linker, was used to generate Bff and J23ff. Some of these deletion mutations were inserted as *Nde*I-*Xho*I restriction fragments into pAR3040 to allow expression in *Escherichia coli*. Plasmid pU contains *Ubx* promoter sequences between nucleotides −185 to +45, inserted at the *Xba*I site of a pBluescript-derived vector. pS1, pS2, and pS4 contain one, two, and four copies of the sequences bound by Sp1 described above, inserted at the *Bam*HI site of pU.

Cell culture and transfections. Schneider line 2 cells (56) were transfected by the calcium-phosphate method (15) as described previously (7, 13). The cells received 0.2 μg of chloramphenicol acetyltransferase (CAT) reporter plasmid, 0.2 μg of a pPac-derived expression plasmid, and 10 μg of pBluescript. All data presented are averages of at least four independent experiments. The data presented in Fig. 2 and Table 2 are averages of six independent experiments. An example of how fold repression was calculated is (relative expression of pXS1/relative expression of pXS1 with pPac eveff) × relative expression of pUbx−185/+45 CAT with pPac eveff. This calculation takes into account binding site-independent repression of pUbx−185/+45 CAT and gives the most conservative value for fold repression.

Purification of proteins. Extracts were prepared from *E. coli* by a modification of the method of Hoey and Levine (24). Fusion proteins except CDff were precipitated from the soluble protein fraction by addition of ammonium sulfate to concentrations between 30 to 55% saturation. The resolubilized pellets were further purified by DNA affinity chromatography (34) as described previously (7). The DNA sequence attached to the resin was the Sp1 DNA recognition sequence described earlier. CDff was prepared from the insoluble protein fraction by dissolving in 6 M guanidine hydrochloride followed by dialysis. The protein was then purified by fractionation over S Sepharose (Pharmacia) and Superdex 75 (Pharmacia). Note that CDff prepared from a soluble protein fraction without denaturation represses transcription. *eve* protein used in the DNA binding experiment was purified from *E. coli* extract by addition of ammonium sulfate to 17% saturation. The pellet was resolubilized in 100 mM KCl HEMG (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-K^+ [pH 7.6], 12.5 mM $MgCl_s$, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol)–0.05% Nonidet P-40 (NP-40), bound to a Pharmacia Mono S resin, and eluted with a gradient of buffer from 100 mM KCl HEMG–0.05% NP-40 to 600 mM KCl HEMG–0.05% NP-40. *zeste* and GAGA proteins were purified to near homogeneity from a *Drosophila* embryo extract by affinity chromatography as previously described (5, 6). Concentrations of these proteins were estimated by comparison of relative band intensities on a silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel.

In vitro transcription and DNA binding assays. In vitro transcription experiments were carried out essentially as described previously (7). However, in most cases, purified fusion proteins were diluted directly into transcription reactions and were not dialyzed subsequent to DNA affinity chromatography. DNase I footprinting was carried out as described previously (7) except that proteins were bound to about 1.5 ng of probe in the presence of 10 ng of poly(dI-dC) carrier DNA per μl. The footprinting reactions shown in Fig. 7 do not contain carrier DNA. The binding reaction mixtures (50 μl) were incubated at 21°C for 10 min prior to DNase I digestion.

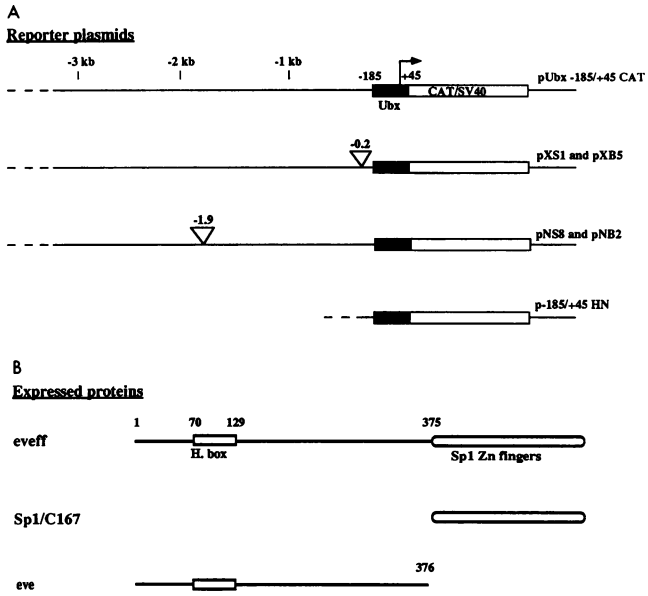

FIG. 1. (A) Diagrams of the reporter plasmids used in transient cotransfection assays. pUbx−185/+45 CAT lacks both Sp1 recognition elements and high-affinity *eve* binding site (same as pUbxΔ3′+45 CAT [7]). pXS1 and pNS8 contain between 12 to 18 Sp1 DNA binding sites at −0.2 and −1.9 kb, respectively. pXB5 and pNB2 contain between 6 to 10 *eve* binding sites at −0.2 and −1.9 kb, respectively. p−185/+45 HN is derived from pUbx −185/+45 CAT and has DNA sequences between −0.2 and −3.5 kb deleted. (B) Diagrams of eveff, Sp1/C167, and *eve* proteins. The protein eveff contains all but the C-terminal amino acid of *eve* fused to the Sp1 DNA binding domain. The protein Sp1/C167 contains the C-terminal 167 amino acids of Sp1, including the Sp1 DNA binding domain (see Fig. 2 in reference 32). The numbers refer to the amino acids of *eve*. The Sp1 Zn fingers and the homeodomain are indicated.

Binding reactions for UV irradiation cross-linking were done under the same conditions as was footprinting but in a total volume of 20 μl. The DNA probe was a double-stranded oligonucleotide containing nucleotides −172 to −126. After binding, samples were UV irradiated for 2 min. Samples were then separated on an SDS–8% polyacrylamide gel.

RESULTS

Previously we have shown that *eve* represses *Ubx* transcription in a DNA binding site-dependent manner in *Drosophila* tissue culture cells (7). To determine which parts of *eve* protein are required for repression, we have examined which regions of *eve*, when fused to the DNA binding domain of the mammalian transcription factor Sp1, can convert this DNA binding domain into an active repressor. Sp1 was chosen because the C-terminal 167 amino acids of Sp1, which contain the DNA binding domain, neither activate nor repress transcription in tissue culture cells and in in vitro transcription reactions containing crude nuclear extract (13, 33).

An *eve*-Sp1 fusion protein silences transcription. Initially, we created a fusion protein, termed eveff, that contained essentially all of *eve* protein joined to the Sp1 DNA binding

TABLE 1. Relative promoter activities of various reporter plasmids in the presence or absence of *eve*, eveff, or Sp1/C167 protein[a]

Reporter promoter	Expression plasmid	Relative CAT expression	Fold repression
pUbx−185/+45 CAT	pPac	1	
	pPac Sp1/C167	1.08	
	pPac eveff	0.48	
	pPac eve	0.40	
pXS1	pPac	1.08	
	pPac Sp1/C167	1.12	1.0
	pPac eveff	0.084	6.2
	pPac eve	0.39	1.0
pNS8	pPac	1.3	
	pPac eveff	0.14	4.5
p−185/+45 HN	pPac	3.2	
pXB5	pPac	0.68	
	pPac eve	0.11	2.5
pNB2	pPac	0.68	
	pPac eve	0.21	1.25

[a] cDNAs encoding these proteins are expressed downstream of the *Drosophila* actin 5C promoter from plasmids named pPac. Reporter plasmids (0.2 μg) were transfected into Schneider 2 cells along with 0.2 μg of either pPac, pPac eve, pPac Sp1/C167, or pPac eveff. The fold repression was calculated as described in the text.

domain (Fig. 1). Reporter plasmids were constructed with DNA sequences recognized by Sp1 (GC boxes) placed at either 0.2 or 1.9 kb upstream of a *Ubx* promoter from which the previously characterized high-affinity *eve* protein binding sites have been removed (Fig. 1). In tissue culture cells, CAT expression from the reporter plasmid pXS1, which contains GC boxes inserted at −0.2 kb, is reduced to 8% of normal levels when eveff is also present in cells (Table 1). eveff also reduces expression of a reporter plasmid lacking GC boxes (Table 1, pUBX−185/+45CAT), but only to 48%

TABLE 2. Relative promoter activities of reporter plasmids with various deletions of eveff[a]

Reporter promoter	Expression plasmid	Relative CAT expression	Fold repression
pUbx−185/+45 CAT	pPac	1	
	pPac eveff	0.48	
	pPac ABCff	0.33	
	pPac ABff	0.68	
	pPac Aff	0.98	
	pPac BCDff	0.55	
	pPac CDff	0.92	
	pPac Dff	1.15	
	pPac Bff	0.80	
	pPac Cff	0.94	
	pPac BCff	0.64	
pXS1	pPac	1.0	
	pPac eveff	0.084	6.2
	pPac ABCff	0.13	2.2
	pPac ABff	0.80	0.74
	pPac Aff	1.32	0.64
	pPac BCDff	0.10	5
	pPac CDff	0.12	6.5
	pPac Dff	0.60	1.7
	pPac Bff	0.62	1.1
	pPac Cff	0.52	1.6
	pPac BCff	0.12	4.6

[a] Transfections were performed as for Table 1.

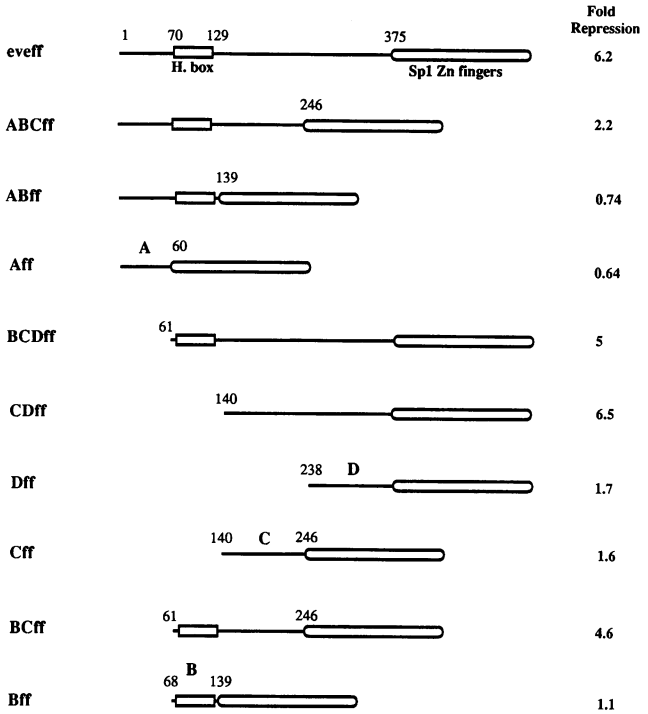

FIG. 2. Domains of *eve* required for repression in Schneider cells. Diagrams of various deletion mutations of eveff protein are shown. The numbers refer to the amino acids of *eve* at the N and C termini of the deletions. The four regions of *eve* (A to D), the Sp1 Zn fingers, and the homeodomain are indicated.

of normal transcription. Thus, eveff actively represses transcription by 6.2-fold ([1.08/0.084] × 0.48 = 6.2; see Materials and Methods), in a manner dependent upon the presence of GC boxes. To demonstrate that the binding site-dependent repression was not due to *eve* protein sequences fortuitously binding the GC boxes, *eve* protein was expressed in cells along with these same two reporter plasmids. No GC box-dependent repression was observed in this case, but mild repression of both plasmids was observed (Table 1). As we show later, the GC box-independent repression appears to be due, at least in part, to the presence of previously uncharacterized low-affinity *eve* binding sites in the *Ubx* proximal promoter. As expected, expression of the Sp1 DNA binding domain alone (Sp1/C167) does not affect CAT expression of either reporter. Thus, eveff must be binding the GC boxes by the Sp1 Zn fingers and repressing transcription as a result of the *eve* protein sequences that it contains.

eveff can also actively repress transcription when bound to GC boxes located 1.9 kb upstream from the RNA start site (pNS8; Table 1). It is unlikely that this repression is due to steric interference with transcriptional activators bound adjacent to the GC boxes, as deletion of the entire upstream region between −0.2 and −3.5 kb does not reduce CAT expression (Table 1, p−185/+45 HN), indicating that no activators are bound to this region. Also, the GC boxes in pNS8 (and in pXS1) have been inserted in bacterial DNA sequences to which *Drosophila* transcription factors are less likely to bind. Thus, repression may occur by interference with the well-characterized activators known to bind the *Ubx* proximal promoter between nucleotides −185 and +45 (8).

We have also examined the ability of wild-type *eve* protein

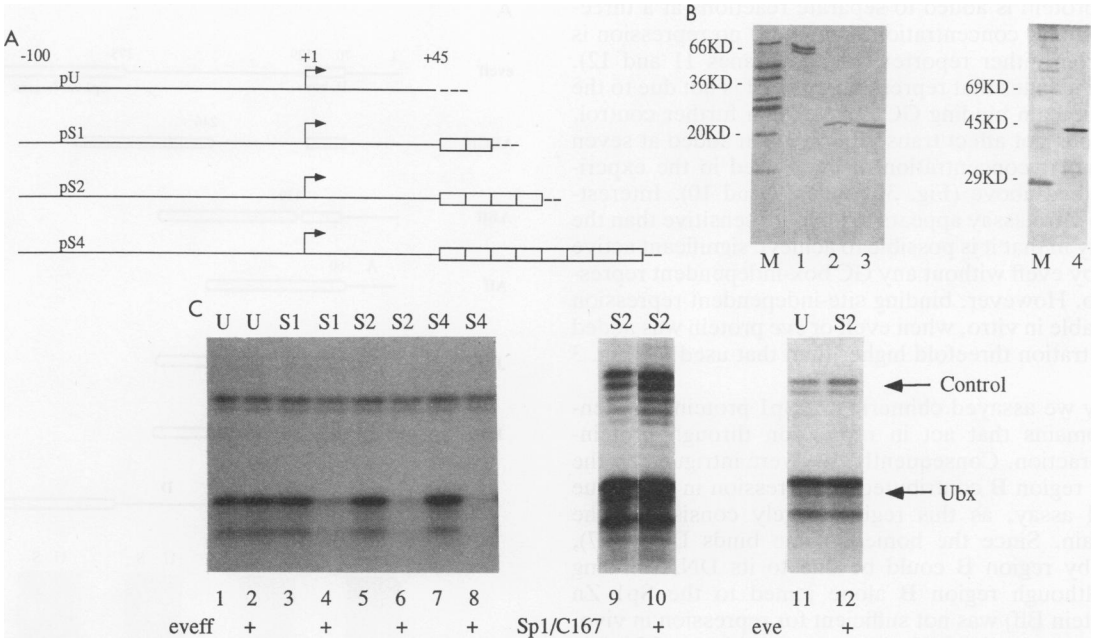

FIG. 3. Repression of transcription in vitro by an *eve*-Sp1 DNA binding domain fusion protein. (A) Diagram of the promoter template DNA used for transcription (pU, pS1, pS2, and pS4). The promoter template pU lacks Sp1 DNA binding sites. The DNAs pS1, pS2, and pS4 contain two, four, and eight Sp1 DNA binding sites, respectively. (B) Analysis of affinity-purified protein by SDS-polyacrylamide gel electrophoresis and silver staining. Lanes: M, markers; 1, eveff protein; 2 and 3, Sp1/C167 protein; 4, *eve* protein. (C) S1 nuclease assay of RNA synthesized from the *Ubx* promoter. Each reaction contained 225 ng of pADHP Δ5′−55 (control template) and 25 ng of either pU (lanes 1, 2, and 11), pS1 (lanes 3 and 4), pS2 (lanes 5, 6, 9, 10, and 12), or pS4 (lanes 7 and 8). All transcription reactions were performed with 116 μg of nuclear extract. Lanes 2, 4, 6, and 8 contained 100 ng of eveff protein (1.6 pmol); lane 10 contained 230 ng of Sp1/C167 protein (12 pmol); lanes 11 and 12 contained 200 ng of *eve* protein (4.8 pmol). The expected RNA initiations are indicated.

lacking Sp1 sequences to silence transcription in tissue culture cells. DNA binding sites recognized by *eve* have been placed at 0.2 kb (pXB5) and 1.9 kb (pNB2) upstream of the *Ubx* RNA start site (Fig. 1). Binding site-dependent repression by *eve* is observed when sites are at −0.2 kb, but the level of repression is only 2.5-fold (Table 1). Little significant active repression is observed when *eve* binding sites are placed at −1.9 kb. We suspect that the affinity of *eve* for the DNA binding sites that we have used is lower than that of eveff for GC boxes, and this is why silencing by *eve* was weak when present at −0.2 kb and insignificant when present at −1.9 kb.

Three regions of *eve* contribute to silencing. To map the domains of *eve* involved in active repression, deletions of *eve* fused to the Sp1 Zn fingers were constructed (Fig. 2). These deletions were tested in the tissue culture cell assay. To describe these results, it is convenient to divide the *eve* protein into four regions, A through D (Fig. 2). Deletion of the most C-terminal region (region D) reduces active repression from 6.4-fold to 2.2-fold (Table 2; compare pPac eveff with pPac ABCff). Further deletion, which removes region C as well, yields a protein that does not silence transcription (Table 2, pPac ABff). These data suggest that both region C and region D contribute to repression. This conclusion is supported by results for three further mutant proteins. CDff actively represses transcription by 6.5-fold and contains only regions C and D fused to the Sp1 Zn fingers. Region C alone or region D alone can actively repress, although only very weakly (Cff or Dff, respectively). From these data, it is not clear whether it is appropriate to consider regions C and D as two halves of one repression domain or two distinct, synergistically acting domains.

Regions C and D are not the only regions which affect repression. A protein containing regions B and C (BCff) is a much more effective repressor than Cff, which contains region C alone (Table 2; 4.6-fold as opposed to 1.6-fold). However, region B alone does not repress when fused to the Sp1 Zn fingers (Table 2, pPac Bff). Thus, there is an apparent synergy between regions C and B, just as there is between regions C and D, but it is not clear whether region B is acting as a distinct repression domain or whether it is affecting repression only by stabilizing the structure of region C. However, data presented below suggest that region B is acting directly in repression.

An in vitro assay for domains required for repression. To determine the mechanisms by which regions of *eve* contribute to repression, we have sought to establish in vitro assays for their action. We previously developed an in vitro transcription assay which establishes the ability of *eve* protein to directly repress *Ubx* transcription (7). This assay has been adapted to test *eve* protein domains fused to the Sp1 Zn fingers in the same promoter context. We constructed *Ubx* promoter templates in which the natural *eve* DNA binding sites downstream of nucleotide +45 (element B) were replaced by GC box sequences recognized by Sp1 (Fig. 3A). These Sp1 DNA binding sites are close to the RNA start site in the in vitro experiments, and repression is no longer at a distance. Nevertheless, we suggest that the mechanisms are probably the same in vitro and in vivo.

eveff, Sp1/C167, and *eve* proteins were expressed in *E. coli* and purified by DNA affinity chromatography (Fig. 3B). Addition of eveff represses transcription from promoters containing GC boxes but not from a promoter lacking these sequences (Fig. 3C; compare lane 2 with lanes 4, 6, and 8).

When *eve* protein is added to separate reactions at a threefold-higher molar concentration than eveff, no repression is observed from either reporter (Fig. 3C, lanes 11 and 12). This result indicates that repression by eveff is not due to the *eve* homeodomain binding GC boxes. As a further control, Sp1/C167 does not affect transcription when added at seven times the molar concentration of eveff used in the experiment described above (Fig. 3C, lanes 9 and 10). Interestingly, the in vitro assay appears to be more sensitive than the in vivo assay in that it is possible to achieve significant active repression by eveff without any GC box-independent repression in vitro. However, binding site-independent repression was observable in vitro, when eveff or *eve* protein was added at a concentration threefold higher than that used for Fig. 3 (4).

Originally we assayed chimeric *eve*-Sp1 proteins to identify *eve* domains that act in repression through protein-protein interaction. Consequently, we were intrigued by the finding that region B contributed to repression in the tissue culture cell assay, as this region largely consists of the homeodomain. Since the homeodomain binds DNA (57), repression by region B could be due to its DNA binding activity. Although region B alone joined to the Sp1 Zn fingers (protein Bff) was not sufficient for repression in vivo, we tested repression by Bff in the more sensitive and flexible in vitro assay. In this assay Bff does actively repress transcription (Fig. 4B, lanes 5 and 6), as do two further proteins that also lack repression domains C and D (ABff and J23ff; Fig. 4A). Although none of these three proteins repress as effectively as does eveff (they are required in higher concentrations), the data provide unambiguous biochemical evidence that region B is sufficient for repression when fused to the Sp1 DNA binding domain.

The protein CDff also actively represses *Ubx* transcription in vitro (Fig. 4B, lanes 7 and 8). Thus, *eve* contains at least two distinct regions which can both convert the Sp1 DNA binding domain into an active repressor in vitro.

The *eve* homeodomain binds activation elements. In an earlier report, we identified an *eve* DNA binding site downstream of nucleotide +45 (element B) that did not overlap activator elements and which was required for repression by intact *eve* protein (7). If Bff's repression activity is due to the homeodomain's DNA binding activity, then there must be further *eve* binding sites on *Ubx*, as element B is not present in the promoter constructs used to assay the *eve*-Sp1 fusion proteins. In this study, DNase I footprinting has been used to show that the homeodomain in Bff does indeed bind to other *Ubx* promoter sequences. On the wild-type *Ubx* promoter, intact *eve* protein (i.e., not a fusion protein) is also shown to bind the same other elements, in a manner dependent upon binding to element B.

Footprinting was performed with DNA probes derived from plasmids pU and pS2, which were used in the in vitro transcription experiments described above. Protein Sp1/C167 (containing only the Sp1 DNA binding domain) specifically binds to the GC boxes in probe pS2. No protection is observed outside of these sequences (Fig. 5, lanes 2 to 4). In contrast, Bff not only protects the GC boxes in this probe but also changes the DNase I cleavage pattern in *Ubx* proximal promoter sequences from positions −170 to +43 (Fig. 5, lanes 9, 10, 18, and 19). Close examination of the pattern reveals that sequences between −170 and −140 (termed site II) and +18 and +43 (termed site I) are protected from digestion, suggesting that the homeodomain in Bff is binding these sequences (Fig. 5; compare lanes 19 and 22). In addition, between nucleotides −115 and +17, an alternating

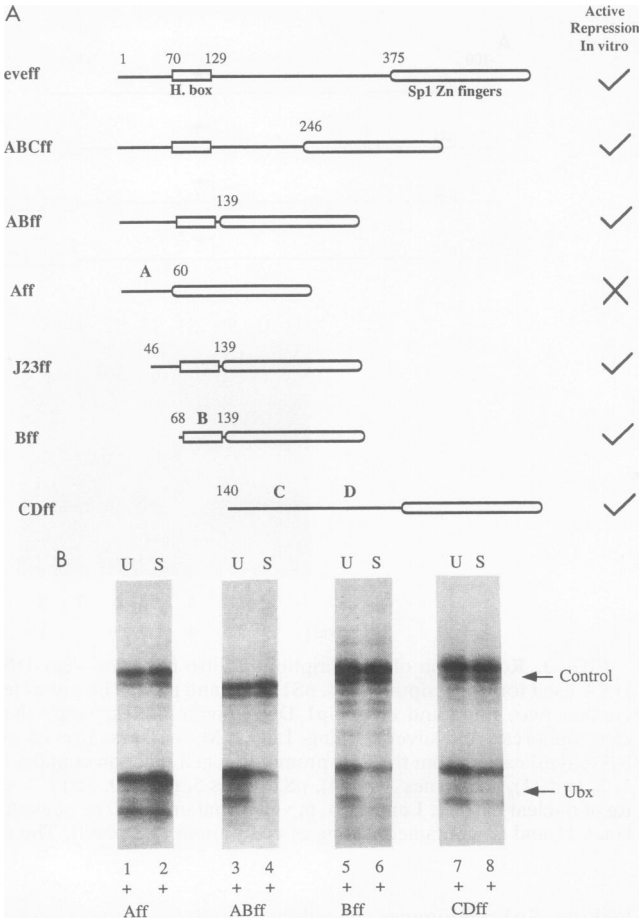

FIG. 4. Domains of *eve* required for repression of *Ubx* transcription in vitro. (A) Diagram of mutants of eveff protein which have been tested by using in vitro transcription assays. The right-hand column indicates whether the mutant protein can actively repress transcription (√) or not (×). (B) Transcriptional properties of several deletion mutants of eveff. In vitro transcription reactions were performed as described for Fig. 3 except that in lanes 5 and 6 only 6 ng of *Ubx* template was included, and in lanes 7 and 8 50 ng of *Ubx* template was used. Lanes 1 and 2 contained 520 ng of Aff (20 pmol); lanes 3 and 4 contained 160 ng of ABff (4.6 pmol); lanes 5 and 6 contained 100 ng of Bff (3.7 pmol); lanes 7 and 8 contained 900 ng of CDff (20 pmol).

pattern of protection and hypersensitive cutting with approximately a 5-bp periodicity is observed (Fig. 5; compare lanes 9 and 10 with the no-protein control in lane 6). An enlarged photograph of the region between −115 and +17 in lanes 6 through 11 of Fig. 5 helps to show this periodic cleavage pattern. This type of altered DNase I cleavage pattern is indicative of bent or looped DNA (16, 38, 48). (Note that a related 10-bp periodic pattern of DNaseI cleavage is observed when straight DNA is in contact with a flat surface [53]. However, in this case hypersensitive cutting should not be observed, as increased cutting is thought to be caused by a widening of the minor groove on the outer edge of a bent DNA. Also, an oozing mechanism of protein binding [52] is not consistent with the data, as the protections observed between −115 and +17 cover only 2 to 4 bp each. These protections are periodically spaced at 10-bp intervals, too close for adjacent binding of protein molecules. Structural studies of homeodomain binding [37] have shown that the

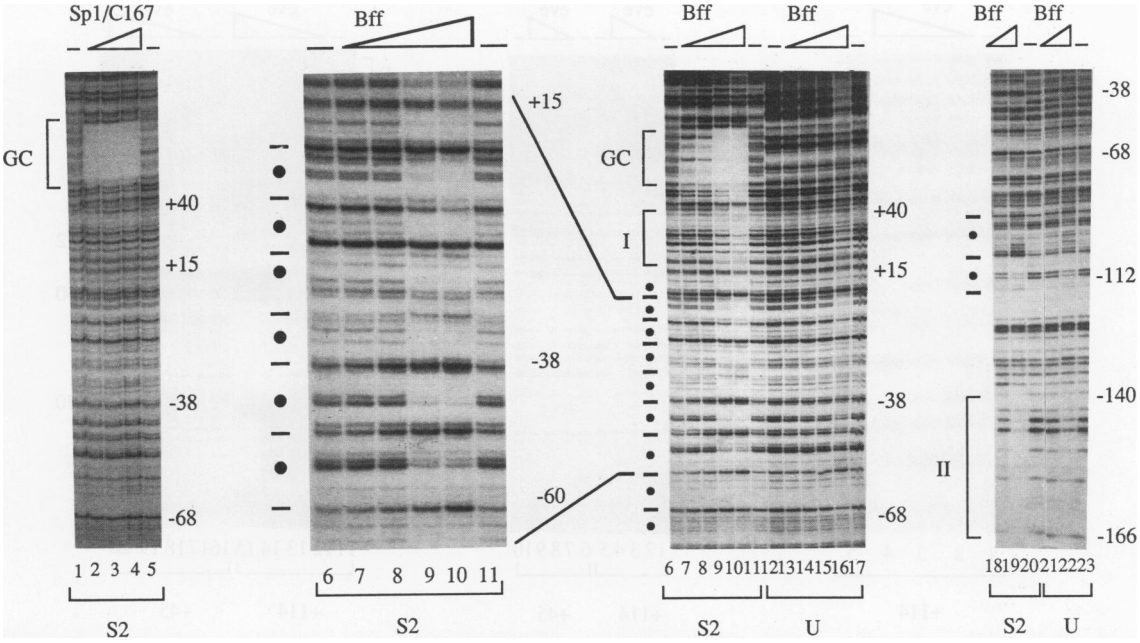

FIG. 5. Binding of the *eve* homeodomain to activator elements. DNase I footprint analysis of Sp1/C167 and Bff proteins binding to DNAs U and S2. Binding reactions were carried out with *Not*I-*Eco*RI fragments of either pU (516 bp) (lanes 12 to 17 and 21 to 23) or pS2 (560 bp) (lanes 1 to 11 and 18 to 20) that were 5′ labeled at the *Not*I site. The footprint patterns obtained with 2, 10, and 40 U of Sp1/C167 (lanes 2, 3, and 4, respectively) are shown. The digestion patterns given by 1 U (lanes 7 and 13), 3 U (lanes 8 and 14), 9 U (lanes 9, 15, 18, and 21), and 28 U (lanes 10, 16, 19, and 22) of Bff protein are also presented. One unit is defined as the amount of protein which just protects the GC box sequences present in 3 fmol of S2 DNA. Control digestion patterns observed in the absence of added DNA binding protein are shown in lanes marked −. The positions of the GC box sequences in probe S2 are indicated together with the locations of sites I and II. DNase I-hypersensitive sites (-) and protections (•) in the presumed looped DNA are marked. The leftmost panel provides a detail of lanes 6 to 11 which shows the region of periodic protection and hypersensitivity more clearly. The nucleotide positions relative to the RNA start site are indicated to the right of the panels.

homeodomain directly contacts 8 bp. DNase I footprinting would show a protection of at least 10 to 15 bp if the protein were actually bound to these sequences.)

The protection of sites I and II suggests one way by which the homeodomain could repress transcription, as these sequences are also known to be bound by activator proteins (5, 6). Thus, the homeodomain may exclude binding of activators to these sequences. As an important control, Fig. 5 shows that Bff, when at a concentration which strongly protects sites I and II on DNA pS2, does not bind these sequences on a DNA lacking the GC boxes (pU) or cause the 5-bp alternating cleavage pattern which is indicative of looped DNA (Fig. 5; compare lanes 9 and 15 and lanes 18 and 21). This finding strengthens the correlation between the DNA binding and transcriptional repressing activities of Bff, as repression by Bff is also dependent on the presence of GC box sequences (Fig. 4). The requirement for binding to the GC boxes in order to obtain binding to sites I and II could result from a single Bff molecule simultaneously binding to both sets of elements, since this protein contains two different DNA binding domains. Alternatively, it could result from cooperative interaction between different molecules of Bff bound at separate sites. In either case, the observed changes in DNase I cleavage and the cooperative binding at a distance are best explained by a looped structure which brings the two promoter regions together.

***eve* binds distant sites cooperatively.** Previously we have shown that *eve* represses *Ubx* transcription in a manner dependent upon the presence of natural high-affinity homeodomain binding sites, which lie between nucleotides +45 and +90 in the wild-type *Ubx* promoter (element B in Fig. 9A) (7). The data presented above suggest that repression involves not only binding to element B but also binding to sites I and II. However, in these experiments, unnatural chimeric proteins, containing the *eve* homeodomain fused to the Sp1 Zn fingers, were used. To establish the importance of binding to sites I and II vis-à-vis a bona fide mechanism by which *eve* protein represses transcription, it is crucial to test the DNA binding properties of native *eve* protein.

When *eve* protein is incubated with a DNA that contains element B (Δ3′+114), *eve* not only binds this high-affinity site but also binds sites I and II and induces the same periodic cleavage pattern between sites I and II as does Bff (Fig. 6, lanes 4 and 14). Again, an enlarged photograph is provided in Fig. 6 to show this cleavage pattern. The same concentration of *eve*, however, does not cause these protections on a DNA lacking element B (Δ3′+45) (Fig. 6, lanes 9 and 19). At higher concentrations of protein, sites I and II are bound on the Δ3′+45 DNA also (Fig. 6, lanes 15 and 20) (56), indicating that these two sites have a lower binding affinity than does element B. Comparison of the binding results for DNAs Δ3′+114 and Δ3′+45 indicates that, as predicted, binding of *eve* to sites I and II is dependent upon binding to element B. Further, since each *eve* molecule contains only one DNA binding domain, cooperative binding to sites I and II must be due to the recruitment of further *eve* molecules to the DNA by those bound downstream of nucleotide +45. The data also predict that if *eve* protein is added to in vitro transcription reactions at concentrations higher than those used in the original experiments, it should

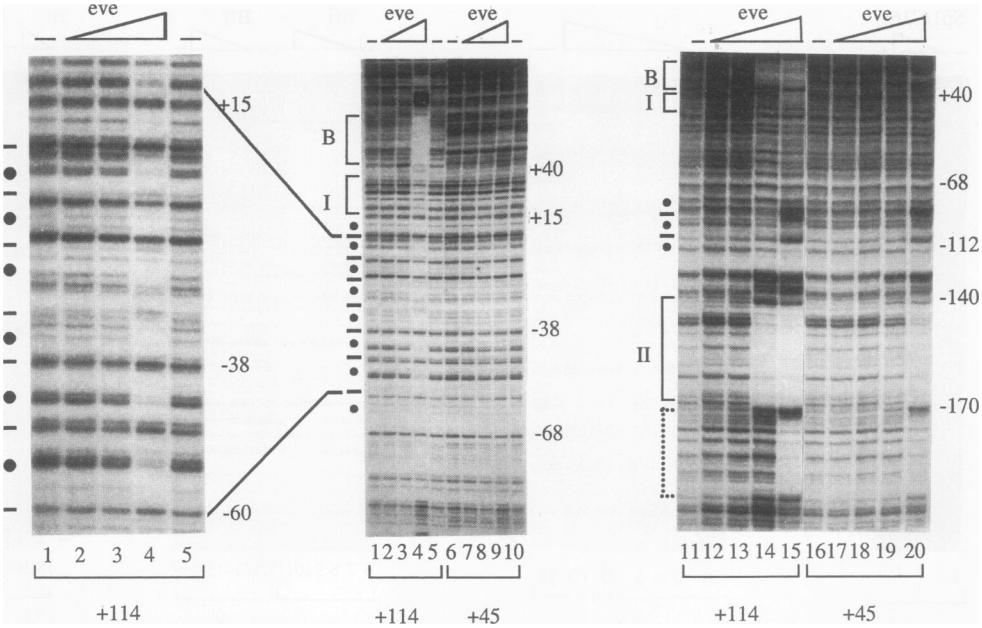

FIG. 6. Evidence that *eve* binds distant sites cooperatively. Shown is DNase I footprinting analysis of *eve* binding to a DNA fragment derived from pUbx Δ3′+114, which contains high-affinity binding sites (lanes 1 to 5 and 11 to 15), or from pUbx Δ3′+45, which lacks these sites (lanes 6 to 10 and 16 to 20). The footprinting patterns obtained with 0.8 ng (lanes 2, 7, 12, and 17), 2.5 ng (lanes 3, 8, 13, and 18), 8 ng (lanes 4, 9, 14, and 19), and 25 ng (lanes 15 to 20) of *eve* protein are shown. The high-affinity *eve* binding sites in DNA Δ3′+114 are indicated (B) together with the two low-affinity sites I and II. Other labeling is as in Fig. 5. A detail of lanes 1 to 5 (leftmost panel) shows the periodic cleavage pattern discussed in the text.

be able to repress *Ubx* promoters lacking element B. The predicted repression of Δ3′+45 transcription has been observed when *eve* protein is present at a concentration only fourfold higher than usual (2). These data further increase the correlation between repression and binding to sites I and II.

Interaction between *eve* and activator proteins. One possible mechanism for repression by *eve* protein bound at the low-affinity sites is that *eve* competes with activator factors at these sites. Site I is an activator element bound by a protein that has not yet been characterized (6). Site II overlaps binding sites for the activator factors *zeste* (*zeste* site 5 [Z5]) and GAGA (GAGA site 3 [G3]) (5, 6). Competition between *eve* protein and either one or both of these factors could contribute significantly to repression, as deletion of the Z5 region reduces transcription in vitro twofold, and deletion of both Z5 and G3 reduce transcription to 17% of normal levels (6).

To investigate the possibility of competition between *eve*, *zeste*, and GAGA proteins at site II, DNase I footprinting was performed with all three proteins. GAGA and *zeste* proteins were purified from a *Drosophila* embryo extract by affinity column chromatography. These two activator proteins were incubated with *Ubx* promoter DNA either singly (Fig. 7, lanes 2 and 8) or together with *eve* protein (Fig. 7, lanes 3, 4, 6, and 7). Comparison of the *zeste* and GAGA footprints with the *eve* protection at site II (Fig. 7, lane 5) shows that the Z5 and G3 protections overlap site II. The order of *zeste* and *eve* protein addition to the binding reaction appears to affect the ability of *zeste* to bind to Z5. Binding of *zeste* protein to the other *zeste* footprint sites is unchanged. The fact that sequences protected by *zeste* protein at Z5 are wholly contained within sequences protected by *eve* protein makes it difficult to determine unam-

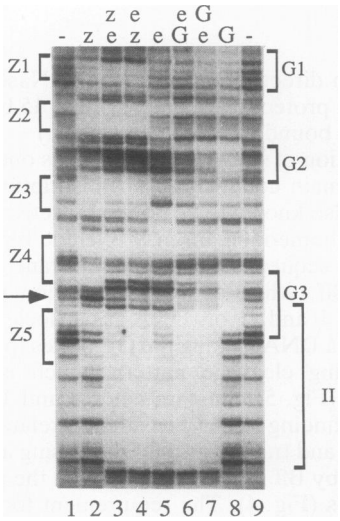

FIG. 7. Evidence that *eve*, *zeste*, and GAGA proteins each bind overlapping regions of the *Ubx* promoter. Shown is DNase I footprint analysis of purified *eve*, *zeste*, and GAGA proteins bound to a *Ubx* promoter sequence between nucleotides −22 and −170. The order of protein addition in lanes containing two proteins is indicated by the letters above the lanes. The top letter indicates the first protein added to the binding reaction; the bottom letter indicates the second. z, *zeste* protein; e, *eve* protein; G, GAGA protein. Lanes: 1 and 9, no protein; 2, 10 ng of *zeste*; 3 and 4, 10 ng of *zeste* and 8 ng of *eve*; 5, 8 ng of *eve*; 6 and 7, 13 ng of GAGA and 8 ng of *eve*; 8, 13 ng of GAGA. Brackets indicate the characterized footprints of each protein (Fig. 6) (5, 6). An arrow indicates the position of the hypersensitive site associated with *zeste* protein binding to site Z5.

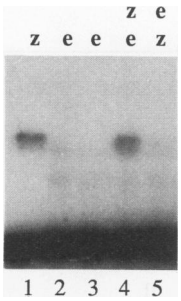

FIG. 8. Competition of *eve* and *zeste* proteins for DNA binding. *zeste* and *eve* proteins were bound to a short DNA probe containing only site II and Z5 from the *Ubx* promoter, cross-linked to the probe by UV irradiation, and separated on an SDS-polyacrylamide gel. The order of protein addition is indicated as in Fig. 7. Lanes: 1, 5 ng of *zeste*; 2, 37 ng of *eve*; 3, 100 ng of *eve*; 4 and 5, 37 ng of *eve* and 5 ng of *zeste*.

biguously whether competition is occurring. However, *zeste* protein binding alone (Fig. 7, lane 3) causes the appearance of a hypersensitive site at the edge of the Z5 footprint region (Fig. 7, lane 2, indicated by an arrow) which is completely absent when *eve* protein is bound alone (Fig. 7, lane 5). The presence of *zeste* protein at this site can be inferred (but not proven) by examining this hypersensitive site. When *zeste* protein is added to the binding reaction before *eve* protein (Fig. 7, lane 3), this hypersensitive band is still apparent but is much weaker. Addition of *eve* protein before *zeste* protein completely abolishes this hypersensitive band (Fig. 7, lane 4). This result seems to indicate that *eve* protein is able to reduce *zeste* protein binding when added to a reaction containing *zeste* protein and that *zeste* protein is unable to bind when *eve* protein is already bound to site II. The result for GAGA protein is inconclusive from this experiment (Fig. 7, lanes 6 to 8), since the *eve* and GAGA protections overlap, and there is no comparable hypersensitive site by which to judge GAGA binding.

A more clear-cut demonstration that *eve* and *zeste* proteins compete for binding to site II was obtained by using a modified polyacrylamide gel mobility shift assay. The conditions of the standard mobility shift assay were unsuitable for analysis of these proteins, as the protein-DNA complexes dissociated inside a nondenaturing gel (60). To solve this problem, binding reactions were subjected to UV irradiation after incubation, to create covalent bonds between protein and DNA. Covalent complexes were then separated by SDS-polyacrylamide gel electrophoresis. For these experiments, *zeste* and *eve* proteins were incubated with a short DNA probe containing nucleotides −172 to −126 which includes only site II and the Z5 binding site. *zeste* protein cross-links very strongly to the DNA probe (Fig. 8, lane 1), but *eve* protein cross-links only weakly (Fig. 8, lanes 2 and 3). *eve* is known to bind to this sequence under these conditions (see above); therefore, the low *eve* signal in this experiment is most likely caused by a low UV cross-linking efficiency. The degree of *zeste* binding in the presence of *eve* has been assessed by order-of-addition experiments. When *zeste* protein is bound first, followed by *eve* protein, *zeste* is cross-linked to the probe but at a lower level than when *zeste* protein is bound alone (Fig. 8; compare lanes 4 and 1). When *eve* protein is bound first (Fig. 8, lane 5), *zeste* protein is apparently unable to bind. As a control, *zeste* protein was also cross-linked to an oligonucleotide containing a different *zeste* binding site from the *Ubx* proximal promoter (nucleotides −61 to −79). Cross-linking of *zeste* was not reduced when *eve* protein was added first to this binding reaction (60), indicating that competition requires a specific *eve* binding site. These results agree with the footprint data, indicating that there is competition between *eve* and *zeste* proteins for binding at site II. The ability of *zeste* protein to remain bound after *eve* protein addition indicates that *zeste* protein has a slow dissociation rate.

DISCUSSION

Silencing of transcription by proteins bound at a distance from the activators which they inhibit is an important means of transcriptional control in eukaryotes (10, 26, 29, 49, 62). We have demonstrated that *eve* protein contains domains that silence *Ubx* transcription when bound up to 1.5 kb upstream of promoter-proximal activator proteins. Three distinct regions of *eve* contribute to active repression, and they appear to act by two different mechanisms. One mechanism involves cooperative binding at a distance between *eve* molecules bound at high- and low-affinity DNA sites. The second mechanism requires a repression domain, which we suggest may interact with the general transcription factors to inhibit their function.

Repression by cooperative binding at a distance. To identify regions of *eve* required for repression but not for initially targeting *eve* protein to the promoter, different portions of *eve* were fused to the DNA binding domain of the Sp1 transcription factor. These chimeric proteins were then assayed in tissue culture cell and in vitro transcription assays. Surprisingly, this analysis indicated that the *eve* DNA binding domain, a homeodomain, acted in repression in a manner dependent upon the fusion protein being bound to the promoter via the Sp1 Zn fingers. Repression was correlated with binding of the homeodomain to two previously uncharacterized low-affinity sites located between nucleotides +43 and +18 (site I) and between nucleotides −140 and −170 (site II) on the *Ubx* proximal promoter (Fig. 9A). In an earlier report, it was shown that repression of *Ubx* by intact *eve* protein (i.e., not a fusion protein) required a high-affinity site between nucleotides +45 and +90 (element B; Fig. 9A) (7). This result indicates that sites I and II are not sufficient to target *eve* protein to the promoter at low protein concentrations. Here, DNA binding experiments have been used to show that binding of intact *eve* protein to sites I and II was dependent upon binding of other *eve* molecules to the higher-affinity element B, as was transcriptional repression. Such cooperative binding explains how *eve* could repress via binding to sites I and II yet require element B for repression. These experiments also demonstrated that cooperative interaction can occur between molecules bound to distant elements, since site II is separated by over 150 bp from the other *eve* DNA binding sites. The data suggest that the DNA between *eve* molecules is looped out (Fig. 9A), as binding of *eve* protein alters the DNase I cleavage pattern between site I and II in a manner indicative of bent DNA (16, 38, 48).

Sequences within site I are essential for *Ubx* transcription in vitro (6), and site II overlaps sequences bound by the *zeste* and GAGA transcriptional activator proteins (Fig. 7 and 9A) (5, 6, 41). *eve* protein bound at sites I and II could repress by competitively inhibiting binding of activators to these sites. This has been shown to occur in the case of site II, where *eve* protein prevents the binding of *zeste* protein. This *zeste* site is known to be important for transcriptional activation, as deletion of the sequences between −154 and −146 removes

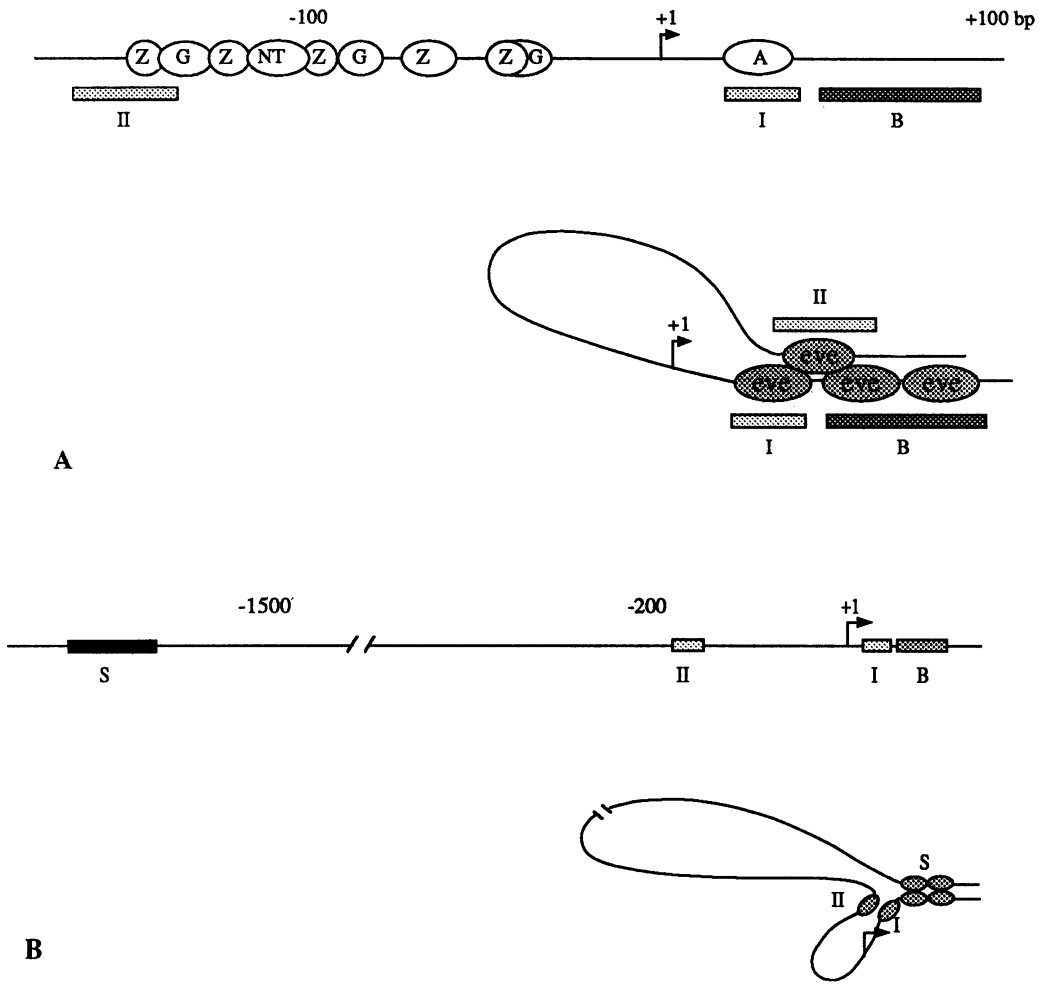

FIG. 9. A mechanism by which *eve* silences transcription. (A) Diagram comparing the binding of activators and *eve* to the *Ubx* proximal promoter. In the upper half, the positions of activator proteins *zeste* (Z), GAGA (G), and NTF (NT) and of the downstream activator factor (A) are indicated by open ellipses. The higher-affinity *eve* binding site (B) and the two low-affinity *eve* binding sites (I and II) are marked by shaded boxes. The lower half shows the proposed structure resulting from the cooperative interaction of *eve* bound to sites B, I, and II. The scale is in base pairs. Note that the precise number of *eve* molecules bound at each site remains to be determined, and it cannot be ruled out that the DNA is wrapped around *eve* molecules. (B) A model for silencing of the *Ubx* proximal promoter by *eve*. The upper half shows the *eve* DNA binding sites at the *Ubx* proximal promoter (B, I, and II) together with a further site (S) over 1,500 bp further upstream. We propose that the silencer element S may be the highest-affinity *eve* DNA binding site and serves to target *eve* to the promoter. *eve* bound at this distant site directs binding of *eve* to sites B, I, and II by looping of DNA and cooperative interaction. The proposed resulting structure is presented in the lower half. A prediction of this model is that if *eve* is initially targeted to promoter sequences well upstream of the RNA start site, it may finally repress via *eve* molecules bound downstream of the RNA start site.

the *zeste* consensus sequence, but not the adjacent GAGA site, and reduces *Ubx* transcription in vitro twofold (see Fig. 3 in reference 6). Also, biochemical and genetic experiments suggest that *zeste* is the only activator protein which acts at this site (5, 41). Therefore, inhibition of *zeste* protein binding accounts in part for the repression caused by *eve*. However, it may also be possible that the bending of DNA itself plays a role in transcriptional repression, as the proposed loop includes other sites bound by *zeste* and GAGA proteins, an NTF-1 activation element, and the RNA start site (Fig. 9A). It is even possible that protein-DNA complex forms a stable structure that prevents elongation by RNA polymerase (see below).

eve protein represses transcription when bound upstream of nucleotide −200 or downstream of nucleotide +45 (this report; 7), and *eve*-Sp1 fusions silence transcription when bound at −1.9 kb. We assume that the mechanisms of repression will be essentially the same for intact *eve* and for the chimeric proteins at all distances. Since *eve* protein can cooperatively bind to sites separated by over 150 bp, we suggest *eve* will be able to silence transcription by a similar mechanism when bound up to several kilobases upstream of the RNA start site (Fig. 9B). Interestingly, genetic and molecular data suggest that the homeodomain proteins *abdominal A*, *Abdominal B*, *engrailed*, and *eve* repress *Ubx* transcription via a regulatory element located 14 kb upstream of the *Ubx* RNA start site (11, 46, 49, 58). Though it is not certain which proteins bind directly to this DNA element in the embryo, we suggest that some of these homeodomain proteins may silence *Ubx* by the mechanism shown in Fig. 9B.

Repression in other systems. Repression by *eve* shares

features with those eukaryotic repressors which do not act at a distance but instead act by binding directly to sequences recognized by activator proteins (35, 51, 54). The difference is that the initial interaction of *eve* protein with promoters is proposed to occur at distant high-affinity sites. Competitive inhibition then occurs at secondary, low-affinity sites. Parallels also exist between the model shown in Fig. 9 and prokaryotic repressors. Studies of prokaryotic repressors first established that action at a distance can occur by interaction between proteins bound at distant sites, with the intervening DNA being looped out (18, 23, 43, 45). Repression of the galactose promoter resembles repression by *eve* in that the galactose repressor also binds both upstream and downstream of the RNA start site. In the case of the galactose repressor, protein binding to both regions forms a loop that encompasses the RNA initiation site. The loop structure formed by the galactose repressor is believed to prevent elongation by RNA polymerase (45).

Another model for silencing in eukaryotes comes from studies in yeast cells. Repression of several genes, including *HMR***a** and *HML*α, correlates with altered nucleosome positioning (50, 55). Since nucleosomes appear to repress transcription in vitro and in vivo (reviewed in reference 20), the stabilizing of nucleosomes across a promoter region may repress transcription. Interestingly, a silencer-binding protein involved in repression of *HML*α, called RAP1, has been proposed to organize higher-order chromatin structure on the basis of its ability to cause DNA loop formation (25). However, since RAP1 can induce DNA loops, it may be that it also represses transcription by the mechanisms proposed in Fig. 9. Although these two models may appear very different, they are not mutually exclusive. Perhaps tertiary protein-DNA structures caused by repressors such as RAP1 and *eve* are further stabilized in cells by nucleosomes and other chromatin-organizing proteins.

A second mechanism by which *eve* silences transcription. A fusion protein containing the C-terminal 236 amino acids of *eve* joined to the Sp1 Zn fingers (termed CDff) represses transcription in tissue culture cells and in vitro (Fig. 2 and 4). Since this protein lacks the homeodomain and does not bind sites I and II (2), it cannot repress by the mechanism shown in Fig. 9. One possibility is that the fusion protein directly contacts other transcription factors to inhibit their action, perhaps by looping out the DNA between these factors and the fusion protein. Since both *eve* protein and CDff repress transcription when bound upstream from a promoter which contains only a TATA box element (2, 9), these other proteins may be the general transcription factors.

A portion of the *Krüppel* and *engrailed* proteins can also convert heterologous DNA binding domains into active repressors, although the mechanism of repression is not known in these cases (28, 42). On the basis of the presence of a large number of alanine residues in this region, it has been suggested that the *Krüppel* domain may be homologous to *eve* amino acids 146 to 177 (42). This portion of *eve* lies within region C (Fig. 2), which is only one of three regions of *eve* contributing to repression in our experiments. The relationship between this homologous sequence and repression by *eve* is being investigated.

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REFERENCES

1. **Adler, S., M. L. Waterman, X. He, and M. G. Rosenfeld.** 1988. Steroid receptor-mediated inhibition of rat prolactin gene expression does not require the receptor DNA-binding domain. Cell **52**:685–695.
2. **Austin, R. J., and M. D. Biggin.** Unpublished data.
3. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61**:49–59.
4. **Biggin, M. D.** Unpublished data.
5. **Biggin, M. D., S. Bickel, M. Benson, V. Pirrotta, and R. Tjian.** 1988. *Zeste* encodes a sequence-specific transcription factor that activates the *Ultrabithorax* promoter *in vitro*. Cell **55**:713–722.
6. **Biggin, M. D., and R. Tjian.** 1988. Transcription factors that activate the *Ultrabithorax* promoter in developmentally staged extracts. Cell **53**:699–711.
7. **Biggin, M. D., and R. Tjian.** 1989. A purified *Drosophila* homeodomain protein represses transcription *in vitro*. Cell **58**:433–440.
8. **Biggin, M. D., and R. Tjian.** 1989. Transcription factors and the control of *Drosophila* development. Trends Genet **5**:377–383.
9. **Biggin, M. D., and R. Tjian.** Unpublished data.
10. **Brand, A. H., G. Micklem, and K. A. Nasmyth.** 1987. A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell **51**:709–721.
11. **Christen, B., and M. Bienz.** 1992. A *cis*-element mediating *Ultrabithorax* autoregulation in the central nervous system. Mech. Dev. **39**:73–80.
12. **Courey, A. J., D. A. Holtzman, S. P. Jackson, and R. Tjian.** 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell **59**:827–836.
13. **Courey, A. J., and R. Tjian.** 1988. Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine rich activation motif. Cell **55**:887–898.
14. **Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. R. Yamamoto.** 1990. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science **249**:1266–1272.
15. **DiNocera, P. P., and I. B. Dawid.** 1983. Transient expression of genes introduced into cultured cells of Drosophila. Proc. Natl. Acad. Sci. USA **80**:7095–7098.
16. **Drew, H. R., and A. A. Travers.** 1985. DNA bending and its relation to nucleosome positioning. J. Mol. Biol. **186**:773–790.
17. **Driever, W., and C. Nüsslein-Volhard.** 1989. The *bicoid* protein is a positive regular of hunchback transcription in the early *Drosophila* embryo. Nature (London) **337**:138–143.
18. **Dunn, T. M., S. Hahn, S. Ogden, and R. F. Schleif.** 1984. An operator at −280 base pairs that is required for repression of araBAD operon promoter: addition of DNA helical turns between the operator and promoter cyclically hinders repression. Proc. Natl. Acad. Sci. USA **81**:5017–5020.
19. **Ghosh, S., and D. Baltimore.** 1990. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **344**: 678–682.
20. **Grunstein, M.** 1990. Nucleosomes: regulators of transcription. Trends Genet. **6**:395–400.
21. **Han, K., M. S. Levine, and J. L. Manley.** 1989. Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. Cell **56**:573–583.
22. **Hayashi, S., and M. P. Scott.** 1990. What determines the specificity of action of *Drosophila* homeodomain proteins. Cell **63**:883–894.
23. **Hochschild, A., and M. Ptashne.** 1986. Cooperative binding of λ repressors to sites separated by integral turns of the DNA helix. Cell **44**:681–687.
24. **Hoey, T., and M. Levine.** 1988. Divergent homeo box proteins

recognize similar DNA sequences in *Drosophila*. Nature (London) **332**:858–861.
25. **Hofmann, J. F.-X., T. Laroche, A. H. Brand, and S. M. Gasser.** 1989. RAP-1 factor is necessary for DNA loop formation in vitro at the silent mating type locus *HML*. Cell **57**:725–737.
26. **Ip, T. Y., R. Kraut, M. Levine, and C. A. Rushlow.** 1991. The *dorsal* morphogen is a sequence-specific DNA binding protein that interacts with a long range repression element in *Drosophila*. Cell **64**:439–446.
27. **Jaynes, J. B., and P. H. O'Farrell.** 1988. Activation and repression of transcription by homeodomain-containing proteins that bind a common site. Nature (London) **336**:744–749.
28. **Jaynes, J. B., and P. H. O'Farrell.** 1991. Active repression of transcription by the *engrailed* homeodomain protein. EMBO J. **10**:1427–1433.
29. **Johnson, A. D., and I. Herskowitz.** 1985. A repressor (MAT α2 product) and its operator control expression of a set of cell type specific genes in yeast. Cell **42**:237–247.
30. **Johnson, F. B., and M. A. Krasnow.** 1990. Stimulation of transcription by an *Ultrabithorax* protein in vitro. Genes Dev. **4**:1044–1052.
31. **Johnston, S. A., J. M. Salmeron, Jr., and S. S. Dincher.** 1987. Interaction of a positive and negative regulatory proteins in the galactose regulon of yeast. Cell **50**:143–146.
32. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor SP1 and functional analysis of the DNA binding domain. Cell **51**:1079–1090.
33. **Kadonaga, J. T., A. J. Courey, J. Ladika, and R. Tijan.** 1988. Distinct regions of SP1 modulate DNA binding and transcriptional activation. Science **242**:1566–1570.
34. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. (USA) **83**:5889–5893.
35. **Kaufman, P. D., and D. C. Rio.** 1991. *Drosophila* P-element transposase is a transcriptional repressor *in vitro*. Proc. Natl. Acad. Sci. USA **88**:2613–2617.
36. **Keleher, C. A., C. Goutte, and A. D. Johnson.** 1988. The yeast cell-type-specific repressor α2 acts cooperatively with a non-cell-type-specific protein. Cell **53**:927–936.
37. **Kissinger, C. R., B. Liu, E. Martin-Blanco, T. B. Kornberg, and C. O. Pabo.** 1990. Crystal structure of an *engrailed* homeodomain-DNA complex at 2.8 Å resolution: a framework for understanding homeodomain-DNA interactions. Cell **63**:579–590.
38. **Knight, J. D., R. Li, and M. Botchan.** 1991. The activation domain of the bovine papillomavirus E2 protein mediates association of DNA-bound dimers to form DNA loops. Proc. Natl. Acad. Sci. USA **88**:3204–3208.
39. **Krasnow, M. A., E. E. Saffman, K. Kornfeld, and D. S. Hogness.** 1989. Transcriptional activation and repression by *Ultrabithorax* proteins in cultured Drosophila cells. Cell **57**:1031–1043.
40. **Kurtz, S., and D. Shore.** 1991. RAP1 protein activates and silences transcription of mating type genes in yeast. Genes Dev. **5**:616–628.
41. **Laney, J. D., and M. D. Biggin.** 1992. *zeste*, a nonessential gene, potently activates transcription of *Ultrabithorax* in Drosophila embryos. Genes Dev. **6**:1531–1541.
42. **Licht, J. D., M. J. Grossel, J. Figge, and U. M. Hansen.** 1990. Drosophila *Krüppel* protein is a transcriptional repressor. Nature (London) **346**:76–79.
43. **Lobell, R. B., and R. F. Schleif.** 1990. DNA looping and unlooping by Ara C protein. Science **250**:528–532.
44. **Ma, J., and M. Ptashne.** 1987. The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80. Cell **50**:137–142.
45. **Mandal, N., W. Su, R. Haber, S. Adhya, and H. Echols.** 1990. DNA looping in cellular repression of transcription of the galactose operon. Genes Dev. **4**:410–418.
46. **Martinez-Arias, A., and R. A. H. White.** 1988. *Ultrabithorax* and *engrailed* expression in *Drosophila* embryos mutant for segmentation genes of the pair-rule class. Development **102**:325–338.
47. **Mitchell, P. J., C. Wang, and R. Tjian.** 1987. Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50**:847–861.
48. **Morrison, A., and N. R. Cozzarelli.** 1981. Contacts between DNA gyrase and its binding site on DNA: features of symmetry and asymmetry revealed by protection from nucleases. Proc. Natl. Acad. Sci. USA **78**:1416–1420.
49. **Müller, J., and M. Bienz.** 1991. Long range expression conferring boundaries of *Ultrabithorax* expression in the *Drosophila* embryo. EMBO J. **10**:3147–3155.
50. **Nasmyth, K. A.** 1982. The regulation of yeast mating-type chromatin structure by *SIR*: an action at a distance affecting both transcription and transposition. Cell **30**:567–578.
51. **Ohkuma, Y., M. Horikoshi, R. G. Roeder, and C. Desplan.** 1990. Binding site-dependent direct activation and repression of in vitro transcription by Drosophila homeodomain proteins. Cell **61**:475–484.
52. **Ptashne, M.** 1986. Gene regulation by proteins acting nearby and at a distance. Nature (London) **322**:697–701.
53. **Rhodes, D., and A. Klug.** 1980. Helical periodicity of NDA determined by enzyme digestion. Nature (London) **286**:573–578.
54. **Rio, D. C., and R. Tjian.** 1983. SV40 T antigen binding site mutations that affect autoregulation. Cell **32**:1227–1240.
55. **Roth, S. Y., M. Shimizu, L. Johnson, M. Grunstein, and R. T. Simpson.** 1992. Stable nucleosome positioning and complete repression by the yeast α2 repressor are disrupted by amino-terminal mutations in histone H4. Genes Dev. **6**:411–425.
56. **Schneider, I.** 1972. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. J. Embryol. Exp. Morphol. **27**:353–365.
57. **Scott, M. P., J. W. Tamkum, and G. W. Hartzell III.** 1989. The structure and function of the homeodomain. Biochim. Biophys. Acta **989**:25–48.
58. **Struhl, G., and R. H. A. White.** 1985. Regulation of the *Ultrabithorax* gene of *Drosophila* by other bithorax complex genes. Cell **43**:507–519.
59. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. J. Mol. Biol. **189**:113–130.
60. **TenHarmsel, A., and M. D. Biggin.** Unpublished data.
61. **Thali, M., M. M. Muller, M. DeLorenzi, P. Matthias, and M. Bienz.** 1988. *Drosophila* homeotic genes encode transcriptional activators similar to OTF-2. Nature (London) **336**:598–601.
62. **Winoto, A., and D. Baltimore.** 1989. αβ lineage-specific expression of the α T cell receptor gene by nearby silencers. Cell **59**:649–655.
63. **Winslow, G. M., S. Hayashi, M. Krasnow, D. S. Hogness, and M. P. Scott.** 1989. Transcriptional activation by the *Antennapedia* and *fushi-tarazu* proteins in cultured *Drosophila* cells. Cell **57**:1071–1030.
64. **Zuo, P., D. Stanojevic, J. Colgan, K. Han, M. Levine, and J. L. Manley.** 1991. Activation and repression of transcription by the gap proteins *hunchback* and *Krüppel* in cultured *Drosophila* cells. Genes Dev. **5**:254–264.