

Two homeo domain proteins bind with similar specificity to a wide range of DNA sites in *Drosophila* embryos

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We have used *in vivo* UV cross-linking to directly measure DNA binding by the homeo domain proteins *even-skipped* (*eve*) and *fushi tarazu* (*ftz*) in *Drosophila* embryos. Strikingly, these two proteins bind at uniformly high levels throughout the length of their genetically identified target genes and at lower, but significant, levels to genes that they are not expected to regulate. The data also suggest that these two proteins have very similar DNA-binding specificities *in vivo*. In contrast, a non-homeo domain transcription factor, *zeste*, is only detected on short DNA elements within a target promoter and not on other genes. These results are consistent with the *in vitro* properties of these various proteins, their respective concentrations in the nucleus, and with earlier predictions of how transcription factors bind DNA *in vivo*. We propose that these data favor the model that *eve*, *ftz*, and closely related homeo domain proteins act by directly regulating mostly the same target genes.

[Key Words: *even-skipped*; *fushi tarazu*; *zeste*; *in vivo* cross-linking; homeo domain proteins]

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In *Drosophila*, the segmentation and homeotic genes play a key role in establishing the body plan of the embryo (Garcia-Bellido 1975; Lewis 1978; Nüsslein-Volhard and Wieschaus 1980; Akam 1987). The segmentation genes are transcribed first, and one of their functions is to initiate the patterns of homeotic gene expression. Both the segmentation and homeotic genes appear to direct morphogenesis by regulating the expression of a great many other genes, some of which have been identified (Garcia-Bellido 1975; Cohen 1990; Immerglück et al. 1990; Gould and White 1992; Vachon et al. 1992; O'Hara et al. 1993; Capovilla et al. 1994).

The homeotic genes and several of the segmentation genes encode proteins that contain a highly conserved DNA-binding domain termed the homeo domain (McGinnis et al. 1984; Scott and Weiner 1984; Desplan et al. 1985). The segmentation proteins *even-skipped* (*eve*), *engrailed*, and *fushi tarazu* (*ftz*) and all the homeotic proteins have a glutamine at position 50 of the homeo domain (Q50 homeo domain proteins), and these proteins bind with similar affinities to the same wide range of specific DNA-binding sites (Desplan et al. 1988; Hoey and Levine 1988; Laughon et al. 1988; Biggin and Tjian 1989a; Hanes and Brent 1989; Treisman et al. 1989; Appel and Sakonju 1993). Homeo domain proteins have also been shown to regulate transcription in tissue culture cells and in *in vitro* transcription reactions, supporting the model that they act as transcription factors to control development (for review, see Biggin and Tjian 1989b; Hayashi and Scott 1990).

To understand the mechanisms by which Q50 homeo domain proteins control development, it is essential to determine which DNA elements they bind in embryos. Identifying these natural target sequences has been extremely difficult for the following reasons (for discussion, see Biggin and Tjian 1989b; Hayashi and Scott 1990). First, the regulatory hierarchy involving homeo domain proteins is highly complex and possibly quite redundant. Therefore, the power of genetic analysis in predicting which interactions may be direct is severely reduced. Second, because different Q50 homeo domain proteins bind the same sequences *in vitro* and are sometimes coexpressed in the same cells, they may compete for DNA-binding sites *in vivo*, and it is difficult to predict the outcome of such competition. Third, it is possible that in the complex environment of the embryo, the DNA-binding specificities of homeo domain proteins are markedly different from those observed *in vitro*.

The above complexities have made it impossible to distinguish among a range of models for the action of Q50 homeo domain proteins in development (e.g., see Winslow et al. 1989; Hayashi and Scott 1990; Dessain et al. 1992; Ekker et al. 1992; Lawrence 1992). At one extreme, it has been suggested that these proteins may all recognize the same DNA sequences *in vivo*, just as they do *in vitro*, and regulate the same targets. At the other extreme, different Q50 homeo domain proteins may bind highly distinct DNA elements *in vivo* and frequently regulate different target genes. This latter type of model has been proposed to account for observations such as

the following: Loss-of-function mutations in different homeo domain proteins do not always affect expression of the same downstream genes (e.g., Carroll and Scott 1986; Harding et al. 1986; Frasch et al. 1988); also, transgenic experiments reveal that when two Q50 homeo domain genes affect expression of the same downstream gene, their respective effects are often mediated through different regulatory elements (e.g., see Müller and Bienz 1992; Vachon et al. 1992; O'Hara et al. 1993). In this paper we have sought to determine whether DNA binding by Q50 homeo domain proteins conforms to either of the above models or to some intermediate model. To this end, we used in vivo cross-linking to directly measure and compare DNA binding by the two Q50 homeo domain proteins *eve* and *ftz* on numerous genes in living embryos.

Results

To identify bona fide DNA targets of homeo domain proteins, we chose an existing in vivo UV cross-linking assay (Gilmour and Lis 1986; Rougvie 1989) that can distinguish DNA binding by members of a family of proteins that recognize the same sites. In this technique, UV light is used to induce covalent bonds between protein and DNA in vivo; protein-DNA complexes are then isolated by immunoprecipitation and characterized by Southern blotting (Fig. 1). A major strength of this approach is that UV light induces covalent bonds only between protein and nucleic acid residues that are in intimate contact with one another (Hockensmith et al. 1991 and references therein; Blatter et al. 1992). Also, cross-linking is directly proportional to the occupancy of a

protein on its binding site (Hockensmith et al. 1991; Williams and Konigsberg 1991). As a result, it has been possible to use in vivo UV cross-linking to locate binding sites of several proteins in vivo (Gilmour and Lis 1986; Gilmour et al. 1986; Rougvie 1989; Champlin et al. 1991). For example, a paused RNA polymerase II molecule has been mapped to high resolution with UV cross-linking in tissue culture cells (Gilmour and Lis 1986), and the accuracy of this mapping was independently confirmed using in vivo footprinting (Giardina et al. 1992) and mapping of partially transcribed RNAs (Rougvie and Lis 1988).

This method has not been employed previously to study sequence-specific transcription factors, and our preliminary attempts to do so were unsuccessful because these factors cross-link to DNA much less efficiently than RNA polymerase II (J. Walter and M.D. Biggin, unpubl.). We therefore improved the sensitivity of the method by 100-fold so that DNA binding of sequence-specific transcription factors could be detected (see Materials and methods).

zeste binds directly to the endogenous *Ubx* gene in embryos

To verify that this modified UV cross-linking method also detects only specific protein-DNA interactions, we first used it to study DNA binding by the non-homeo domain transcription factor *zeste*. Unlike homeo domain proteins, *zeste* is not a member of a gene family, and its DNA binding is very specific in vitro (Benson and Pirrotta 1987, 1988; Biggin et al. 1988; Laney and Biggin 1992). These properties have made it possible to identify a cluster of five high affinity *zeste* protein-binding sites in the *Ultrabithorax* (*Ubx*) proximal promoter that are very probably bound by *zeste* protein in the embryo (Biggin et al. 1988; Laney and Biggin 1992). The modified cross-linking method was tested by determining whether *zeste* protein could be specifically cross-linked to this cluster of sites.

Embryos in which the *Ubx* gene is actively transcribed and which express high levels of *zeste* protein were UV irradiated, and their chromatin was extracted and restriction digested. Figure 2, lane 7, shows that a 3.5-kb *Ubx* proximal promoter fragment is immunoprecipitated from this chromatin by an affinity-purified anti-*zeste* antibody. Comparison of this signal with a dilution series of the total DNA present in the reaction prior to the addition of antibody (referred to as "total DNA"; Fig. 2, lanes 1–4) shows that the level of recovery is .005%. In the absence of *zeste* antibodies, the yield of promoter fragments is at least 50-fold lower (Fig. 2, lane 8). To demonstrate the specificity of the antibody for the *zeste* protein, cross-linking was carried out on homozygous *z^{ae(bx)}* mutant embryos. These embryos carry an inversion that removes the 3' half of the *zeste* gene, abolishing the epitopes that the antibody recognizes (Benson and Pirrotta 1987; Pirrotta et al. 1987). As expected, no *Ubx* promoter sequences are precipitated from these embryos in the presence of *zeste* antibody (Fig. 2, lane 5). As a

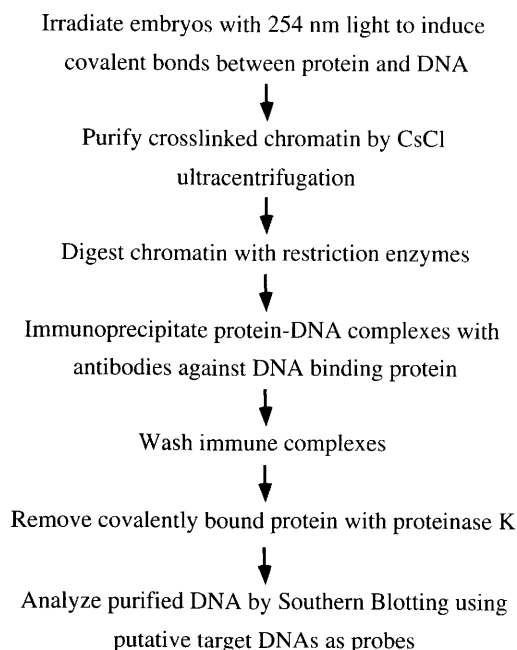


Figure 1. The in vivo UV cross-linking method of Gilmour and Lis (1985, 1986).

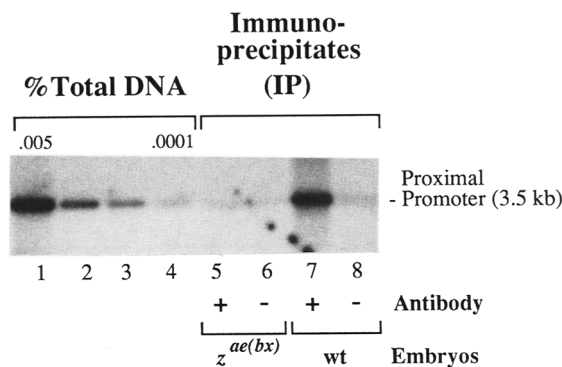


Figure 2. Endogenous *zeste* protein binds to the *Ubx* promoter in living embryos. Southern blotting shows the results of a UV cross-linking experiment carried out on 8- to 10-hr-old embryos (for details, see Materials and methods). Wild-type (lanes 7,8) or homozygous *z^{ae(bx)}* mutant (lanes 5,6) embryos were irradiated with UV light to induce protein-DNA cross-linking, and their chromatin was extracted and purified by CsCl density ultracentrifugation. Chromatin was digested with *Eco*RI, and 450- μ g aliquots were immunoprecipitated with affinity-purified anti-*zeste* antibody (lanes 5,7) or mock-precipitated in the absence of antibody (lanes 6,8). Protein-DNA complexes were isolated using fixed *Staphylococcus aureus* (Staph A) cells and washed extensively. The complexes were then eluted from the Staph A cells and digested with proteinase K, and the purified DNA was separated on a 0.7% agarose gel. The DNA was analyzed by Southern blotting using a radioactive probe generated by random priming of a 3.5-kb *Eco*RI *Ubx* promoter fragment [genomic clone 3102; Bender et al. 1983]. For quantitation, lanes 1–4 contain .005%, .001%, .0005%, and .0001%, respectively, of the total DNA present in a single immunoprecipitation reaction prior to the addition of antibody (total DNA).

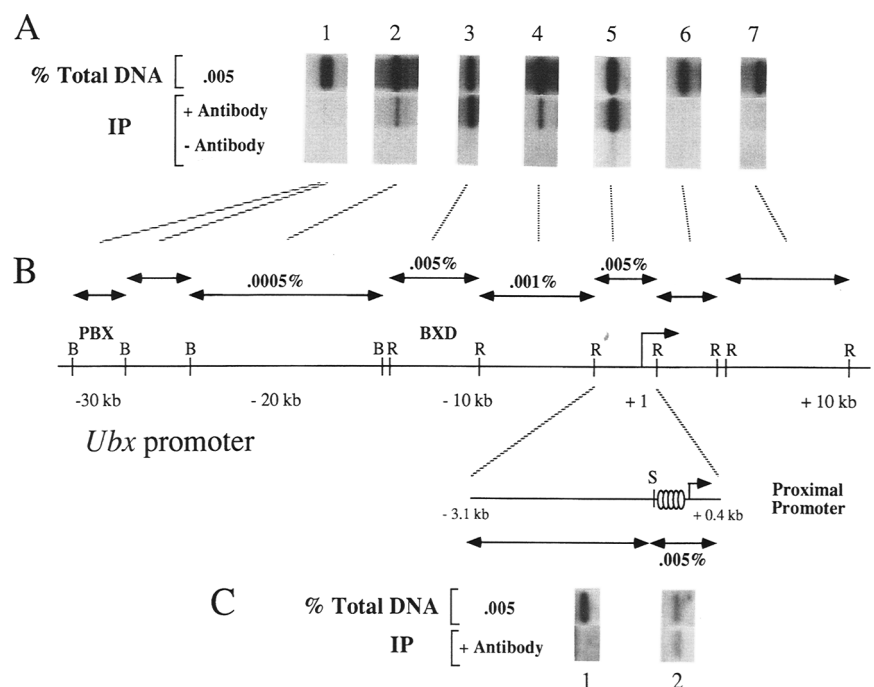
control, it was determined that cross-linking by RNA polymerase II to the *actin 5C* gene was unaffected in these embryos (data not shown). These results demonstrate that endogenous *zeste* protein interacts directly with the *Ubx* promoter.

To map *zeste* cross-linking further within the 3.5-kb *Ubx* proximal promoter fragment, chromatin from wild-type embryos was digested with both *Eco*RI and *Stu*I. This separates the promoter into a 1-kb fragment surrounding the RNA start site, which includes the cluster of five *zeste* protein-binding sites, and a 2.5-kb fragment

lacking *zeste*-binding sites (see expanded diagram of the proximal promoter shown in Fig. 3B; see also Benson and Pirrotta 1987). The 1-kb fragment is precipitated just as efficiently as the entire 3.5-kb *Eco*RI fragment (Fig. 3C, column 2), whereas no recovery of the 2.5-kb fragment is observed (Fig. 3C, column 1). Thus, the cross-linking of *zeste* protein in vivo maps to the region expected based on biochemical and molecular genetic experiments.

Next, we examined the in vivo distribution of *zeste* protein on a more extensive region of the *Ubx* gene and found an excellent correlation with previous in vitro im-

Figure 3. *zeste* cross-links to specific regions in 40 kb of the *Ubx* promoter. (A) Each vertical column shows the result of a UV cross-linking experiment using chromatin from irradiated wild-type 8- to 10-hr old embryos, which was digested with either *Bam*HI (columns 1,2) or *Eco*RI (columns 3–7). The top lane in each column contains .005% of total DNA; the lower two lanes show the results of immunoprecipitations, either in the presence or absence of affinity-purified anti-*zeste* antibody. Southern blots in columns 1–7 were probed with the genomic *Eco*RI or *Bam*HI fragment indicated below each column as a double-headed arrow (genomic fragments 3104, 3103, 3105, 3106, 3102, 3108, and 3109, respectively; Bender et al. 1983). (B) Schematic representation of the *Ubx* gene from –30 to +10 kb relative to the start site of transcription at +1. Double-headed arrows represent genomic fragments that were assayed in UV cross-linking experiments, and those that were recovered above background have numbers denoting the efficiency of recovery as a percentage of total DNA. The approximate locations of the PBX and BXD enhancers (Müller and Bienz 1991) are indicated. The 3.5-kb proximal promoter *Eco*RI fragment is expanded to show the five high affinity *zeste* protein-binding sites as open ellipses and the two *Eco*RI–*Stu*I fragments. (C) Mapping of *zeste* protein within the *Ubx* proximal promoter. UV cross-linking was carried out as in A except that chromatin was digested with *Eco*RI and *Stu*I. The blot was probed with a 3.5-kb *Eco*RI proximal promoter fragment (3102, Bender et al. 1983) that hybridizes to a 2.5-kb promoter fragment (column 1) and a 1-kb promoter fragment (column 2).



munoprecipitations that mapped *zeste* protein-binding sites on the same genomic region (Benson and Pirrotta 1988). Four DNA fragments that are bound by *zeste* protein in vitro were examined, and binding to all these fragments was detected in vivo (Fig. 3A; columns 2–5). Furthermore, three fragments that are not bound by *zeste* protein in vitro were not recovered above the background of .00005% in the UV cross-linking assay (Fig. 3A, columns 1,6,7). The cross-linking per kilobase on each of these latter three fragments is several hundred-fold lower than the value on the 1-kb fragment surrounding the *Ubx* transcription start site, suggesting very specific binding by *zeste* protein in vivo.

Binding of zeste is not detected on the ftz, rosy, actin 5C, or eve genes

To further assess the distribution of *zeste* protein in vivo, we examined a series of other promoters. The binding of *zeste* protein to the *ftz* promoter was examined because *zeste* protein does not bind to this promoter in vitro (Benson and Pirrotta 1988). Consistent with this, there was no cross-linking of *zeste* protein above the background on two 3.5-kb *ftz* promoter fragments that span most of the regulatory and transcribed region (Fig. 4A). We also examined binding to sizable portions of the *rosy*, *actin 5C*, and *eve* promoters. These promoters have not been assayed for *zeste* protein-binding sites in vitro, and it is not known whether they are regulated by *zeste*. None of these promoter fragments were recovered above background levels in UV cross-linking experiments with *zeste* antibody, suggesting that *zeste* protein does not bind significantly to these promoters in the embryo (Fig. 4B–D).

In summary, cross-linking by *zeste* protein maps to characterized *zeste* protein-binding sites in the *Ubx* gene, and no cross-linking is detected on numerous DNA fragments in *Ubx* and *ftz* to which no binding by *zeste* protein is seen in vitro. This agrees with earlier results showing that in vivo cross-linking is limited to the region of contact between protein and DNA (Gilmour and Lis 1986; Rougvie and Lis 1988).

eve binds directly to its own promoter in cellular blastoderm and gastrulating embryos

Having established the specificity of our more sensitive UV cross-linking assay, experiments were initiated to understand how homeo domain proteins bind to DNA in the embryo. We chose to examine DNA binding by the segmentation proteins *eve* and *ftz*, both of which contain Q50 homeo domains. Both of these proteins regulate their own expression, and they also regulate the segmentation gene *engrailed* and many of the homeotic genes (Akam 1987; Hiromi and Gehring 1987; Frasch et al. 1988). As described in the introductory section, it has been difficult to establish whether these interactions are direct. Also, it is unclear whether related homeo domain proteins like *eve* and *ftz* have similar or different DNA-binding specificities in vivo.

We first examined the binding of *eve* protein on the *eve* gene, as it is one of the most studied potential targets of direct *eve* regulation in vivo (Frasch et al. 1988; Goto et al. 1989; Harding et al. 1989; Jiang et al. 1991). *eve* is required to maintain high levels of its own expression in narrow stripes of cells during gastrulation, and it is necessary for repression of its own promoter in other cells during the same period. Although promoter elements that mediate this positive and negative autoregulation have been shown to lie within 8 kb of DNA upstream of the RNA start site, both direct and indirect mechanisms have been invoked to explain this regulation (Jiang et al. 1991; Manoukian and Krause 1992).

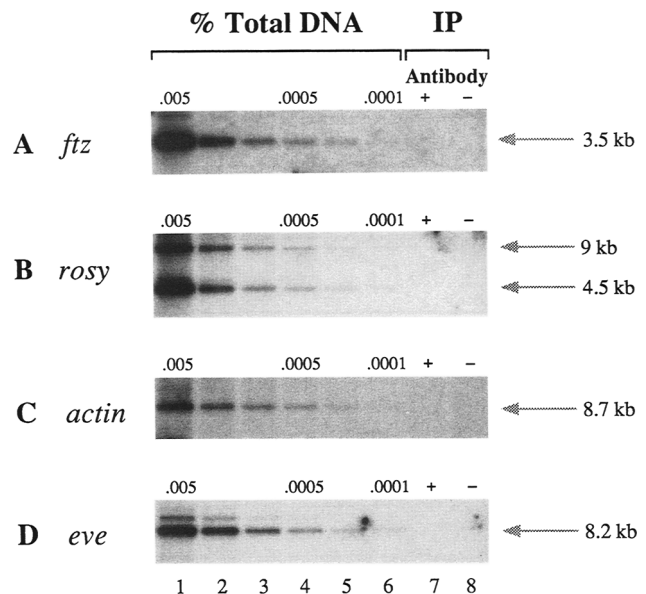


Figure 4. Binding of *zeste* protein is not detected on the *rosy*, *actin 5C*, *ftz*, or *eve* genes. (A–D) The results of UV cross-linking experiments using *EcoRI*-digested chromatin from irradiated 8- to 10-hr embryos (as described in Fig. 2). Lanes 1–6 in each blot contain .005%, .002%, .001%, .0005%, .0002%, and .0001%, respectively, of total DNA; lanes 7 and 8 show the results of immunoprecipitations in the presence (lane 7) or absence (lane 8) of anti-*zeste* antibody. The blot in A was probed with a 6.8-kb *KpnI*–*Sall* promoter fragment from the *ftz* gene (from plasmid pFK3; provided by Henry Krause, University of Toronto, Canada). It hybridizes to three contiguous *EcoRI* fragments extending from –6 kb to about +2 kb relative to the RNA start site (Weiner et al. 1984). Two of these comigrate at a size of 3.5-kb (for a diagram, see Fig. 8C). No cross-linking is seen to the third fragment, which is 1.2 kb in size (data not shown). (B) Probed with a 7.3-kb *HindIII* fragment from the *rosy* gene (from plasmid pHZ50PL; Hiromi and Gehring 1987), which hybridizes to two *EcoRI* fragments (9 and 4.5 kb) extending from about –7 kb to +6 kb relative to the RNA start site (Coté et al. 1986). (C) Probed with *actin 5C* sequences (from plasmid pPac U + *NdeI*; Biggin and Tjian 1989a) that hybridize to an 8.7-kb *EcoRI* genomic fragment containing the *actin 5C* transcription unit (Fyrberg et al. 1980). (D) Probed with *eve* promoter sequences from –0.4 to –6.4 kb relative to the RNA start site, which hybridize to an 8.2-kb *EcoRI* *eve* promoter fragment surrounding the RNA start site (Frasch et al. 1987).

To determine whether *eve* protein binds directly to promoter sequences mediating autoregulation, UV cross-linking was carried out on 4- to 5-hr-old embryos (corresponding to the latter half of stage 5, and stages 6–8 according to Campos-Ortega and Hartenstein 1985). In these embryos, *eve* expression is at its highest and both positive and negative autoregulation are occurring. Using chromatin from irradiated 4- to 5-hr embryos, an antibody specific for the *eve* protein precipitates a 7.3-kb *Bgl*I promoter fragment that includes the sequences that mediate positive and negative autoregulation (Fig. 5A, lane 1). The recovery is equivalent to .005% of total DNA. In the absence of antibody, or when the embryos are not irradiated, no *eve* promoter fragment is isolated (Fig. 5A, lanes 2,3). The antibody used in this experiment (amino-terminal *eve* antibody) was affinity purified from a serum raised against the amino-terminal two-thirds of the *eve* protein. This serum gives the characteristic seven stripe pattern of *eve* expression in whole mount embryos with extremely low background (see Materials and methods).

To confirm the specificity of the *eve* antibody, cross-linking was examined in older embryos in which *eve* expression is very low. As expected, cross-linking by *eve* protein was virtually undetectable in two populations of older embryos in which *eve* expression is either off or limited to a small number of cells (Fig. 5B, lanes 2 and 3, respectively). As a control, we verified that cross-linking of RNA polymerase II to the *actin 5C* gene is virtually the same in old and young embryos (data not shown). To confirm independently that *eve* protein is bound to its own promoter, we used an affinity-purified polyclonal antibody that recognizes a different portion of the *eve* protein, the carboxy-terminal region (carboxy-terminal *eve* antibody; see Materials and methods). This antibody also precipitated *eve* promoter DNA in a stage-dependent manner, albeit with about twofold lower yields and a slight background in 8- to 10-hr embryos (Fig. 5C, cf. lanes 1 and 2 with lanes 3 and 4). Also, serum that has been depleted of *eve*-specific antibodies by affinity chromatography has no activity in the UV cross-linking assay (Fig. 5C, lane 5).

To provide rigorous proof that this UV cross-linking assay can detect *eve*-specific cross-linking, the experiment was carried out in *Drosophila* tissue culture cells. These cells do not normally express *eve* protein. However, transfection of these cells with an *eve* expression plasmid leads to repression of a reporter plasmid containing the *Ubx* proximal promoter (Biggin and Tjian 1989a). Under these conditions, the UV cross-linking method detects *eve* protein binding to the 1.1-kb *Ubx* proximal promoter fragment present in the reporter plasmid (Fig. 5D, lane 1). If the *eve* expression plasmid is not cotransfected with the reporter plasmid, background levels of the *Ubx* promoter fragment are isolated (Fig. 5D, lane 2). Because the only parameter altered in this experiment is the level of *eve* expression, this shows rigorously that the *eve* antibody detects *eve*-specific cross-linking.

Taken together, these results argue strongly that *eve* protein interacts directly with its own promoter and, fur-

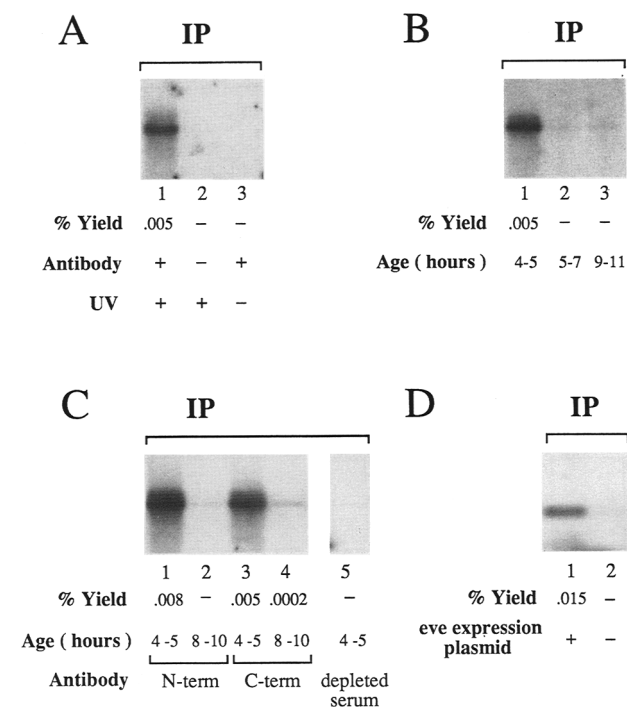


Figure 5. In vivo UV cross-linking of *eve* protein. In the four UV cross-linking experiments shown in A–D, immunoprecipitation yields are indicated based on comparisons with dilution series of total DNA, which are not shown. (–) The signal observed was background. Southern blots in A, B, and C were probed with the same probe used in Fig. 4D, and the DNA fragment detected is a 7.3-kb *eve* promoter fragment extending from –0.3 to –7.6 kb (see Fig. 6A). (A) Binding of *eve* protein to the *eve* promoter. Chromatin was isolated from irradiated 4- to 5-hr old (lanes 1,2) or unirradiated 4- to 5-hr (lane 3) embryos and digested with *Bgl*I. Affinity purified amino-terminal *eve* antibody was present in immunoprecipitations 1 and 3, but not in 2. (B) Binding of *eve* protein to the *eve* promoter at different stages in development. Immunoprecipitations as described in A were carried out with irradiated chromatin from 4- to 5-hr (lane 1), 5- to 7-hr (lane 2; contains stages 9 and 10), or 9- to 11-hr (lane 3; contains predominantly stages 11 and 12) embryos. Though we have defined as background the low levels of *eve* promoter fragments that are immunoprecipitated from older embryos [equivalent to ~.0001% of total DNA], this could represent DNA-binding by the low level of *eve* protein being expressed in these embryos. (C) *eve* cross-linking is also detected with a second-affinity purified antibody. Immunoprecipitations were performed with *Bgl*I-digested chromatin from irradiated 4- to 5-hr (lanes 1,3,5) or 8- to 10-hr (lanes 2,4) embryos. Reactions contained amino-terminal *eve* antibody (lanes 1,2), carboxy-terminal *eve* antibody (lanes 3,4), or serum that had been depleted of *eve*-specific antibodies by affinity chromatography (lane 5). (D) *eve* protein cross-linking in tissue culture cells. UV cross-linking was carried out on Schneider cells (see Materials and methods) that were transfected with a CAT reporter plasmid containing *Ubx* proximal promoter sequences from –600 to +360 relative to the RNA start site. Cells used for the reaction in lane 1 were cotransfected with an *eve* expression plasmid, pPac U+NdeI *eve*; cells used for the reaction in lane 2 were transfected with a control plasmid. The chromatin in both reactions was digested with both *Eco*RI and *Bam*HI and the blot was probed with the same probe used in Fig. 3A.

thermore, that any cross-linking above the background of .0001% in embryos represents *eve* molecules bound to DNA. It is not known which *eve*-expressing cells give rise to the cross-linking signals. However, the relatively undifferentiated state of the tissue and the fact that *eve* appears to control *eve* expression very similarly in all seven *eve* stripes makes it likely that the cross-linking observed is representative of binding in most or all *eve*-expressing cells.

eve protein binds throughout 10-kb of the *eve* promoter

To map more closely the regions to which *eve* protein binds, ~10 kb of the *eve* promoter extending from -7 to +3 kb relative to the RNA start site was divided into ~1.5-kb fragments using different restriction enzymes. Cross-linking of *eve* protein to each fragment was determined in 4- to 5-hr embryos and, as a negative control, in 8- to 10-hr embryos. In Fig. 6B, lanes 6 and 7 show the results of immunoprecipitations using the amino-terminal *eve* antibody, and these data are represented schematically in Figure 6A. Strikingly, all fragments examined were isolated in the immunoprecipitation, including ones containing the transcribed region. Furthermore, the yield of each fragment is roughly proportional to its size, with all fragments being cross-linked at ~.001%/kb. Because percentage cross-linking is likely to be directly proportional to occupancy for proteins such as *eve* that recognize a wide range of sites, our results suggest that *eve* protein binds in a highly uniform pattern throughout many kilobase pairs of the *eve* promoter. To provide independent confirmation of this result, cross-linking to the three largest fragments (I, II, and III) was also examined using the carboxy-terminal *eve* antibody (Fig. 6B, lanes 8,9). The results were the same, except that the yield of each fragment was slightly lower, as expected for this antibody.

The widespread distribution of *eve* protein on the *eve* promoter differs from expectations based on previous experiments: Transgenic promoter deletion studies indicate that sequences required for both positive and negative autoregulation lie between -5.2 and -8 kb upstream of the RNA start site (Goto et al. 1989; Harding et al. 1989; see Fig. 6A). Importantly, transcription from a promoter containing only the region between -5.2 kb and the RNA start site is practically unaffected in *eve* mutant embryos (Harding et al. 1989). Thus, in the endogenous gene, *eve* protein binds strongly to a promoter region that has no known *eve*-dependent regulatory activity when separated from the rest of the promoter. It will now be important to determine whether *eve* protein is bound at high levels to this isolated promoter construct.

Another striking aspect of our data is that efficient cross-linking occurs on fragments that according to in vitro studies do not contain high affinity binding sites (fragments V and IX, Fig. 6A). This observation may be explained by the finding that in vitro, *eve* molecules bound to high affinity sites can, through cooperative interactions, cause efficient binding of *eve* molecules to

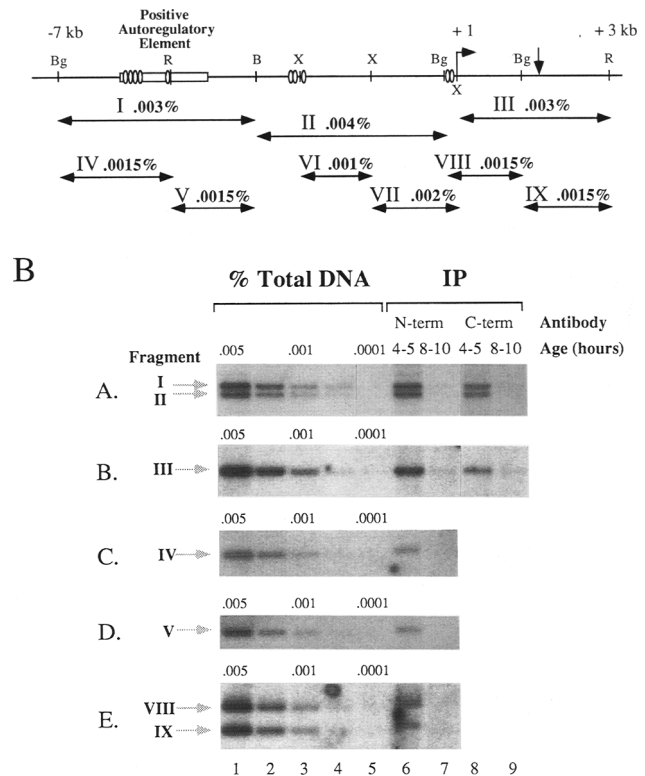


Figure 6. *eve* protein binds to extensive regions of the *eve* promoter. (A) Schematic representation of 10 kb of the *eve* promoter shows the positive autoregulatory element as an open box (Goto et al. 1989; Harding et al. 1989), the transcription start site as a bent arrow, the polyadenylation site as a vertical arrow, and high affinity *eve* protein-binding sites determined by in vitro immunoprecipitation and footprinting analysis as open ellipses (Hoey and Levine 1988; Hoey 1989; M. Levine, pers. comm.). Double-headed arrows represent genomic fragments that were assayed for binding by *eve* protein, with the average level of recovery in UV cross-linking experiments expressed as a percentage of total DNA above each fragment. (Bg) *Bgl*I; (R) *Eco*RI; (X) *Xho*I. (B) UV cross-linking was carried out on 4- to 5-hr (lanes 6,8) and on 8- to 10-hr (lanes 7,9) embryos to determine cross-linking efficiencies to restriction fragments I-IX shown in A. Data for fragments VI and VII are not shown. Lanes 1-5 on each blot contain a dilution series of total DNA. In lanes 6 and 7, the amino-terminal *eve* antibody was used; in lanes 8 and 9, the carboxy-terminal *eve* antibody was used. Blot A was probed as in Fig. 4D; B was probed with *eve* cDNA sequences from an *Eco*RV-*Nde*I fragment derived from plasmid pAR3040 *eve* (Hoey and Levine 1988); C was probed with *eve* sequences from -4.7 kb to -6.4 kb; D was probed with the same sequences as C; E was probed with the same sequences as B.

lower affinity *eve* sites located at a distance (TenHarmel et al. 1993). We suggest that such cooperative interactions between *eve* protein molecules may lead to the filling of many moderate affinity sites in vivo and thus cause the pattern of cross-linking observed on the *eve* promoter (see Discussion).

eve protein is detected on promoters not expected to be targets of *eve*

Cross-linking was also examined on various genes that we did not initially expect to be bound by *eve* protein in vivo (Fig. 7). Strikingly, low but significant binding by *eve* protein was detected on all of the following fragments: a 7.3-kb fragment encompassing the *rosy* gene, two *Alcohol dehydrogenase* (*Adh*) fragments spanning the *Adh* transcription unit, various fragments from the uninduced *hsp70* promoter and transcription unit, an 8.7-kb fragment spanning the *actin* 5C transcription

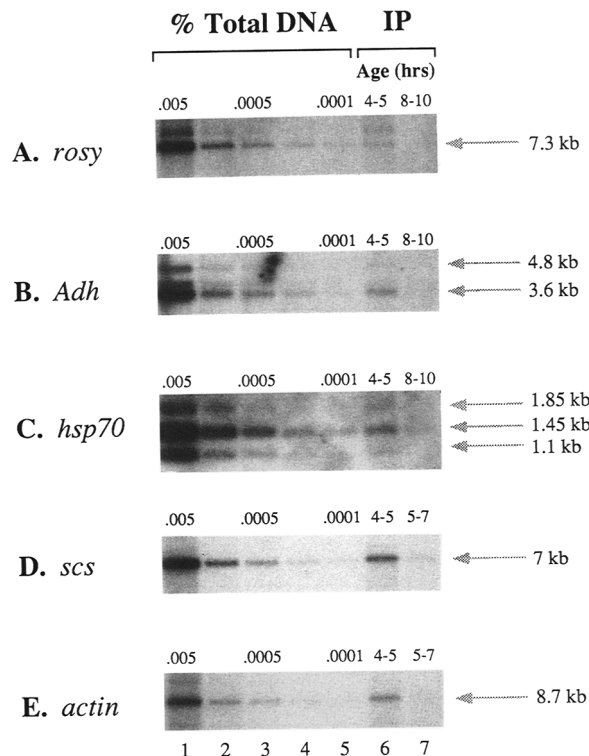


Figure 7. *eve* protein binds detectably to randomly chosen genes. UV cross-linking was carried out on 4- to 5-hr (lane 6, A-E), on 8- to 10-hr (lane 7, A-C), and on 5- to 7-hr (lane 7, D and E) embryos using the amino-terminal *eve* antibody. Lanes 1-5 in blots A-E contain a dilution series of total DNA. (A) Chromatin was digested with *Hind*III and probed as in Fig. 4B, revealing a 7.3-kb genomic *rosy* fragment. (B) Digested with *Sal*I, the blot was probed with a genomic 4.7-kb *Eco*RI *Adh* fragment (plasmid p13E-3; Heberlein et al. 1985) that hybridizes to 4.8- and 3.6-kb genomic fragments surrounding the RNA start site (Goldberg 1980). The intermediate sized species is a partial digestion product. (C) Digested with *Bam*HI and *Xho*I and probed with a 2.6-kb *Xho*I *hsp70* fragment (from plasmid p70X2.6; Rougvie and Lis 1988). The probe hybridizes with three restriction fragments which together contain the transcription units of the five *hsp70* genes (Gilmour and Lis 1986). (D) Digested with *Sal*I and probed with a 1.8-kb *scs* element (Kellum and Schedl 1991) that hybridizes to a 7-kb genomic fragment from the proximal side of the *hsp70* 87A locus that lies outside of the transcription unit (Udvardy et al. 1985). (E) Digested with *Eco*RI and probed as in Fig. 4C, revealing an 8.7-kb genomic fragment containing the *actin* 5C transcription unit.

unit, and a 7-kb fragment containing a putative chromatin domain boundary located several kilobase pairs away from the *hsp70* transcribed region (*scs*, Kellum and Schedl 1991). The average cross-linking per kilobase on these unexpected targets is .0001%/kb, or 10-fold lower than on the *eve* gene (see also Fig. 10, below); the range is from 26-fold lower on *rosy* to 6-fold lower on *hsp70*.

The following considerations argue strongly that the cross-linking signals seen on these genes are *eve* protein specific: The results were obtained using affinity-purified antibodies, and they are highly reproducible; binding by *eve* protein on the *actin* 5C gene and on the *scs* element was also detected using the carboxy-terminal *eve* antibody (data not shown); serum that has been depleted of *eve*-specific antibodies using affinity chromatography does not precipitate these fragments (data not shown; see also Fig. 5C, lane 5) ruling out the possibility that some genomic regions, when irradiated, have an inherent tendency to be precipitated in this procedure; finally, cross-linking signals follow closely the levels of *eve* protein expression in development.

Although the significant level of binding on these genes was initially very surprising, we argue in the Discussion that this observation fits previous predictions that the majority of transcription factors not bound to their functional targets would bind randomly at low-levels throughout the rest of the genome (von Hippel et al. 1974; Lin and Riggs 1975). These predictions generally assume that low-level binding throughout the genome involves very low affinity binding to completely random DNA sequences (nonspecific sites). Even if this is the case for *eve* protein, cross-linking to genes such as *rosy* is still directly comparable to cross-linking on the *eve* promoter where many of the *eve* molecules are almost certainly binding to specific sites. This is because, given similar occupancies, the average cross-linking by transcription factors to specific and nonspecific binding sites is very similar and shows the same rate of increase over a time course of UV irradiation (Lin and Riggs 1974). We therefore interpret the 10-fold lower level of cross-linking to genes such as *rosy* and *actin* as a ~10-fold lower occupancy of *eve* protein on these genes.

eve protein also binds throughout the *Ubx* and *ftz* genes

Next, we examined binding by *eve* protein to two other putative *eve* targets, *Ubx* and *ftz*, to determine whether *eve* protein binds at high levels to these promoters and, if so, whether the binding is as widespread as on the *eve* promoter. Previous experiments showed that *eve* protein can directly repress transcription from the *Ubx* proximal promoter in vitro (Biggin and Tjian 1989b), but *eve*'s repression of *Ubx* in the embryo can also be explained by an indirect mechanism through the *engrailed* gene (Martinez-Arias and White 1988). Consistent with a direct mechanism of repression, *eve* protein binds at levels significantly above background to genomic fragments covering 40 kb of the *Ubx* gene, including the proximal promoter and two enhancers (Fig. 8B; results summarized in

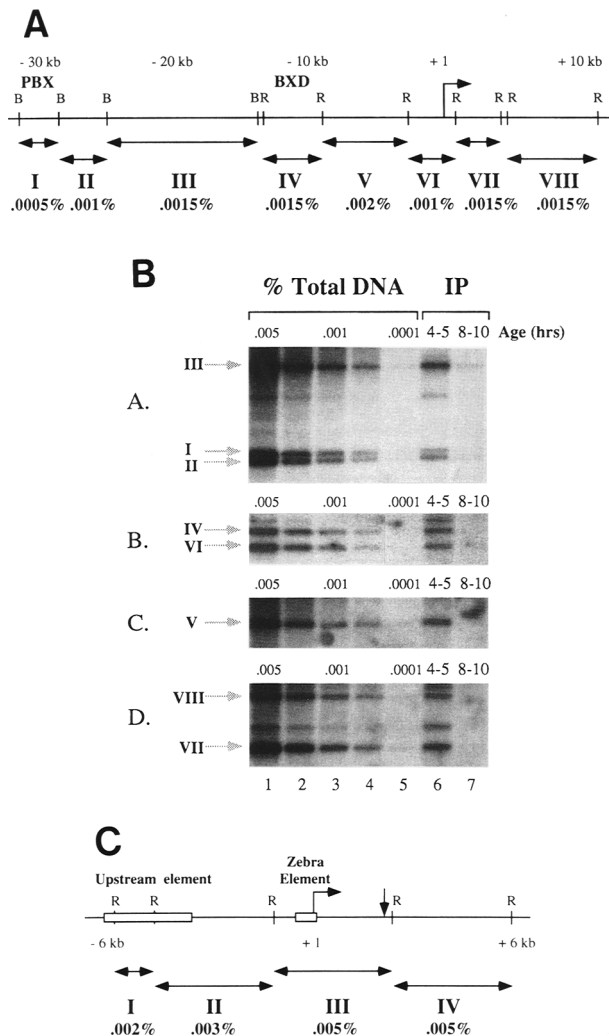


Figure 8. *eve* protein also cross-links to large regions of the *Ubx* and *ftz* genes. (A) Schematic representation of the *Ubx* gene similar to the one shown in Fig. 3B; cross-linking by *eve* protein to each fragment is expressed as a percentage of total DNA. (B) UV cross-linking was carried out on 4- to 5-hr (lane 6) and on 8- to 10-hr (lane 7) embryos using the amino-terminal *eve* antibody to determine the cross-linking efficiencies to genomic fragments I–VIII shown in A. (Lanes 1–5) A dilution series of total DNA. Blot A was probed with *Ubx* genomic fragments 3103 and 3104 (see legend to Fig. 3 (B); Bender et al. 1983); B was probed with 3102 and 3105; C was probed with 3106; D was probed with 3108 and 3109. Arrows indicate products of complete digestion with restriction enzymes; other bands represent partial digestion products. Partial digestion occurs because of excessive UV exposure; this also explains why partial digestion products are preferentially immunoprecipitated. (C) Schematic diagram of the *ftz* promoter (derived from Hiromi and Gehring 1987) showing the zebra and upstream enhancer elements and the results of *eve* protein cross-linking to four genomic fragments spanning this locus. These results were obtained in UV cross-linking experiments on 4- to 5-hr embryos using the amino-terminal *eve* antibody. Eight to 10-hr embryos served as a negative control.

Fig. 8A). The cross-linking/kb of *eve* protein on *Ubx* is three- to fourfold lower than on the *eve* promoter, and

about threefold higher than the average on genes such as *actin 5C* and *rosy* (see Fig. 7). The level of cross-linking is likely to be significant, as it is consistently higher than the level on *rosy* and *actin 5C* for many DNA fragments over a very large region.

We also examined the binding of *eve* protein to the *ftz* gene, which it appears to regulate in a largely redundant manner. [Throughout this paper, the term redundant is used to describe a regulatory interaction that is potentially occurring in wild-type embryos but that is dispensable because other proteins can perform the same function; (see also Laney and Biggin 1992; Tautz 1992; Rudnicki et al. 1993)]. At the cellular blastoderm stage, *eve* and *ftz* proteins are expressed in a series of alternating stripes (Hafen et al. 1984; Harding et al. 1986). Strong loss-of-function mutations in the *eve* gene cause derepression of *ftz* transcription in only a small subset of the cells in which *eve* is normally expressed (Carroll and Scott 1986; Harding et al. 1986). However, ectopic expression of *eve* protein throughout the embryo at approximately wild-type levels leads to rapid and complete repression of all *ftz* stripes (Manoukian and Krause 1992). Thus, in wild-type embryos *eve* may bind to and repress the *ftz* promoter in all *eve*-expressing cells, but this function might be redundant with that of other proteins in all but a few cells. In vivo cross-linking in 4- to 5-hr-old embryos shows that *eve* protein is bound at high levels to four contiguous fragments spanning 12 kb of the *ftz* promoter (data not shown; results summarized in Fig. 8C). This region includes the upstream *ftz* autoregulatory element, the “zebra element” required to initiate the striped *ftz* pattern, and the transcription unit (Hiromi and Gehring 1987). At .0013% cross-linking/kb, the level of cross-linking is slightly higher than on the *eve* promoter. Therefore, if *eve* protein were only bound to the *ftz* promoter in the few cells in which *ftz* expression is perturbed by *eve* loss-of-function mutations, this level of cross-linking would not have been detected. Thus, our data support the idea that *eve* represses *ftz* redundantly in many cells.

ftz protein binds to the same DNA fragments as *eve* protein in vivo

We then wanted to examine cross-linking by *ftz* protein to the same DNA fragments that were assayed for binding by *eve* molecules. This would allow us to determine whether conditions in the embryo cause these two Q50 homeo domain proteins to bind different sequences, or whether their binding in vivo is largely the same as it is in vitro. In support of the latter possibility, we found that *ftz* protein binds strongly to the same four *ftz* promoter fragments that the *eve* protein occupies at high levels (Figs. 9A and 8C). Although *ftz* protein cross-links at about twofold lower levels to these fragments than *eve* protein (.0007% / kb vs. .00125% / kb), this difference is observed on virtually all fragments that were assayed for binding by both proteins (see below). The binding of *ftz* to fragment II of the *ftz* promoter (Fig. 9A) is of particular interest, as a second site suppression experiment

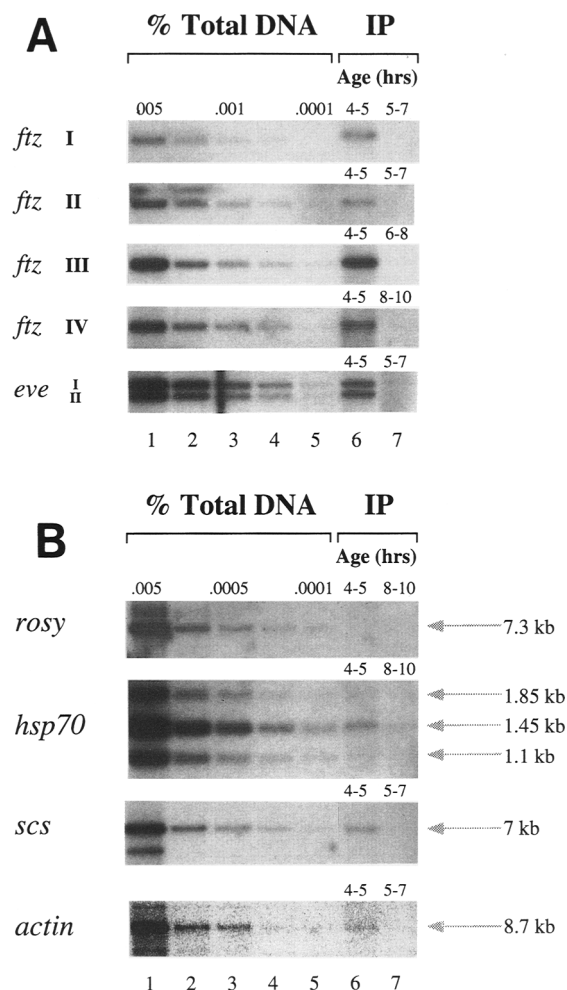


Figure 9. In vivo UV cross-linking by the *ftz* protein. Cross-linking was carried out on populations of 4- to 5-hr old embryos (lane 6) and on older embryos (lane 7) using affinity-purified anti-*ftz* antibodies. Like *eve* protein, *ftz* expression peaks between 4 and 5 hr after egg laying and then drops sharply, so that older embryos serve as a negative control. Lanes 1–5 of each blot contain dilution series of total DNA. (A) *ftz* protein binds strongly to large portions of the *ftz* and *eve* promoters. (*ftz* I–IV) *ftz* promoter fragments I–IV shown in Fig. 8C; (*eve* I,II) *eve* promoter fragments I and II diagrammed in Fig. 6A. Chromatin used in all *ftz* blots was digested with *Eco*RI. Blots *ftz* I and *ftz* II were probed with a 2.8 kb *Bam*HI–*Kpn*I *ftz* promoter fragment located 5 kb upstream of the RNA start site; *ftz* III was probed with a 3.5-kb *Eco*RI fragment centered around the RNA start site; *ftz* IV was probed with the next downstream *Eco*RI fragment (3.5 kb). All *ftz* probes were derived from plasmid pfK3, which contains a 12-kb *Kpn*I insert covering the *ftz* transcription unit. *eve* I, II was probed the same as blot A in Fig. 6B. (B) *ftz* protein binds detectably to randomly chosen promoters. Chromatin was restriction digested and probed exactly as described for the corresponding blots in Fig. 7. The faster migrating fragment in lane 1 of the *scs* blot is due to a sample of *Sall*-digested total DNA that was preloaded onto the agarose gel. The actin blot image was obtained using a PhosphorImager.

strongly suggests that *ftz* protein activates its own expression by binding directly to sequences located in this

fragment (Schier and Gehring 1992). Thus, this level of cross-linking is very likely to be functionally significant. Putative *ftz* autoregulatory sequences are also located in fragment I (Pick et al. 1990), whereas it is not known whether fragments III and IV possess autoregulatory activity. Like the *eve* protein, the *ftz* protein also cross-links strongly to three fragments covering 10 kb of the *eve* gene [.001% on fragment *eve* I; .0015% on fragment *eve* II; .0015% on fragment *eve* III (Fig. 9A and data not shown; see Fig. 6A for schematic representation of promoter fragments)]. The average cross-linking over this region (.00045%/kb) is again ~2.5-fold lower than the cross-linking by *eve* protein to the same region. Like the effect of *eve* on *ftz*, genetic evidence indicates that *ftz* regulates *eve* in a redundant fashion (Harding et al. 1986; Kellerman et al. 1990), and our finding that *ftz* cross-links strongly to the *eve* gene is consistent with such a mechanism.

We also determined the level of binding by *ftz* protein to genes that are not expected to be regulated by *ftz*. Similar to *eve* protein, *ftz* protein was detected at low but significant levels on the *hsp70* (.00015%), *scs* (.0004%), and *actin* 5C (.0003%) fragments (Fig. 9B). On the *rosy* and *Adh* gene fragments, *ftz* cross-linking was barely detectable above background levels (Fig. 9B; data not shown). These latter two are also the fragments on which *eve* cross-linking/kb was lowest (Figs. 7 and 10). The average cross-linking of *ftz* protein on the fragments shown in Figure 9B (including *Adh*) is .00003%/kb or 20-fold lower than the average cross-linking to the *ftz* gene and 14-fold lower than cross-linking to the *eve* gene (Fig. 9A; data not shown). In summary, like *eve*, *ftz* binds at high levels throughout large regions of its genetic targets and at low but significant levels to genes that are not expected to be regulated by *ftz*. Although *eve* protein cross-links two- to threefold more strongly to almost all fragments than *ftz* protein, both proteins cross-link with very similar relative preferences to all 12 fragments on which binding by both was examined (see Fig. 10). Because *eve* and *ftz* proteins bind mostly the same sequences in vitro (Biggin and Tjian 1989b), we infer that the similar cross-linking of these proteins to their genetic targets results from both proteins binding the same array of DNA sites in vivo.

Discussion

We have improved the sensitivity of an in vivo UV cross-linking assay (Gilmour and Lis 1986), so that it is now possible to directly measure DNA binding of sequence-specific transcription factors in living tissue. Using this technique to identify DNA fragments that are bound directly by the Q50 homeo domain proteins *eve* and *ftz* gave three striking results. First, strong binding by these two proteins is detected at remarkably uniform levels throughout the length of their genetically identified target genes. This includes some target genes that appear to be controlled redundantly by these proteins. Second, both proteins are cross-linked on average at only 10- to 20-fold lower levels to genes that they are generally not

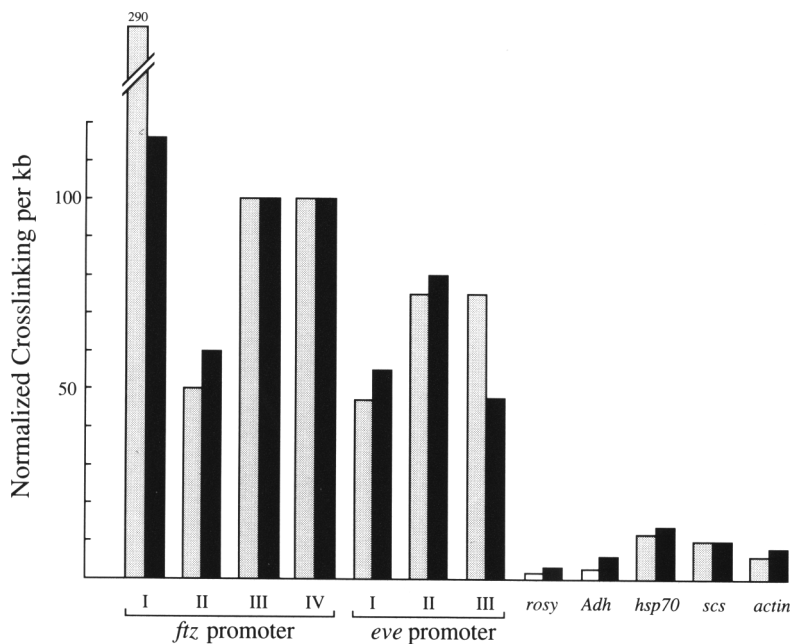


Figure 10. Normalized cross-linking efficiencies of *eve* and *ftz* proteins. To compare the relative preferences of *eve* and *ftz* proteins for different DNA fragments, the percent cross-linking of each protein to each fragment was divided by the size of the fragment to give percent cross-linking/kb. The cross-linking/kb calculated for both *eve* and *ftz* proteins on fragment III of the *ftz* promoter was then arbitrarily assigned a value of 100. Cross-linking/kb of *eve* and *ftz* proteins on all other fragments was expressed on this scale and plotted as shaded (*ftz* protein) or solid (*eve* protein) bars on the graph. Because the binding of *ftz* protein to *Adh* and *rosy* was so close to the background, the estimated values for these interactions are somewhat unreliable and are therefore indicated as open bars.

expected to regulate, suggesting that *eve* and *ftz* proteins may bind at low levels throughout much of the genome. Third, *eve* and *ftz* proteins appear to have the same DNA-binding specificities in vivo, as their relative efficiency of cross-linking to any given DNA fragment is very similar. In contrast, the non-homeo domain transcription factor *zeste* was found to bind to short DNA elements within a target promoter (*Ubx*), and not to other genes. Below, we discuss mechanisms that can explain the binding of *eve* and *ftz* proteins in vivo, and we suggest that our observations support the view that Q50 homeo domain proteins act by directly regulating largely the same target genes.

Mechanisms of widespread binding by eve and ftz proteins

It is generally thought that sequence-specific transcription factors bind to localized regions within the promoters they regulate (Mitchell and Tjian 1989; McKnight and Yamamoto 1992). This was also thought to be the case for Q50 homeo domain proteins because deletion studies indicated that homeo domain responsive promoter elements can be relatively short (e.g., Jiang et al. 1991; Schier and Gehring 1992; Vachon et al. 1992) and because in vitro DNA-binding studies showed preferential binding to certain regions within promoters (Desplan et al. 1985; Beachy et al. 1988; Hoey and Levine 1988; Laughon et al. 1988; Regulski et al. 1991). However, some of the same binding studies also showed that at lower stringency, many more promoter fragments were bound by homeo domain proteins (Desplan et al. 1985; Beachy et al. 1988), indicating the presence of many moderate affinity sites throughout promoters. Our finding that *eve* and *ftz* proteins cross-link uniformly

throughout their genetic targets suggests that they bind efficiently to these moderate affinity sites in vivo ($K_d = 10^{-8}$ M) in addition to occupying high affinity sites ($K_d \leq 10^{-9}$ M). A mechanism for how this could be achieved is suggested by in vitro studies of how *eve* protein represses the *Ubx* promoter (TenHarmsel et al. 1993). Repression requires the binding of *eve* protein to moderate affinity sites, and this binding is stabilized by cooperative interactions with other *eve* molecules bound to distant high affinity sites, causing the intervening DNA to bend. Such cooperativity, which may be a common feature of homeo domain proteins (Beachy et al. 1993), could act to stabilize binding to moderate affinity sites throughout large promoter regions, thereby forming a series of miniloop structures. In this capacity, these proteins may influence profoundly the structure of their target genes and thus may act as chromatin-organizing proteins.

The observation that the *eve* and *ftz* proteins cross-link at only ~10 to 20-fold lower levels to promoters that they are not expected to regulate is consistent with previous thermodynamic calculations carried out to model the binding of sequence-specific DNA-binding proteins in living cells (von Hippel et al. 1974; Lin and Riggs 1975). Such calculations take into account the specific and nonspecific DNA-binding constants of transcription factors, and the concentration of these proteins and genomic DNA in a cell. They predict that the vast majority of transcription factor molecules not bound to high affinity specific binding sites would bind to the excess of nonspecific DNA sites present in a cell, leaving very little protein free in the nucleoplasm. In the case of a prokaryotic protein such as the λ repressor, this would lead to the binding of one repressor molecule every 30 kb. For homeo domain proteins such as *eve* and *ftz*,

which are present at 50,000 molecules per cell (Krause et al. 1988; J. Walter, and M.D. Biggin, unpubl.), the same calculations predict a background binding of one molecule every 4 kb if one disregards potential effects of chromatin structure. We suggest that the detection of low-level binding by *eve* and *ftz* proteins on genes such as *rosy* and *actin* provide direct support for these earlier models. However, we cannot determine whether the background binding by homeo domain proteins is attributable to low affinity interactions with completely random DNA sequences or binding to fortuitously occurring specific sites. The reason for this is that unlike prokaryotic regulators, homeo domain proteins recognize an array of specific DNA sequences that occur quite frequently in nontarget DNAs (Desplan et al. 1985; J. Walter and M.D. Biggin, unpubl.).

By comparison with *eve* and *ftz* proteins, the *zeste* transcription factor does not bind detectably to randomly chosen DNA fragments in vivo. Consistent with this observation, *zeste* protein discriminates between specific and nonspecific DNA sites in vitro much better than do homeo domain proteins (cf. Benson and Pirrotta 1987, 1988 and Biggin et al. 1988 with Affolter et al. 1990 and Desplan et al. 1985), and the concentration of *zeste* protein in embryonic nuclei appears to be ~10-fold lower than that of *eve* or *ftz* (Biggin et al. 1988; Krause et al. 1988). These two properties probably explain why any background binding by *zeste* protein is below the limit of detection in our assay.

Binding vs. function

The finding that *eve* and *ftz* proteins bind at low levels to genes such as *rosy*, *hsp70*, and *actin* raises the possibility that these genes could be regulated by *eve* or *ftz*. However, we suspect that most or all of these interactions are not functional, as *eve* and *ftz* probably bind at low levels to thousands of genes throughout the genome, and it is difficult to envision a function for such indiscriminate regulation by homeo domain proteins. We suggest that *eve* and *ftz* proteins may not be able to regulate genes such as *rosy* and *actin* because effective regulation may require sequence-specific cofactors that do not bind to *rosy* and *actin*, or because binding by *eve* and *ftz* to these genes may be too transient to cause transcriptional regulation. In contrast, we believe the high level of binding throughout genetic targets is likely to be functionally significant, as it is observed with both *eve* and *ftz* proteins on all genetic targets examined. This includes a DNA fragment of the *ftz* promoter that is a strong candidate for direct regulation by *ftz* protein in vivo (fragment II, Fig. 9A; Schier and Gehring 1992) and other promoter fragments that are considered possible direct targets of *eve* and/or *ftz* protein (Biggin and Tjian 1989a; Harding et al. 1989; Pick et al. 1990).

Given the high level binding throughout their target promoters, it is important to note that *eve* and *ftz*, in some cases, regulate almost wild-type patterns of gene expression when acting through very short regulatory elements in transgenic promoter studies (Goto et al.

1989; Harding et al. 1989; Pick et al. 1990; Müller and Bienz 1992; Schier and Gehring 1992). Also, other large promoter regions from the same genes do not appear to be significantly regulated by *eve* or *ftz* in the same type of experiment. An explanation for these observations is that the *eve* and *ftz* positive autoregulatory elements contain binding sites for other transcription factors that are also required for enhancer function (Jiang et al. 1991; Han et al. 1993; Schier and Gehring 1993). If these cofactors bind only within the autoregulatory element, it would explain why other promoter regions, when isolated from the rest of the gene, are not regulated by these homeo domain proteins. However, we suggest that in the context of intact genes, the binding of homeo domain proteins across the entire promoter may be essential to significantly affect transcription. *eve*, *ftz*, and other Q50 homeo domain proteins are master regulators that control a wide range of complex promoters in many cell types. In this capacity, they may have to influence the action of a particularly large number of other transcription factors, and binding throughout the length of promoters may be a general mechanism that accomplishes this. In addition, high level binding throughout target genes may serve to communicate the effects of enhancers and silencers to the proximal promoter region or it may have a subtle or redundant role in augmenting the effect of regulatory elements.

Two Q50 homeo domain proteins appear to have the same DNA-binding specificity in vivo

eve, *ftz*, *engrailed*, and the homeotic proteins contain a glutamine at position 50 of the homeo domain (Q50 homeo domain proteins) and bind to largely the same array of DNA sites in vitro (Biggin and Tjian 1989b; Hayashi and Scott 1990). Because we have shown that two of these proteins also have similar DNA-binding specificities in vivo, we conclude that all the members of this group may have the intrinsic ability to bind the same DNA sites in vivo. This has two important implications. First, in those cells of the embryo that coexpress several Q50 homeo domain proteins, competition for DNA sites will probably play an important role in determining how these proteins bind to their targets (see also Desplan et al. 1988; Hoey and Levine 1988; Ohkuma et al. 1990; Lamka et al. 1992). Second, virtually all members of the Q50 family of proteins are uniquely expressed in at least some cells in the embryo; here, the effects of competition can be ignored, and therefore it is likely that these proteins all bind strongly to a common set of target genes. Although in some cases high levels of binding may be nonfunctional, in general we believe this will not be the case, and we favor the view that different Q50 homeo domain proteins regulate predominantly the same target genes (for similar models, see Garcia-Bellido 1975; Lawrence 1992). If this is true, the chief differences between Q50 homeo domain proteins would lie in the degree to which they activate or repress the same genes. Presumably, such differences would arise through their differential interactions with the unique combinations

of other transcription factors that are known to bind to these target genes (Biggin and Tjian 1989b; Ohkuma et al. 1990; Han et al. 1993; TenHarmsel et al. 1993) and also with proteins that may not directly contact DNA themselves (Tanaka et al. 1988; Mendel et al. 1991).

Genetic experiments provide at least partial support for the above model, as mutations in different Q50 homeo domain genes frequently lead to altered expression of the same downstream genes (Garcia-Bellido 1975; Harding et al. 1986; Akam 1987; Frasch et al. 1988; Gould and White 1992; Lawrence 1992; Capovilla et al. 1994). However, in some instances, expression of a gene downstream of one homeo domain gene is not affected by mutations in other Q50 homeo domain genes (e.g., see Harding et al. 1986; Hayashi and Scott 1990). We suggest that many of these latter instances can be explained if some targets are regulated redundantly by some homeo domain proteins. For example, we have shown that *eve* and *ftz* proteins bind at high levels to genes whose expression is not significantly affected by loss-of-function mutations in *eve* or *ftz*, and in the Results section we detail the evidence that these interactions are functional and redundant, rather than nonfunctional. Thus, if one takes redundancy into account, the available genetic evidence in no way contradicts the view that Q50 homeo domain proteins largely regulate the same targets.

Materials and methods

Antibodies

Using standard techniques, the amino-terminal *eve* antibody was affinity purified from a polyclonal rabbit serum directed against amino acids 1–246 of the *eve* protein (M. Frasch, pers. comm.), and the carboxy-terminal *eve* antibody was affinity purified from a polyclonal guinea pig serum directed against amino acids 238–376 of *eve* (R. Warrior, pers. comm.). Both crude sera give the characteristic seven-stripe *eve* expression pattern in whole mount antibody staining of embryos (Stanojevic et al. 1989; Manoukian and Krause 1992). Affinity-purified rabbit anti-*zeste* antibodies are directed against the carboxy-terminal region of the *zeste* protein (region III; Benson and Pirrotta 1987, 1988; Bickel 1991). *ftz* antibodies were affinity purified from a polyclonal rabbit serum raised against wild-type *ftz* protein; the affinity-purified antibodies give the canonical *ftz* pair-rule pattern with low background in whole mount antibody staining of embryos (S. Carroll, pers. comm.).

Embryo irradiation and chromatin isolation

The original UV cross-linking procedure (Gilmour and Lis 1985 1986; Rougvie 1989) was designed to study RNA polymerase, which cross-links to DNA ~100-fold more efficiently than sequence-specific transcription factors (J. Walter and M.D. Biggin, unpubl.). This is probably because RNA polymerase interacts with single-stranded DNA, a much better substrate for UV cross-linking. To detect DNA binding by sequence-specific factors, many changes to the original cross-linking protocol were made, and those that significantly improved the sensitivity of the method are indicated below. All experiments were performed at least twice, but most experiments were repeated three to five times, and the variability in yields of immunoprecipitated DNA fragments rarely exceeded 25%.

Embryos were collected for 1 or 2 hr from population cages of adult flies maintained at 60% humidity and 24.5°C and then aged for the desired time at 24.5°C. The embryos were weighed, dechorionated in fresh 50% Clorox bleach to increase UV exposure, and resuspended in ice-cold 0.1% Tween 20. Aliquots of embryos (2–5 grams) were irradiated at 4°C using a Fotodyne DNA transfer lamp with four 15W bulbs generating 254 nm light. The embryo suspension was placed at a distance of 3 cm from the bulbs, irradiated for 30 min with periodic shaking, and frozen immediately in liquid nitrogen.

Embryos (8–10 grams) were partially thawed, homogenized in 30 ml of cold NIB buffer (0.3 M sucrose, 15 mM NaCl, 5 mM MgCl₂, 15 mM Tris at pH 7.5, 60 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) with three strokes of a motorized B Dounce homogenizer at 800 rpm, and filtered through Miracloth (Calbiochem). While being stirred, 20% Triton X-100 was added to a final concentration of 0.3% and the suspension was centrifuged at 4000 rpm and 4°C for 15 min in a Sorvall SS34 rotor (Beckman) to pellet nuclei. Nuclei were resuspended in nuclei lysis buffer (100 mM NaCl, 10 mM Tris at pH 8.0, 1 mM EDTA, 0.1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride) by manual homogenization in a B Dounce homogenizer, 1/10 volume of 20% Sarkosyl was added to lyse nuclei, and the chromatin was sheared by one passage through an 18-gauge needle and two passages through a 25-gauge needle. Sheared chromatin from up to 23 grams of 4- to 5-hr embryos and up to 7.5 grams of 8- to 10-hr embryos in a volume of 9 ml was purified on 26-ml CsCl step gradients (Gilmour and Lis 1985). Chromatin was harvested from the gradient by insertion of an 18-gauge needle near the bottom of the tube and collection of 1-ml fractions. Peak fractions were pooled and dialyzed for 7 hr against three changes of dialysis buffer (2 mM EDTA, 50 mM Tris at pH 8.0). Chromatin yields are 200 µg/gram of 4- to 5-hr embryos, 500 µg/gram of 5- to 7-hr embryos, and 800 µg/gram of 8- to 10-hr embryos. Dialyzed chromatin is frozen in liquid nitrogen and stored at –70°C where it remains stable for months.

Chromatin digestion and immunoprecipitation

For digestion with restriction endonucleases, chromatin was supplemented to contain final concentrations of 150 mM NaCl, 10 mM Tris at pH 7.5, 10 mM MgCl₂, 50 µg/ml of RIA grade BSA, and .01% Triton X-100. Aliquots of 450 µg of chromatin were prewarmed for 15 min at 37°C and then restriction digested with two additions of 100 units of enzyme over 12 hr. For the last 2 hr of the digestion, RNase was added to 60 µg/ml. The digests were stopped by the addition of 1/25 volume of 0.5 M EDTA. Sarkosyl was added to a final concentration of .05% and Triton-X 100 to a final concentration of 0.3%. The chromatin was centrifuged for 15 min in a microcentrifuge at 4°C and transferred to fresh tubes. The appropriate affinity purified primary antibody (1–2 µg) was added (except in the experiment shown in Fig. 5D, where 1 µl of crude serum against *eve* residues 1–246 was used), and the mixture was rocked for 3 hr at 4°C and then centrifuged again for 15 min at 4°C. The supernatants were individually passed through 0.22-µm syringe filters directly into fresh tubes to remove nonspecifically precipitated chromatin. Twenty-five microliters of a 20% suspension of *Staphylococcus aureus* (Staph A) cells (see below) was added to each reaction and incubated for no more than 15 min at room temperature; longer incubations contribute to nonspecific backgrounds. Staph A cells were pelleted and washed twice in 1.4 ml of dialysis buffer containing 0.2% Sarkosyl, and four times in 1.4 ml of immunoprecipitation buffer (100 mM Tris at pH 9.0, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid). Washes were carried out at room temperature, and during the last wash, the

Staph A cells were transferred to fresh tubes to remove chromatin stuck to plastic. Immune complexes were eluted from Staph A cells by vortexing the cells for 10 min in 100 μ l of elution buffer (50 mM NaHCO₃, 1% SDS, 1.5 μ g/ml of sonicated calf thymus DNA at pH10), spinning down cells, and harvesting the supernatant. This was repeated two more times. The eluted DNA was mixed with 200 μ l of 0.3% SDS, 50 mM Tris at pH 7.5, 10 mM EDTA, and 1 mg/ml of proteinase K (added fresh) and incubated at 60°C for 12 hr, after which it was precipitated by the addition of 50 μ l of 3 M sodium acetate (pH 5.3), 40 μ g of yeast carrier RNA, and 1.25 ml of ethanol.

Staph A cells were prepared as follows to reduce nonspecific adsorption of chromatin: Lyophilized Staph A cells (Boehringer Mannheim) were resuspended as a 5% solution in PBS, 3% SDS, and 10% β -mercaptoethanol and boiled for 30 min. After washing, they were resuspended as a 5% suspension in chromatin purified from irradiated 8- to 10-hr-old embryos that had been sonicated to an average length of 2 kb. The cells were rocked for 3 hr at 4°C, spun down, and resuspended as a 20% solution in dialysis buffer containing 0.2% Sarkosyl.

UV cross-linking on tissue culture cells

Unless specified otherwise, the procedure was the same as for embryos. Schneider line 2 cells (8×10^7 ; Schneider 1972) were transfected by the calcium phosphate method (DiNocera and Dawid 1983) as described previously (Biggin and Tjian 1989a). Twenty-four micrograms of pUbx CAT -600/+360 and either 16 μ g of pPac U+NdeI or 16 μ g of pPac U + NdeI eve (Biggin and Tjian 1989a) were added to the cells. After 48 hr, cells were mixed with $\frac{1}{500}$ volume of 12% HCl to dissolve calcium phosphate precipitates, pelleted, washed in 0.7 volumes of 137 mM NaCl, 2.7 mM KCl, and 5 mM bisTris (pH 6.0), pelleted again, and resuspended in 1 volume of cold PBS. Cells (2.4×10^8) were irradiated using the same arrangement as for embryos except that irradiation was for 4 min. After irradiation, the cells were spun down and resuspended in 2.7 ml of nuclei lysis buffer. Cells were lysed with 300 μ l of 20% Sarkosyl, and the chromatin was sheared by five passages through a 25-gauge needle and loaded onto an 8.3-ml CsCl step gradient (4.5 ml of 1.75 g/ml CsCl, 2.3 ml 1.5 g/ml CsCl, 1.5 ml 1.3 g/ml CsCl). Gradients were centrifuged for 36 hr at 37,000 rpm and 20°C in a Beckman SW41 rotor. Each immunoprecipitation was carried out with chromatin from 4×10^7 cells essentially as described above.

Southern blotting

Because of the small amounts of DNA being detected and the large quantities of carrier DNA used to purify immunoprecipitates, it was essential to employ this Southern blotting protocol, which gives high signals and low backgrounds. DNA was separated on a 0.7% agarose gel, denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min, neutralized in 1.5 M NaCl, 0.5 M Tris at pH 7.2, 1 mM EDTA for 30 min, and transferred to Hybond-N (Amersham, uncharged nylon membrane) for 12 hr using capillary transfer in $2 \times$ SSPE (3.6 M NaCl, 0.2 M NaPO₄, 20 mM EDTA at pH 8.0). After transfer, membranes were washed for 5 min in $2 \times$ SSPE, gently blotted dry, baked for 30 min at 80°C in a vacuum oven, and irradiated for 5–30 sec (depending on the batch of membrane) with a Fotodyne DNA transfer lamp. The membrane was then incubated for 16–24 hr at 42°C in prehybridization solution (50% formamide, $6 \times$ SSPE, 90 μ g/ml of sonicated calf thymus DNA, half of which had been denatured by boiling, 10 μ g/ml of sheared *Escherichia coli* DNA, $5 \times$ Denhardt's solution, 10% dextran sulfate, 5% SDS, 1% Sarkosyl; filter through 0.45- μ m syringe filter). Radioactive probe with a

sp. act. of $\sim 5 \times 10^9$ cpm/ μ g (generated by random priming using an Amersham kit) was added to the blot at 1.6×10^7 cpm/ml of hybridization solution and hybridized for 20 hr. Blots were washed for 30 min in $2 \times$ SSPE, 0.1% SDS, at room temperature, for 15 min in $1 \times$ SSPE, 0.1% SDS, at 65°C, for 4–24 hr in $0.1 \times$ SSPE and 1.0% SDS, at 65°C and exposed to film once the filter background dropped below 1 count per second. A 4- to 5-day exposure was typically required to detect .0001% of total DNA in a reaction originally containing 450 μ g of chromatin.

A more detailed protocol is available upon request.

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Two homeo domain proteins bind with similar specificity to a wide range of DNA sites in *Drosophila* embryos.

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