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DNA binding specificity of two homeodomain proteins *in vitro* and in *Drosophila* embryos

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ABSTRACT **In previous experiments, the homeodomain proteins even-skipped and fushi-tarazu were found to UV cross-link to a surprisingly wide array of DNA sites in living *Drosophila* embryos. We now show that UV cross-linking gives a highly accurate measure of DNA binding by these proteins. In addition, the binding of even-skipped and fushi-tarazu proteins has been measured *in vitro* to the same DNA fragments that were examined *in vivo*. This analysis shows that these proteins have broad DNA recognition properties *in vitro* that are likely to be important determinants of their distribution on DNA *in vivo*, but it also shows that *in vitro* DNA binding specificity alone is not sufficient to explain the distribution of these proteins in embryos.**

Many of the proteins that regulate *Drosophila* development are transcription factors that share a homologous DNA binding domain termed the homeodomain. As with many other eukaryotic transcription factors, it has been difficult to determine which DNA sites are bound by homeodomain proteins *in vivo*. This is due to the complex and redundant nature of the regulatory network in which homeodomain proteins act and because many of these proteins recognize the same DNA sites (1).

To allow identification of DNA sites bound by sequence-specific transcription factors *in vivo*, we previously improved the sensitivity of an established *in vivo* cross-linking protocol (2, 3). This method involves immunoprecipitation of proteins that have been cross-linked to DNA with UV light *in vivo* and subsequent characterization of the attached DNAs by southern blotting. Using this approach to study the DNA binding of the homeodomain proteins even-skipped (eve) and fushi-tarazu (ftz) gave the following striking results (3): (*i*) both proteins cross-linked at nearly uniform levels throughout the length of several genetically identified target genes; (*ii*) cross-linking was also detected at lower but significant levels to a series of randomly chosen genes for which there was no evidence suggesting that they were regulated by eve or ftz; and (*iii*) eve and ftz proteins appeared to have very similar DNA binding specificities *in vivo*. In contrast, a nonhomeodomain transcription factor, zeste, was detected only on short DNA elements within a target promoter and not on other genes. Although the binding pattern of eve and ftz proteins is much broader than predicted by earlier models, it is consistent with the relatively high abundance of these proteins in embryos. We also suggest that these data support models in which related homeodomain proteins act by regulating largely the same target genes (3–5).

An important question is what are the mechanisms determining the distribution of eve and ftz proteins *in vivo*? In contrast to the binding throughout promoters observed *in vivo*, most *in vitro* binding studies with eve, ftz, and similar homeodomain proteins have been interpreted as showing binding only to localized regions within genetic target genes (refs. 6–11; for an exception, see ref. 12). However, in most of these *in vitro* studies, binding was generally assayed under very stringent conditions, no quantitative comparisons were made among the various fragments assayed, and binding was measured only to a subset of the genes that have been examined *in vivo* by UV cross-linking. Therefore, to better understand what role the intrinsic DNA binding specificities of eve and ftz proteins play in establishing their distributions *in vivo*, we decided to systematically measure the relative affinities of these proteins for the same DNA fragments that were examined *in vivo*. In addition, control experiments have been carried out to verify that the results of UV cross-linking are indeed directly comparable to conventional *in vitro* DNA binding assays.

EXPERIMENTAL PROCEDURES

DNA Fragments. The following plasmids were digested, mixed together in equimolar amounts, and radioactively labeled for use in Fig. 1*A*: an 8.75-kb *Eco*RI genomic Actin 5C fragment (13) cloned into pUC12 and digested with *Eco*RI; a 7.3-kb *Hin*dIII rosy genomic fragment cloned into pUC12 and digested with *Hin*dIII; a 4.75-kb *Adh* genomic *Eco*RI fragment cloned into pUC13 and digested with *Eco*RI; a 3.5-kb *Ubx* genomic *Eco*RI fragment containing the RNA start site cloned into pUC12 and digested with *Eco*RI; a 2.6-kb genomic *hsp70* *Bam*HI fragment cloned into pUC13 and digested with *Bam*HI and *Xho* I, yielding a 1.1- and a 1.45-kb fragment. References for these DNA fragments are contained in ref. 3. DNA fragments used in other experiments are described in the figure legends.

Purification of Proteins. ftz protein was overexpressed in a 1-liter bacterial culture and inclusion bodies were isolated, washed twice in RIPA buffer, and resuspended in 2.5 ml of 8 M guanidine·HCl. After addition of 2.5 ml of 50 mM Tris/12.5 mM glycine, pH 9.6, ftz protein was purified on an S300 column equilibrated in 4 M guanidine·HCl/25 mM Tris/6.25 mM glycine, pH 9.6. Glycerol (0.2 vol) was added to the peak of ftz protein, which was then dialyzed for 12 hr against 100 vol of 1 M guanidine/50% glycerol/25 mM Tris/6.25 mM glycine, pH 9.6/1 M NaCl; then against two changes (12 hr each) of the same buffer lacking guanidine; and finally, against 20% glycerol/100 mM NaCl/25 mM Tris/6.25 mM glycine/12.5 mM $MgCl_2$, pH 9.6. eve protein was purified to near homogeneity as described (14); purified Dfd protein was a gift of W. McGinnis (University of California at San Diego) and was purified similarly to eve protein. zeste protein was overexpressed in bacteria and then purified essentially as described (15).

Immunoprecipitation Assay. Protein and DNA were incubated together for 30 min on ice in a total vol of 50 μl. In addition to 150 or 210 mM NaCl, reaction mixtures also contained 20 mM Tris (pH 7.5), 0.25 mM EDTA, 10% glycerol, 6.25 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM dithiothreitol (DTT), 50 μg of sonicated herring sperm DNA per ml, and 10

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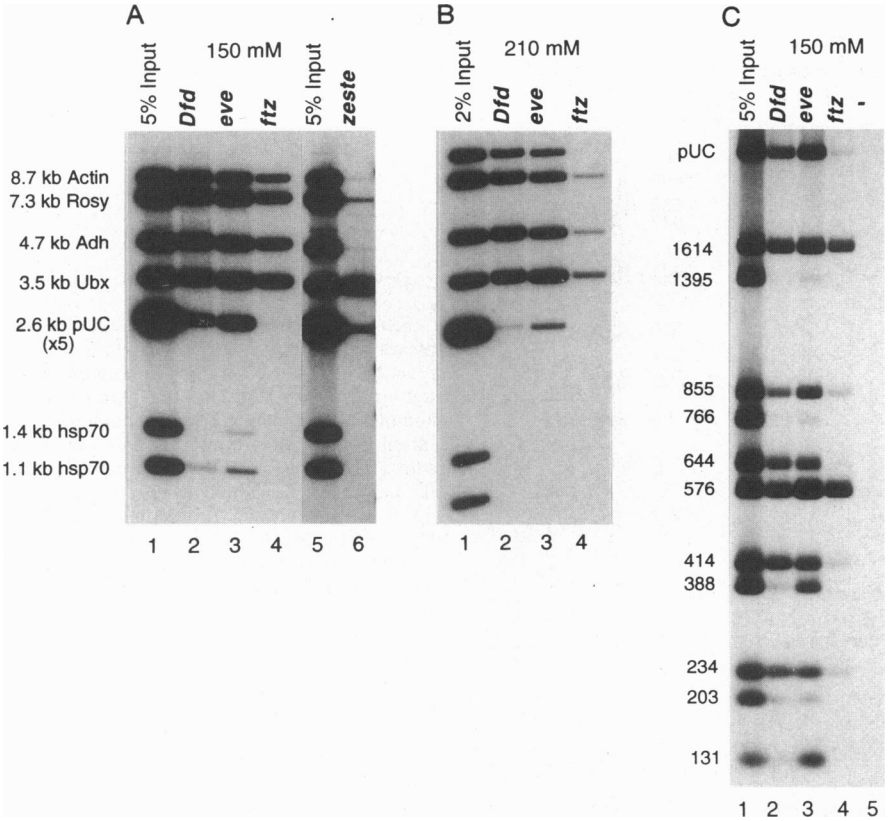

FIG. 1. Binding of purified proteins to two mixtures of DNA fragments in 150 mM (*A* and *C*) or 210 mM (*B*) NaCl using an immunoprecipitation assay. Binding was carried out using 120 ng of Dfd (lane 2), 150 ng of eve (lane 3), or 400 ng of ftz protein (lane 4), except that in *B* 800 ng of ftz protein was used. Binding in *A* (lane 6) used 1200 ng of zeste protein. Lanes 1 in *A–C* and lane 5 in *A* contain a percentage of the input DNA. Lane 5 in *C*, no homeodomain protein was added, but the reaction mixture contained eve antibodies. The sizes of DNA fragments used are indicated. The fragments in *C* were derived from a *Hin*dIII, *Apo* I, and *Bgl* II digest of the rosy plasmid used in *A*.

fmol of each ^{32}P phosphorylated DNA fragment. In reaction mixtures containing zeste protein shown in Fig. 1, the carrier DNA used was poly(dI-dC) (50 μg/ml). [Note that when binding by eve and ftz proteins is assayed using poly(dI-dC) (50 μg/ml) as a competitor, their binding is slightly less specific than when herring sperm DNA is used (data not shown).] To precipitate the homeodomain protein DNA complexes, 150 ng of affinity-purified anti-Dfd antibody (a gift of W. McGinnis), 160 ng of anti-ftz antibody (a gift of S. Carroll, University of Wisconsin at Madison), or 300 ng of an antibody raised against amino acids 1–139 of the eve protein (unpublished results) was preincubated for 30 min with 10 μl of 20% staphylococcal A cells (prepared as described in ref. 3). The washed staphylococcal A–antibody complexes were then added to the binding reaction mixture for 30 min at room temperature. To immunoprecipitate the zeste protein–DNA complexes, 8 μl of a crude serum raised against a zeste β-galactosidase fusion protein was used (16). Immune complexes were collected and washed two times with the binding buffer lacking protein, DTT, and DNA. The DNA was extracted with phenol chloroform, ethanol precipitated, separated on agarose gels, and visualized by autoradiography. For eve protein, it was verified that the amount of protein used gives maximal specificity of binding.

***In Vitro* UV Cross-Linking.** Binding reaction mixtures (as for immunoprecipitation) were irradiated for 3 min on parafilm-covered chilled metal blocks at a distance of 3 cm from the bulbs of the UV lamp described (3). The reactions were then mixed with 200 μl of a buffer that disrupts noncovalent protein–DNA complexes: 150 mM NaCl/10 mM Tris, pH 7.5/10 mM $MgCl_2$/1% Triton X-100/0.2% Sarkosyl/100 μg of RIA grade bovine serum albumin (BSA) per ml (Sigma); 4.5 μg of purified anti-eve antibody (see above) was added for 1.5 hr at 4°C before addition of 10 μl of 20% staphylococcal A cells. After 30 min of incubation, staphylococcal A cells were collected by centrifugation and washed (3) to further disrupt noncovalent interactions. After eluting the DNA (3), it was digested with proteinase K for 30 min (3), extracted once with phenol chloroform, once with chloroform, and ethanol precipitated.

Filter Binding. eve protein was incubated with 10 fmol of each DNA fragment in 150 mM NaCl/20 mM Hepes, pH 7.6/5 mM $MgCl_2$/0.25 mM EDTA/5% dimethyl sulfoxide/125 μg of acetylated BSA per ml (New England Biolabs)/1 mM DTT/50 μg of sonicated herring sperm DNA per ml in a total vol of 50 μl for 30 min at 4°C (17). Filtration of reactions was carried out as described (17), except that filters were washed with 0.5 ml of binding buffer lacking DTT, DNA, and BSA. DNA was removed from the filters by shaking them for 30 min in 500 μl of 20 mM Tris, pH 8/0.8% SDS/4 mM EDTA/1 μg of sonicated calf thymus DNA per ml containing 0.1 mg of proteinase K per ml. It was then extracted with phenol/chloroform and chloroform and ethanol precipitated.

RESULTS AND DISCUSSION

We first measured *in vitro* DNA binding by eve and ftz proteins to the group of genes which, *in vivo*, are cross-linked by eve and ftz proteins on average at levels 10-fold lower than known genetic targets and which have not generally been considered to be regulated by these proteins (Fig. 1*A*). For comparison, *in vitro* binding was also measured to the genetic target, Ultrabithorax (*Ubx*). The *Ubx* promoter fragment used contains high-affinity binding sites that are required for regulation of this promoter by eve and ftz proteins in transcription assays (18, 19), and the dissociation constant of eve protein for these sites is $\approx 5 \times 10^{-9}$ M (ref. 18; unpublished data). To determine whether our observations apply more generally to other similar homeodomain proteins, *in vitro* DNA binding of the homeotic regulator Deformed (*Dfd*) has also been examined. Since zeste protein crosslinks specifically to the *Ubx* promoter fragment *in vivo* and not to the other genes (3), we also examined binding of this protein *in vitro*.

Interestingly, immunoprecipitation assays show that, eve, ftz, and Dfd proteins bind to rosy, Actin 5C, and Alcohol dehydrogenase (*Adh*) gene fragments on average only 2-fold

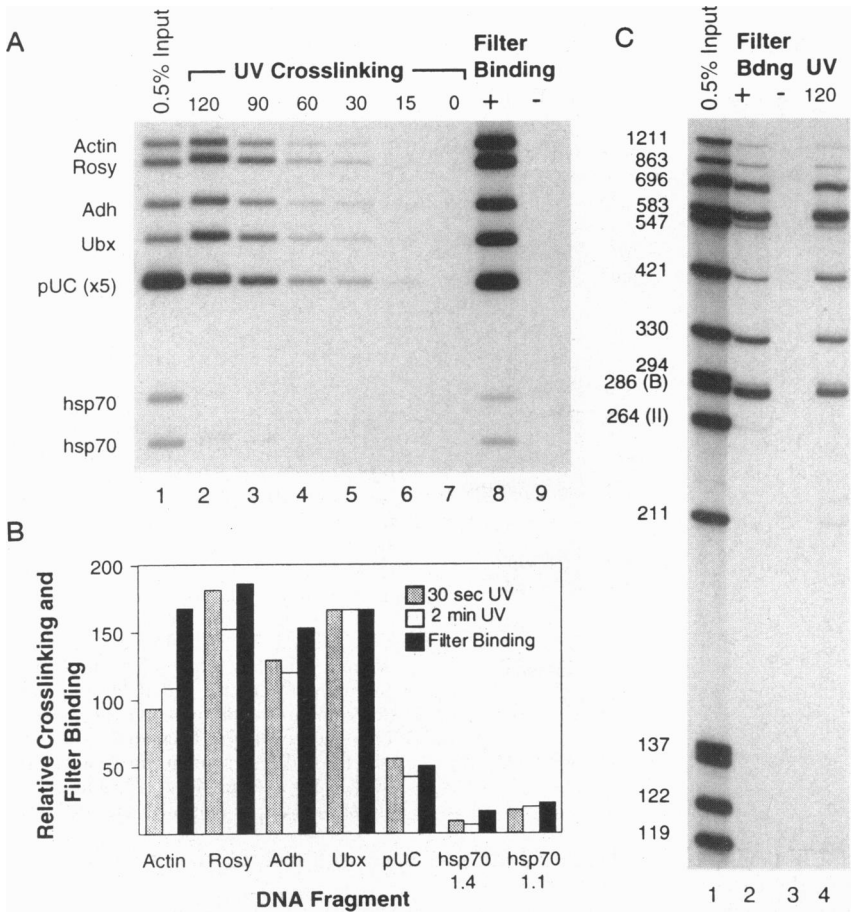

FIG. 2. (*A*) Covalent protein–DNA complexes were induced by UV irradiating binding reaction mixtures containing 400 ng of eve protein (as in Fig. 1*A*) for 0–120 sec, and the complexes were characterized by immunoprecipitation and gel electrophoresis (lanes 2–7). Also shown are 3% of the DNA fragments bound in the presence (lane 8) and absence (lane 9) of 400 ng of eve protein in a filter binding assay. (*B*) PhosphorImager quantitation of selected lanes in *A*. Recovery of the *Ubx* fragment was set to be equal in all three cases and other values were adjusted accordingly (the result calculated for pUC was also divided by 5). (*C*) Filter binding with (lane 2) and without (lane 3) protein and UV cross-linking (lane 4) were carried out exactly as in *A* except that DNA fragments were derived from a *Bsa*JI, *Bss*HII, and *Sca* I triple digest of the 3.5-kb *Ubx* proximal promoter fragment, and products were separated on a 6% sequencing gel. Only 1.6% of the filter-bound fragments were loaded onto the gel (lanes 2 and 3). Fragments containing the high-affinity element B and the moderate-affinity site II of the *Ubx* promoter (15) are indicated.

less well than to *Ubx*, and that eve and Dfd proteins, but not ftz protein, bind detectably to *hsp70* DNA (Fig. 1*A*). The same result was observed using a filter binding assay (Fig. 2*A*; data not shown), and at even higher stringency, all three proteins still bind to *Adh* and rosy almost as well as they do to *Ubx* (Fig. 1*B*). By contrast, zeste protein binds strongly to the *Ubx* promoter fragment but hardly at all to the other genes, consistent with the previous *in vivo* and *in vitro* binding studies with this protein (3, 20). Several lines of evidence suggest that the binding of the three homeodomain proteins to *Adh* and rosy is sequence specific. (*i*) Dfd, eve, and ftz proteins bind on average much more weakly to bacterial plasmid DNA (Fig. 1 *A* and *B*). (*ii*) Fig. 1*C* demonstrates that precipitation of the 7-kb rosy fragment is due to the presence of several high and moderate affinity sites spread throughout this fragment. (*iii*) Binding by eve protein to the three highest affinity rosy fragments is virtually as tight as binding to the three highest affinity fragments derived from the *Ubx* gene (Figs. 1*C* and 2*C*; data not shown). The poor discrimination of these proteins *in vitro* between the characterized genetic target *Ubx* and the other genes is surprising. In addition, there is no correlation between *in vitro* binding and *in vivo* cross-linking to these genes. This is best illustrated by the fact that eve and ftz proteins cross-link *in vivo* to the rosy and *Adh* genes at the lowest level among this group of genes, whereas their binding to these genes *in vitro* is the highest (see also Fig. 4).

To verify that the different *in vitro* and *in vivo* preferences are not due to biases inherent in the UV cross-linking method, *in vitro* UV cross-linking of eve protein to a mixture of DNA fragments was compared with a filter binding assay. This experiment shows that the efficiency of UV cross-linking to each fragment in this mixture is directly proportional to its retention in the filter binding assay (Fig. 2 *A* and *B*), and this result applies to a wide range of UV irradiation times. Furthermore, the proportionality between binding and cross-linking also applies when much smaller DNA fragments are examined (Fig. 2*C*) or when the nonhomeodomain transcription factor, zeste, is used (data not shown). Thus, *in vitro* UV cross-linking is proportional to DNA binding for two different transcription factors on a wide range of sequences. The excellent correspondence between the relative levels of binding and cross-linking is probably due to the presence of multiple binding sites on each fragment so that any differences in cross-linking efficiency between individual sites are averaged out. These results strongly suggest that the relative levels of *in vivo* UV cross-linking reported previously for eve, ftz, and zeste proteins (3) directly reflect the relative level of DNA binding by these proteins to different DNA fragments *in vivo*. We suggest, however, that the proportionality between binding and cross-linking should be examined anew when other classes of transcription factors are examined by UV cross-linking.

From the above results it is clear that the DNA binding preferences of eve and ftz proteins *in vivo* and *in vitro* are different.† It is possible that these differences can be explained by the influences of chromatin structure on DNA binding *in vivo*. For example, rosy and *Adh* are both transcriptionally inactive at the developmental stage assayed in the UV cross-linking experiments (22, 23), and such inactive genes are thought to be in a closed chromatin state which can prevent binding by regulatory molecules (24). rosy and *Adh* are the two genes that are bound most weakly *in vivo*, and thus binding to the high-affinity sites located in these genes may be inhibited

†It is unlikely that the observed differences are due to our use of bacterially expressed proteins rather than proteins purified from insect cells since proteins derived from these two sources exhibit the same *in vitro* DNA binding specificity (refs. 11 and 21, M.D.B., unpublished results).

by a closed chromatin structure *in vivo*. Conversely, Actin 5C is heavily transcribed, and *hsp70* is known to be in an "open" chromatin conformation (13, 25), which might account for the fact that binding to these genes *in vivo* is higher than to rosy or *Adh*, despite the fact that Actin 5C and *hsp70* are the more weakly bound genes *in vitro*.

We next examined *in vitro* binding to DNA fragments spanning the length of two genetic targets of eve and ftz, the *eve* and *ftz* genes themselves. In embryos, strong and relatively uniform cross-linking by eve and ftz proteins was seen on many kilobase pairs of these two targets *in vivo* (3). In contrast, earlier *in vitro* studies reported that ftz protein binds only to a 2-kb upstream autoregulatory region of the *ftz* promoter (8, 9), and that eve protein binds only to three short regions of the *eve* promoter (6, 7). Significantly, at 150 mM NaCl, we observed binding of eve, ftz, and Dfd proteins to other regions of these promoters as well, and, in most cases, only 2- to 3-fold differences in binding are seen between many fragments across a promoter (Fig. 3 *A, C*, and *D*). At higher NaCl concentrations, however, much greater differences in binding are seen between DNA fragments, especially in the case of the ftz protein (Fig. 3*B*), giving a pattern of binding more similar to that of the earlier reports (8, 9). Thus, the less selective binding observed at 150 mM NaCl *in vitro* is more similar to that observed *in vivo*, and so these conditions may provide a more relevant description of these proteins' DNA binding properties. However, even under these conditions, some differences between binding *in vitro* and *in vivo* are still observed, as illustrated in Fig. 3*D*.

Fig. 4 shows a quantitative comparison of binding *in vivo* and *in vitro* to the various genes examined in this paper. In addition to the points made earlier, this figure illustrates that binding to a genetically characterized regulatory target *in vitro* can be comparatively weak. Specifically, eve and ftz proteins bind to the *eve* gene more weakly *in vitro* than to genes such as rosy and *Adh*, even though *in vivo* the reverse preference is observed. These results suggest that binding to the *eve* gene *in vivo* could be facilitated by the kinds of cooperative interactions with other transcription factors that have been noted in other systems (26, 27). In this regard, it is interesting that ftz molecules lacking the DNA binding domain have been shown to still regulate transcription in embryos, suggesting that interactions with other proteins may indeed be able to recruit ftz protein to some promoters (28). However, it is also possible that eve and ftz proteins bind more strongly to the *eve* gene than to genes such as rosy and *Adh* entirely because of differences in the chromatin structure and hence accessibility of these genes.

The experiments presented here appear to argue against one of the models by which homeodomain proteins have been suggested to act. This model envisions that the modest differences in DNA binding specificity observed *in vitro* between homeodomain proteins might be greatly increased *in vivo*, causing these proteins to occupy mostly different DNA sites (reviewed in refs. 1 and 29). *In vitro*, we observe that eve, ftz, and Dfd proteins all bind to largely the same high-affinity sites with similar specificity, but that ftz protein binds very weakly or not at all to many sites which Dfd and eve bind with moderate affinity (see Figs. 1 and 3). In contrast, the relative levels of binding of eve and ftz proteins to all DNAs examined *in vivo* are virtually identical (3). Thus, instead of observing an amplification of differences in DNA binding specificity, the data indicate that such modest differences are actually reduced *in vivo*. The similar and widespread binding of eve and ftz proteins *in vivo* also differs dramatically from the distribution predicted by another model of homeodomain targeting (27). In this model, the homeotic proteins (which include Dfd) interact differentially with cofactors to achieve unique and specific

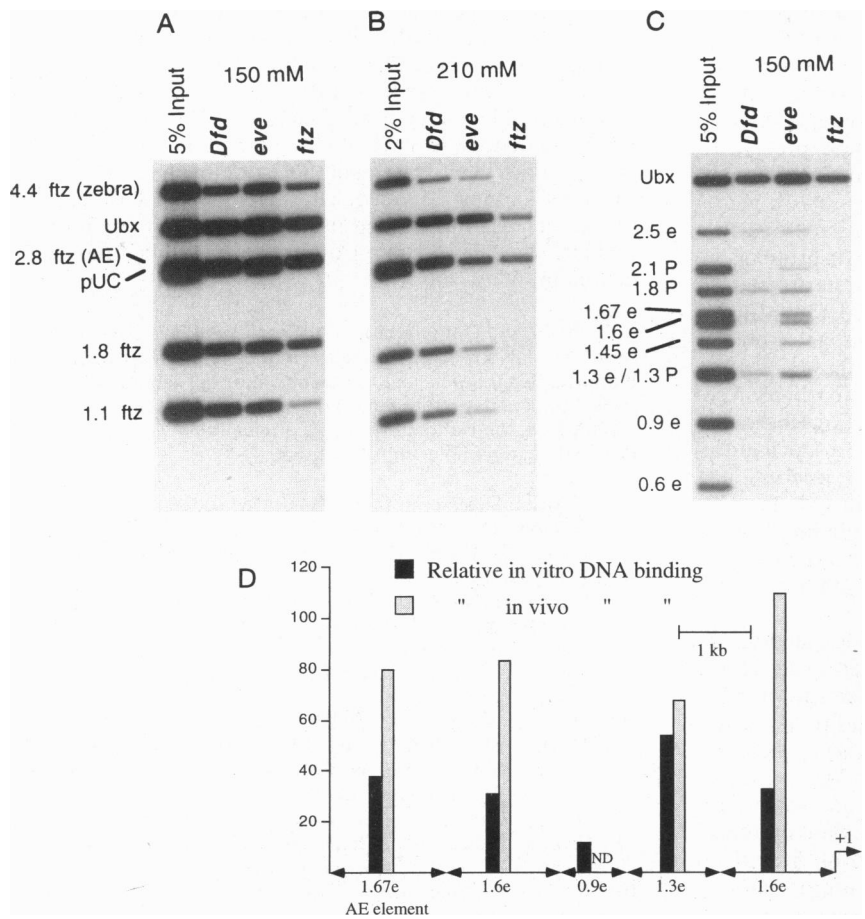

FIG. 3. Binding of homeodomain proteins to *ftz* and *eve* gene fragments using the same procedure as in Fig. 1. *ftz* promoter fragments (used in *A* and *B*) were derived from a *Bam*HI digest of a genomic *Kpn* I fragment extending from −6 to +4 kb relative to the RNA start site, giving, in the same order as on the chromosome, a 2.8-kb fragment which includes the upstream autoregulatory (AE) element, a 1.8-kb fragment, a 4.4-kb fragment including the zebra element and the transcription unit, and a 1.1-kb fragment. eve promoter fragments (used in *C*) were generated by digesting a plasmid (pEL3) that contains *eve* DNA from −8.9 to +0.15 kb relative to the RNA start site with *Bgl* I, *Bam*HI, *Xho* I, and *Eco*RI. The sizes of each fragment and whether it corresponds to *eve* (e) or vector (P) DNA are indicated, and the locations of some of these fragments in the *eve* gene are shown in *D*. All reaction mixtures in *A–C* also contained the 3.5-kb *Ubx* proximal promoter fragment. (*D*) Comparison of the relative *in vitro* binding at 150 mM NaCl (solid bars) and *in vivo* cross-linking (shaded bars) (3) of eve protein to *eve* DNA fragments, which are shown as double-headed arrows with the same nomenclature as in *C*. See Fig. 4 for conventions used to plot the data. The *in vitro* data are derived from *C*, except that data for fragments 1.3e and the 3′ most 1.6e fragment are not shown. While eve protein binds quite uniformly throughout the *eve* gene, ftz protein binds detectably only to fragment 1.3e at a relative value of 11.

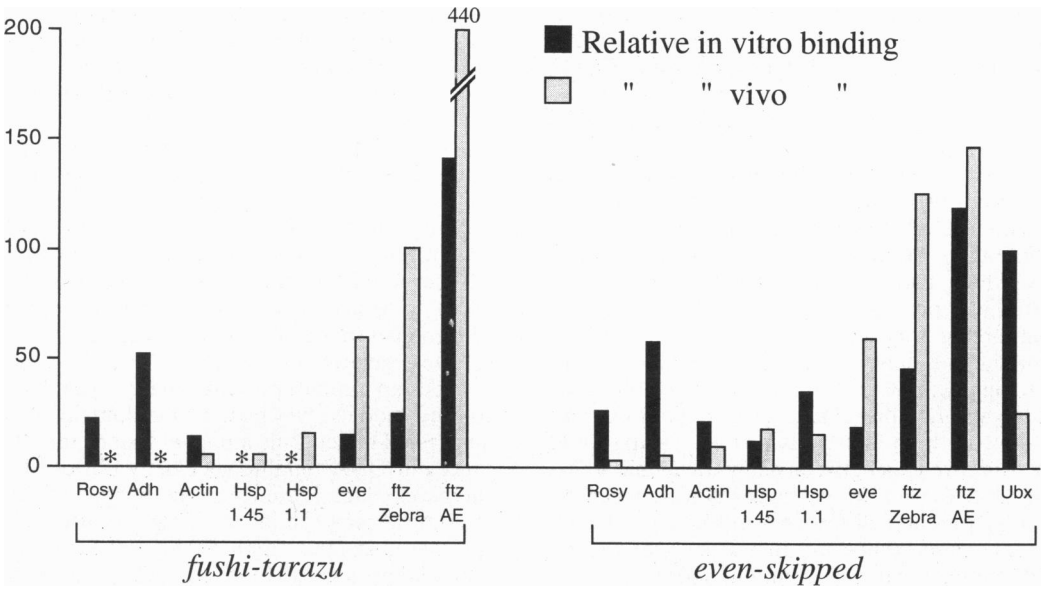

FIG. 4. Comparison of relative *in vitro* binding (solid bars) and *in vivo* cross-linking (shaded bars) of eve and ftz proteins to various DNA fragments. Relative *in vitro* DNA binding to each fragment was divided by the size of the fragment and then normalized to the 3.5-kb *Ubx* control fragment, which was assigned a value of 100. Absolute cross-linking values were also divided by the size of each fragment (3) and plotted so that high values for eve and for ftz were both set at 100; all other fragments were normalized to this value. Asterisk denotes background binding or UV cross-linking. The *eve* promoter fragment is a 7-kb fragment extending from the RNA start site to −7.3 kb. In the case of the *ftz* gene, the zebra and autoregulatory fragments examined *in vivo* are in both cases fully contained within the corresponding fragments examined *in vitro* (see Fig. 3*A* and ref. 3). In the case of the *hsp70* gene, the 1.45- and 1.1-kb fragments examined *in vitro* are derived from only one (I32E3) of the several *hsp70* gene copies that exist and that were examined together *in vivo* (3). The *in vitro* binding data were derived from PhosphorImager quantitation of the experiments shown in Figs. 1 and 3 at 150 mM NaCl, which represent typical results.

patterns of DNA binding in embryos. Given these different views, it will now be important to directly examine the binding of homeotic selector proteins *in vivo* by UV cross-linking.

In summary, the *in vitro/in vivo* comparison presented in this paper suggests that a reassessment of the DNA binding properties of eve, ftz and perhaps other homeodomain proteins is necessary. The *in vitro* binding data show that these proteins bind many sites throughout their genetic targets, including many moderate affinity sites that have previously been overlooked. Furthermore, many high- and moderate-affinity sites have been found in virtually all *Drosophila* genes we have tested. Since these broad DNA recognition properties fit well with the broad DNA binding of eve and ftz proteins on chromatin, they are likely to be an important determinant of these proteins' binding *in vivo*. Binding to moderate-affinity sites is probably driven by the high concentrations of homeodomain proteins in embryonic nuclei (3, 30, 31, 32). In addition, binding to these lower-affinity sites is probably also facilitated by homomeric cooperative interactions between homeodomain proteins bound to distant sites, a mechanism that has been shown to be important for transcriptional regulation of various promoters by eve protein (14, 15, 33). Most likely, the differences observed between binding *in vitro* and *in vivo* are due to the influence of chromatin structure and perhaps also to cooperative interactions with other proteins that bind only to some genes.

The functional significance of many of the homeodomain binding sites identified here remains to be determined. However, moderate-affinity sites have previously been shown to be functionally important for the action of eve and ftz proteins, suggesting that many of the moderate-affinity sites reported here could be functionally significant (7, 14, 15, 34). Currently it is not known what percentage of *Drosophila* genes are directly regulated by eve, ftz, or other homeodomain proteins. However, estimates that predict that at least 25% of *Drosophila* genes are expressed in segmentally repeating patterns (35, 36) suggest that this number could be very large. This observation is consistent with our finding that high-affinity homeodomain binding sites are very abundant in *Drosophila* genes.

In vivo UV cross-linking with sequence-specific transcription factors is a generally applicable method that has now been carried out successfully with four transcription factors containing three distinct DNA binding domains (3, 37). In this paper, we have presented further evidence that UV cross-linking provides a quantitative measure of binding to different DNA sequences *in vivo*. As a method that also provides unambiguous identification of the factor involved in binding, *in vivo* cross-linking is unique. Thus, this method has the potential to increase our limited understanding of the parameters that influence DNA binding of sequence-specific transcription factors in living cells.

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