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A comparison of *in vivo* and *in vitro* DNA-binding specificities suggests a new model for homeoprotein DNA binding in *Drosophila* embryos

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Little is known about the range of DNA sequences bound by transcription factors *in vivo*. Using a sensitive UV cross-linking technique, we show that three classes of homeoprotein bind at significant levels to the majority of genes in *Drosophila* embryos. The three classes bind with specificities different from each other; however, their levels of binding on any single DNA fragment differ by no more than 5- to 10-fold. On actively transcribed genes, there is a good correlation between the *in vivo* DNA-binding specificity of each class and its *in vitro* DNA-binding specificity. In contrast, no such correlation is seen on inactive or weakly transcribed genes. These genes are bound poorly *in vivo*, even though they contain many high affinity homeoprotein-binding sites. Based on these results, we suggest how the *in vivo* pattern of homeoprotein DNA binding is determined.

Keywords: Bicoid/*Drosophila*/homeoproteins/Paired/UV cross-linking

Introduction

Many metazoan transcription factors belong to protein families that bind *in vitro* to short, degenerate DNA sequences that occur frequently in the majority of genes (Faisst and Meyer, 1992; Pabo and Sauer, 1992; Heinemeyer *et al*., 1998; http://transfac.gbf.de/transfac). In addition, members of the same family often show similar DNA specificities *in vitro*. What are the range of genes that these proteins bind in cells? One theory suggests that these transcription factors must bind much more selectively *in vivo* than they do *in vitro* and that, in cells, the different members of a family will each bind to a different set of genes (e.g. Johnson, 1992; Mann and Chan, 1996). However, this theory is based on indirect evidence. Directly determining the range of genes bound *in vivo* requires that binding of endogenous proteins be measured in living cells. Consequently, we previously developed an *in vivo* UV cross-linking method that accurately quantitates DNA binding and used it to compare binding of two members of the selector homeoprotein family of transcription factors in *Drosophila* (Walter *et al*., 1994).

The selector homeoproteins are an evolutionarily conserved group of homeoprotein that include the Hox (or homeotic) proteins, the Eve- and Engrailed-like proteins (Burglin, 1994; Biggin and McGinnis, 1997). *In vitro*, all of these molecules have nearly identical DNA-binding specificities, showing similar preferences for variants of the consensus sequence NNATTA (Gehring *et al*., 1994; Biggin and McGinnis, 1997). Our UV cross-linking experiments suggest that, in embryos, the selector homeoproteins Eve and Ftz bind with similar specificities to DNA sites throughout the length of the majority of genes (Walter *et al*., 1994). Most genes are bound at lower levels than the best characterized genetically defined targets of these proteins, but only at 2- to 20-fold lower levels. Other experiments indicate that Eve, Ftz and the other selector homeoproteins have broad regulatory properties in embryos that are consistent with much of the *in vivo* DNA binding being functional (Liang and Biggin, 1998). Over the genes tested, quantitative differences in DNA binding correlate with quantitative differences in gene regulation. Even the most weakly bound genes are affected detectably by changes in *eve* expression (Liang and Biggin, 1998).

In this study, we have extended this analysis by examining DNA binding by two *Drosophila* homeoproteins, Bicoid and Paired, which are evolutionarily diverged from the selector homeoproteins. The amino acid at position 50 of the homeodomain makes specific contacts with the two bases 5′ of the ATTA core recognition sequence (Gehring *et al*., 1994; Hirsch and Aggarwal, 1995). All of the selector homeoproteins have a glutamine at this position, whereas Bicoid has a lysine and Paired has a serine. These different residues give Bicoid and Paired unique preferences for variants of the NNATTA consensus sequence (Treisman *et al*., 1989; Percival-Smith *et al*., 1990; Wilson *et al*., 1993). For example, Bicoid binds *in vitro* >10 times more strongly than the selector homeoproteins to the sequence GGATTA but binds at least 10 times more weakly than the selector homeoproteins to the sequence CCATTA (Percival-Smith *et al*., 1990). In addition, Paired contains a second DNA-binding domain, the paired domain. This domain recognizes an entirely different 10–14 bp sequence, which is found adjacent to homeodomain recognition sites in Paired target elements (Fujioka *et al*., 1996; Jun and Desplan, 1996).

We wished to determine how the distinct *in vitro* preferences of these three classes of homeoprotein are related to their DNA binding *in vivo*. Our results indicate that, in embryos, Paired and Bicoid bind most strongly to known target elements within a promoter and that, like the selector homeoproteins, they may also bind at significant levels to the majority of genes. Based on a comparison of *in vitro* and *in vivo* DNA-binding preferences, we suggest how the *in vivo* pattern of binding by these proteins is determined and we propose that DNA binding by other families of metazoan transcription factors may be determined in a similar manner.

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Results and discussion

UV cross-linking accurately reflects relative levels of DNA binding

UV cross-linking is a well characterized method that only covalently couples proteins to DNA sequences to which they are bound directly (Hockensmith *et al.*, 1991; Blatter *et al.*, 1992; Walter and Biggin, 1997). Control *in vitro* experiments demonstrate that the amounts of Eve protein UV cross-linked to a series of different affinity DNA fragments accurately reflect the relative levels of protein bound to DNA; also, Eve does not UV cross-link to short restriction fragments that do not contain specific homeoprotein recognition sequences (Walter and Biggin, 1996). Therefore, because UV cross-linking is a good measure of relative DNA binding by Eve *in vitro*, this method should also provide an accurate quantitation of Eve's binding to specific DNA sites *in vivo*.

Before examining cross-linking of Paired and Bicoid in embryos, we have first ensured that UV cross-linking gives an accurate measure of DNA binding for these proteins. Figure 1 compares the results of a standard *in vitro* DNA-binding experiment with those of an *in vitro* UV cross-linking assay. Paired and Bicoid bind with different relative affinities to a series of DNA fragments that each contain a number of Paired and Bicoid recognition motifs. The levels of UV cross-linking closely follow these DNA-binding profiles (Figure 1B). Among the DNA fragments tested, the mean difference between the DNA-binding and UV cross-linking data is $\pm$ 20%, and the most extreme difference on any DNA fragment is ~2-fold. Therefore, *in vivo* UV cross-linking should be a good measure of binding by endogenous Paired and Bicoid at specific DNA sites in embryos.

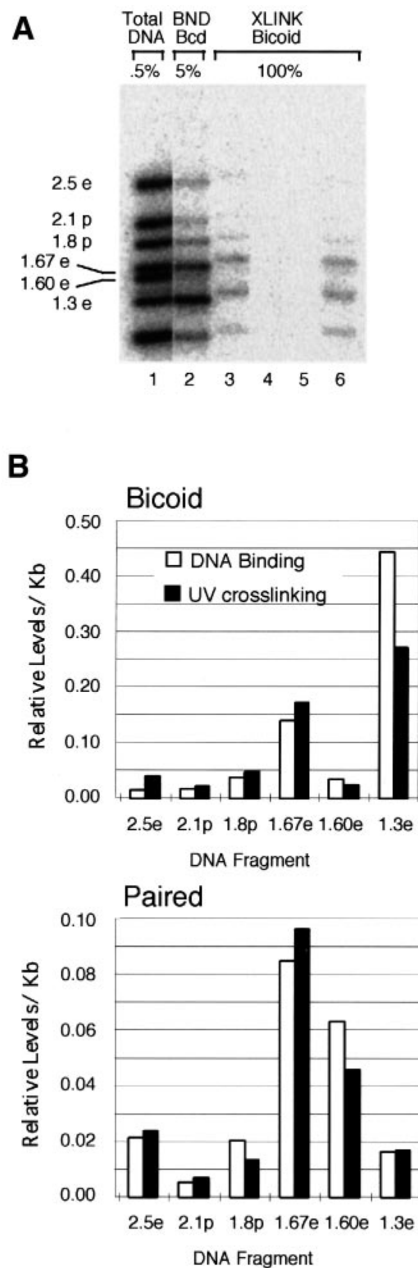


Fig. 1. UV cross-linking is a good measure of DNA binding. (**A**) For all experiments, DNA-binding reactions contained 300 ng of purified Bicoid protein and 10 fmol of a *Bgl*I, *Bam*HI, *Eco*RI and *Xho*I restriction digest of plasmid pEL3. In a standard DNA-binding reaction (lane 2), protein–DNA complexes were purified by immunoprecipitation using anti-Bicoid antibodies bound to *Staphylococcus aureus* cells; then precipitated complexes were washed briefly in binding buffer, the bound DNA fragments were eluted and 5% of the recovered DNA fragments was analyzed by electrophoresis on an agarose gel and exposure to an imaging plate. In UV cross-linking experiments, DNA-binding reactions were either UV irradiated for 2 min (lanes 3, 5 and 6) or were not irradiated (lane 4); then protein–DNA complexes were isolated by immunoprecipitation, uncross-linked DNA was removed by washing precipitated complexes in a buffer containing 1% Triton X-100 and 0.2% Sarkosyl, covalently coupled protein–DNA complexes were eluted from the *S.aureus* cells and digested with proteinase K, and the recovered DNAs were analyzed by electrophoresis. In the UV cross-linking experiment shown in lane 5, NaCl was added to reactions at a final concentration of 1 M prior to UV irradiation. In the experiment shown in lane 6, NaCl was added to a final concentration of 1 M after UV irradiation. The sizes of the DNA fragments present in reactions, and whether they are plasmid (p) or *eve* (e) sequences, are indicated on the left. (**B**) Quantitation of the relative levels of DNA binding (open bars) and UV cross-linking (dark bars) by Paired and Bicoid proteins to the DNA fragments shown in (A). The data are expressed as the density of binding or UV cross-linking per kb of DNA. The data are normalized to place them on the same scale.

Paired and Bicoid cross-link to known target genes in vivo

Paired and Bicoid directly activate transcription of the *eve* gene via enhancer elements located in a 7 kb region just upstream of the *eve* mRNA start site (Small *et al.*, 1992; Fujioka *et al.*, 1996). Bicoid activates *hunchback* by binding to sites just 5′ of the P2 transcription initiation site (Driever and Nusslein-Volhard, 1989; Margolis *et al.*, 1995). Since these are some of the best characterized targets of these homeoproteins, we initially determined if our *in vivo* UV cross-linking assay could detect the interaction of Paired and Bicoid with these elements, first examining binding to the *eve* gene.

paired is transiently expressed in a significant number of cells from 3.5 to 5 h after fertilization (Kilchherr *et al.*, 1986; Gutjahr *et al.*, 1993). The Paired target element (PTE) in the *eve* gene lies 5.5 kb upstream of the RNA start site (Figure 2A; Fujioka *et al.*, 1996). To determine whether Paired binds to this region *in vivo*, chromatin was purified from UV-irradiated 4–5 h embryos (stages 5b–8; Campos-Ortega and Hartenstein, 1997), then the DNA was restriction digested and immunoprecipitated with

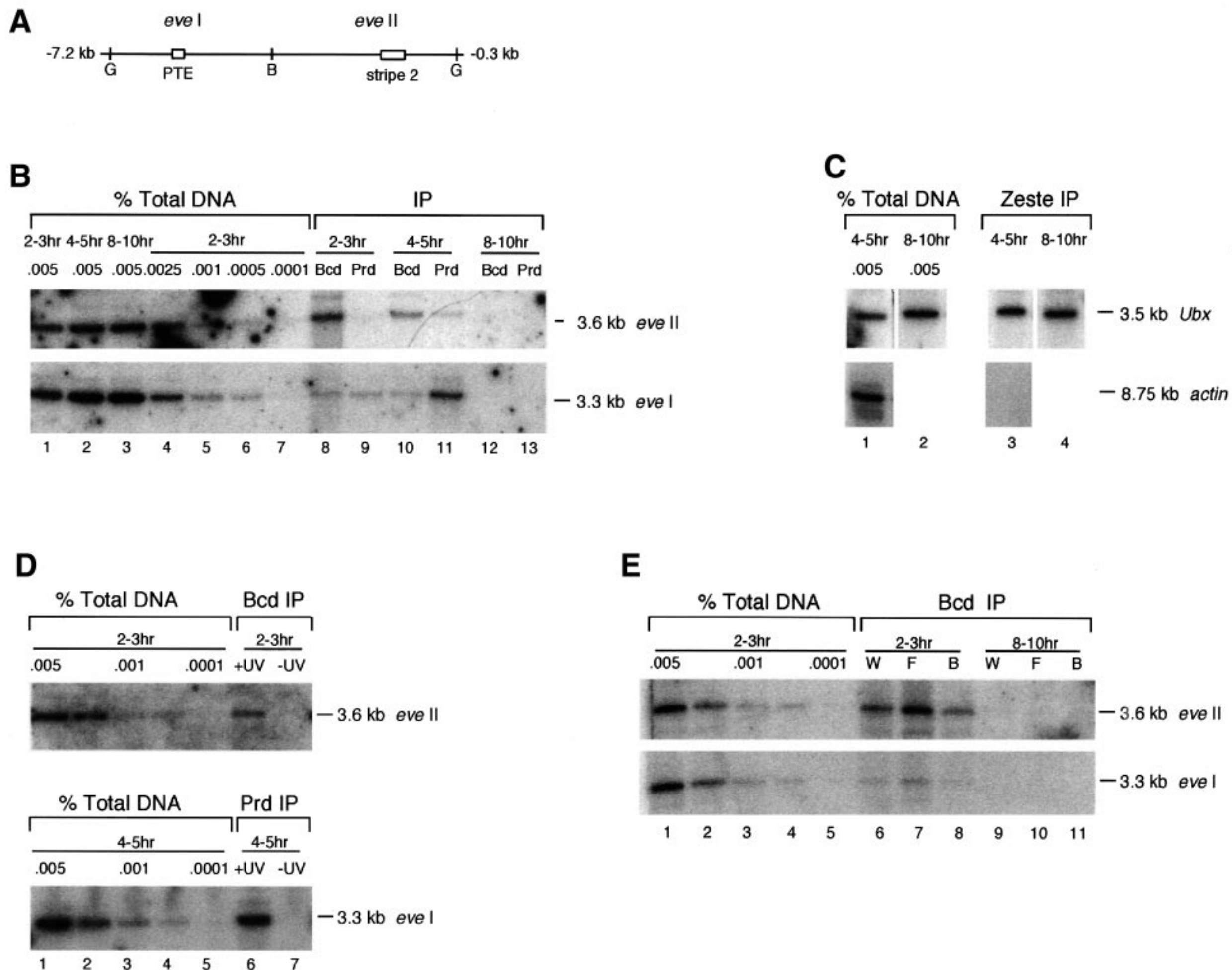


Fig. 2. Paired and Bicoid proteins UV cross-link to the *eve* promoter *in vivo*. (**A**) Diagram of the *eve* gene showing two restriction fragments to which cross-linking has been assayed: a 3.6 kb *Bgl*I–*Bam*HI fragment (*eve* II), located between nucleotides –0.3 and –3.9 kb, and a 3.3 kb *Bgl*I–*Bam*HI fragment (*eve* I), which spans nucleotides –3.9 to –7.2 kb. The Paired target element (PTE), the Bicoid responsive stripe 2 element (stripe 2) and the positions of *Bgl*I (G) and *Bam*HI (B) restriction sites are shown. (**B**) Chromatin was extracted from UV-irradiated 2–3, 4–5 and 8–10 h embryos and purified by CsCl buoyant density ultracentrifugation. Then 300–450 µg of this chromatin was digested with *Bgl*I and *Bam*HI and immunoprecipitated using *S.aureus* cells and either anti-Paired (lanes 9, 11 and 13) or anti-Bicoid (lanes 8, 10 and 12) antibodies. The precipitated protein–DNA complexes were then eluted from the *S.aureus* cells and digested with proteinase K. The recovered DNA was electrophoresed on an agarose gel and then transferred to a nylon membrane for Southern blot analysis. The same Southern blot was probed successively with DNAs that hybridize with either fragment *eve* I (lower panel) or *eve* II (upper panel) (Walter *et al*., 1994). To allow quantitation, the figure shows titrations from 0.005 to 0.0001% of the total amount of DNA present in an immunoprecipitation reaction (% Total DNA, lanes 1–7). The ages of the embryos from which chromatin was derived are given above each lane. (**C**) Zeste cross-links to a target DNA with equal efficiency in 4–5 and 8–10 h embryos. UV-irradiated chromatin from 4–5 or 8–10 h embryos was restriction digested, then precipitated using anti-Zeste antibodies (Walter *et al.*, 1994). Southern blots were probed either with sequences that hybridize to a 3.5 kb *Eco*RI fragment from the *Ubx* proximal promoter or with sequences that hybridize to an 8.75 kb *Eco*RI fragment containing the transcription unit of the *actin 5C* gene (Walter *et al.*, 1994). On the left is shown 0.005% of the total DNA present in immunoprecipitation reactions of chromatin from 4–5 (lane 1) or 8–10 h (lane 2) embryos, the DNA recovered from these immunoprecipitation reactions is shown on the right (lanes 3 and 4). Consistent with earlier results (Water *et al.*, 1994), Zeste cross-links strongly to the *Ubx* promoter (upper panel) but not to the *actin 5C* gene (lower panel). (**D**) Immunoprecipitation of *eve* promoter DNA is dependent upon UV irradiation. Chromatin was extracted from 2–3 or 4–5 h embryos that had been either UV irradiated for 30 min (lane 6) or not irradiated (lane 7). Chromatin from 2–3 h embryos was immunoprecipitated using anti-Bicoid antibodies (upper panel), and chromatin from 4–5 h embryos was immunoprecipitated using anti-Paired antibodies (lower panel). Procedures and conventions are described in (B). (**E**) Antibodies raised to different epitopes of Bicoid give similar results. Chromatin from 2–3 and 8–10 h UV-irradiated embryos was precipitated with one of the following affinity-purified anti-Bicoid antibodies: W, which is directed against amino acids 56–489 (lanes 6 and 9); F, which is directed against amino acids 56–330 (lanes 7 and 10); and B, which is directed against amino acids 330–489 (lanes 8 and 11). Otherwise, methods and conventions are as described in (B).

affinity-purified anti-Paired antibodies that recognize a 95 amino acid portion of Paired. In these experiments, a 3.3 kb restriction fragment (*eve* I) that contains the PTE is cross-linked to Paired protein *in vivo* (Figure 2B, lane 11, lower panel). An adjacent 3.6 kb *eve* fragment (*eve* II) is also cross-linked to Paired, but at 10-fold lower levels (Figure 2B, lane 11, upper panel). Consistent with the temporal pattern of Paired expression, the anti-Paired antibodies do not detectably precipitate UV cross-linked chromatin purified from 8–10 h embryos (stages 11 and 12) and only weakly bring down DNA from 2–3 h embryos (stage 4) (Figure 2B, lanes 9 and 13). This failure to immunoprecipitate chromatin from 8–10 h embryos is not simply because this material is poorly cross-linked. Another transcription factor, Zeste, which is expressed at equal levels in 4–5 and 8–10 h embryos, is cross-linked

specifically at equal levels to a known target element in chromatin from embryos of both ages (Figure 2C; Walter *et al*., 1994; Laney and Biggin, 1997).

Bicoid is transiently expressed for only the first 3.5 h of embryogenesis and activates *eve* transcription via a cluster of five high affinity binding sites located within the stripe 2 element (Figure 2A; Driever and Nusslein-Volhard, 1988; Small *et al*., 1992). Anti-Bicoid antibodies were used to immunoprecipitate chromatin purified from UV-irradiated 2–3 h embryos. Figure 2B shows that fragment *eve* II, which includes the stripe 2 element, is cross-linked to Bicoid approximately five times more efficiently than the adjacent promoter region, fragment *eve* I (Figure 2B, lane 8, compare upper and lower panels). Chromatin prepared from 4–5 or 8–10 h embryos gives weak or undetectable Bicoid cross-linking signals on these same DNA fragments (Figure 2B, lanes 10 and 12), in agreement with the expression profile of Bicoid.

Chromatin prepared from unirradiated embryos is not immunoprecipitated detectably by either anti-Paired or anti-Bicoid antibodies, indicating that the immunoprecipitation of DNA is dependent upon covalent coupling of proteins to DNA *in vivo* (Figure 2D). Separate affinity-purified antibodies directed against either the N- or C-terminal halves of Bicoid both immunoprecipitate five times more fragment *eve* II than fragment *eve* I (Figure 2E). Likewise, antibodies recognizing either amino acids 355–450 or 450–613 of Paired give similar results to each other (data not shown). Non-specific rabbit anti-mouse antibodies do not precipitate detectably either region of the *eve* promoter (data not shown). Therefore, our assays specifically detect only Paired or Bicoid.

Paired and Bicoid also bind at appreciable levels to a third region of the *eve* gene that includes the entire transcription unit. Figure 3A compares the relative levels of cross-linking of these two proteins on all three *eve* gene fragments. This figure illustrates the distinct preferences of Paired and Bicoid for different promoter regions, and shows that these two proteins bind most strongly to their known target elements. On the *hunchback* gene, Bicoid cross-links at similar levels to a 3.4 kb fragment containing the Bicoid target element as it does to the *eve* stripe 2 element (Figure 3, compare *hunchback* II with *eve* II). In addition, just as on the *eve* gene, Bicoid is found at significant levels on regions of *hunchback* that flank its known target element (Figure 3B).

The fact that Paired and Bicoid cross-link at appreciable levels to DNA fragments adjacent to their characterized target elements is not entirely unexpected. Paired and Bicoid recognition motifs are found throughout the length of many genes, and the selector homeoproteins Eve and Ftz cross-link to sites throughout the length of their best characterized target genes *in vivo* (Walter *et al.*, 1994). This broad cross-linking across genes is not an artifact caused by the *in vivo* UV assay misrepresenting the pattern of homeoprotein DNA binding. In addition to the *in vitro* control experiments described earlier, the following result demonstrates the specificity of the method *in vivo*: the transcription factor Zeste is UV cross-linked *in vivo* to 200–500 bp regions that contain high affinity Zeste sites and not to adjacent gene fragments that lack Zeste recognition sites (Walter *et al.*, 1994; Walter and Biggin, 1996; Laney and Biggin, 1997). Thus, the broad cross-linking

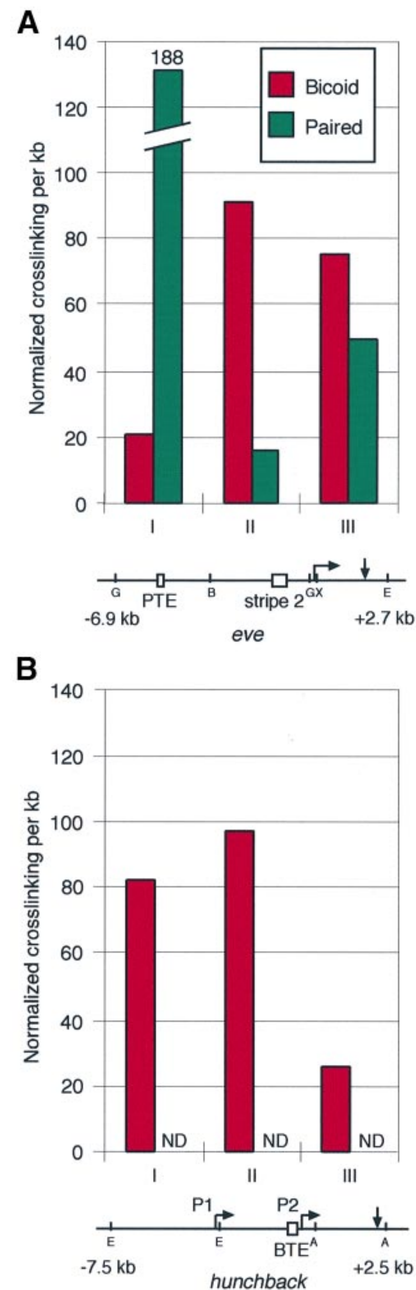


Fig. 3. Bicoid binds at similar levels to the *eve* and *hunchback* genes *in vivo*. (**A**) The relative levels of Paired (green) and Bicoid (red) cross-linking to three adjacent fragments of the *eve* gene. All data are derived by dividing the average immunoprecipitation signals for a DNA fragment by the length of the DNA fragment in kb and thus represent the relative density of cross-linking per kb of DNA. At the bottom is a diagram showing the three DNA fragments, the transcription start site (horizontal arrows), the mRNA polyadenylation site (vertical arrows), the PTE and the stripe 2 element. The positions of *Bgl*I (G), *Bam*HI (B), *Eco*RI (E) and *Xho*I (X) restriction sites are also indicated. *eve* fragments I and II are described in Figure 2A; *eve* fragment III is a 2.7 kb *Xho*I–*Eco*RI fragment that encodes the entire transcription unit. (**B**) Relative cross-linking per kb of Bicoid (red) to three regions of the *hunchback* gene. The methods and conventions are as described in (A) except that the positions of the Bicoid target element (BTE), the *Xba*I (A) restriction sites, and the P1 and P2 mRNA start sites are also shown. The three DNA fragments to which cross-linking was measured are a 4.1 kb *Eco*RI fragment including the P1 promoter (I), a 3.4 kb *Eco*RI–*Xba*I fragment containing the P2 promoter (II) and a 2.4 kb *Xba*I fragment containing the entire protein coding sequence (III). The DNA fragments used to detect these three promoter regions in Southern blots were derived from the 12 kb KG fragment described in Margolis *et al*. (1995).

of homeoproteins *in vivo* must be caused by proteins bound directly to specific sites present throughout genes.

Paired and Bicoid bind to a wide range of genes in vivo

Previous experiments established that Eve and Ftz cross-link in embryos to their well characterized targets *eve*, *ftz* and *Ubx* at only 2- to 20-fold higher levels than they do to four unexpected targets, *Adh*, *actin 5C*, *hsp70* and *rosy* (Walter *et al.*, 1994). Subsequently, it was shown that, contrary to previous claims in the literature, these four unexpected targets are regulated by Eve and probably by the other selector homeoproteins as well (Liang and Biggin, 1998), suggesting that these homeoproteins may bind and regulate a large percentage of genes. This widespread DNA binding by Eve and Ftz is consistent with the relatively high concentrations of these two proteins *in vivo* (at least 50 000 molecules per nucleus; Walter *et al.*, 1994) and, because Paired and Bicoid are expressed at similarly high levels, we wished to determine if they also bind to a wide array of genes in embryos. Consequently, we quantified the mean cross-linking per kb of DNA of Paired and Bicoid to the same series of DNA fragments used in the studies of Eve and Ftz.

Paired and Bicoid cross-link at levels above the limit of detection of our assay to almost all gene fragments tested (Figures 4 and 5); only the interactions of Bicoid with *Adh* and of Paired with *rosy* and the *hsp70* transcription unit are too weak to be detected in our assay. Thus, like Eve and Ftz, Paired and Bicoid may bind at appreciable levels to most genes in *Drosophila*.

Eve and Ftz cross-link with very similar specificity to all DNA fragments tested, whereas Paired and Bicoid show different patterns of cross-linking, both from each other and from Eve and Ftz (Walter *et al.*, 1994; Figure 5). For example, Paired binds more weakly than the other homeoproteins to the *hsp70* transcription unit, yet Paired binds more strongly than these other proteins to *eve* fragment I. Our data also show, however, that although the DNA-binding specificities of these proteins differ, these differences represent no more than a 5- to 10-fold variations in cross-linking to any given DNA fragment and, on some fragments, all four proteins cross-link at comparable levels (Figure 5).

The density of cross-linking for each homeoprotein is highest on all *eve* and *ftz* gene fragments and is generally lowest on *rosy* and *Adh* (Figure 5). Later, we argue that this pattern of binding may be due to chromatin structure varying from gene to gene: homeoprotein recognition sites at weakly bound genes could be less accessible than those at strongly bound genes.

The in vitro and in vivo DNA-binding specificities of homeoproteins are broadly similar across the eve gene

The preceding experiments establish the *in vivo* pattern of DNA binding by Paired and Bicoid in comparison with that of Eve and Ftz. We are interested in the relationship between this *in vivo* binding and the intrinsic *in vitro* DNA-binding specificities of these three classes of homeoprotein. To examine this question, we first compared *in vitro* and *in vivo* DNA binding on the *eve* gene as this gene contains well characterized target elements for Paired,

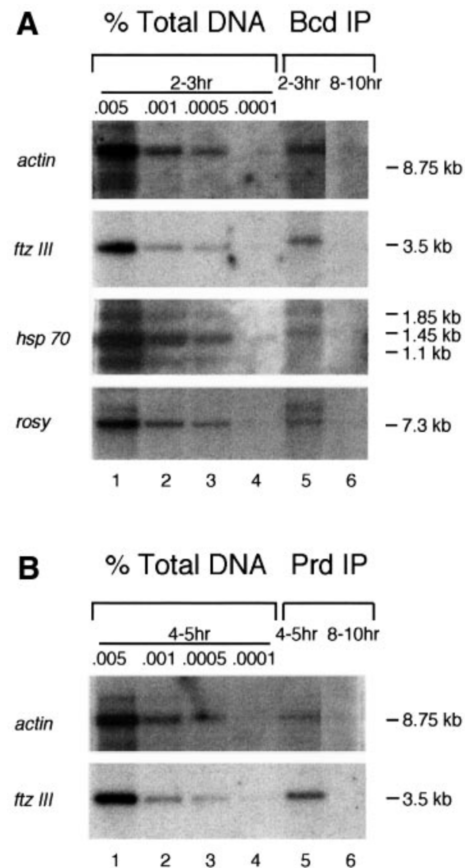


Fig. 4. Bicoid and Paired bind to a wide range of genes in embryos. (**A**) Cross-linking of endogenous Bicoid protein to a number of gene fragments was assayed as described in Figure 2 and Walter *et al.* (1994). The DNA fragments cross-linked to Bicoid are an 8.75 kb genomic fragment that includes the *actin 5C* transcription unit, a 3.5 kb *Eco*RI genomic fragment (*ftz* III) that contains the *ftz* transcription unit, three *Bam*HI–*Xho*I genomic restriction fragments that contain the promoters and transcription units of the five *hsp70* genes and a 7.3 kb *Hin*dIII fragment that contains the entire *rosy* gene. Titrations of the total DNA used in immunoprecipitation reactions of 2–3 h chromatin are shown in lanes 1–4, and the DNA recovered after immunoprecipitation is shown in lane 5. The DNA recovered from immunoprecipitation of 8–10 h chromatin is shown in lane 6. (**B**) UV cross-linking of endogenous Paired protein to the same *actin 5C* and *ftz* III gene fragments shown in (A). Methods and conventions are as described earlier.

Bicoid and Eve. We divided the upstream region of *eve* into four similarly sized restriction fragments, each of ~1.5 kb (Figure 6B). Figure 6A shows the results of an *in vitro* immunoprecipitation assay demonstrating the different affinities of Eve, Paired and Bicoid for some of these DNA fragments.

Eve binds at roughly similar levels to all four promoter fragments both *in vitro* and *in vivo* (Figure 6; Walter *et al.*, 1994; Walter and Biggin, 1996). *In vitro*, Eve has been shown to recognize most variants of the NNATTA consensus sequence with similar affinity (TenHarmsel *et al.*, 1993; D.Dalma-Weiszhausz and M.D.Biggin, unpublished data). Figure 6B shows that these sites are found at comparable frequencies in each of the four upstream regions: between 10 and 14 sites are found per kb of DNA.

Bicoid binds most strongly both *in vitro* and *in vivo* to a fragment containing the stripe 2 element (Figure 6B). Only fragment *eve* II_A shows a large difference between

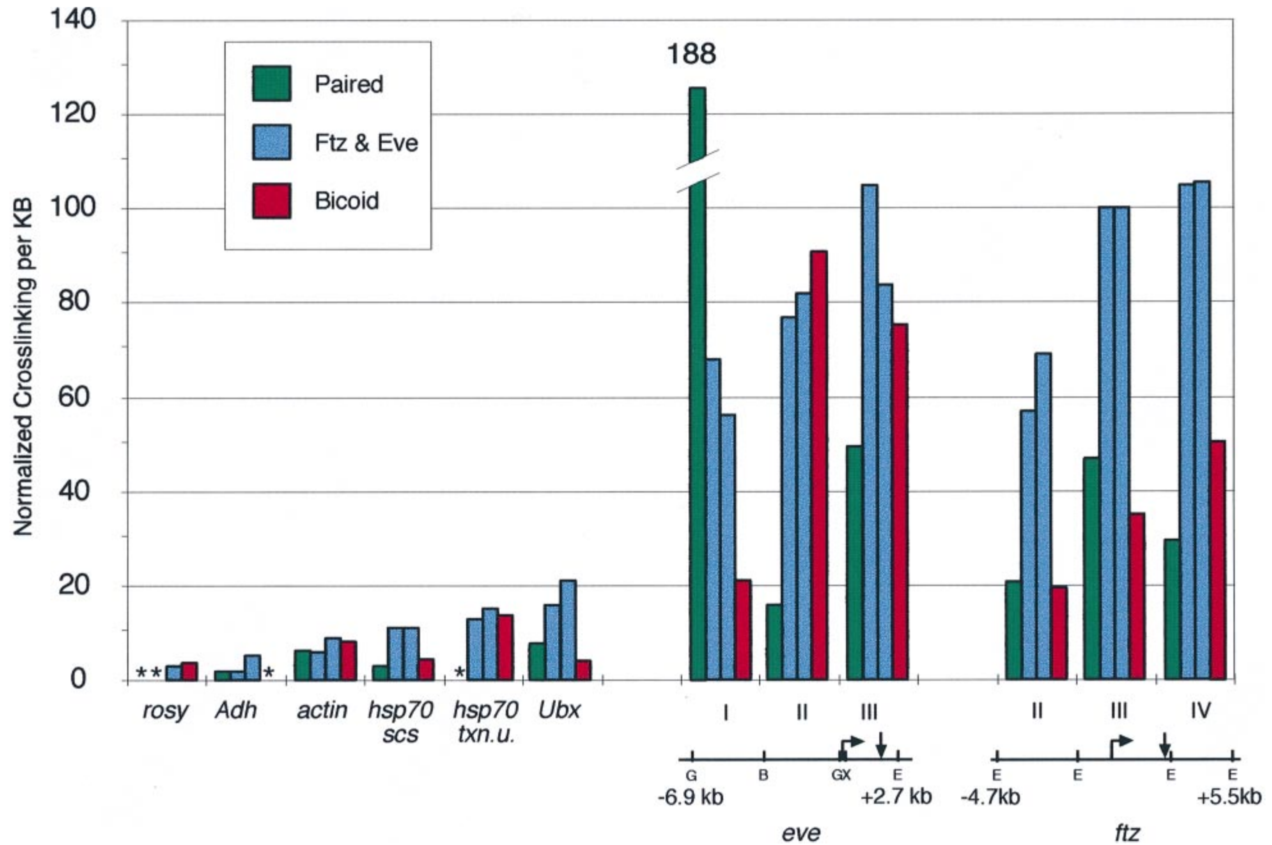


Fig. 5. Paired and Bicoid bind to a wide range of genes *in vivo* but with different specificities from each other and from Eve and Ftz. Cross-linking of Paired (green), Bicoid (red), Ftz (blue, left) and Eve (blue, right) to DNA fragments from a number of genes. All data are expressed as the relative density of cross-linking per kb of DNA. The DNA fragments for which data is presented include those described in Figures 2, 3 and 4, together with a 4.7 kb *Eco*RI fragment containing the *Adh* gene, a 7 kb genomic fragment including the scs region from the proximal side of the *hsp70* 87A locus, a 3.2 kb *Eco*RI upstream fragment containing the *ftz* autoregulatory element (*ftz* II) and a 3.5 kb *Eco*RI fragment containing sequences downstream of the *ftz* transcription unit (*ftz* IV). Schematic representations of the *eve* and *ftz* genes show the positions of several DNA fragments to which cross-linking was assayed using the conventions and symbols described in Figure 3. The data for the Eve and Ftz proteins were collected previously and are shown here for comparison (Walter *et al*., 1994).

the *in vitro* and *in vivo* data, being bound 35-fold more weakly *in vitro* than *in vivo*. Bicoid only binds strongly *in vitro* to the sequences GGATTA, GCATTA, AGATTA and CGATTA (Driever and Nusslein-Volhard, 1989; Percival-Smith, 1990; Small *et al*., 1992). Importantly, the frequency of these sites in each promoter region correlates with the relative affinity of Bicoid for these regions (Figure 6B). Thus, the distinct preferences of Eve and Bicoid for high affinity DNA sites appear to be the major determinant of their preferences for fragments across the *eve* gene.

A close correspondence between *in vivo* UV cross-linking and *in vitro* DNA binding is also seen for Paired on all four DNA fragments (Figure 6B). Unfortunately, there is not sufficient information available to predict accurately the DNA sequences bound by Paired. However, from the available data, it is likely that all four promoter regions contain a number of high and moderate affinity sites (Hoey and Levine, 1988; Fujioka *et al.*, 1996; Jun and Desplan, 1996).

In vitro and *in vivo* specificities have also been compared across other genes. On the *hunchback* gene, Bicoid cross-links *in vivo* more strongly to a promoter region containing several clusters of high affinity Bicoid-binding sites than it does to the transcription unit, which contains only low affinity sites (Figure 2B, compare *hunchback* fragments II and III; Driever and Nusslein-Volhard, 1989). Across the *ftz* gene, each promoter region is bound at roughly similar levels *in vitro* and *in vivo* by all four homeoproteins examined (Figure 5; Walter and Biggin, 1996; unpublished data), the only exception being that *ftz* fragment III is bound significantly more weakly *in vitro* by Paired and Bicoid than it is *in vivo*. A close correspondence between *in vitro* and *in vivo* DNA-binding specificity is also seen for the non-homeodomain transcription factor Zeste (Walter and Biggin, 1996; Laney and Biggin, 1997). Thus, for all interactions measured, the intrinsic DNA-binding specificities of transcription factors is a good but not precise guide to the distribution of these proteins across their best characterized target genes *in vivo*.

Interestingly, the few interactions that show a significant discrepancy between *in vitro* and *in vivo* binding all occur on DNA fragments that are bound more weakly *in vitro* than they are *in vivo*. Later, we argue that in these cases, and only in these cases, cooperative interactions with other transcription factors may play a major role in

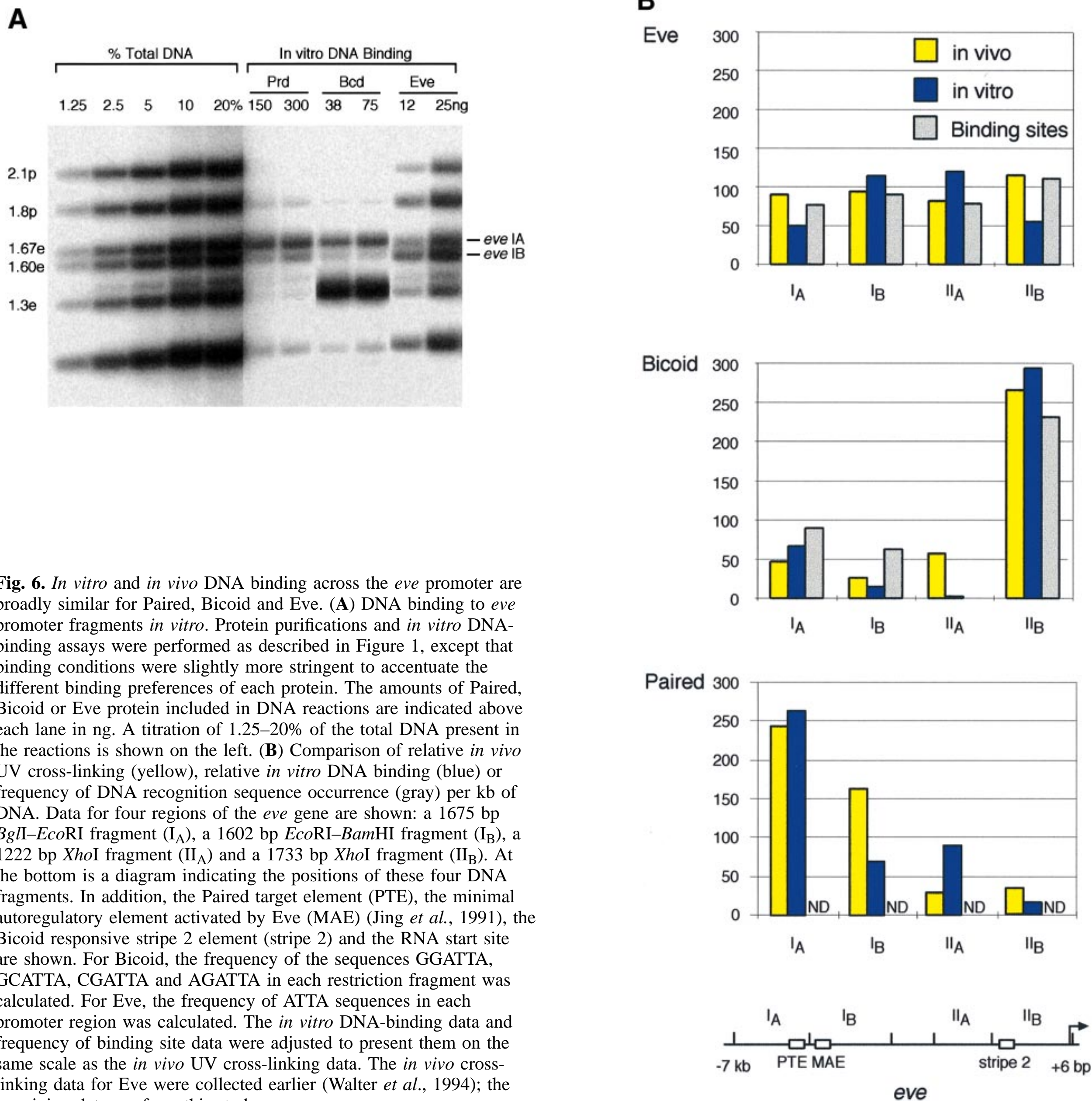


Fig. 6. *In vitro* and *in vivo* DNA binding across the *eve* promoter are broadly similar for Paired, Bicoid and Eve. (**A**) DNA binding to *eve* promoter fragments *in vitro*. Protein purifications and *in vitro* DNA-binding assays were performed as described in Figure 1, except that binding conditions were slightly more stringent to accentuate the different binding preferences of each protein. The amounts of Paired, Bicoid or Eve protein included in DNA reactions are indicated above each lane in ng. A titration of 1.25–20% of the total DNA present in the reactions is shown on the left. (**B**) Comparison of relative *in vivo* UV cross-linking (yellow), relative *in vitro* DNA binding (blue) or frequency of DNA recognition sequence occurrence (gray) per kb of DNA. Data for four regions of the *eve* gene are shown: a 1675 bp *Bgl*I–*Eco*RI fragment (I_A), a 1602 bp *Eco*RI–*Bam*HI fragment (I_B), a 1222 bp *Xho*I fragment (II_A) and a 1733 bp *Xho*I fragment (II_B). At the bottom is a diagram indicating the positions of these four DNA fragments. In addition, the Paired target element (PTE), the minimal autoregulatory element activated by Eve (MAE) (Jing *et al.*, 1991), the Bicoid responsive stripe 2 element (stripe 2) and the RNA start site are shown. For Bicoid, the frequency of the sequences GGATTA, GCATTA, CGATTA and AGATTA in each restriction fragment was calculated. For Eve, the frequency of ATTA sequences in each promoter region was calculated. The *in vitro* DNA-binding data and frequency of binding site data were adjusted to present them on the same scale as the *in vivo* UV cross-linking data. The *in vivo* cross-linking data for Eve were collected earlier (Walter *et al*., 1994); the remaining data are from this study.

determining the level of occupancy *in vivo* by increasing the level of DNA binding.

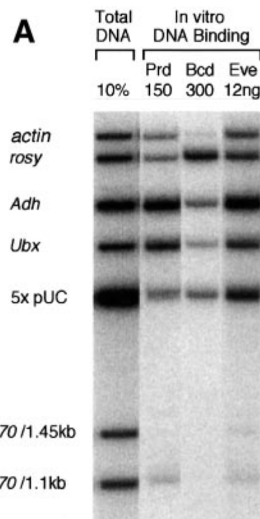
Comparison of in vivo cross-linking and in vitro binding to different loci

The genes for which there is a good correlation between *in vitro* and *in vivo* DNA-binding preferences, such as *eve* and *ftz*, are all bound strongly in embryos. However, when genes bound weakly *in vivo* are also included in such an analysis, no correlation is seen between *in vitro* and *in vivo* DNA-binding specificities (Walter and Biggin, 1996; Figure 7).

Figure 7A shows *in vitro* binding of Eve, Paired and Bicoid to some of the gene fragments to which binding was tested in the *in vivo* studies. Each protein shows a different preference for these genes. Figure 7B compares relative levels of binding *in vitro* to relative levels of UV cross-linking *in vivo* to a range of genes. There is no simple relationship between the two sets of data. For example, a fragment containing the *rosy* gene is one of the most strongly bound by Bicoid *in vitro* but is one of the most weakly bound *in vivo*. Similarly, Paired cross-links most strongly *in vivo* to the 6.9 kb *eve* upstream region but binds this fragment more weakly *in vitro* than it binds the *Ubx* or *Adh* promoter fragments.

A model for homeoprotein DNA binding in vivo

The above discrepancies between homeoprotein DNA binding *in vitro* and *in vivo* indicate that conditions in the embryo affect the preferences of homeoproteins for different genes. For the reasons described below, we suggest that the major factor affecting DNA binding *in vivo* is the inhibition of binding at some gene loci by chromatin structure. We believe that cooperative inter-



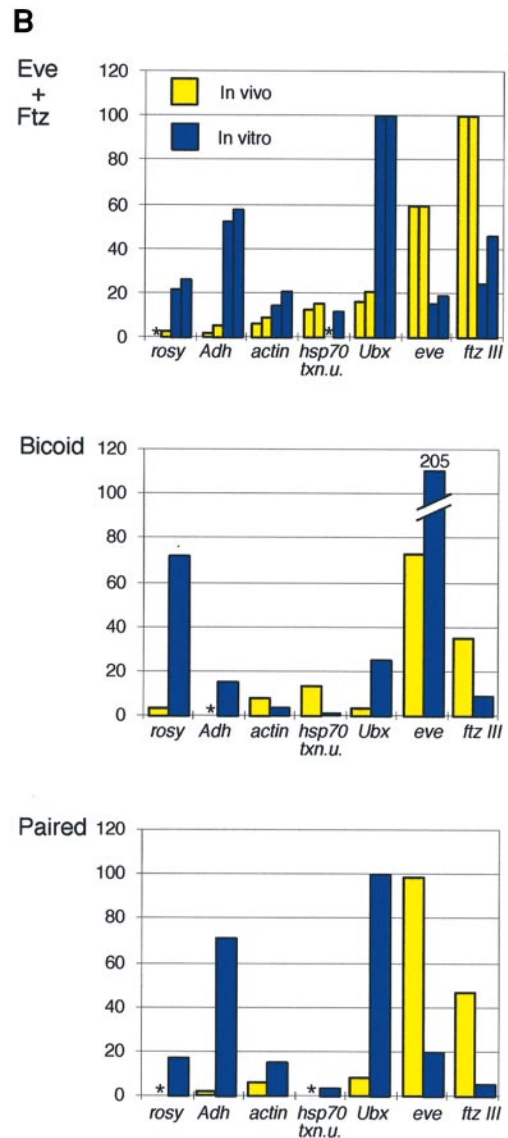


Fig. 7. *In vitro* and *in vivo* DNA-binding preferences differ when compared on a range of genes. (**A**) Binding of Eve, Paired and Bicoid *in vitro*. DNA-binding reactions were performed as described in Figure 6 and contained a cocktail of the following DNA fragments: an 8.75 kb *Eco*RI *actin 5C* fragment, a 7.3 kb *Hin*dIII *rosy* fragment, a 4.7 kb *Eco*RI *Adh* fragment, a 3.5 kb *Eco*RI *Ubx* proximal promoter fragment, 1.45 and 1.1 kb fragments from a *Bam*HI–*Xho*I digest of a 2.6 kb *Bam*HI *hsp70* fragment, and pUC plasmid DNA (Walter and Biggin, 1996). Purified proteins were added at the amount indicated in ng; 10% of the DNA used in the DNA-binding reactions is shown on the left. (**B**) Comparison of *in vitro* DNA binding (blue) and *in vivo* UV cross-linking (yellow) per kb of DNA for four homeoproteins. In the panel showing data for Eve and Ftz, bars on the left of each pair show binding or cross-linking of Ftz and bars on the right show data for Eve. The DNA fragments for which data are presented are described in Figures 4, 5 and 7A, except for the *eve* gene, for which data is presented for a 6.9 kb *Bgl*I fragment spanning nucleotides –7.2 to –0.3 kb. The *in vitro* data are normalized to give a percentage of binding to a 3.5 kb *Ubx* proximal promoter fragment: Paired, Eve and Ftz binding to the *Ubx* DNA is set at 100; Bicoid binding is set at 25. Data for Paired and Bicoid were collected in this study; data for Eve and Ftz were collected earlier (Walter *et al*., 1996).

actions with other transcription factors (cofactors) play only a minor role by increasing DNA binding at a limited number of lower affinity sites within genes.

At the stage of embryogenesis examined in the UV cross-linking experiments, the *Adh* gene is not transcribed and the *rosy* gene is inactive in most cells (Liang and Biggin, 1998). These two genes are bound most weakly *in vivo* by Eve, Ftz, Bicoid and Paired, even though these two genes are bound relatively well *in vitro* (Figure 7B). The chromatin structure of transcriptionally inactive genes is thought to inhibit DNA binding by certain classes of transcription factor (Wallrath *et al*., 1994; Beato and Eisfeld, 1997; Kadonaga, 1998). Therefore, closed chromatin structure could explain the reduced binding to *Adh* and *rosy*. The *Ubx* gene is only weakly transcribed at cellular blastoderm, and the *hsp70* fragment shown in Figure 7 is only open to transcription factor binding over part of its length *in vivo* (Wu, 1984; Akam and Martinez-Arias, 1985; O'Brien *et al*., 1995). Thus, partially open chromatin structure may explain the intermediate levels of UV cross-linking to *Ubx* and *hsp70 in vivo*. The *eve*, *ftz* and *hunchback* genes are all highly transcribed. Thus, their chromatin structure may be fully permissive to homeoprotein binding, and this could explain why they are the most highly bound genes. [The transcriptional state of the *actin 5C* gene at cellular blastoderm has not been determined because high levels of perduring maternal transcripts obscure any zygotic *actin 5C* transcription (Liang and Biggin, 1998).]

Our model readily explains the similarity between *in vitro* and *in vivo* DNA binding across *eve*, *ftz* and *hunchback*. If homeoproteins can bind to most sites on actively transcribed genes without the help of cooperative interactions with cofactors, then homeoproteins would be distributed across these genes in the same manner *in vitro* and *in vivo*, as we see. In contrast, if homeoproteins could only bind DNA via cooperative associations with cofactors, as others have proposed (e.g. Johnson, 1992; Mann and Chan, 1996), then the *in vitro* and *in vivo* homeoprotein DNA-binding profiles across actively transcribed genes would probably differ; homeoproteins would be distributed *in vivo* in a manner dependent upon the DNA-binding specificities of their cofactors.

Transgenic promoter constructs containing only high

affinity Bicoid recognition sequences are activated by Bicoid in embryos (Hanes *et al.*, 1994; Simpson-Brose *et al.*, 1994). Thus it seems unlikely that endogenous Bicoid needs to form heteromeric complexes with cofactors in order to bind to Bicoid recognition sites *in vivo*. By extension, it is not unreasonable to propose that other homeoproteins that are expressed at levels similar to Bicoid and that bind DNA with comparable efficiency may also bind accessible recognition sites without the aid of cofactors.

The activities of homeoproteins such as Ubx, Eve and Ftz are significantly affected by combinatorial interactions with other transcription factors. However, the available data suggest that these cofactors do not act by substantially increasing homeoprotein DNA binding in embryos through cooperative interactions (Biggin and McGinnis, 1997). Instead, these cofactors probably act in alternative ways. For example, our data indicate that conditions in the embryo modify the DNA-binding preferences of Eve and Ftz in essentially the same way (Figure 7B); yet a cofactor important for Ftz activity *in vivo*, Ftz-F1, has no affect on *eve* function (Guichet *et al.*, 1997; Yu *et al.*, 1997). If the *in vivo* distribution of Eve and Ftz was determined primarily by cooperative DNA binding with cofactors, then another cofactor with the same DNA-binding specificity as Ftz-F1 would be required to position Eve in the same manner as Ftz. We suggest that it is simplest to assume that cooperative interactions with Ftz-F1 do not influence DNA binding by Ftz at most sites and that Ftz-F1 acts by some other mechanism. Combinatorial interactions between other transcription factors have been shown to occur by synergistic interactions with different components of the general transcriptional machinery (Sauer *et al.*, 1995; Ptashne and Gann, 1997). Such a mechanism could therefore explain how cofactors might influence homeoprotein activity without affecting their DNA binding.

In yeast, homeoproteins do appear to require heteromeric association with cofactors in order to bind DNA at functionally significant levels (Johnson, 1992). Differential interactions with cofactors are thought to cause these yeast homeoproteins to bind much more selectively and differently from each other *in vivo* than they do *in vitro*, allowing each to bind and regulate different target genes (Johnson, 1992). We suggest that the reason why *Drosophila* homeoproteins may not bind in this way is because their biological functions are different from those of the yeast homeoproteins. In *Drosophila*, homeoproteins control diverse processes such as cell size, cell proliferation, cell shape, cell movement and differentiation (Biggin and McGinnis, 1997). These global functions may require *Drosophila* homeoproteins to bind broadly and to regulate the expression of a large percentage of genes (Liang and Biggin, 1998).

Transcription factor DNA binding in vivo

It is difficult to assess what fraction of transcription factors will show widespread DNA binding *in vivo*. We strongly suspect that other classes of homeoproteins in *Drosophila* as well as homeoproteins in other animals will bind to a very broad range of genes *in vivo*. Cross-linking studies suggest that the *Drosophila* GAGA factor and the human c-Myc factor also bind very widely in cells (O'Brien *et al.*, 1995; Boyd *et al.*, 1998). In contrast, Zeste cross-links *in vivo* to short regions of the *Ubx* promoter at at least 100-fold higher levels than it does to other genes (Walter *et al.*, 1994; Laney and Biggin, 1997). Studies of proteins bound to polytene chromosomes also suggest that some transcription factors bind selectively to only a small number of genes (Urness and Thummel, 1990; Yao *et al.*, 1993). We suggest that metazoan transcription factors will show a spectrum of DNA binding, from factors that bind very selectively to those that bind as broadly as Bicoid, Paired, Eve and Ftz.

The majority of transcription factor molecules in prokaryotes are predicted to be bound to DNA. Most molecules are thought to be bound in a sequence-independent manner at very low levels throughout the genome because sequence-specific DNA-binding proteins can bind any DNA sequence weakly via electrostatic interactions and because the concentration of DNA in cells is very high (von Hippel *et al*., 1974; Lin and Riggs, 1975; Ptashne, 1992; Yang and Nash, 1995). We suggest that there are several key differences between these predictions and the widespread DNA binding of homeoproteins in *Drosophila*. First, in contrast to the poor discrimination between most genes shown by homeoproteins, prokaryotic regulators are predicted to bind to their target genes at levels at least 100–1000 times higher than they bind to any other region of the genome (Lin and Riggs, 1975; Biggin, 1998). Secondly, many prokaryotic transcription factors bind with high affinity to 14–20 bp specific sequences that occur rarely in the genome, whereas homeoproteins bind to degenerate 6 bp sequences that are found in most *Drosophila* genes at a density of 5–10 sites per kb of DNA (Walter and Biggin, 1996; D.Dalma-Weiszhausz and M.D.Biggin, unpublished data). Thus, unlike prokaryotic regulators, the majority of homeoprotein molecules in a cell may be bound at specific high affinity sites. Thirdly, the low levels of prokaryotic regulators bound to most genes do not affect transcription, whereas the widespread binding of homeoproteins may play a direct role in regulating the expression of a large proportion of genes (Liang and Biggin, 1998). Understanding how homeoproteins control development will require a detailed analysis of how this widespread DNA binding affects transcription.

Materials and methods

Antibodies

Crude serum and affinity-purified rabbit antibodies raised against a truncated Paired protein containing amino acids 355–613 but lacking the paired repeat (amino acids 552–572) were provided by C.Desplan. This C-terminal portion of Paired does not include either the homeodomain or the Paired domain. Two additional preparations of anti-Paired antibodies directed against non-overlapping regions of Paired protein (amino acids 355–450 and 450–613) were purified from the above crude serum using standard techniques. The majority of the *in vivo* and *in vitro* UV cross-linking and DNA-binding data for Paired were collected using antibodies affinity purified against amino acids 355–450. Antibodies directed against non-overlapping regions of Bicoid (amino acids 56–330 and 330–489) were affinity purified from a crude anti-Bicoid rabbit serum directed against amino acids 56–489 (G.Struhl, personal communication). A third set of antibodies was purified with a Bicoid protein containing amino acids 56–489. This set was used to collect nearly all of the Bicoid *in vivo* and *in vitro* cross-linking and DNA-binding data.

In vivo UV cross-linking

Embryos aged 2–3 h (primarily stage 4; Campos-Ortega and Hartenstein, 1997), 4–5 h (primarily stages 5b–8) and 8–10 h (stages 11 and 12)

were collected from standard size population cages. Embryos were UV irradiated and chromatin was purified as described previously (Walter *et al.*, 1994; Walter and Biggin, 1997; Biggin, 1999; Carr and Biggin, 1999). After digestion with the specified restriction enzyme(s), chromatin was immunoprecipitated and the recovered DNA detected by Southern blot. Immunoprecipitations of chromatin from 8–10 h embryos with anti-Bicoid antibodies gave background signals that were on average ~0.00003% of total DNA. In contrast, the signal obtained from immunoprecipitation of fragment *eve* II_A from 2–3 h chromatin using the same antibody was 0.0046%. Thus, the highest signals obtained are >100-fold above the background in the assay. A slightly higher background of ~0.00005% of total DNA was found in Paired immunoprecipitations of chromatin from 8–10 h embryos.

All DNA fragments for which cross-linking values are given were consistently immunoprecipitated more efficiently than chromatin from 8–10 h embryos. Additionally, similar data are obtained when two separate antibodies recognizing non-overlapping regions of each protein are used in independent immunoprecipitation reactions: multiple anti-Bicoid or anti-Paired antibodies have been used to compare cross-linking to a total of seven DNA fragments from two or three genes with similar results (data not shown). For almost all interactions, relative cross-linking per kb of DNA was calculated from the mean of at least three independent immunoprecipitation experiments.

Protein purification

Full-length Paired and Bicoid proteins were both expressed as His-tag fusion proteins from pET 19B vectors. After induction, cells were pelleted, frozen at –70°C, and then thawed and resuspended in sonication buffer (25 mM HEPES pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 100 mM KCl, 20% glycerol, 1 mM dithiothreitol). Samples were sonicated and then centrifuged at 4°C for 40 min at 25 000 r.p.m. in a Beckman Type 70.1 TI rotor. Pelleted inclusion bodies contained most of the expressed protein for both Paired and Bicoid. The insoluble material was resuspended in RIPA buffer [1× phosphate-buffered saline (PBS); 0.1% (w/v) SDS; 1% (v/v) Triton X-100; 1% (w/v) deoxycholate], dounced and then centrifuged at 4°C for 15 min at 12 000 r.p.m. in a SS34 rotor. This wash was repeated twice more with RIPA buffer and then twice with 20 mM Tris (pH 8.0). After the last centrifugation, pellets were resuspended in 4 M guanidine and 20 mM Tris (pH 8.0). This suspension was centrifuged at 4°C for 15 min at 12 000 r.p.m. in a SS34 rotor and filtered through a 5 µm filter prior to its application to a Ni^{2+} Sepharose column (Qiagen). The column was washed with 4 M guanidine, 10 mM Tris (pH 6.0) and 0.1 M NaH_2PO_4. Proteins were eluted with 4 M guanidine, 10 mM Tris (pH 4.5) and 0.1 M NaH_2PO_4. Eluted fractions containing the most protein were combined and then dialyzed into dialysis buffer 1 (4 M guanidine, 25 mM Tris/6.25 mM glycine pH 9.5, 0.1% Triton X-100, 1 M NaCl) and then into dialysis buffer 2 [1 M guanidine, 25 mM Tris/6.25 mM glycine pH 9.5, 1 M NaCl, 20% (v/v) glycerol]. Protein samples were flash frozen in liquid nitrogen and stored at –70°C at a concentration of 300 µg/ml. Eve protein was prepared as previously described (TenHarmsel *et al.*, 1993).

In vitro DNA binding and in vitro UV cross-linking

In vitro protein–DNA binding and *in vitro* UV cross-linking assays were carried out as described earlier (Walter and Biggin, 1996, 1997) with the following modifications. DNA-binding reactions contained the following amounts of protein: 75–300 ng of Paired, 19–150 ng of Bicoid or 12–50 ng of Eve protein. Each binding reaction also contained 50 µg/ml sonicated calf thymus DNA. To immunoprecipitate DNA fragments, 0.5 µg of either anti-Paired, anti-Bicoid or anti-Eve affinity-purified antibody was added. *Staphyloccus aureus* cells were prepared without a boiling step: they were resuspended from a lyophilized state, washed once in 1× PBS and once in 50 mM Tris (pH 8.0) 2 mM EDTA, then stored frozen in 50 mM Tris (pH 8.0), 2 mM EDTA and 0.2% Sarkosyl. After immunoprecipitation of protein–DNA complexes, *S.aureus* cell immune complexes were washed twice quickly with 500 µl of 1× binding buffer prior to elution of the DNA. In the UV cross-linking assay shown in Figure 1, DNA-binding reactions were irradiated for 2 min. The *in vitro* DNA-binding data for Eve, Paired and Bicoid shown in Figure 6B were collected from separate reactions containing either the DNA digest shown in Figure 6A or an *Xho*I digest of pEL3. Binding to fragments I_A and I_B was determined from the former digest whereas binding to fragments II_A and II_B was measured from the latter.

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