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Binding of the Wheat Basic Leucine Zipper Protein EmBP-1 to Nucleosomal Binding Sites Is Modulated by Nucleosome Positioning

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To investigate interactions of the basic leucine zipper transcription factor EmBP-1 with its recognition sites in nucleosomal DNA, we reconstituted an abscisic acid response element and a high-affinity binding site for EmBP-1 into human and wheat nucleosome cores in vitro. DNA binding studies demonstrated that nucleosomal elements can be bound by EmBP-1 at reduced affinities relative to naked DNA. EmBP-1 affinity was lowest when the recognition sites were positioned near the center of the nucleosome. Binding was achieved with a truncated DNA binding domain; however, binding of full-length EmBP-1 caused additional strong DNase I hypersensitivity flanking the binding sites. Similar results were observed with nucleosomes reconstituted with either human or wheat histones, demonstrating a conserved mechanism of transcription factor–nucleosome interactions. We conclude that positioning of recognition sequences on a nucleosome may play an important role in regulating interactions of EmBP-1 with its target sites in plant cells.

INTRODUCTION

In eukaryotic cells, DNA is wrapped around an octamer of histones (two H2A-H2B dimers and one $H3_2$ plus $H4_2$ tetramer) to form nucleosome cores, which can be further packaged into higher levels of chromatin structure. During the last decade, both genetic and biochemical evidence have established that nucleosome particles play an essential role in gene regulatory mechanisms (reviewed by Felsenfeld, 1992; Svaren and Hörz, 1993; Wolffe, 1994). Nucleosomes can present formidable obstacles to gene activation by transcription factors, and their presence has been correlated with gene repression (Grunstein, 1990; Kornberg and Lorch, 1991). Thus, when *cis*-regulatory elements in enhancers and/or promoters are organized into nucleosomes, overcoming nucleosome repression is a necessary step for gene activation (Workman and Buchman, 1993). One mechanism by which this can be achieved is via displacement of nucleosomes and recruitment of the transcription factors necessary for formation of a transcription complex.

Alterations in positioning of nucleosomes upon transcriptional activation have been observed in the promoter regions of a number of genes. The mouse mammary tumor virus promoter is incorporated into positioned nucleosomes, which include glucocorticoid response elements (Richard-Foy and Hager, 1987). Induction by glucocorticoids results in binding of the glucocorticoid receptor to the glucocorticoid response elements and nucleosome disruption (Perlmann and Wrange, 1991; Archer et al., 1992). Similarly, the yeast phosphate-regulated *PHO5* promoter is organized into nucleosomes, which are disrupted during transcription activation by the PHO2 and PHO4 regulatory proteins (Almer et al., 1986; Schmid et al., 1992; Svaren et al., 1994b). Chromatin structural changes have also been associated with gene activation in plant systems (Spiker et al., 1983; Ashraf et al., 1987; Kaufman et al., 1987; Paul et al., 1987; Wurtzel et al., 1987; Frommer and Starlinger, 1988; Görz et al., 1988; Thompson and Flavell, 1988; Lund et al., 1995). Evidence for differences in nucleosome configurations correlating with gene activation status has been shown for the Arabidopsis alcohol dehydrogenase (*Adh*) gene promoter (Vega-Palas and Ferl, 1995).

The interactions of transcription factors with nucleosomal templates and their role in such interactions in chromatin remodeling have been studied with mammalian and yeast factors (Kornberg and Lorch, 1995). Current studies indicate that the ability to bind nucleosomal DNA is an intrinsic property of individual factors (Owen-Hughes and Workman, 1994). Although factors with diverse DNA binding domains (i.e., binuclear Zn clusters, Zn fingers, and basic helix-loop-helix leucine zipper and Rel domains) have been shown to bind nucleosomal DNA under some circumstances, the affinity of different factors for recognition sites contained in nucleosomes varies (Owen-Hughes and Workman, 1994). However, there is little information available regarding the interactions of basic leucine zipper (bZIP) proteins with nucleosomal DNA. No binding was detected in one attempt to analyze nucleosome binding by a bZIP protein (Svaren et al., 1994a).

The inherent ability of a transcription factor to recognize and

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bind its cognate site in chromatin is thought to be determined by nucleosome positioning (Simpson, 1991; Wolffe, 1994). Nucleosome positioning has two main components: translational positioning, which refers to the position of the binding site with respect to the nucleosome dyad axis of symmetry; and rotational phasing, which refers to the orientation of the DNA helix with respect to the underlying histone octamer surface. Translational positioning has been shown to affect the binding of a number of transcription factors, including the glucocorticoid receptor, GAL4 derivatives, the upstream stimulatory transcription factor (USF), and NF-κB (Li and Wrange, 1993; Vettese-Dadey et al., 1994; Adams and Workman, 1995). Rotational positioning has been shown to affect the binding of the TATA binding protein and glucocorticoid receptor to nucleosomes (Imbalzano et al., 1994; Li and Wrange, 1995). The implications of nucleosome positioning have also been demonstrated in vivo. When a yeast autonomously replicating sequence was moved from the periphery to the center of a nucleosome, the copy number of the plasmid was dramatically reduced from ~100 copies to one copy per cell, reflecting a reduction in autonomously replicating sequence function (Simpson, 1990).

The bZIP proteins are a major group of eukaryotic transcription factors, many of which have been isolated from plant species (Foster et al., 1994). The majority of plant bZIP proteins have been shown to bind the sequence containing CACGTG (G-box core) with high affinity (Izawa et al., 1993). Sequences with a G-box core have been shown to be functionally important in numerous promoters of plant genes, including those regulated by light (the ribulose bisphosphate carboxylase small subunit gene *rbcS-1A*), abscisic acid (ABA; *Em*), UV light (the chalcone synthase gene), and an anaerobically regulated gene (*Adh*; reviewed by de Vetten and Ferl, 1994; Menkens et al., 1995). The commonalty of the G-box in promoters of such diverse regulatory properties has led to the hypothesis that multiple protein interactions lead to response specificity. Interactions with nucleosomes must be taken into account in such a model to address the in vivo DNA–protein configuration. To understand how bZIP proteins function on nucleosome templates, we have examined nucleosome binding by the bZIP transcription factor EmBP-1.

EmBP-1 is a plant transcription factor implicated in ABA-induced gene expression in wheat (Guiltinan et al., 1990;

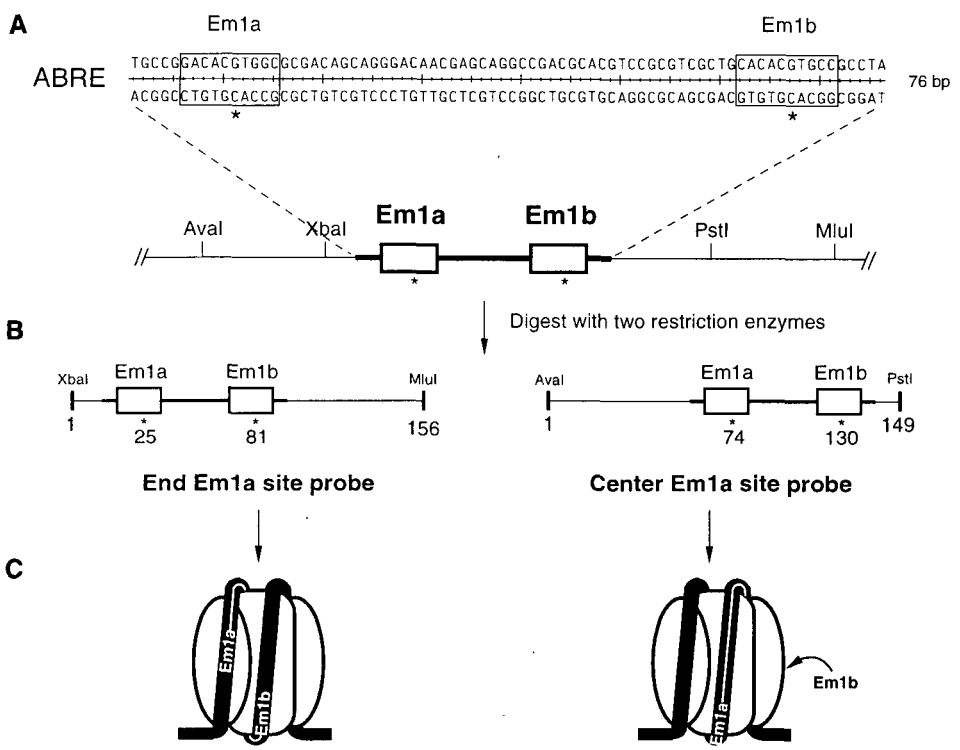

Figure 1. Schematic Illustration of Nucleosome Reconstitution with ABRE Fragments.

The diagrams are not drawn to scale.

(A) Nucleotide sequence of the ABRE and flanking restriction sites in pXN300. The Em1a and Em1b sites are boxed. The stars indicate restriction enzyme MaeII sites at the centers of Em1a and Emlb sites.

(B) Two ABRE fragments used in nucleosome reconstitution. The DNA fragment at the left, in which the Em1a site is close to the probe end (End Em1a site probe), was generated by digestion with XbaI and MluI. The DNA fragment at the right, in which the Em1a site is near the probe center (Center Em1a site probe), was generated by digestion with AvaI and PstI. The stars are as in **(A)**.

(C) Nucleosome structures showing the location of the Em1a and Em1b sites. When the two probes in **(B)** were reconstituted into nucleosomes, the Em1a site was located near the nucleosome edge or near the nucleosome dyad, respectively.

Quatrano et al., 1992). Its DNA binding specificity and DNA binding and dimerization domains have been characterized, indicating that EmBP-1 is a typical bZIP transcription factor that shares many common properties with the well-characterized yeast transcription factor GCN4 (Guiltinan and Miller, 1994; Niu and Guiltinan, 1994). EmBP-1 binds to the G-box with the highest known specificity of any plant bZIP protein (Izawa et al., 1993). EmBP-1 may also interact with VIVIPAROUS1, a maize regulatory protein that is involved in the response to ABA during maize embryo development (Vasil et al., 1995). VIVIPAROUS1 interaction enhances EmBP-1's ability to bind an ABA response element (ABRE), supporting a multiple protein interaction model (Hill et al., 1996).

We show that EmBP-1 can bind ABRE and G-box elements in reconstituted HeLa or wheat nucleosomes but with reduced affinity relative to naked DNA. Nucleosome binding was strongly affected by the translational position of the binding sites but to a lesser extent by the rotational phasing of the DNA helix on the surface of the nucleosome. Although the minimal DNA binding domain of EmBP-1 was sufficient for nucleosome binding, the addition of the N-terminal putative transactivation domain resulted in a strong DNase I hypersensitivity surrounding the binding sites. Similar effects on EmBP-1 affinity were manifested by both HeLa and wheat nucleosomes. These results suggest a functional role of the G-box binding proteins in the recognition of *cis* elements in the context of chromatin structure.

RESULTS

Because histones are highly conserved among mammalian and plant cells, we initially studied interactions of the wheat bZIP protein EmBP-1 with reconstituted nucleosomes by using a well-established system developed with HeLa histones. To investigate whether EmBP-1 can also interact with homologous plant nucleosomes, we also reconstituted nucleosomes with wheat histones.

Both Full-Length and Minimal DNA Binding Domains of EmBP-1 Can Bind ABRE Elements Reconstituted into HeLa Nucleosomes

The naturally occurring *cis* element ABRE in the *Em* gene promoter has been shown to be capable of activating gene expression upon ABA induction in transient expression assays using rice protoplasts (Marcotte et al., 1988, 1989; Guiltinan et al., 1990). The ABRE consists of a high-affinity binding site, Em1a, and a low-affinity binding site, Em1b, for EmBP-1, which are 56 bp apart. For nucleosome reconstitution, the ABRE (76 bp) (Guiltinan et al., 1990) was cloned into a pBend derivative pTK401 (Kerppola and Curran, 1991) (Figure 1A). A DNA fragment of 156 bp was purified from this plasmid, radiolabeled, and reconstituted into single nucleosomes. The center of the Em1a site was 25 bp from the fragment end (end Em1a site probe in Figure 1B). When this fragment was reconstituted into a nucleosome, the Em1a site was located near the edge of the nucleosome (Figure 1C).

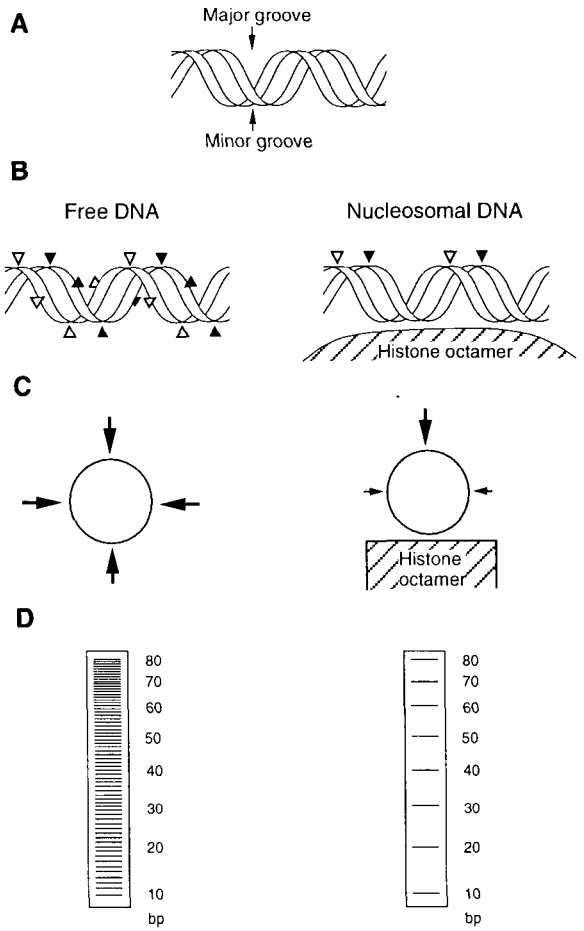

Figure 2. Depiction of DNase I Cleavage Patterns on Naked and Nucleosomal DNA.

(A) Two opposite faces of a helical double-stranded DNA. The major groove and the minor groove are indicated.

(B) Side views of DNase I cuts on free and nucleosomal DNA. DNase I cuts free DNA within the minor groove from all sides. DNase I cleavage on nucleosomal DNA is restricted to the minor groove facing outward from the histone octamer surface. Cuts on different strands are indicated by open and filled triangles.

(C) End views of DNase I cuts on free and nucleosomal DNA. DNase I cuts are indicated by arrows. Small arrows on the nucleosomal DNA indicate less frequent cuts.

(D) Depiction of sequencing gels displaying DNase I digestion patterns on free and nucleosomal DNA. DNase I cutting of naked DNA produces a ladder with cuts at every position, whereas nucleosomal DNA produces an ~10- or 11-bp ladder.

To analyze nucleosome binding by EmBP-1, we initially used DNase I footprinting. DNase I cuts randomly within the minor groove of a DNA helix (see Figure 2A, and Figures 2B and 2C at left). When DNA is wrapped around a nucleosome, the minor groove on one side of DNA helix contacts the histone

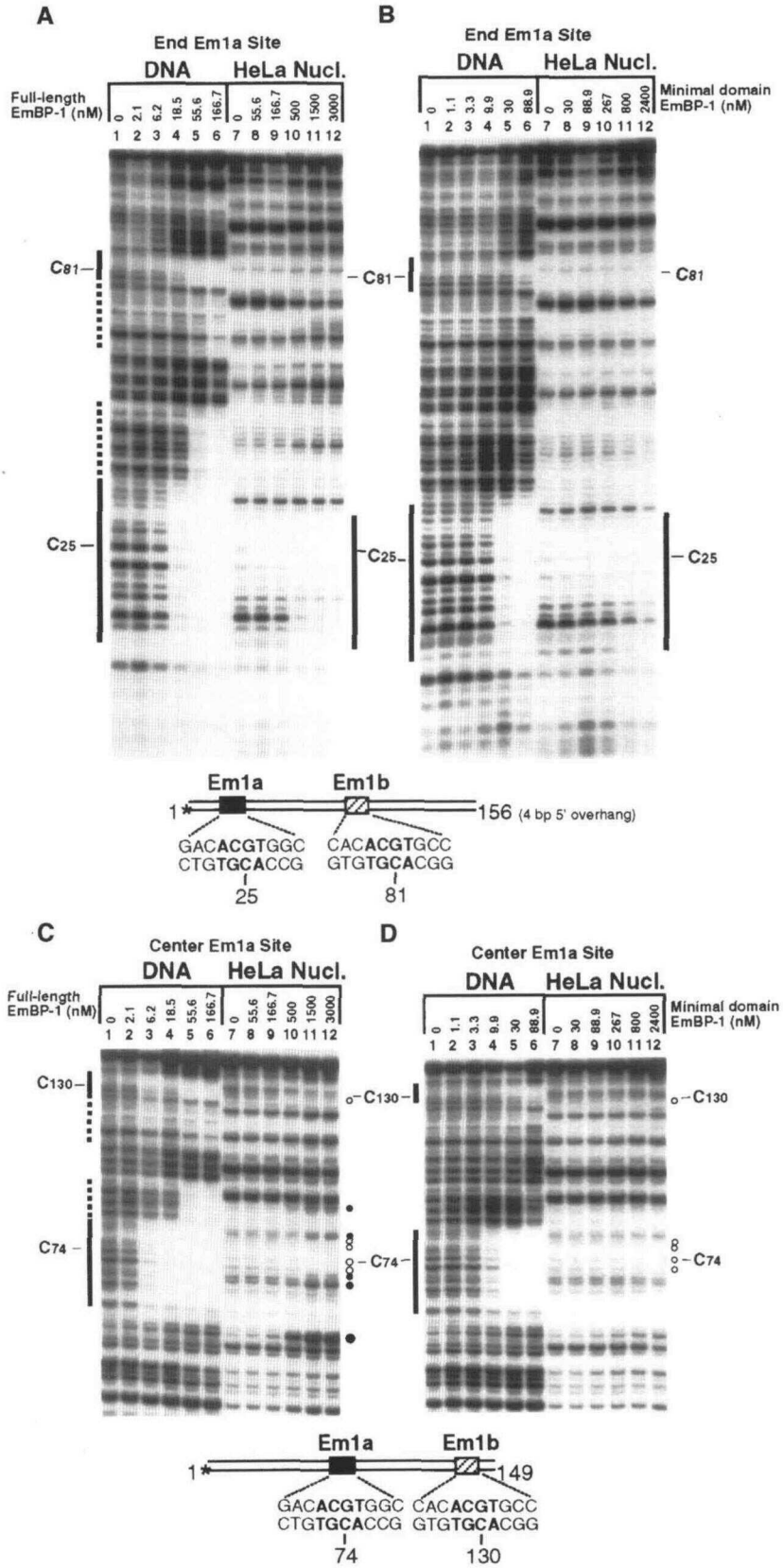

Figure 3. DNase I Footprinting Titration of Full-Length EmBP-1 and Mini-EmBP-1 Protein Interactions with HeLa Nucleosome-Reconstituted ABRE Fragments.

octamer surface every 10 to 11 bp, and these positions are less accessible to DNase I attack (see Figures 2B and 2C at right). Therefore, the most frequent cutting by DNase I occurs where the minor groove is not in contact with histone octamer and faces away from the octamer surface (see Figures 2B and 2C at right). When there is a sequence preference for one side of a DNA helix to face the histone octamer, DNase I cuts at approximately every 10 to 11 bp (one turn of the DNA helix) along the sequence of the DNA fragment, generating a 10-bp ladder on a gel (see Figure 2D). Thus, DNase I footprinting can be used to monitor the quality and positioning of a reconstituted nucleosome preparation.

As shown in Figure 3, nucleosomes reconstituted with the ABRE and HeLa histones demonstrated an ~10-bp periodicity of DNase I cleavage patterns, which was not apparent when naked DNA was digested (compare lane 1 and lane 7 in Figure 3A). The reconstituted HeLa nucleosomes were also verified by an electrophoretic mobility shift assay (EMSA), which illustrated that more than 80% of the fragment was contained in the nucleosome-reconstituted band, with less than 20% remaining as free DNA. The DNase I cleavage pattern in Figure 3A indicates that the minor groove at the center of the Em1a site (C25 in Figure 3A) in the nucleosomal ABRE faced toward the octamer surface, as indicated by the low frequency of DNase I cleavage at this position relative to adjacent sites. This also means that the major groove at the center of the Em1a site (C25) in the nucleosomal ABRE faces outward from the octamer surface (see Figures 4A, and 4B and 4C at left).

DNase I footprinting demonstrated that the full-length EmBP-1 protein could bind specifically to the Em1a site reconstituted into nucleosomes (compare protection in DNA and in nucleosomes in Figure 3A). At least a 30-fold higher concentration of protein was required to observe the protection over the binding site within the nucleosome compared with binding to naked DNA, indicating a significant inhibition of EmBP-1 binding by nucleosomes. However, because bZIP proteins bind the major groove (Ellenberger et al., 1992) and DNase I cleaves the minor groove, the protection appears to be less conspicuous when the major groove of the binding site faces outward from the nucleosome core. Therefore, quantitative estimates on nucleosome binding affinity based merely on DNase I footprinting for this class of proteins should be made with caution. However, used in conjunction with DNase I footprinting, an EMSA can be used quantitatively because it is not subject to the limitations of DNase I–based footprinting on nucleosomal DNA (Figure 5).

At higher concentrations where the Em1a site was almost completely protected, EmBP-1 also bound the lower affinity Em1b site on naked DNA (Figure 3A, lanes 5 and 6). In nucleosomes, however, the Em1b site, which was close to the nucleosomal dyad (C81 in Figure 3A), showed little protection by EmBP-1, even at high protein concentrations (Figure 3A, lanes 10 to 12). According to the DNase I cleavage pattern, the major groove at the center of the Em1b site was projected parallel to the octamer surface (see Figures 4A, and 4B and 4C at right).

Even at protein concentrations that gave nearly full protection of the Em1a site on these nucleosomes, the nucleosomal 10-bp periodicity of DNase I cleavage was still apparent elsewhere in the sequence (Figure 3A, lane 12). This demonstrates that EmBP-1 at high concentrations did not cause dissociation of the DNA from the histone octamer, consistent with the formation of a ternary complex (three factor–associated complex) containing the bound factor, histones, and DNA (Piña et al., 1990; Workman and Kingston, 1992; Li et al., 1994).

To investigate the domains of EmBP-1 required for nucleosome binding, we analyzed the ability of the minimal DNA binding domains of the EmBP-1 protein to bind nucleosome cores. The minimal domain of EmBP-1 (mini-EmBP-1) contains only the basic region and four leucine repeats from amino acids 249 to 308 and binds specifically to the ABRE probe with affinity similar to the full-length EmBP-1 protein (Guiltinan and Miller, 1994). The recombinantly expressed mini-EmBP-1 protein was purified (a total of 98 amino acids with a 60–amino acid mini-EmBP-1; see Methods) and tested for nucleosome binding under the same conditions as described for the full-length protein. Binding of mini-EmBP-1 to the naked ABRE probe is illustrated in Figure 3B (lanes 1 to 6). The concentration required for protection of the Em1a site by mini-EmBP-1

Figure 3. (continued).

In **(A)** to **(D)**, binding of EmBP-1 to naked DNA is shown in lanes 1 to 6 and binding to nucleosomal (Nucl.) DNA is shown in lanes 7 to 12. The nanomolar protein concentrations in each reaction are indicated at top. Solid bars indicate protected regions, and the extended dashed bars indicate weak interactions at the Em1a and Em1b sites seen at high protein concentrations. Positions of C bases (C25, C81, C74, and C130) in the bottom strand of the ACGT core sequences of the Em1a and Em1b sites are indicated as determined by MaeII partial digestion (not shown). The probes used for DNase I footprinting are depicted at the bottom. Phosphorus-32 label (asterisk), probe length (in bp), positions of Em1a (filled box) and Em1b (diagonally striped box), and Em1a and Em1b sequences (ACGT cores are in boldface) are shown. In **(C)** and **(D)**, sequencing gels were run 1 hr longer to resolve protection on the distal sequence from the labeled end.

(A) Binding of the full-length EmBP-1 protein to the end Em1a site ABRE fragment.

(B) Binding of the mini-EmBP-1 protein to the end Em1a site ABRE fragment.

(C) Binding of the full-length EmBP-1 protein to the center Em1a site ABRE fragment. The open and closed circles at right indicate protected and hypersensitive bases in nucleosomes, respectively, as a result of specific binding by the full-length EmBP-1 protein.

(D) Binding of mini-EmBP-1 protein to the center Em1a site ABRE fragment. Open circles at right indicate protected bases in nucleosomes as a result of specific binding by the mini-EmBP-1 protein.

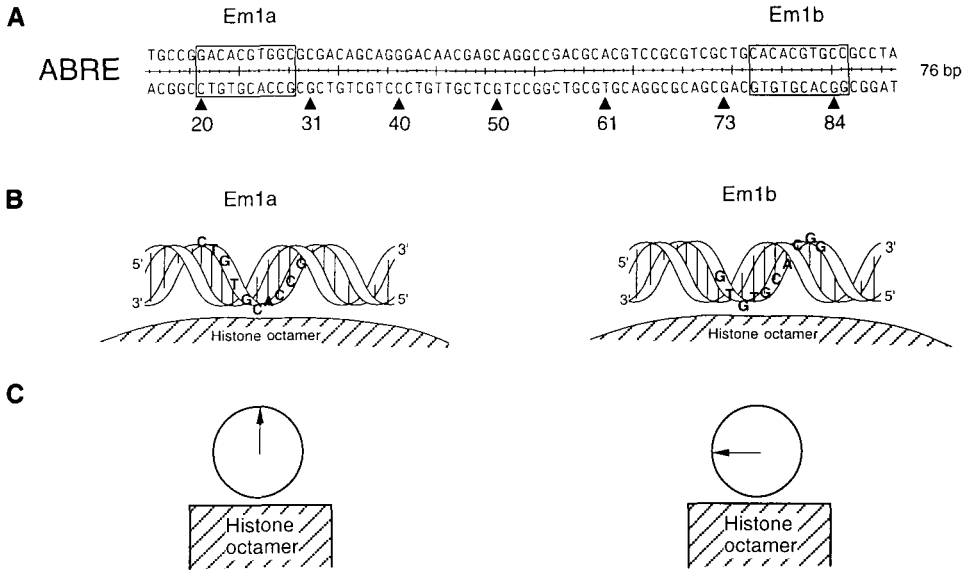

Figure 4. Positioning of the ABRE Sequence in Nucleosomes.

(A) Shown are both strands of the ABRE sequences and DNase I cleavage sites on the bottom strand as determined by footprinting in Figure 3A and chemical sequencing (data not shown). The Em1a and Em1b sites are boxed. The numbering starts from the labeled end of the ABRE end site probe shown in Figure 3A.

(B) Em1a and Em1b site positioning on nucleosomes. The bottom strand sequences of the Em1a and Em1b sites are depicted.

(C) End views of Em1a and Em1b site positioning on nucleosomes. The arrows indicate the directions of the major groove at the center of Em1a and Em1b sites, respectively.

was slightly higher than for full-length EmBP-1; however, mini-EmBP-1 was also able to bind the Em1a site in reconstituted nucleosomes, as shown in Figure 3B (lanes 7 to 12). Because of the smaller size of mini-EmBP-1, it presented less steric hindrance for interactions of DNase I with the minor grooves that are exposed on the surface of nucleosomes, resulting in a smaller protected region. Consistent with this conclusion, protection of nucleosome bands was more constrained to the major groove (compare the protected regions from lanes 8 to 12 in Figures 3A and 3B). Higher concentrations of mini-EmBP-1 were required for nucleosome binding than for naked DNA binding, consistent with the findings for full-length EmBP-1. Interestingly, full-length EmBP-1 induced DNase hypersensitivity at positions flanking the Em1a binding site. This was not seen using the minimal version of EmBP-1, even at high concentrations (compare lanes 10 to 12 in Figures 3A and 3B). This indicates that the N-terminal domain of EmBP-1 may establish interactions with nucleosomes that cause structural changes upon binding in addition to steric protection of DNA from DNase I attack.

EmBP-1 Binding to Nucleosomal DNA Is Subject to a Nucleosome Translational Positioning Effect

To investigate how the positioning of binding sites within nucleosomes affects binding by EmBP-1, we made a second 149-bp ABRE probe in which the center of Em1a is 74 bp from the probe end (center Em1a site probe in Figure 1B at right). When reconstituted into nucleosomes, the center of the Em1a site was near the nucleosomal dyad (see Figure 1C at right and C74 in Figures 3C and 3D). DNase I footprinting in Figures 3C and 3D indicates that in reconstituted nucleosomes, the orientation of the DNA helix (rotational phasing) for the ABRE fragment was similar to that observed when the ABRE Em1a site was near the edge of the nucleosome, that is, the major groove at the center of the Em1a site faces outward from and Em1b is parallel to the octamer surface (see Figure 4). The fact that the ABRE assumes similar rotational phasing at two locations within nucleosomes implies that the ABREs and surrounding sequences may induce rotational positioning on the nucleosomes. Full-length EmBP-1 was able to bind the Em1a site in this nucleosomal template, but the affinity for this centered site was lower than for the Em1a site located near the edge of nucleosome (compare lanes 7 to 12 in Figures 3A and 3C). The nucleosome binding to this centered site by EmBP-1 is characterized by weak protection and strong hypersensitivity around the region. The positional effect on EmBP-1 binding is further illustrated by the weaker binding site Em1b, which is located 20 bp from the probe end. As opposed to the lack of binding to this site in the more centrally located position (Figure 3A), full-length EmBP-1 bound this site (C130 in Figure 3C) both in naked DNA and in the nucleosomes at high protein concentrations, as indicated by DNase I footprinting titration.

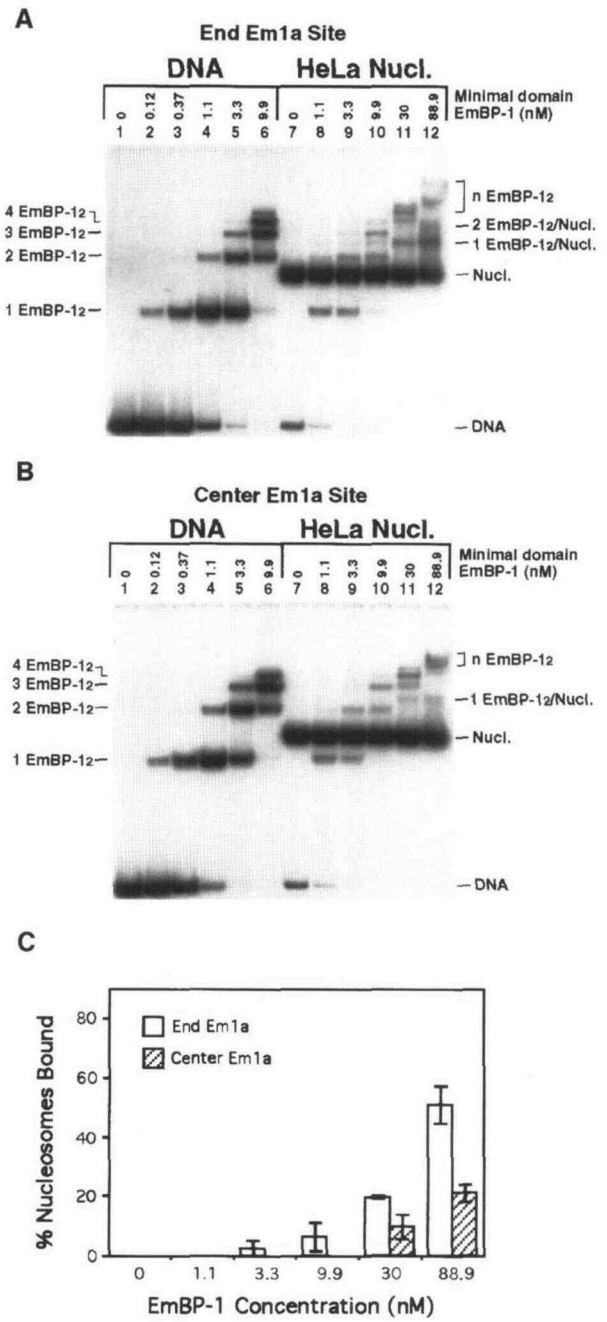

Figure 5. Positional Effect on EmBP-1 Binding to HeLa Nucleosome Cores.

An EMSA of mini-EmBP-1 protein binding to the ABRE fragments as naked DNA (lanes 1 to 6) or as nucleosome (lanes 7 to 12) is shown. The nanamolar concentrations of EmBP-1 tested are indicated above the lanes. The formation of EmBP-1–naked DNA complexes (1 EmBP-1_2 is one EmBP-1 dimer; 2 EmBP-1_2, two EmBP-1 dimers; 3 EmBP-1_2, three EmBP-1 dimers; 4 EmBP-1_2, four EmBP-1 dimers) are indicated at left. The positions of naked DNA (DNA), nucleosomes (Nucl.), the EmBP-1–nucleosome complex (EmBP-1_2/Nucl.), and multimers of EmBP-1–naked DNA complexes (n EmBP-1_2) are indicated at right.

Binding to the centered Em1a site by mini-EmBP-1 is shown in Figure 3D. Protection of nucleosomal Em1a and Em1b by the mini-EmBP-1 protein was similar to full-length EmBP-1, but most noticeably, mini-EmBP-1 does not cause the adjacent hypersensitivity upon nucleosome binding.

The positional effect on nucleosome binding by EmBP-1 was further assessed by an EMSA with the same ABRE fragments used in DNase I footprinting. Mini-EmBP-1 binding to naked DNA is illustrated in Figures 5A and 5B (lanes 1 to 6). With increased EmBP-1 concentration, incremental numbers of EmBP-1 dimers bound to the free DNA probe. As shown by the DNase I footprinting analysis (Figure 3A, lanes 1 to 6), as the concentration of EmBP-1 increases, EmBP-1 bound the Em1a site first; then the Em1b site and weak interactions around the Em1a and Em1b sites occurred at the highest concentration. In these binding reactions, nonspecific competitive DNA was not used. Multiple forms of EmBP-1–naked DNA complexes served as references for determination of EmBP-1 binding to nucleosomal DNA. EMSAs of EmBP-1 binding to nucleosomal DNA are illustrated in Figures 5A and 5B (lanes 7 to 12). Similar to the free DNA lanes (Figures 5A and 5B, lanes 1 to 6), DNA–EmBP-1 complexes can be seen in identical positions but at lower levels, due to the low amount of free DNA in these binding reactions (lanes 7 to 12).

Nucleosomes bound by EmBP-1 were well resolved from complexes composed of naked DNA and EmBP-1 alone. At the same protein concentrations, more nucleosomes were bound by EmBP-1 with the end Em1a site probe (Figure 5A) than with the center Em1a site probe (Figure 5B), indicating a specificity of nucleosome binding consistent with the footprinting results. At the highest concentration tested, nucleosomes with the end Em1a site probe were bound by a second EmBP-1 dimer (Figure 5A, lane 12), consistent with footprinting results and indicating that EmBP-1 can also interact weakly with the Em1b site in nucleosomes. Replicate sets of EMSA gels were quantified to estimate the binding affinities of EmBP-1 to these two nucleosomal templates (Figure 5C). At the same protein concentrations, the percentage of nucleosomal ABRE bound was two to three times higher when

(A) Binding to the ABRE fragment with an end Em1a site (25 bp from the end, as shown in Figures 3A and 3B).

(B) Binding to the ABRE fragment with a center Em1a site (74 bp from the end, as shown in Figures 3C and 3D).

(C) Bar graph of the percentage of nucleosomes bound by EmBP-1 at increasing EmBP-1 concentrations, with end and center Em1a binding sites. The open bars represent average values of nucleosomes bound with an end Em1a site from the experiment in **(A)**; the diagonally striped bars represent mean values of nucleosomes bound with a center Em1a site from the experiment in **(B)**. The mean values are derived from three independent experiments. The standard deviations are also indicated.

the Em1a site was located near the edge of nucleosome as opposed to the center Em1a site. This affinity was ~100-fold less than for naked DNA.

EmBP-1 Binds to the G-Box Site with Higher Affinity than to the Em1a at the Same Locations in *Nucleosomes*

The bZIP protein EmBP-1 demonstrated nucleosome binding abilities that appear to be less than that of GAL4 derivatives and Sp1 (Taylor et al., 1991; Li et al., 1994; Vettese-Dadey et al., 1994) but similar to that of USF (Adams and Workman, 1995), which also showed ~100-fold difference in relative affinity for free DNA versus nucleosome cores when their binding sites were positioned at approximately the same location in nucleosomes. Our previous studies with naked DNA have shown that a palindromic G-box (GCCACGTGGC) is a stronger binding site for EmBP-1 than is the Em1a site found within the native context of the ABRE (GACACGTGGC) (Niu and Guiltinan, 1994). This suggested that EmBP-1 may also bind to the G-box with higher affinity than to Em1a within nucleosomes.

To test this hypothesis, we made two probes containing the G-box site in which the center of the G-box is 26 and 84 bp from the ends of the probes, respectively (Figures 6A and 6B). When reconstituted into nucleosomes, the translational positioning of the G-box was approximately the same as the Em1a site used in Figure 3: the G-box site was located either at the edge or at the dyad of the nucleosomes. However, the G-box sequence did not tend to position rotationally in a similar manner as the ABRE, that is, with the major groove at the center of the binding site facing out from the nucleosome (data not shown). To create rotational phasing for the G-box probes similar to that of the ABRE probes, we included a 25-bp bent DNA sequence (see Methods) consisting of alternating (A)5 and (C/G)5 tracts (Kerppola and Curran, 1991). The (A)5 tract contracts its minor groove and therefore has a widened major groove, whereas the (C/G)5 tract tends to contract its major groove and has a widened minor groove. The alternating (A)5 and (C/G)5 tracts produce an intrinsic bend in the DNA fragment (Kerppola and Curran, 1991). This bent sequence defines the orientation of the DNA helix when wrapped (bent) around the surface of the histone octamer.

As shown in Figures 6A and 6B, the major groove at the G-box center faced outward for both probes when reconstituted into nucleosomes. This is indicated by the 10-bp periodicity of DNase I cleavage flanking the center of the G-box dyad. Specific binding of the nucleosomal G-box by EmBP-1 was observed when the G-box was located at either the edge or the center of nucleosomes. Protection of the end nucleosomal G-box was observed at an EmBP-1 concentration of 55.6 nM; however, ~500 nM of EmBP-1 was required to observe protection of the center nucleosomal G-box. Binding of full-length EmBP-1 to the center nucleosomal G-box also resulted in adjacent DNase hypersensitivity as with the Em1a probe,

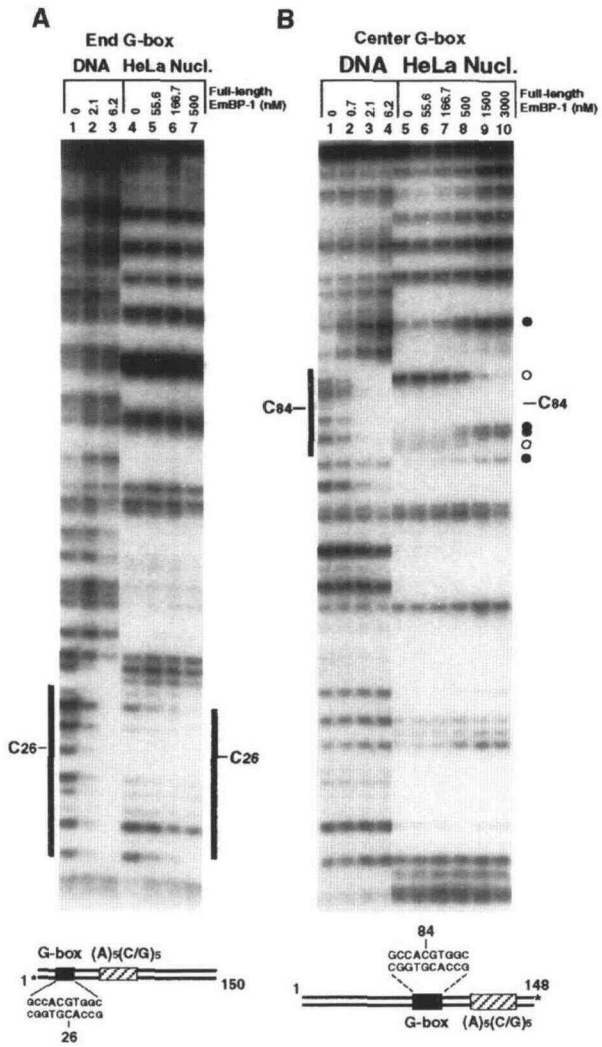

Figure 6. *DNase I Footprinting Titration* of Full-Length EmBP-1 Protein Interactions with G-Box Fragments Reconstituted into HeLa Nucleosomes.

Labeling is as given in Figure 3. DNA probes used for DNase I footprinting are depicted below the footprinting gels, with an indication of G-box (solid box) and bent sequence (diagonally striped box, (A)5(C/G)5).

(A) Binding of the full-length EmBP-1 protein to the end G-box fragment as naked DNA (lanes 1 to 3) and as reconstituted nucleosome (Nucl.) cores (lanes 4 to 7).

(B) Binding of the full-length EmBP-1 protein to the center site G-box fragment as naked DNA (lanes 1 to 4) and as reconstituted nucleosome cores (lanes 5 to 10).

which was absent upon binding by mini-EmBP-1 at high concentrations (data not shown).

An EMSA of EmBP-1 binding to these G-box probes is illustrated in Figure 7. EmBP-1 binding to naked DNA is shown at left in Figures 7A and 7B (lanes 1 to 6). As described above,

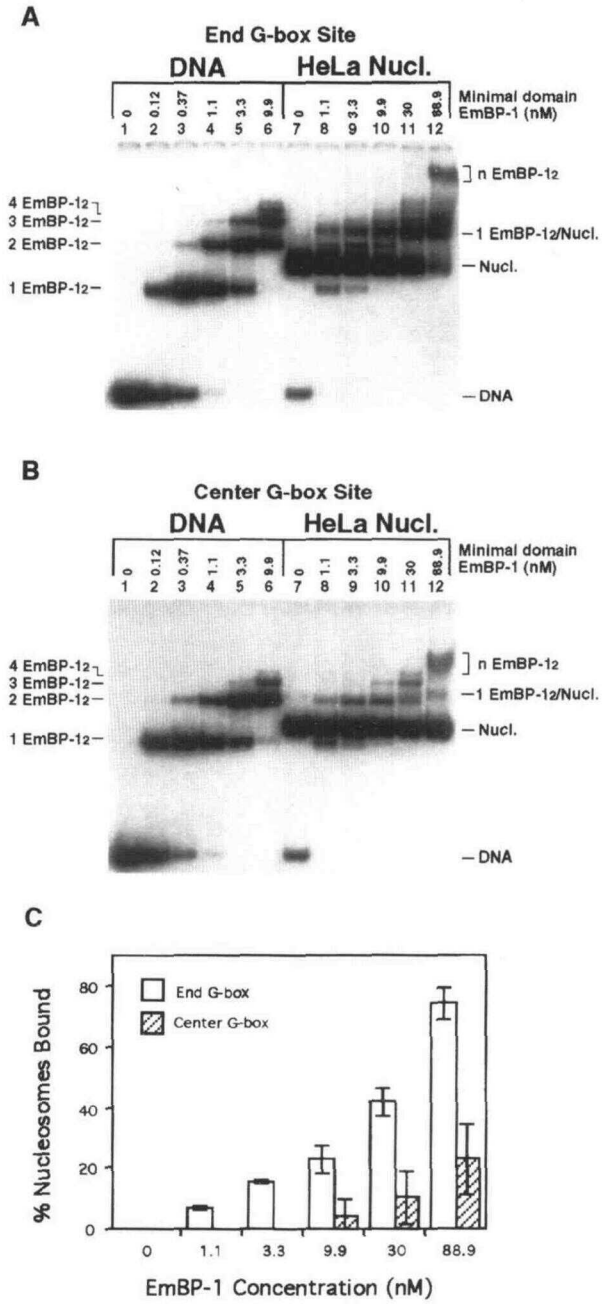

Figure 7. EMSA of Mini-EmBP-1 Protein Binding to G-Box Fragments Reconstituted into HeLa Nucleosomes.

An EMSA of mini-EmBP-1 protein binding to the G-box fragments as naked DNA (lanes 1 to 6) or as nucleosomes (Nucl.; lanes 7 to 12) is shown. Binding reactions were run on 5.5% native polyacrylamide gel. The end and center G-box site probes are shown in Figure 6. Labeling for protein concentrations and bands on the gels is the same as given in Figure 5.

(A) Binding to the end G-box site fragment.

(B) Binding to the center G-box site fragment.

(C) Bar graph of the percentage of nucleosomes bound by EmBP-1 at increasing EmBP-1 concentrations with end and center G-box binding sites. The open bars represent average values of nucleosomes bound with an end G-box site from the experiment in **(A)**; the diagonally striped bars represent average values of nucleosomes bound with a center G-box site from the experiment in **(B)**. The average values are derived from three independent experiments. The standard deviations are also indicated.

with increased EmBP-1 concentration, incremental numbers of EmBP-1 dimers bound to the DNA probes. EmBP-1 binding to nucleosomal DNA is shown at right in Figures 7A and 7B (lanes 7 to 12). At the same protein concentration, nucleosomes reconstituted with the end site G-box probe were bound with much higher affinity by EmBP-1 than by nucleosomes reconstituted with the center G-box probe. Quantification of these results is presented in Figure 7C. At the same protein concentration, the percentage of the end site nucleosomal G-box bound was four to five times higher than with the center nucleosomal G-box. The effect of translational positioning of the binding site on EmBP-1 affinity to nucleosomal DNA is clearly demonstrated in this experiment. These data also indicate that the relative affinity of EmBP-1 for the G-box in naked DNA is ~100 times higher than in nucleosomal DNA when the binding site is at the external location.

A comparison of Figures 5C and 7C indicates that EmBP-1 had a higher affinity for a nucleosomal G-box than for the ABRE when the binding sites were located near the edge of nucleosomes (Figures 5C and 7C). This is similar to the relative EmBP-1 affinities for the G-box and ABRE in naked DNA, as shown in this study and in Niu and Guiltinan (1994). When the binding sites were located near the nucleosome dyad, binding was greatly inhibited. Interestingly, the binding affinity for the centered nucleosomal G-box sites and ABRE Em1a site by EmBP-1 is approximately the same; in other words, nucleosomal inhibition of centered site binding is less for the ABRE than for the G-box. Binding to the centered ABRE is likely to be the result of EmBP-1 interactions with both Em1a and Em1b sites as shown by DNase I footprinting.

A comparison of EmBP-1 binding to the nucleosomal end Em1a site (Figure 5A) and center G-box site (Figure 7B) also demonstrates how nucleosome positioning could significantly modulate EmBP-1 affinity for the DNAs. With these nucleosome positions, EmBP-1 bound to the Em1a site better than to the G-box. Quantifications in Figures 5C and 7C indicate that the affinity of EmBP-1 for the nucleosomal end Em1a site is two to three higher than for the nucleosomal center G-box site. Therefore, the affinity of EmBP-1 for G-box and Em1a sites in free DNA is reversed by differential nucleosome positioning.

Reconstitution of Wheat Nucleosomes in Vitro

Our initial results demonstrated that EmBP-1 could bind HeLa nucleosomes. To extend these findings into a homologous

system, we next investigated interactions of the wheat bZIP protein EmBP-1 with wheat nucleosomes. Wheat oligonucleosomes were first prepared from wheat germ according to published methods (Simon and Becker, 1976; Moehs et al., 1992; Côté et al., 1995). We found that reconstitution of wheat nucleosomes with radiolabeled DNA fragments by the octamer transfer method (see Methods for details) was not efficient. Only approximately half of the DNA probe could be reconstituted into nucleosomes under the same conditions used for HeLa nucleosome reconstitution.

An alternative approach was tested using purified wheat histone octamers. Figure 8A shows an SDS protein gel of purified HeLa oligonucleosomes and wheat core histone octamers used in nucleosome reconstitution. The purified wheat core histone octamers are >90% pure with only minor amounts of histone H1 present. The nonstoichiometric appearance of wheat histones on the SDS gel is likely due to overlapping of histones H2 and H3 and preferential staining by Coomassie Brilliant Blue R 250, as seen previously (Moehs et al., 1992). Unlike HeLa histones H2A and H2B, a number of wheat histone H2A and H2B variants can be observed as bands of broad ranges on the SDS protein gel, consistent with previous reports (Spiker, 1982; Spiker et al., 1987). Those studies reported five forms of wheat H2A histones and six forms of wheat H2B histones, with estimated molecular weights of 15,300 to 19,000 (Spiker, 1982). In addition, at least four H2A and five H2B histone variants of wheat (cDNA clones) have been isolated (GenBank accession numbers D38087, D38088, D38090, and D38091 for H2A; D37942 to D37945, and X59873 for H2B). They encode proteins with calculated molecular weights of 13,934 to 16,435. It is has also been shown that the expression of wheat histone variants do not significantly change during wheat development (Spiker et al., 1987). Therefore, all of these histone variants would be expected to be present in our histone preparations, consistent with the multiple bands we observed (Figure 8A).

Figure 8B illustrates wheat nucleosomes reconstituted with a DNA probe using a salt dilution method (see Methods). Nearly 80% of the probe DNA was reconstituted into wheat nucleosomes. Even though the wheat octamers are heterogeneous, reconstituted wheat nucleosomes ran as a single band on mobility shift gels. Consistent with a previously published result (Arwood and Spiker, 1990), reconstituted wheat nucleosomes also ran slower than did HeLa nucleosomes on the gel. The difference in electrophoretic mobility of the wheat and chicken nucleosomes was proposed to be due to conformational differences of plant and mammalian nucleosomes (Arwood and Spiker, 1990).

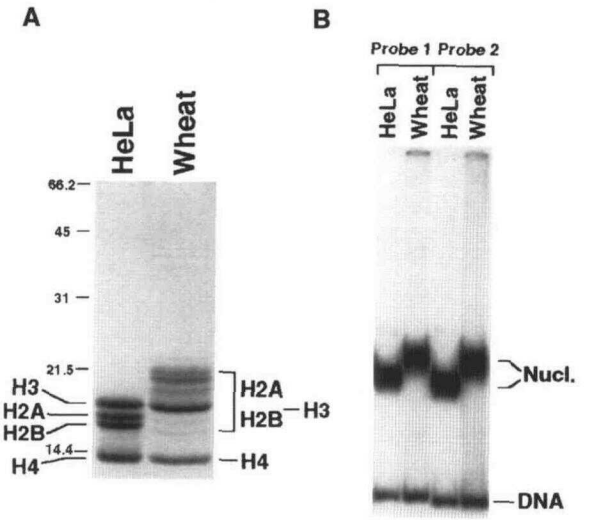

Figure 8. Reconstitution of Wheat Nucleosomes with Purified Wheat Histone Octamers.

(A) Comparison of purified HeLa and wheat histone octamers. Approximately 15 μg of purified HeLa and wheat histones was run on an 18% SDS–polyacrylamide gel and stained with Coomassie blue. The positions of HeLa histones H3, H2A, H2B, and H4 are indicated at left. The positions of wheat histones H2A, H2B, H3, and H4 are indicated at right. Wheat histone H2A and H2B variants are collectively indicated with a bracket. The positions of protein makers (in kilodaltons) (Bio-Rad, low range) are also shown at left.

(B) An EMSA of HeLa and wheat nucleosomes reconstituted with two different probes. Probe 1 and probe 2 are the end and center Em1a site ABRE probes, respectively, as shown in Figure 9. The reconstituted nucleosomes were run on a 5% native polyacrylamide gel. The positions of reconstituted nucleosomes (Nucl.) and free DNA (DNA) are indicated at right.

EmBP-1 Can Also Bind ABRE Elements within Wheat Nucleosomes

To investigate the interactions of the bZIP protein EmBP-1 with wheat nucleosomes, we made two probes containing the ABRE fragment in which the center of the Em1a site is 25 and 74 bp from the ends. When the probes were reconstituted into nucleosomes, the Em1a site was located near the edge or the center of nucleosomes, respectively (see Figures 1B and 1C). For the ABRE end Em1a site probe (158 bp), DNase I cleavage patterns in Figures 9A and 9B indicate that it was reconstituted into a nucleosome position similar to that found in the HeLa nucleosomes (compare Figures 3A and 3B with 9A and 9B). However, there are some differences in the band intensity within the 10-bp periodicity and additional minor DNase I cleavage sites. For the ABRE center site probe (155 bp), DNase I cleavage patterns in Figures 9C and 9D indicate that it was reconstituted into a nucleosome position quite different from that of HeLa nucleosomes (compare Figures 3C and 3D with 9C and 9D). First, although nucleosome reconstitution with wheat histones altered the DNase I pattern of this probe as compared with DNase pattern of naked DNA, the 10-bp DNase I cleavage pattern was not obvious for nucleosomal DNA. This suggests that wheat nucleosomes with this

sequence may have multiple rotational positions. Second, DNase I cleavage occurred at the center of the Em1a site (C74 in Figures 9C and 9D) in the wheat nucleosomes but not in the HeLa nucleosomes.

Similar to the results with HeLa nucleosomes, DNase I footprinting in Figures 9A and 9B indicates that both full-length and minimal-domain EmBP-1 proteins were able to bind the end Em1a site in the wheat nucleosomes. At least 30-fold more EmBP-1 protein was required to generate protection on nucleosomal DNA relative to naked DNA. In addition, binding of full-length EmBP-1 to wheat nucleosomes caused hypersensitivity around the Em1b site, indicating some interactions at this site as well.

Despite multiple rotational frames in wheat nucleosomes containing the DNA probe with the centered Em1a site, DNase I footprinting in Figure 9C indicates that full-length EmBP-1 did bind the center Em1a site but with a lower affinity than to the end site, as shown in Figure 9A. Full-length EmBP-1 also generated a major hypersensitive site and two minor sensitive sites, as seen for binding to the HeLa nucleosome containing this probe. Again, mini-EmBP-1 did not produce the hypersensitivity flanking the binding site. Protection of the centered Em1a site by the mini-EmBP-1 was not as apparent on the wheat nucleosomes (Figure 9D) as it was with the HeLa nucleosomes. This might be due to the heterogeneity in rotational phasing of this sequence in wheat nucleosomes or conformational differences between wheat and HeLa nucleosomes. However, at the highest concentration of mini-EmBP-1, protection at the centered Em1a site was still apparent in the wheat nucleosomes.

To verify EmBP-1 binding to wheat nucleosomes, we also performed an EMSA. Figures 10A and 10B show that mini-EmBP-1 could bind to the end nucleosomal Em1a site, but binding to the center nucleosomal Em1a site was not detectable at the concentrations used. These results are consistent with footprinting titration, indicating reduced binding at the centered site (Figure 9). Taken together, translational positioning effects on EmBP-1 binding to wheat nucleosomes were clearly demonstrated.

EmBP-1 Is Capable of Binding to Nucleosomal G-Box with Different Rotational Phasing Positions

Because bZIP proteins bind the major groove of DNA (Ellenberger et al., 1992), we reasoned that rotating the major groove so that the center of the binding site is facing the nucleosome might interfere with accessibility to EmBP-1. To test this possibility, we prepared three probes containing the nucleosome phasing sequence, (A)5 and (G/C)5 tracts (see above), in which the center of the G-box is 26 bp from the probe end, as shown in Figure 11A. Of these two probes, the G-box at position −2 and the G-box at position +4 had either 2 bp deleted or 4 bp inserted between the binding site and the phasing sequence relative to the G-box (at position 0) probe. These probes were reconstituted into wheat nucleosomes and analyzed by DNase I footprinting, as shown in Figure 11A. As we determined by DNase I cleavage periodicity, the major grooves at the center of the G-box (position −2) binding site faced outward and at an angle from the histone octamer surface; the G-box (position 0) binding site faced outward and was upright from histone octamer surface; the G-box (position +4) binding site faced inward (see Figure 11B). DNase I footprinting titration indicates that EmBP-1 bound the G-box sites as naked DNA with approximately the same affinity (Figure 11A, lanes 1 to 4). EmBP-1 was also able to bind to these sites as nucleosomes, regardless of the rotational phasing of the binding sites (Figure 11A, lanes 5 to 9). Gel mobility shift assays indicated that there might be a small rotational phasing effect on EmBP-1 binding but that it is much less than the translational positioning effect (data not shown). DNase I footprinting titration and gel shift assays of EmBP-1 binding to reconstituted HeLa nucleosomes made with these same probes gave similar results (data not shown).

DISCUSSION

EmBP-1 is a member of the bZIP family of transcription factors (Guiltinan et al., 1990; Guiltinan and Miller, 1994; Niu and Guiltinan, 1994). This class of transcription factors is highly conserved in eukaryotes (Johnson and McKnight, 1989; Pabo and Sauer, 1992). EmBP-1 has been implicated in the mechanisms of ABA-regulated gene expression of the wheat late embryo-abundant *Em* gene because it binds specifically to the ABRE located within the *Em* promoter (Guiltinan et al., 1990). Furthermore, EmBP-1 shares a similar binding specificity with a subclass of plant bZIP proteins known as the G-box binding proteins (GBFs). A diverse array of GBFs has been found, and they interact with various promoter elements having a wide array of functional activities (Menkens et al., 1995). One possible role of the GBFs in regulating gene expression could involve remolding of chromosomal structure via nucleosomal interactions. We have demonstrated in this study that EmBP-1 is capable of binding to nucleosomes containing its high-affinity DNA recognition sequences. Translational positioning of the binding sites relative to the histone octamer surface can modulate the apparent affinity of EmBP-1 for nucleosomal DNA.

Even though EmBP-1 is capable of binding nucleosomes, its affinity is very sensitive to the positioning of nucleosomes over its cognate binding site. Consistently, we observed a clear effect of translational positioning of the ABRE or G-box elements on binding affinity. Thus, the affinity of EmBP-1 for nucleosomal DNA is affected by nucleosome positioning in a manner analogous to that of the glucocorticoid receptor (Li and Wrange, 1993), GAL4 derivatives (Vettese-Dadey et al., 1994), Sp1 (Li et al., 1994), USF, and NF-κB (Adams and Workman, 1995). Nucleosome binding by bZIP proteins and

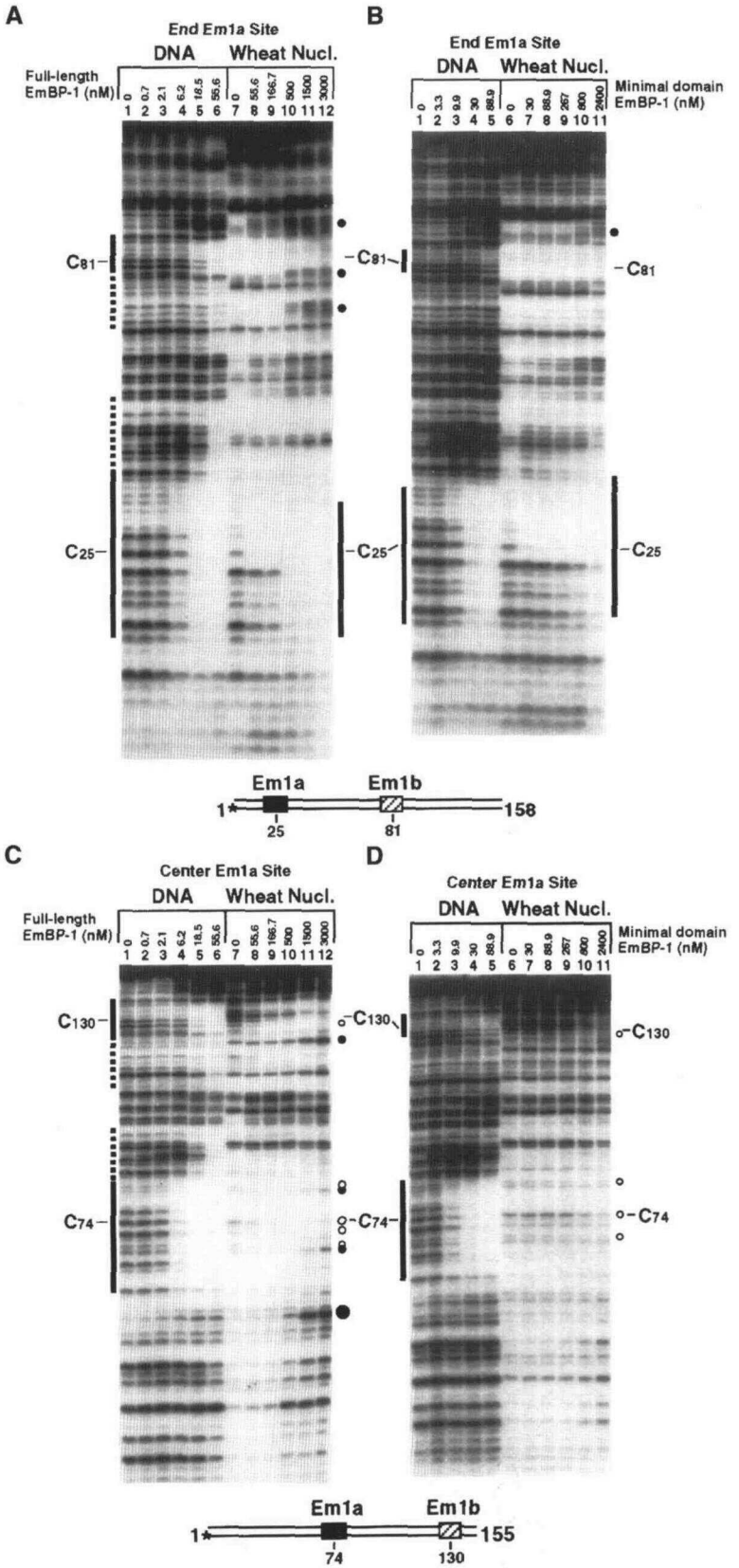

Figure 9. DNase I Footprinting Titration of Full-Length EmBP-1 and Mini-EmBP-1 Protein Interactions with ABRE Fragments Reconstituted into Wheat Nucleosomes.

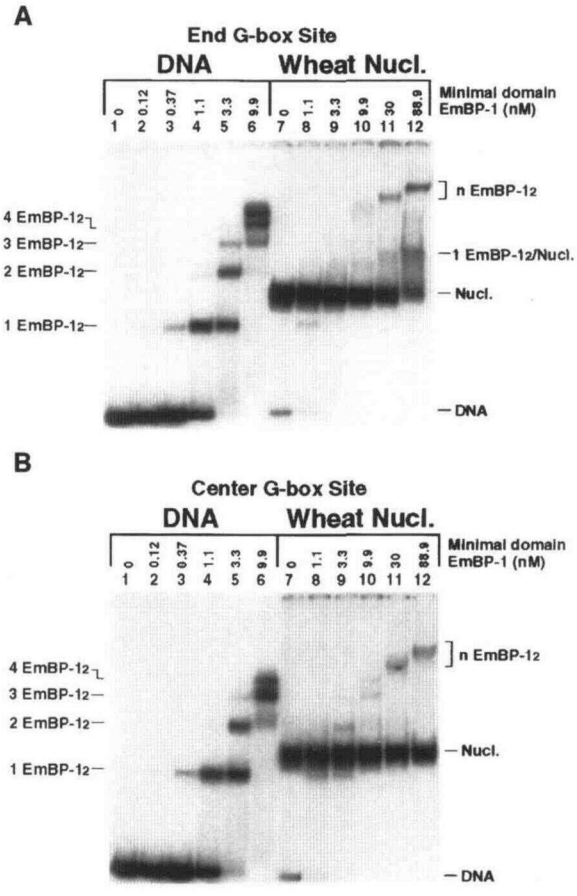

Figure 10. EMSA of Mini-EmBP-1 Protein Binding to the ABRE Fragments Reconstituted into Wheat Nucleosomes.

Labeling is as given in Figure 5, except that wheat nucleosomes (Nucl.) were tested. Binding reactions were run on a 5% native polyacrylamide gel. The end and center Em1a site ABRE probes are the same as given in Figure 9.

(A) Binding to the end Em1a site ABRE fragment.

(B) Binding to the center Em1a site ABRE fragment.

these other diverse transcription factors appears to be modulated by similar constraints imposed by nucleosome structure. These results collectively show the potential importance of nucleosome positioning on transcription factor access and gene activation.

The DNase I cleavage patterns near the edges of nucleosomal DNA appear to be more like those of naked DNA than do more internal positions, indicating weaker DNA–histone interactions near the edges. This has also been demonstrated by physicochemical studies of thermally induced changes in nucleosome structure (Simpson, 1979). Consistently, when the higher affinity G-box binding site was reconstituted into nucleosomes at an external location, an increased affinity of EmBP-1 for nucleosomes was observed relative to the Em1a site. This difference was reduced when the binding sites were located near the center of nucleosomes because of increased inhibition of EmBP-1 in general at this location. Therefore, it appears that the binding of EmBP-1 to G-box and the Em1a sites at two nucleosome positions reveals the same mechanism governing nucleosome translational position effects. Interestingly, EmBP-1 affinity for the G-box binding site located near nucleosome dyad was lower than for the Em1a site located near the edge of nucleosomes (see Figures 5A and 7B).

Nucleosomal positioning effects on EmBP-1 recognition of its binding sites could have important implications in vivo. Studies with Drosophila, yeast, and mammalian cells have revealed important roles of nucleosomes in gene regulation (Elgin, 1995). Chromatin structure has also been suggested as the basis for such regulatory phenomenon as gene silencing, paramutation (Patterson et al., 1993; Bestor et al., 1994), and proper developmental regulation of the bean phaseolin promoter (Frisch et al., 1995). One level of chromatin structure that could be involved in these processes is the positioning of nucleosomes. Interestingly, the Arabidopsis *Adh* promoter has been shown to have a positioned nucleosome on the G-box binding site in the cells in which the *Adh* gene is expressed (Vega-Palas and Ferl, 1995). The G-box was previously shown to have functional importance in high-level expression of this gene (McKendree and Ferl, 1992). Adjacent to the nucleosome containing the G-box binding site, nucleosome-free regions

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Figure 9. (continued).

Labeling is as given in Figure 3, except that wheat nucleosomes (Nucl.) were tested.

(A) Binding of the full-length EmBP-1 protein to the end Em1a site ABRE fragment as naked DNA (lanes 1 to 6) and as nucleosomes (Nucl.; lanes 7 to 12). The closed circles at right indicate hypersensitive bases in nucleosomes around the Em1b site.

(B) Binding of the mini-EmBP-1 protein to the end Em1a site ABRE fragment as naked DNA (lanes 1 to 5) and as nucleosomes (lanes 6 to 11). The closed circle at right indicates hypersensitive bases in nucleosomes around the Em1b site.

(C) Binding of full-length EmBP-1 protein to the center Em1a site ABRE fragment as naked DNA (lanes 1 to 6) and as nucleosomes (lanes 7 to 11). The open and closed circles at right indicate protected and hypersensitive bases in nucleosomes, respectively, as a result of specific binding by full-length EmBP-1 protein.

(D) Binding of the mini-EmBP-1 protein to the center Em1a site ABRE fragment as naked DNA (lanes 1 to 5) and as nucleosomes (lanes 6 to 11). Open circles at right indicate protected bases in nucleosomes, as a result of specific binding by mini-EmBP-1 protein.

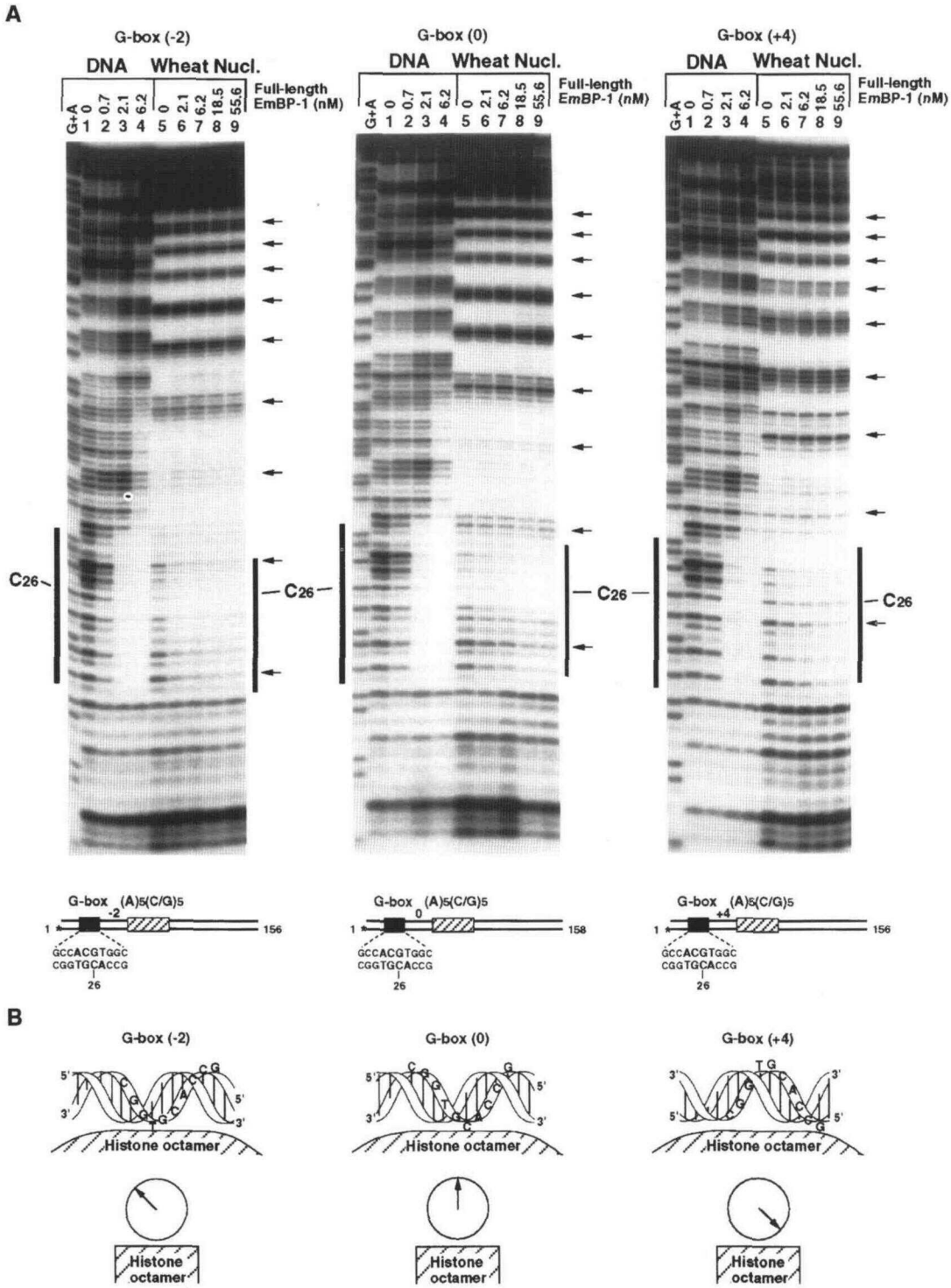

Figure 11. DNase I Footprinting Titration of Full-Length EmBP-1 Binding to G-Box Sites with Three Rotational Phasing Positions in Reconstituted Wheat Nucleosomes.

(A) Binding of full-length EmBP-1 protein to the G-box fragments (G-box at position −2, G-box at position 0, and G-box at position +4) as naked DNA (*lanes 1 to 4*) and as reconstituted wheat nucleosome (Nucl.) cores (lanes 5 to 9). Labeling is as given in Figure 3. DNA probes used for DNase I footprinting are depicted below the footprinting gels, with an indication of the G-box (solid box) and bent sequence (diagonally striped box, (A)5(C/G)5. The arrows to the right of each gel indicate ~10- to 11-bp periodicities of DNase I cuts on nucleosomal DNA.

(B) Schematic presentation of three rotational phasing positions of the G-box site on nucleosomes. The bottom strand sequence of the G-box site is depicted. The arrows in the end views indicate the directions of the major groove at the centers of the G-box binding sites.

were found that resulted in DNase I hypersensitivity upon gene transcription (Vega-Palas and Ferl, 1995).

We have shown that the mini-EmBP-1 required for DNA binding (Guiltinan and Miller, 1994) is capable of nucleosome binding. Similarly, only the minimal domains required for DNA binding by GAL4 and Sp1 are required for nucleosome binding (Taylor et al., 1991; Workman and Kingston, 1992; Li et al., 1994). However, we have observed that the patterns of DNase I cleavage are quite different when full-length and mini-EmBP-1 proteins bind to nucleosomal DNA (compare Figures 3A with 3B and Figures 3C with 3D). DNase I hypersensitivity was detected upon binding of the full-length protein but not with mini-EmBP-1. This effect was not observed with naked DNA. The N-terminal portion of EmBP-1 is proline- and serine-rich and contains a leucine–proline repeat of unknown function. It is possible that this domain interacts with the histone octamer or directly alters the DNA structure in some way so that DNase I hypersensitivity is induced (Alevizopoulos et al., 1995). It is important to note that EmBP-1 formed a distinct complex upon nucleosome binding that is "supershifted" relative to naked DNA–protein complexes. This indicates that EmBP-1 formed a ternary complex with bound histones on the same fragment of DNA. It is possible that further disturbance of nucleosomal DNA by protein moieties beyond the DNA binding and dimerization domains will play a role in further destabilizing the nucleosome (Workman and Kingston, 1992) and/or enhancing the cooperative binding of additional factors to the same nucleosome (Adams and Workman, 1995).

In a previous study analyzing the internal enhancer binding factor (IBF), which is a bZIP transcription factor, no binding to nucleosome cores was detected (Svaren et al., 1994a). Our results with EmBP-1 demonstrate that the extent of EmBP-1 inhibition depends on nucleosome positioning. When an EMSA and DNase I footprinting titration were used, at least 100-fold more protein was needed to observe nucleosome binding when the binding sites were located near the nucleosomal dyad. It is likely that the IBF would similarly demonstrate nucleosome binding if analyzed by using higher concentrations of protein, as was done in this study; however, it is possible that these proteins may exhibit different nucleosome binding abilities due to amino acid differences in DNA binding and dimerization domains. For example, USF, Max, and c-Myc belong to the same family of transcription factors (basic helix-loop-helix leucine zipper); however, these proteins exhibit differences in affinity for nucleosomal DNA (Chen et al., 1994; Wechsler et al., 1994; Adams and Workman, 1995). Moreover, changing the dimerization domain of c-Myc to that of GCN4 enables c-Myc to bind to nucleosomal DNA. Thus, EmBP-1 and IBF may similarly differ in nucleosome binding due to the difference in DNA binding and/or dimerization domains.

Even though certain transcription factors, including IBF, were not found to bind nucleosomes in vitro, it is possible that they can recognize their binding sites within nucleosomes in vivo where additional activities facilitate transcription factor binding. For example, it has been shown that histone acetylation increases transcription factor binding to nucleosomes (Lee et al., 1993; Vettese-Dadey et al., 1994). In addition, ATP-dependent nucleosome remodeling factors, such as SWI/SNF (Côté et al., 1994; Imbalzano et al., 1994) and the nucleosome remodeling factor (Tsukiyama and Wu, 1995), have been purified and shown to stimulate transcription factor binding to nucleosomes.

Other mechanisms enabling weak nucleosome binding factors to function in vivo have been hypothesized. Potentially, transcription factors can bind to DNA during replication when nucleosomes are perturbed during passage of the replication fork before nucleosome reassembly (Wolffe, 1991). Alternatively, a weaker nucleosome binding factor could bind cooperatively with a strong nucleosome binding factor (Adams and Workman, 1995).

In this study, we have also demonstrated that EmBP-1 can bind both HeLa and wheat nucleosomes to similar extents, perhaps reflecting the high degree of evolutionary conservation of the histones. However, wheat histones H2A and H2B are different from their HeLa counterparts in size and consist of a number of variants; thus, nucleosome positioning could potentially be different between HeLa and wheat nucleosomes and even among wheat nucleosomes with differing H2A and H2B variants. Consistent with this idea, some minor differences were observed in the binding of EmBP-1 to HeLa and wheat nucleosomes. These may have been due to a conformational difference between plant and mammalian nucleosomes or to alternative rotational frames resulting from histone variants. The latter possibility seems unlikely because only weak rotational phasing effects on EmBP-1 binding were observed. Despite the differences between wheat and HeLa histones H2A and H2B, we have demonstrated that full-length EmBP-1 is capable of binding to its high-affinity sites in both types of nucleosomes, and binding affinities are modulated by nucleosome positioning in a similar manner. Plant histones H2A and H2B consisting of closely related variants that are larger than their mammalian counterparts have been reported for several plant species, including wheat, pea, and Arabidopsis (Spiker and Isenberg, 1977; Spiker, 1982; Moehs et al., 1988). This reflects evolutionary relatedness and conservation of plant H2A and H2B genes and implies a functional significance for this variation. However, the physiological roles of distinct plant histone H2A and H2B variants remain unknown. Based on our studies of EmBP-1 binding to HeLa and wheat nucleosomes, we predict that the nucleosome positioning effects that have been shown in yeast and animal systems are generally applicable to interactions of transcription factors with nucleosomes of plant species, and these interactions play important roles in gene regulation.

METHODS

Protein Expression and Purification

The full-length recombinant EmBP-1 protein expressed in *Escherichia coli* with a hexahistidine fusion was purified to 90% homogeneity by single-step nickel ion chromatography as described by Niu and

Guiltinan (1994). A truncated version of EmBP-1 was also expressed. It contained the minimal domain of EmBP-1 (mini-EmBP-1; 60 amino acids) (Guiltinan and Miller, 1994) and encodes amino acids 249 to 308. A portion of the EmBP-1 sequence was amplified by polymerase chain reaction (PCR) with two primers: the N-terminal primer consisted of an EcoRI site followed by GGC (glycine) and the sequence downstream of amino acid 249 of EmBP-1a (5′-AGTGGATCCGGAATTCGGCATGGATGAACGGGAACTGAAGAGG-3′); the C-terminal primer includes the sequence immediately upstream from amino acid 308 of EmBP-1a followed by CCA (proline) and an EcoRI site (5′-TTGGGTACCGAATTCTGGGGTTTTGCAGTCCTTCTTAAGCTG-3′). The PCR product was cloned into an EcoRI site of a histidine fusion expression vector, pV2b (Van Dyke et al., 1992), resulting in a 27–amino acid N-terminal fusion containing six histidines and an 11–amino acid C-terminal fusion to the mini-EmBP-1, creating the construct pXN320, which was verified by sequencing. The mini-EmBP-1 protein was expressed in *E. coli* and purified as described for the full-length protein.

DNA Probe Preparation

The abscisic acid (ABA) response element (ABRE) and G-box binding sites were amplified with a pUC forward primer (5′-ACGGCCAGTGCCAAGCT-3′) and a pUC reverse primer (5′-CAGGAAACAGCTATGAC-3′) by using plasmids pMG76.155 (Guiltinan et al., 1990) and pXN16.2 (Niu and Guiltinan, 1994) as templates. The PCR products were repaired with the Klenow fragment of DNA polymerase I, cut with XbaI, gel purified, and ligated into the SalI-cut, Klenow-filled, and XbaI-cut pBEND derivative pTK401 (Kerppola and Curran, 1991). Two plasmids, pXN300 and pXN303, were produced, and they harbored the ABRE and G-box fragments, respectively.

For rotational phasing studies, pXN303, containing the G-box binding site, was cut with HindIII, treated with mung bean nuclease (four nucleotides of single-stranded DNA and 1 bp of double-stranded DNA were removed from the ends), and then cut with PstI. The fragment containing the G-box was gel purified and ligated into XbaI-cut, Klenow-filled, and PstI-cut pTK401-26 and pTK401-28 (Kerppola and Curran, 1991), generating two plasmids, pXN330-26 and pXN330-28, respectively. pXN330-28 was cut at a unique SalI site located between the G-box and bent sequence, filled in with the Klenow fragment, and religated, generating plasmid pXN330-32. All plasmid constructs were verified by sequencing. For simplicity, the DNA fragments generated from pXN330-26, pXN330-28, and pXN330-32 are referred to as the G-box (at position −2), G-box (at position 0), and G-box (at position +4), respectively. The partial DNA sequences for these fragments are as follows. G-boxes are italicized, bent sequences are underlined, and sequence changes among these fragments are in lowercase letters. G-box (−2) is TTAT*GCCACGTGGC*ACTAGATGCTGACTCATTGTCGACGCAAAAACGGGCAAAAACGGGCAAAAACTCGAC; G-box (0), TTAT*GCCACGTGGC*ACTAGATGCTGACTCATTGTCGAcaCGCAAAAACGGGCAAAAACGGGCAAAAACTCGAC; G-box (+4), TTAT*GCCACGTGGC*ACTAGATGCTGACTCATTGTCGAtcgacaCGCAAAAACGGGCAAAAACGGGCAAAAACTCGAC.

DNA probes were produced by cutting plasmids (pXN300, pXN330-26, pXN330-28, and pXN330-32) with two different restriction enzymes, as indicated in the text. Labeling was with α-^{32}P-dCTP or α-^{32}P-dATP at one end with the Klenow fragment, followed by gel purification on 8% polyacrylamide gels, according to standard protocols (Sambrook et al., 1989).

HeLa and Wheat Nucleosome Reconstitution, Mobility Shifts, and DNase I Footprinting

Oligonucleosomes used for HeLa nucleosome reconstitution were purified from nuclear pellets, as described previously (Vettese-Dadey et al., 1994). HeLa nucleosome core reconstitution was achieved by octamer transfer (Rhodes and Laskey, 1989) as follows. A trace amount of radiolabeled probe DNA (5 to 10 ng) was mixed with a large amount of H1-depleted oligonucleosomes (10 μg containing 5 μg of histones and 5 μg of DNA) in a 10-μL reaction volume at a high-salt concentration (1 M NaCl). The reaction was incubated at 37°C for 20 min. At this salt concentration, histone octamers are mobile and can be transferred to DNA. Following the incubation, transfer reactions were serially diluted (five steps) to 0.2 M NaCl (50 μL) with 10 mM Hepes, pH 8.0, 0.5 mM EDTA, with a 30-min incubation at 30°C between each step. These dilutions gradually reduce the salt concentration and therefore restablize the association of histone octamers with the DNA. Following a final twofold dilution to 0.1 M NaCl (100 μL total volume) with the above-mentioned buffer containing 1 mM phenylmethylsulfonyl fluoride and 10% glycerol, the samples were placed on ice and aliquoted for binding reactions. For mock reconstitution, TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was substituted for the radiolabeled DNA in the initial transfer reaction. Following a fivefold serial dilution to 0.2 M NaCl, probe DNA was added to the final dilution step, making the concentration of probe DNA identical to that in the legitimately reconstituted samples.

To purify wheat core histones, we purified wheat embryo chromatin from wheat germ (kindly provided by General Mills, Minneapolis, MN) by using the higher ionic strength method (Simon and Becker, 1976), with the modifications as described by Moehs et al. (1992). The resulting "purified chromatin" was used to purify the wheat core histone octamer by one-step hydroxyapitite column chromatography, as described by Côté et al. (1995). To reconstitute wheat nucleosomes, we mixed 5 μg of calf thymus DNA (average length of 600 bp; Sigma), 5 to 10 ng of probe, and ~10 μg of purified wheat core histones in a 10-μL reaction volume containing 1 M NaCl. The salt concentration was reduced to 0.1 M NaCl by serial dilution, as described above for reconstitution of HeLa nucleosomes.

Binding reactions for both gel mobility shift and DNase I footprinting were performed in the binding buffer, as described previously (Guiltinan and Miller, 1994), with the following modifications. All binding reactions contained 0.1% Nonidet P-40, 0.2 mg/mL BSA, and no poly(dI-dC) in a 20-μL final volume and were incubated at room temperature for 20 min. For electrophoretic mobility shift assays (EMSAs), 2 μL of reconstituted nucleosomes was included in each reaction, loaded on acrylamide (acrylamide–bisacrylamide, 29:1 [w/w])–0.5 × TBE (Tris-borate-EDTA) gels, and run in 0.5 × TBE at 150 V for 3.5 hr at room temperature. Gels were dried and exposed to Kodak XAR 5 film. For DNase I footprinting, 3 μL of reconstituted nucleosomes was included in each reaction. DNase I digestion (1 to 3 min) and sequencing gels were as previously described (Côté et al., 1994). For positional markers, the ABRE and G-box probes were partially digested with MaeII and run next to the footprinting reactions so that the C bases in the center of the ACGT cores of the EmBP-1 binding sites could be located. For some probes, a G+A ladder was generated by chemical sequencing according to a standard protocol (Sambrook et al., 1989).

Quantification

Gels were scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Quantification of the images was performed using

Image Quant software (Molecular Dynamics). The term volume (*Vol*) denotes the total number of counts per minute for a particular band on the gels. The bound fraction or percentage of nucleosomes bound in Figures 5C and 7C is calculated as

$$\text{Bound fraction} = 1 - \frac{(Voln/Volt)\text{EmBP}-1}{(Voln/Volt)\ \text{control}}$$

where *Voln* is the volume for the nucleosome band that remained unbound by EmBP-1 (see Figures 5 and 7) and *Volt* is the total volume in the same lane. The relative unbound nucleosome in each lane $(Voln/Volt)_{\text{EmBP-1}}$ was normalized to the lane in which no EmBP-1 was added, giving the term (*Voln*/*Volt*)EmBP-1/(*Voln*/*Volt*)control in the equation. The principle for the equation provided above is similar to that used for quantitative footprinting analysis (Brenowitz et al., 1986).

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