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Binding of Disparate Transcriptional Activators to Nucleosomal DNA Is Inherently Cooperative

CHRISTOPHER C. ADAMS AND JERRY L. WORKMAN*

Department of Biochemistry and Molecular Biology and The Center for Gene Regulation, The Pennsylvania State University, University Park, Pennsylvania 16802-4500

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To investigate mechanisms by which multiple transcription factors access complex promoters and enhancers within cellular chromatin, we have analyzed the binding of disparate factors to nucleosome cores. We used a purified in vitro system to analyze binding of four activator proteins, two GAL4 derivatives, USF, and NF-κB (KBF1), to reconstituted nucleosome cores containing different combinations of binding sites. Here we show that binding of any two or all three of these factors to nucleosomal DNA is inherently cooperative. Thus, the binuclear Zn clusters of GAL4, the helix-loop-helix/basic domains of USF, and the *rel* domain of NF-κB all participated in cooperative nucleosome binding, illustrating that this effect is not restricted to a particular DNA-binding domain. Simultaneous binding by two factors increased the affinity of individual factors for nucleosomal DNA by up to 2 orders of magnitude. Importantly, cooperative binding resulted in efficient nucleosome binding by factors (USF and NF-κB) which independently possess little nucleosome-binding ability. The participation of GAL4 derivatives in cooperative nucleosome binding required only DNA-binding and dimerization domains, indicating that disruption of histone-DNA contacts by factor binding was responsible for the increased affinity of additional factors. Cooperative nucleosome binding required sequence-specific binding of all transcription factors, appeared to have spatial constraints, and was independent of the orientation of the binding sites on the nucleosome. These results indicate that cooperative nucleosome binding is a general mechanism that may play a significant role in loading complex enhancer and promoter elements with multiple diverse factors in chromatin and contribute to the generation of threshold responses and transcriptional synergy by multiple activator sites in vivo.

Mounting evidence suggests that controlling the dynamic equilibrium between competing functional and structural proteins (i.e., transcription factors versus nucleosome formation) plays a crucial role in regulating gene activity (20, 62, 75). It is well established that nucleosomes are potent inhibitors of transcription in vitro and can directly interfere with transcription factor binding (reviewed in references 34 and 77). However, increasing evidence from both yeast and mammalian systems illustrates that nucleosomes are susceptible to replication-independent disruption initiated by transcription factor binding in vivo (reviewed in references 1 and 64). For example, nucleosomes located over the mouse mammary tumor virus and rat tyrosine aminotransferase promoters are disrupted by glucocorticoid receptor binding (3, 7, 38, 55, 57, 58, 81). A sequence-positioned nucleosome within the integrated human immunodeficiency virus type 1 5′ long terminal repeat is disrupted following tetradecanoyl phorbol acetate induction (71). In *Saccharomyces cerevisiae*, heat shock factor disrupts a rotationally phased nucleosome from the TATA box and initiation site of the *HSP82* heat shock gene upon transcriptional activation (20). The DNA-binding domain of the GAL4 protein can directly disrupt nucleosomes to which it binds (46), while its activation domains lead to disruption of an adjacent nucleosome on the *GAL1* promoter (4). Similarly, binding of the PHO4 activator to the *PHO5* promoter disrupts adjacent nucleosomes, in a process facilitated by the PHO4 activation domain (60, 66). Thus, studies with *S. cerevisiae* suggest a role of DNA-binding domains in binding or disruption of underlying nucleosomes and a function of activation domains which is most apparent in disruption of adjacent nucleosomes (perhaps indirectly).

Consistent with the in vivo studies of replication-independent chromatin remodeling, complementary in vitro studies have directly demonstrated the binding of transcription factors to nucleosomal DNA and the disruption of underlying nucleosomes. GAL4 derivatives (67, 72, 78), glucocorticoid receptor (2, 40, 50, 51, 53), Sp1 (39), TFIIIA (37), USF (12), and Max/Max and Myc/Max dimers (74) have all been shown to be capable of nucleosome binding under some circumstances. However, many variables govern the affinity of a given transcription factor for nucleosomal DNA (73). The inherent affinity of a factor for its cognate site on a nucleosome appears to be largely a property of its DNA-binding motif but is further influenced by dimerization domains and partners (67, 74). Nucleosome binding is affected by the location of binding sites within nucleosomes, the availability of accessory proteins which participate in nucleosome disruption, and the chemical modification of the histone amino-terminal tails (2, 12, 13, 37, 39, 40, 53). In addition, the binding of GAL4 derivatives to nucleosomes is cooperative (67). The binding of multiple GAL4 dimers overcomes inhibition from the core histone amino termini, alleviating nucleosome position effects (72).

Chromatin assembly greatly enhances the ability of multiple GAL4-VP16 activators to mount a synergistic transcriptional response in vitro (11). The requirement for multiple GAL4-binding sites can provide for synergistic interactions of multiple activation domains with target proteins that form transcription initiation complexes at the core promoter (9, 42; reviewed in reference 24), a rate-limiting step on nucleosomal templates (32, 33, 44, 76, 79, 80). In addition, multiplicity of GAL4-

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, 306 Althouse Laboratory, The Pennsylvania State University, University Park, PA 16804-4500. Phone: (814) 863-8256. Fax: (814) 863-0099. Electronic mail address: JLW10@psuvm.psu.edu.

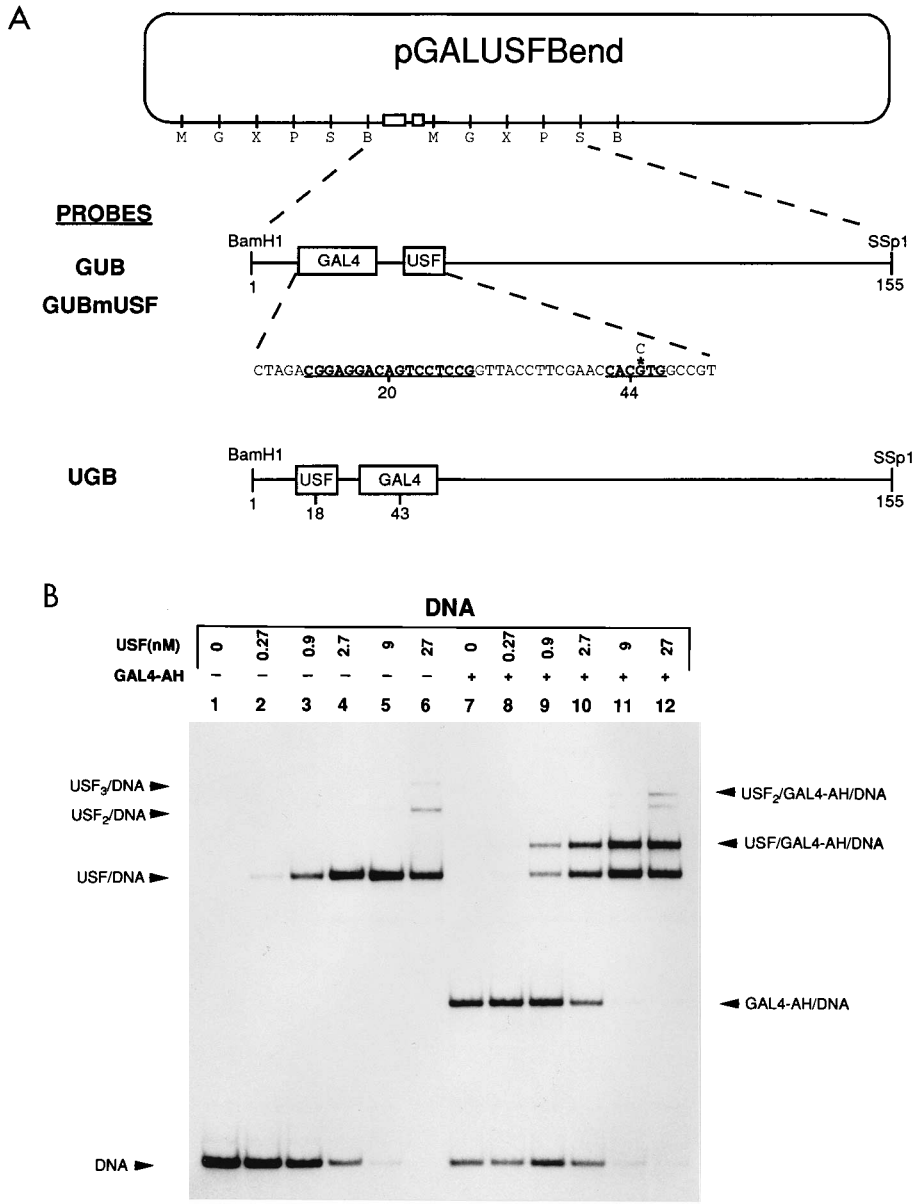
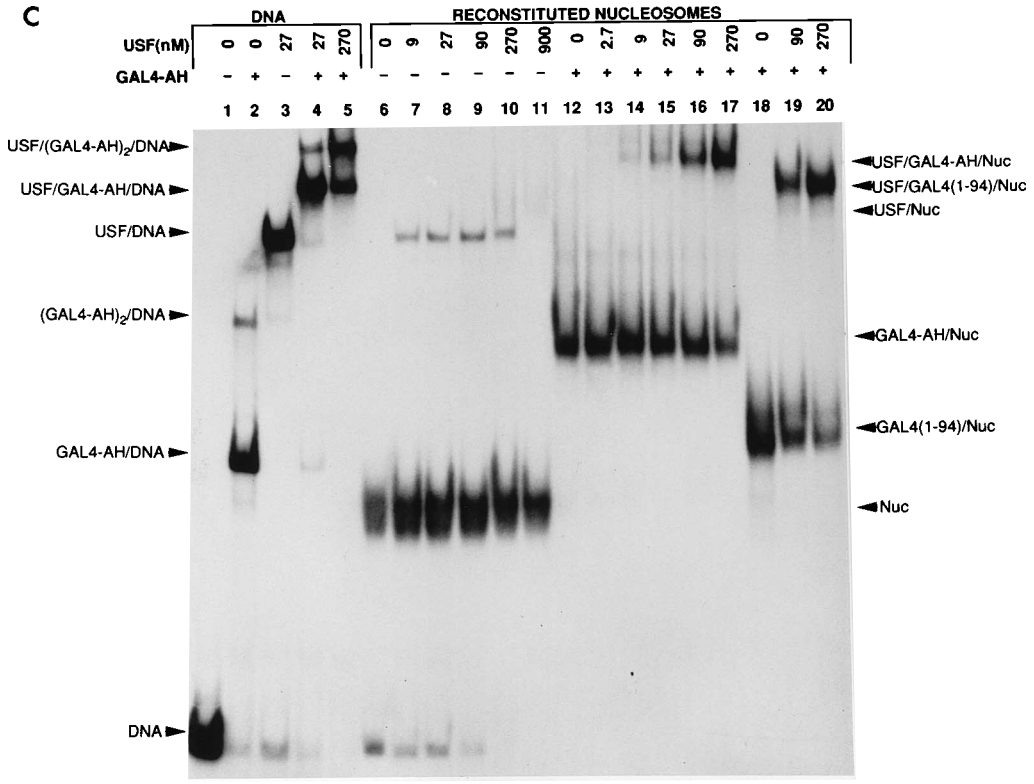


FIG. 1. Unrelated transcription factors USF and GAL4 bind cooperatively to nucleosome cores but not to naked DNA. (A) Diagram of nucleosome-length DNA fragments, containing GAL4 and USF binding sites, used as probes for binding studies. Probes GUB and UGB are identical except for the orientation of the oligonucleotide, containing both binding sites, that is shown. Probe GUBmUSF is identical to GUB except for the single base substitution within the 6-bp core USF-binding site. Nucleotides are numbered from the *Bam*HI site (bp 1) used to excise all probe fragments from their respective plasmids (see Materials and Methods). Nucleotides used to denote the center of each binding site in all three fragments are indicated. M, *Mlu*I; G, *Bgl*II; X, *Xho*I; P, *Pvu*II; S, *Ssp*I; B, *Bam*HI. (B) EMSA analysis of USF binding to probe GUB as mock-reconstituted naked DNA. Probe DNA was labeled at the *Bam*HI site by Klenow polymerase. For mock reconstitution, labeled DNA probe was added to HeLa oligonucleosomes, after dilution to 0.1 M NaCl, such that the final concentrations of donor nucleosomes and template DNA are the same as in the reconstituted samples (see Materials and Methods). USF was titrated into binding reaction mixtures with (+; lanes 7 to 12) or without (−; lanes 1 to 6) 40 nM GAL4-AH. The relative concentration of USF protein in each binding reaction mixture is shown. Protein/DNA complexes were resolved by 4% PAGE and visualized by autoradiography. Complexes representing free probe DNA and USF and/or GAL4 bound to DNA are labeled, DNA, USF/DNA, GAL4-AH/DNA, and USF/GAL4-AH/DNA, respectively. At high USF concentrations, nonspecific binding of additional USF proteins to the DNA is detected (USF_2/DNA, USF_3/DNA, and USF_2/GAL4-AH/DNA; lanes 6 and 12). These complexes represent nonspecific binding of additional USF dimers and/or binding of USF tetramers. (C) EMSA (4% PAGE) analysis of USF binding to probe GUB reconstituted into nucleosome cores. Probe DNA was labeled at the *Bam*HI site as described above. USF was titrated into binding reaction mixtures containing radiolabeled probe DNA that had been mock reconstituted (DNA; lanes 1 to 5) or reconstituted into nucleosome cores (lanes 5 to 20), in the absence (−) or presence (+) of GAL4 derivatives GAL4-AH (lanes 2, 4, 5, and 12 to 17) and GAL4(1-94) (lanes 18 to 20). The concentration of USF protein in each binding reaction is given. The concentrations of GAL4-AH used in binding reactions were 123 nM for naked DNA samples (lanes 2 and 4) and 2.1 μM for nucleosome samples; the concentration of GAL4(1-94) was 2.0 μM. Complexes representing proteins bound to free DNA (DNA) are labeled on the left, while complexes resulting from proteins bound to nucleosome cores (Nuc) are labeled on the right. (D) Comparison of USF binding to DNA probes GUB (lanes 1 to 7) and GUBmUSF (lanes 8 to 14) reconstituted into nucleosomes. USF titration, GAL4-AH concentration, and conditions of EMSA are the same as for panel C.


C
DNA
RECONSTITUTED NUCLEOSOMES
USF(nM)
0 0 27 27 270 0 9 27 90 270 900 0 2.7 9 27 90 270 0 90 270
GAL4-AH
– + – + + – – – – – – + + + + + + + + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
USF/(GAL4-AH)2/DNA
USF/GAL4-AH/DNA
USF/DNA
(GAL4-AH)2/DNA
GAL4-AH/DNA
DNA
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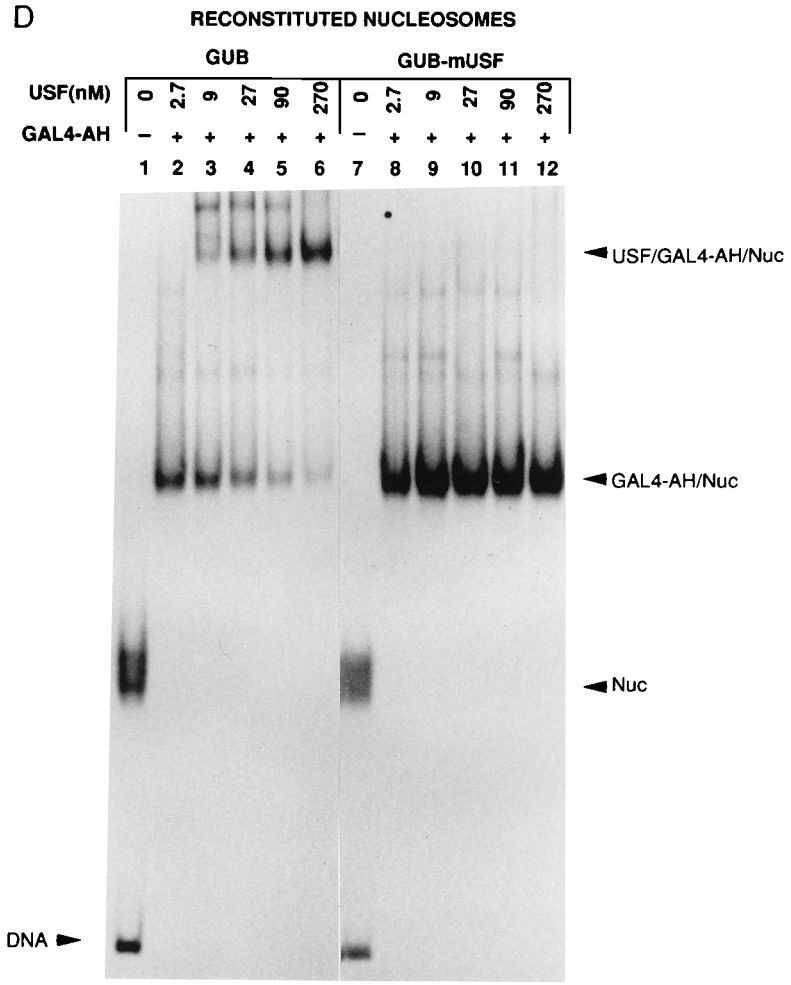
GAL4(1-94)/Nuc
Nuc


D
RECONSTITUTED NUCLEOSOMES
GUB
GUB-mUSF
USF(nM)
0 2.7 9 27 90 270 0 2.7 9 27 90 270
GAL4-AH
– + + + + + – + + + + +
1 2 3 4 5 6 7 8 9 10 11 12
USF/GAL4-AH/Nuc
GAL4-AH/Nuc
Nuc
DNA

binding sites also provides for cooperative binding of GAL4 derivatives to recognition elements in nucleosomes (67, 72). Together, these non-mutually exclusive mechanisms (cooperative binding and activation domain synergism) may lead to substantial synergistic effects and threshold responses from cellular promoters and enhancers in vivo.

A common feature of most enhancers and promoters is the presence of multiple binding sites (enhansons), often for disparate regulatory factors (reviewed in reference 16). Cooperative binding of regulatory factors can result from direct protein-protein interactions between the factors. For example, such interactions of Sp1 may mediate cooperative binding with itself and additional factors (14, 18, 22, 26, 41, 48, 49). In addition, cooperative binding might occur indirectly, via perturbations of nucleosome structure. The combined effect of multiple bound factors on histone DNA contacts could lead to a significant increase in the affinity of each factor for nucleosomal DNA. Consistent with this possibility, cooperative nucleosome binding by GAL4 derivatives requires only the minimum domains of the protein necessary for DNA binding (DNA-binding and dimerization domains [67]).

In principle, cooperative nucleosome binding could provide a mechanism to increase the binding of multiple disparate factors to complex enhancer and promoter elements without requiring direct factor-factor interactions. Such a general mechanism would require that cooperative nucleosome binding occur between different unrelated transcription factors. To address this question, we have used a purified system utilizing recombinant transcription factors (GAL4 derivatives, USF, and NF-κB) to assess the generality of cooperative transcription factor binding to nucleosomes. We demonstrate that cooperative nucleosome binding occurs between any pair or all three of these disparate transcription factors and that this cooperativity can increase the affinity of these factors for nucleosomal DNA by more than 2 orders of magnitude.

MATERIALS AND METHODS

Plasmid construction and DNA probe purification. To construct plasmids pGALUSFBend and pUSFGALBend, the 46-bp oligonucleotide containing the GAL4- and USF-binding sites (shown in Fig. 1A) was inserted into a unique *Xba*I site in plasmid pTK401 (29). For plasmid pGUBmUSF, the inserted oligonucleotide was identical except for a single base change (CACGTG changed to CACCTG) within the USF-binding site. Plasmid pGUB-NFx1 was made by inserting the 20-bp oligonucleotide (5′-CGTAGGGGACGTCCCCGTAT-3′) containing an NF-κB binding site that has a high affinity for p50/p50 homodimers into a unique *Bst*BI site between the GAL4 and USF sites in pGALUSFBend. Probes GUB, GUBmUSF, and UGB were generated by digesting the appropriate plasmid with *Bam*HI, labeling the ends by Klenow incorporation of [^{32}P]ATP, and subsequently digesting with *Ssp*I to yield nucleosome-length fragments (Fig. 1A). Probes GNUB and NUB were both excised from pGUB-NFx1 (see Fig. 4A). Probe GNUB was radiolabeled at the *Bam*HI end and NUB was radiolabeled at the *Bst*EII end by Klenow incorporation of [^{32}P]dATP or [^{32}P]dCTP. All probe DNA fragments were purified by polyacrylamide gel electrophoresis (PAGE) (8% polyacrylamide gels) as described previously (59).

Protein purification. All transcription factors were overexpressed in *Escherichia coli* by using the pET system (Novagen). The fusion protein GAL4-AH, containing the N-terminal DNA-binding and dimerization domains of GAL4 (147 amino acids) and an artificial 15-amino-acid putative amphipathic helix, was purified by the method of Lin et al. (43). The GAL4 derivative GAL4(1-94) was also purified by this method. Purified GAL4 derivatives were serially diluted with buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 1 mM EDTA, 10 mM 2-mercaptoethanol, 10 μM $ZnCl_2$, and 1 mg of bovine serum albumin (BSA) per ml. The 43-kDa recombinant USF protein was purified as described by Pognonec et al. (54). The NF-κB (p50) protein used in this study was the 41.5-kDa truncated p50 derivative (35). NF-κB was also purified by the method of Pognonec et al. (54). Both USF and NF-κB proteins were serially diluted in buffer containing 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1 mM dithiothreitol, 5 μg of leupeptin per ml, and 1% (vol/vol) aprotinin. Oligonucleosomes used for octamer transfer onto probe DNA templates were purified from HeLa nuclear pellets as described previously (72). Typically, purified oligonucleosomes ranged in size from monomers to trimers.

Nucleosome reconstitution and transcription factor binding. Nucleosome core reconstitution was achieved by octamer transfer (56). Approximately 25 ng of end-labeled probe DNA (1×10^5 to 2×10^5 cpm) was mixed with H1-depleted oligonucleosomes (0.2 to 0.3 mg/ml, final concentration) at 1 M NaCl in a 20-μl reaction volume. Following incubation at 37°C for 30 min, the transfer reaction mixtures were serially diluted (five steps) to 0.2 M NaCl (100 μl, total volume) with 10 mM HEPES (pH 8.0)–1 mM EDTA, with a 25-min incubation at 30°C between each dilution step. A final twofold dilution to 0.1 M NaCl (200 μl, total volume) with buffer containing 10 mM Tris (pH 7.8), 1 mM EDTA, 0.1% Nonidet P-40, 5 mM dithiothreitol, 2-mercaptoethanol, and 20% glycerol was performed; the samples were placed on ice and subsequently aliquoted for binding reactions. For mock reconstitutions, TE buffer (10 mM Tris-Cl [pH 7.5]–0.5 mM EDTA) was substituted for the radiolabeled probe DNA in the initial transfer reaction. Following 10-fold serial dilution to 0.1 M NaCl, probe DNA was added after the final dilution step such that the concentration of probe DNA was identical to that in the legitimately reconstituted samples. Several lines of evidence indicate that complete nucleosome cores result from the octamer transfer protocol. (i) Binding reaction mixtures contain equal amounts of all four core histones (i.e., histones were not lost during reconstitution steps [28]). (ii) Cross-linking of core histones after reconstitution with dimethylsuberimidate results in cross-linked octamers without the appearance of cross-linking-resistant histone hexamers or tetramers (72a). (iii) Nucleosome reconstitution protects 150 bp of probe DNA (nucleosome core length) from micrococcal nuclease digestion (72). (iv) DNase I digestion of nucleosome core-reconstituted probes with strong rotational phasing (i.e., containing bent DNA sequences) results in 10- to 11-bp digestion repeats extending up to 140 bp (data not shown).

All binding reaction mixtures contained 100 mM NaCl, 0.2 mg of BSA per ml, 10 mM Tris-Cl (pH 7.5), 10 μM $ZnCl_2$, 0.2 mM $MgCl_2$ (for electrophoretic mobility shift assays [EMSAs]) or 3 mM $MgCl_2$ (for DNase I footprinting), 5 mM dithiothreitol, 5% glycerol, 0.2 mM HEPES (pH 7.5), 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 2.5 μg of leupeptin per ml, 0.5% (vol/vol) aprotinin, 0.05% Nonidet P-40, 100 ng of poly(dI-dC) DNA (Pharmacia), and the amount of each transcription factor indicated in the figures, in a total volume of 20 μl. All transcription factors were added to the binding reaction mixtures at essentially the same time (i.e., within 1 min of each other). Binding reaction mixtures were incubated at 30°C for 20 min and subsequently analyzed by either EMSA or DNase I footprinting (see below).

EMSA, DNase I footprinting, and restriction enzyme accessibility assays. For EMSA, binding reaction mixtures were supplemented with tracking dyes, loaded directly onto 4% acrylamide (acrylamide/bisacrylamide, 29:1)–0.5× Tris-borate-EDTA (TBE) gels (20 by 25 cm), and run at 150 V (constant voltage) at room temperature for 7 h. This extended electrophoresis was necessary to resolve some bands with one or two factors bound to nucleosomes. However, it also resulted in a broadening of the nucleosome bands (which migrated 15 to 20 cm). The breadths of the nucleosome bands in some cases appear to indicate split bands (i.e., Fig. 1C), which are most likely artifacts of the extended electrophoresis since they are not observed with samples electrophoresed for only 3 h (Fig. 8C). This finding might either reflect small heterogeneous changes of octamer position on the probes or indicate that some of the nucleosome cores lost histones (i.e., an H2A/H2B dimer), both of which could occur during extended electrophoresis at room temperature. Gels were dried and subjected to autoradiography. In addition, each gel was quantitated with a Betascope blot analyzer (Betagen Corporation).

For DNase I footprinting, binding reaction mixtures were cooled to room temperature and digested with DNase I (Boehringer Mannheim) for 3 min. Samples containing reconstituted templates were digested with 1.2 U of DNase I enzyme, while mock-reconstituted samples were digested with 0.12 U of enzyme. Digestion was terminated with 1 volume of 20 mM Tris (pH 7.5)–50 mM EDTA–2% sodium dodecyl sulfate–0.25 mg of yeast tRNA (Sigma) per ml–200 mg of proteinase K (Sigma) per ml. Reaction mixtures were then incubated at 50°C for 1 to 3 h, and the DNA was precipitated with 0.3 volume of 10 M ammonium acetate and 3 volumes of absolute ethanol. DNA pellets were washed with 80% ethanol, dried, and resuspended in 2 μl of double-distilled H_2O and 3 μl of formamide loading buffer (59). Samples were incubated at 95°C for 5 min, quenched on ice, and resolved on 8% acrylamide–8 M urea sequencing gels. Gels were transferred to blotting paper and subjected to autoradiography at −80°C.

For the restriction enzyme digestion analysis of nucleosome positioning, 20-μl binding reaction mixtures, identical to those used for DNase I digestion, were prepared. Following incubation at 30°C to allow for GAL4 binding, 1 μl (10 U) of the appropriate restriction enzyme was added instead of the DNase I nuclease. Digestion was terminated, and the DNA was purified as described for DNase I analysis. The lyophilized DNA pellets were dissolved in 10 to 20 μl of TE (pH 8.0) with 1× glycerol loading buffer (59) and resolved on 10 to 12% polyacrylamide–0.5× TBE gels. Gels were dried and subjected to autoradiography. In addition, each gel was quantitated with a PhosphorImager (Molecular Dynamics).

RESULTS

The binding of a single GAL4-AH dimer greatly enhances USF binding to nucleosomal DNA. To investigate whether disparate transcription factors would bind cooperatively to nu-

cleosomal DNA, we reconstituted a 155-bp fragment containing binding sites for both GAL4-AH and USF into nucleosome cores. The GAL4-binding site was centered 20 bp from one end, while the adjacent USF-binding site was centered 44 bp from the same end (see Fig. 1A and Materials and Methods). Following reconstitution of this DNA fragment into nucleosome core particles by the octamer transfer method (see Materials and Methods), the binding of GAL4-AH and USF to mock-reconstituted (naked DNA) and reconstituted nucleosomes was monitored by EMSA and DNase I footprinting. As shown in Fig. 1B, titration of USF onto naked DNA, in the absence (lanes 1 to 6) or presence (lanes 7 to 12) of GAL4-AH binding, resulted in the formation of a distinct shifted complex, representing specific binding of USF to the DNA probe or the probe also bound by GAL4-AH (compare lanes 3 to 6 with lanes 9 to 12). While demonstrating that GAL4-AH and USF are capable of close binding to adjacent sites without detectable physical occlusion, this experiment also indicates that the binding of these two factors to naked DNA is not cooperative. In contrast, binding of USF to nucleosome cores was greatly enhanced by the binding of an adjacent GAL4-AH dimer (Fig. 1C). Titration of USF onto nucleosomes in the absence (lanes 6 to 11) or presence (lanes 12 to 17) of GAL4-AH revealed that the affinity of USF for its cognate site on nucleosomal DNA can be significantly enhanced by the adjacent binding of GAL4-AH. Binding of USF to its cognate site at this position on the nucleosome core was difficult, and very little binding was observed (USF/Nuc; lanes 10 and 11). However, when the same nucleosome cores were bound by GAL4-AH, significant USF binding was observed (USF/GAL4-AH/Nuc; lanes 13 to 17). The magnitude of this effect is evidenced by the fact that while only 8% of the nucleosome cores were bound by USF in the absence of GAL4-AH at 900 nM USF (USF/Nuc; lane 11), at a 100-fold-lower USF concentration, more than 10% of the GAL4-AH/nucleosome complexes were subsequently bound by USF (USF/GAL4-AH/Nuc; lane 14). Thus, GAL4-AH binding enhanced the affinity of USF for its binding site, at this location on a nucleosome core, by more than 2 orders of magnitude, allowing significant USF binding at physiological concentrations. Nearly 50% of the GAL4-AH-bound nucleosome cores are bound by USF at 90 nM (lane 16), while the intranuclear concentrations of USF have been estimated to be greater than 500 nM (17). It is important to note that as the USF concentration increased, the appearance of the more slowly migrating complex, not present in the mock-reconstituted controls (USF/GAL4-AH/Nuc), was concurrent with a simultaneous decrease in the level of GAL4-AH bound nucleosomes (GAL4-AH/Nuc).

In vivo studies of the GAL1 promoter indicate that activation domains of GAL4 can participate in the disruption of a nucleosome adjacent to the bound GAL4 (4). To address whether activation domains were required for GAL4 derivatives to enhance binding of USF to the same nucleosome, we investigated whether the DNA-binding and dimerization domains of GAL4 [derivative GAL4(1-94)] were sufficient for cooperative binding with USF. Binding reactions corresponding to those using GAL4-AH shown in lanes 12, 16, and 17 in Fig. 1C were repeated with the GAL4(1-94) protein (Fig. 1C, lanes 18 to 20). As can be seen, the results with GAL4(1-94) were identical to those observed with GAL4-AH, with the exception of the slightly reduced size of all protein/DNA complexes containing this truncated derivative. Therefore, activation domains do not appear to be necessary for cooperative binding of transcription factors to nucleosomes, suggesting that a mechanism responsible for this cooperativity is linked to the DNA-binding domains of the activator proteins.

At high USF concentrations, binding of a second or third USF dimer to naked DNA can be observed (Fig. 1B). These complexes may represent nonspecific binding of additional dimers to the DNA and/or the binding of USF tetramers which can form at physiological USF concentrations (17). To exclude the possibility that nonspecific binding of USF was responsible for the appearance of the USF/GAL4-AH/nucleosome complex (Fig. 1C), we tested the dependence of USF binding to the GAL4-AH/nucleosome complex on the presence of an intact USF recognition site. This analysis used another DNA probe containing a single base substitution within the USF core recognition sequence (CACGTG mutated to CACcTG; probe GUBmUSF in Fig. 1A). This mutation totally abolished specific binding of USF to this DNA fragment (data not shown). Following reconstitution of both wild-type and mutant probes into nucleosome cores, binding reaction mixtures were incubated with increasing concentrations of USF in the presence of saturating GAL4-AH (Fig. 1D). As seen in Fig. 1C, the addition of USF to nucleosomes, bearing the CACGTG site and bound by GAL4-AH, resulted in the formation of a novel supershifted complex indicative of USF binding (USF/GAL4-AH/Nuc; Fig. 1D, lanes 2 to 6). In contrast, while GAL4-AH bound equally well to the mutant probe, USF was unable to bind and no corresponding complex was formed (lanes 9 to 14), demonstrating that USF binding to the GAL4-AH/nucleosome complex was sequence specific. Taken together, these results illustrate that binding of GAL4-AH to its cognate site on a nucleosome can alleviate the nucleosome-mediated inhibition of USF binding to an internal site on the same nucleosome.

USF binding stimulates the binding of GAL4-AH to nucleosomes. In the reciprocal experiment, we examined whether USF binding to the more external site on a nucleosome could facilitate GAL4-AH binding to an internal site. By simply inverting the orientation of the oligonucleotide containing the GAL4- and USF-binding sites used to construct the probe GUB discussed above, an identical restriction fragment was generated, except that the positions of the USF and GAL4 sites were switched. The USF site was now closest to the end, centered 18 bp from the *Bam*HI site, and the GAL4 site was located 43 bp from the same end (Fig. 1A). As shown in Fig. 2, binding of GAL4-AH to this internal site on a nucleosome was significantly reduced compared with the level of binding observed when the same site was located 20 bp closer to the end (compare lanes 6 to 9 in Fig. 2 with lane 12 in Fig. 1C). This nucleosome-mediated reduction in GAL4-AH binding to this internal site is identical to the position-dependent modulation of GAL4-AH binding efficiency previously reported (72). Conversely, USF binding to its site at this external location was greatly increased, indicating a similar nucleosome position effect on USF binding.

In the presence of USF protein, a high level of GAL4-AH binding at this location on the nucleosome was observed (GAL4-AH/USF/Nuc; Fig. 2, lanes 11 to 14). Titration of GAL4-AH into binding reaction mixtures containing reconstituted nucleosome cores, in the presence or absence of USF, led to the gradual formation of a novel, larger complex (lanes 6 to 9 and 11 to 14) not seen under identical conditions with mock-reconstituted probe DNA (lanes 1 to 4). Furthermore, as this novel complex appeared, the complex representing USF-bound nucleosomes (USF/Nuc) disappeared, indicating that GAL4-AH binding was supershifting this protein-DNA complex. Binding of USF was found to enhance the subsequent affinity of GAL4-AH for this internal site by up to 2 orders of magnitude. In the absence of USF binding, GAL4-AH bound only approximately 10% of the total nucleosome population at

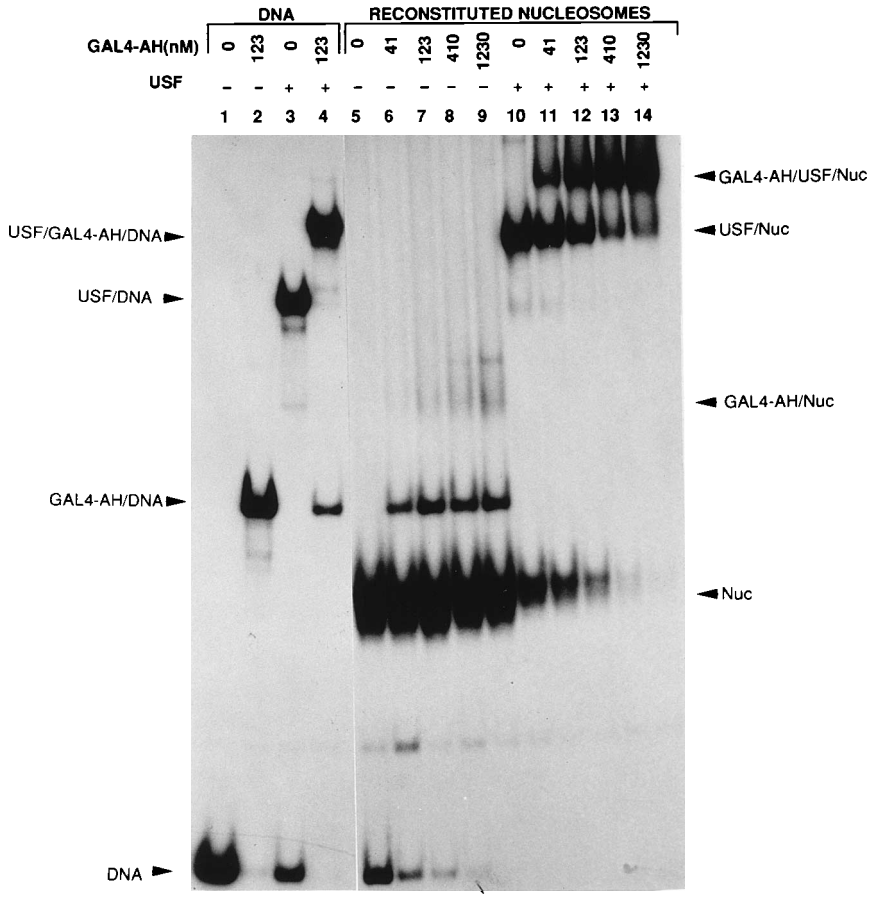


FIG. 2. Cooperative binding of USF and GAL4 to nucleosomes is independent of the orientation of the two binding sites. (A) EMSA analysis of GAL4-AH binding to probe UGB (155 bp) as free DNA or nucleosomes in the presence (+) or absence (−) of USF. Probe DNA was labeled at the *Bam*HI site by Klenow enzyme. GAL4-AH was titrated into binding reaction mixtures (final concentrations are shown) containing radiolabeled probe that had been mock reconstituted (DNA; lanes 1 to 4) or reconstituted into nucleosome cores (lanes 5 to 14). The concentrations of USF used in binding reactions were 27 nM for naked DNA samples (lanes 3 and 4) and 4.5 μM for nucleosome samples (lanes 10 to 14). Protein/DNA complexes were resolved by 4% PAGE and visualized by autoradiography. Complexes representing proteins bound to free DNA (DNA) are labeled on the left, while complexes resulting from proteins bound to nucleosome cores (Nuc) are labeled on the right.

the highest GAL4 concentration (lane 9). However, in the presence of USF binding, more than 25% of the total nucleosome population, composed of nucleosomes plus USF-bound nucleosomes, was bound by GAL4-AH at a 30-fold-lower GAL4 concentration (lane 11). It is important to note that the residual nucleosome complex, i.e., that population of nucleosome cores not initially bound by USF (lane 10), also disappeared as the concentration of GAL4-AH increased, which is similar to what was observed for the USF/nucleosome complex. This finding indicates that the effect of USF in enhancing GAL4-AH binding is due to increased stability (i.e., a lower K_d) rather than a kinetic effect. The ternary complex containing both transcription factors bound to a nucleosome core was more stable than either factor bound alone. Thus, as the GAL4 concentration was increased, the ratio of unbound nucleosomes to USF-bound nucleosomes did not change; the levels of both simply dropped while the level of the GAL4/USF/nucleosome complex increased. Thus, GAL4-AH also stimulated the binding of USF to the external site. This is also confirmed in the DNase I footprinting experiment shown in Fig. 3. As the GAL4-AH concentration increased, increased protection over the USF site on nucleosomes is seen even though the USF protein concentration remained constant.

DNase I footprinting reveals cooperative factor binding to nucleosomal DNA. To further confirm cooperative binding of GAL4-AH and USF to nucleosomes and to determine whether both USF and GAL4-AH were indeed binding in a sequence-specific manner to their respective sites, we analyzed binding reactions by DNase I footprinting. Shown in Fig. 3 is the DNase I cleavage profile of GAL4-AH binding to nucleosome cores reconstituted on the UGB probe, and mock-reconstituted DNA, in the presence and absence of USF binding. Shown in lanes 1 to 4 are USF and GAL4-AH binding to mock-reconstituted (naked) DNA. As expected, DNase I cleaved at nearly every base pair along the 155-bp probe except where the sequence is protected by bound transcription factors (footprinted regions). The reconstituted nucleosome samples showed a different cleavage profile of hypersensitive cleavages spaced at approximately 10-bp intervals, indicative of rotationally phased nucleosome cores. While very little detectable protection was observed at the GAL4 site in the absence of USF binding (lanes 5 to 11), total protection of the entire GAL4 site was achieved when USF was also bound (lanes 12 to 18). In the absence of USF, even at a GAL4-AH concentration of 1,230 nM (lane 11), only partial GAL4-AH binding to its cognate site on the nucleosome occurred and only a weak footprint was seen. In contrast, adjacent binding of USF increased the affinity of GAL4-AH for this internal site by approximately 2 orders of magnitude, as a partial footprint of GAL4-AH on the USF/nucleosome complex was apparent at 12.3 nM GAL4-AH.

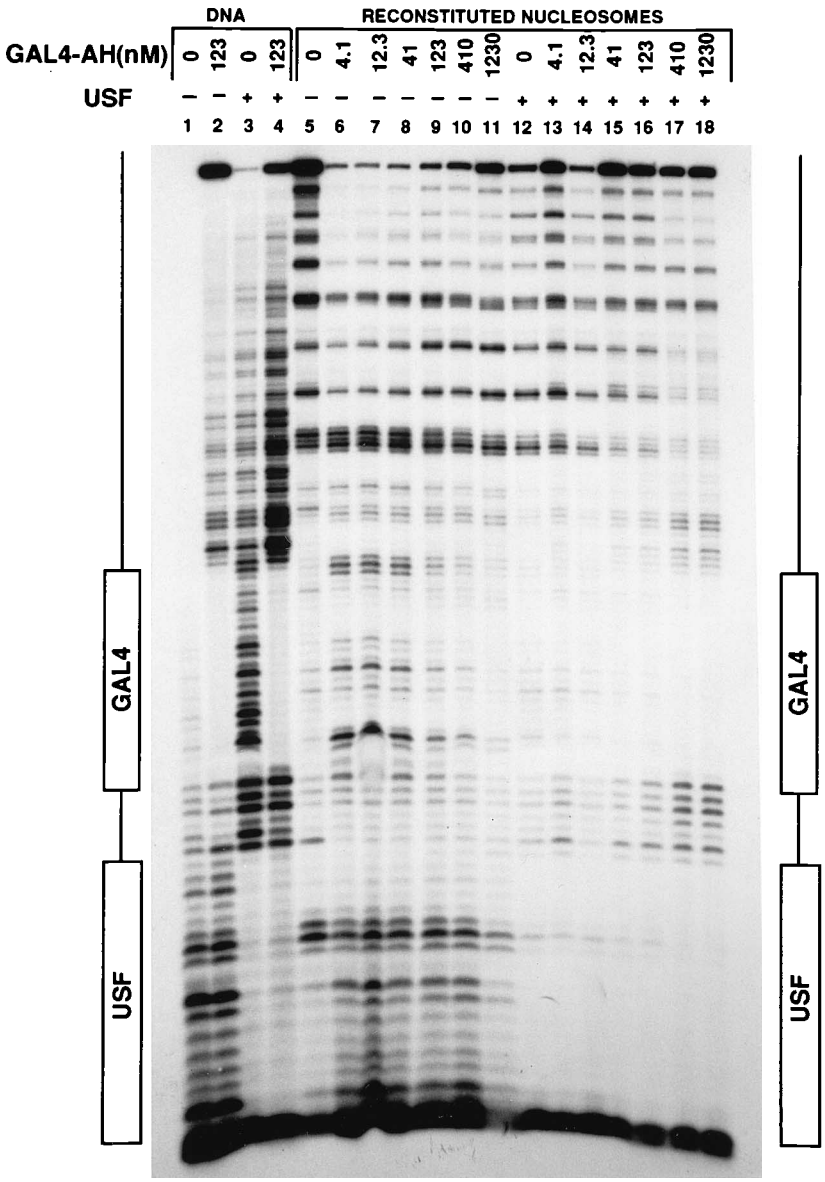


FIG. 3. Cooperative binding of USF and GAL4 to nucleosomes requires sequence-specific binding of both proteins. DNase I footprinting of GAL4-AH binding to probe UGB as free DNA (lanes 1 to 4) or nucleosomes (lanes 5 to 18) in the absence (−) or presence (+) of USF binding. Binding reaction mixtures identical to those analyzed by EMSA in Fig. 2 were digested with DNase I. The concentrations of USF were 27 nM for samples with naked DNA (lanes 3 and 4) and 4.5 μM for samples with nucleosomes (lanes 12 to 18). The concentration of GAL4-AH used in each binding reaction is given. Following DNase I digestion, the DNA was purified, resolved on an 8% sequencing gel, transferred to blotting paper, and subjected to autoradiography. DNase I cleavage sites on the lower strand of probe GUB are visualized. Footprinted sequences (those protected from DNase I cleavage), which include the GAL4- and USF-binding sites and flanking nucleotides, are illustrated.

These data illustrate that cooperative binding of USF and GAL4-AH to adjacent sites on a nucleosome core occurred regardless of which protein was bound on the outside, required sequence-specific binding of both factors, and resulted in the formation of a stable ternary complex containing both transcription factors and some fraction of the histone octamer.

Cooperative nucleosome binding of NF-κB with GAL4-AH and/or USF. To determine whether additional unrelated transcription factors were capable of cooperative binding to nucleosomes, we analyzed binding of NF-κB (p50/p50 homodimers) and USF or NF-κB and GAL4-AH to adjacent sites on reconstituted nucleosome cores. Once again, nucleosome-length DNA fragments containing two adjacent binding sites (Fig. 4A) were excised from plasmid pGUB-NFx1 (see Materials and Methods), reconstituted into nucleosome cores, and assayed for transcription factor binding by EMSA or DNase I footprinting. An EMSA examining USF binding to an internal site on nucleosomes (centered 36 bp from one end), with or without simultaneous binding of NF-κB to an external adjacent site (centered at 18 bp from the same end), is shown in Fig. 4B. As previously demonstrated in Fig. 1C, a barely detectable level of USF binding to its cognate site at this location on reconstituted nucleosome cores was seen (lanes 6 to 11). Only USF binding to the residual naked DNA was apparent. At most, 10% of the total population of nucleosome cores was bound by USF at the highest concentration of USF protein (900 nM). However, when NF-κB was also included in the binding reaction mixtures, the affinity of USF for its cognate

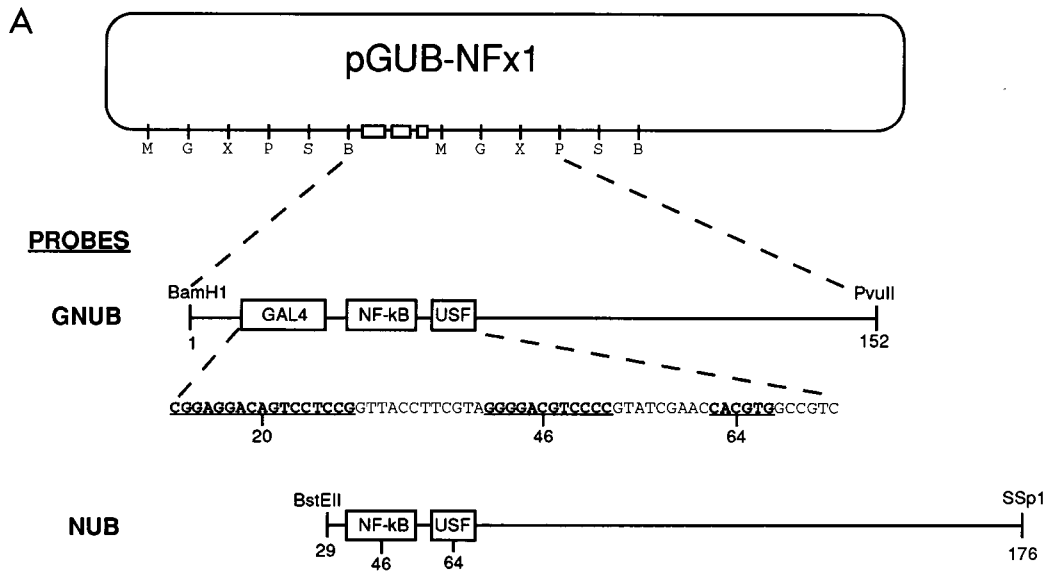
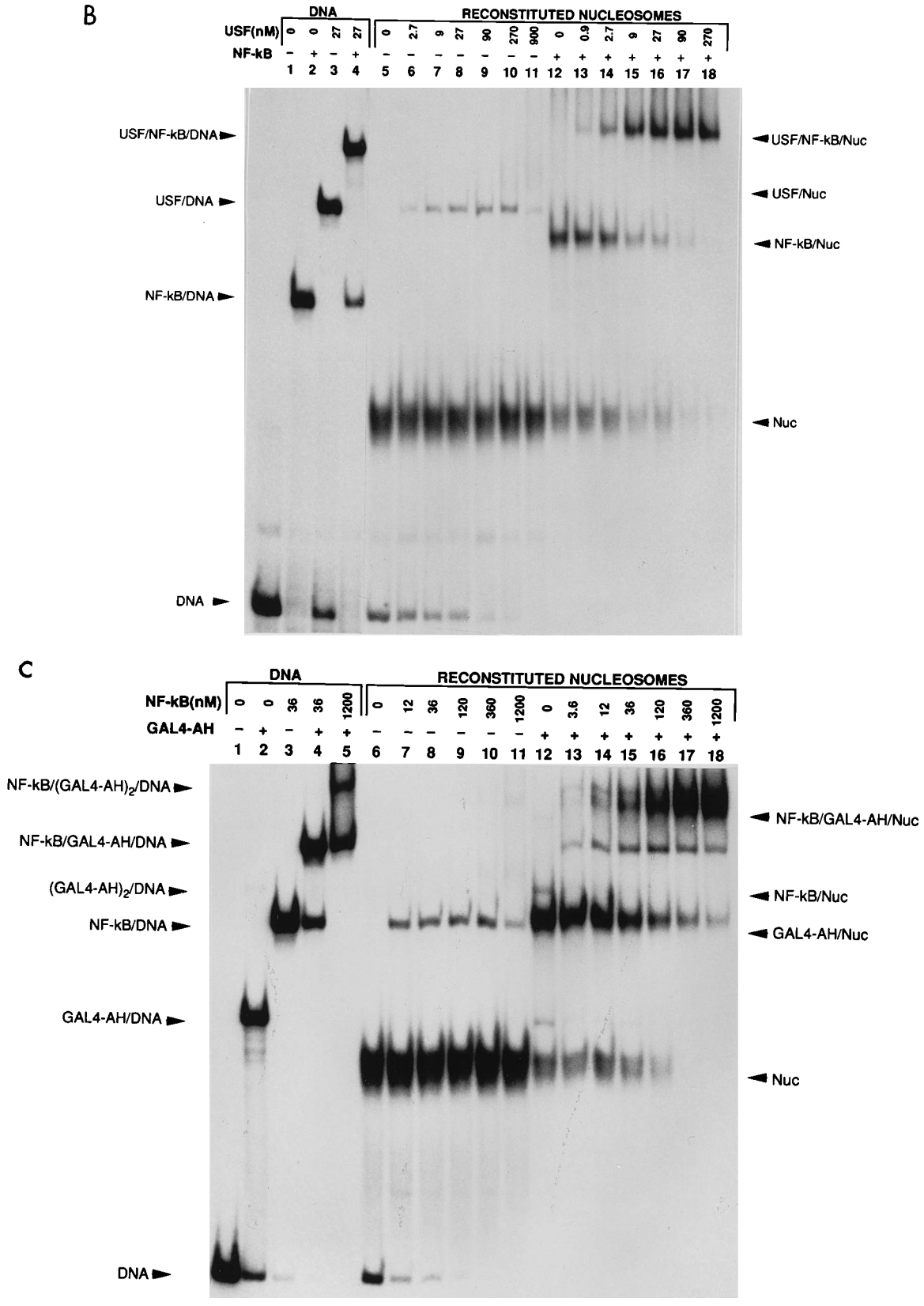


FIG. 4. NF-κB binds cooperatively with GAL4 or USF to nucleosome cores. (A) Diagram of nucleosome-length DNA fragments, GNUB and NUB, used as probes for binding studies. Both probes are derived from the same plasmid, pGUB-NFx1 (shown). Probe GNUB is 152 bp long and contains adjacent GAL4 and NF-κB binding sites centered 20 and 46 bp from the *Bam*HI end (bp 1), respectively. All nucleotides are numbered from the *Bam*HI site used to excise the GNUB probe fragment. Probe NUB was generated by cutting at a unique *Bst*EII site, between the GAL4 and NF-κB sites in plasmid pGUB-NFx1, and a downstream *Pvu*II site, giving rise to a 147-bp fragment containing adjacent NF-κB and USF sites centered 18 and 36 bp from the *Bst*EII end, respectively. The sequences of the GAL4-, NF-κB-, and USF-binding sites are shown. M, *Mlu*I; G, *Bgl*II; X, *Xho*I; P, *Pvu*II; S, *Ssp*I; B, *Bam*HI. (B) EMSA (4% PAGE) analysis of USF binding to probe NUB (147 bp), in the absence (−) or presence (+) of NF-κB binding. USF was titrated into binding reaction mixtures containing radiolabeled probe DNA that had been mock reconstituted (DNA; lanes 1 to 4) or reconstituted into nucleosomes (lanes 5 to 18). The concentration of USF protein in each binding reaction is given. The concentrations of NF-κB used in binding reactions were 36 nM for naked DNA samples (lanes 2 and 4) and 1.2 μM for nucleosome samples. Complexes representing proteins bound to free DNA (DNA) are labeled on the left, while complexes resulting from proteins bound to nucleosome cores (Nuc) are labeled on the right. (C) EMSA (4% PAGE) analysis of NF-κB binding to probe GNUB (152 bp), in the absence (−) or presence (+) of GAL4 binding. NF-κB was titrated into binding reaction mixtures containing radiolabeled probe DNA that had been mock reconstituted (DNA; lanes 1 to 4) or reconstituted into nucleosomes (lanes 5 to 8). The concentration of NF-κB protein in each binding reaction is given. The concentrations of GAL4-AH used in binding reactions were 123 nM for naked DNA samples (lanes 2 and 4) and 2.1 μM for nucleosome samples. Complexes representing proteins bound to free DNA (DNA) are labeled on the left, while complexes resulting from proteins bound to nucleosome cores (Nuc) are labeled on the right.

site was greatly enhanced. Titration of USF into binding reaction mixtures also containing NF-κB resulted in the formation of a novel supershifted complex consisting of both transcription factors bound to a nucleosome core (USF/NF-κB/Nuc; lanes 13 to 18). Furthermore, the population of nucleosomes (Nuc) as well as NF-κB-bound nucleosomes (NF-κB/Nuc) disappear at a rate consistent with the appearance of this novel supershifted complex. Moreover, the fact that nucleosome cores not bound by NF-κB (Nuc) were also shifted into the complex containing both bound factors (USF/NF-κB/Nuc) indicates that the presence of USF enhanced NF-κB binding to these nucleosomes (see also Fig. 5).

In Fig. 4C, an identical EMSA was performed to examine GAL4-AH and NF-κB binding to the 152-bp probe pGUB-NFx1 containing a GAL4-binding site (centered 20 bp from the closest end) and an adjacent internal NF-κB-binding site (centered 46 bp from the same end; Fig. 4A). Probe DNA was reconstituted into nucleosome cores and added to binding reaction mixtures containing increasing amounts of NF-κB, with or without added GAL4-AH protein. Titration of NF-κB onto nucleosomes resulted in the formation of only a small amount of supershifted complex representing NF-κB binding to nucleosomes (NF-κB/Nuc; lanes 6 to 11), indicating a low affinity of NF-κB for a binding site at this internal location on a nucleosome core. At an NF-κB concentration of 1,200 nM, at most 10% of the total population of nucleosomes was bound by NF-κB. In contrast, binding of GAL4-AH to these nucleosomes promoted efficient binding of NF-κB to this internal adjacent site, leading to the formation of a novel complex containing both transcription factors and the nucleosome core (NF-κB/GAL4-AH/Nuc; lanes 13 to 18). Again, NF-κB binding was apparent not only by the appearance of this novel complex but also by the disappearance of the GAL4-AH-bound nucleosomes (GAL4-AH/Nuc). The facilitated binding of NF-κB by GAL4-AH was also confirmed by DNase I footprinting (see Fig. 7). These results suggest that the cooperative binding of disparate transcription factors to nucleosomes is a universal mechanism for alleviating nucleosome-mediated repression of transcription factor binding to DNA.

Reciprocity of binding enhancement depends on the factor binding the more accessible position. In all of the foregoing experiments, high concentrations of at least one transcription factor were always present in the binding reaction mixtures in order to demonstrate that the binding of one transcription factor to nucleosomes at an outside site can stimulate the binding of additional factors to internal sites on the same nucleosome. To further characterize this stimulation, we examined the stimulation observed when the factor binding the outside site was at lower concentrations. To do this, we simply examined the binding of USF (90 nM) to an internal binding site (DNA probes GUB and NUB as in Fig. 1A and C and 4A and B, respectively) while varying the concentrations of the factor binding the outside site (GAL4-AH or NF-κB). As shown in Fig. 5A, the binding of USF to nucleosomes reconstituted on DNA probe GUB was stimulated by GAL4-AH over a broad range of GAL4-AH concentrations (as low as 4.1 nM; lane 7). As the concentration of GAL4-AH in the binding reaction mixtures increased, from 4.1 to 410 nM, so did the


B
DNA
RECONSTITUTED NUCLEOSOMES
USF(nM) 0 0 27 27 0 2.7 9 27 90 270 900 0 0.9 2.7 9 27 90 270
NF-kB – + – + – – – – – – – + + + + + + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
USF/NF-kB/DNA
USF/DNA
NF-kB/DNA
DNA
USF/NF-kB/Nuc
USF/Nuc
NF-kB/Nuc
Nuc
C
DNA
RECONSTITUTED NUCLEOSOMES
NF-kB(nM) 0 0 36 36 1200 0 12 36 120 360 1200 0 3.6 12 36 120 360 1200
GAL4-AH – + – + + – – – – – – + + + + + + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
NF-kB/(GAL4-AH)2/DNA
NF-kB/GAL4-AH/DNA
(GAL4-AH)2/DNA
NF-kB/DNA
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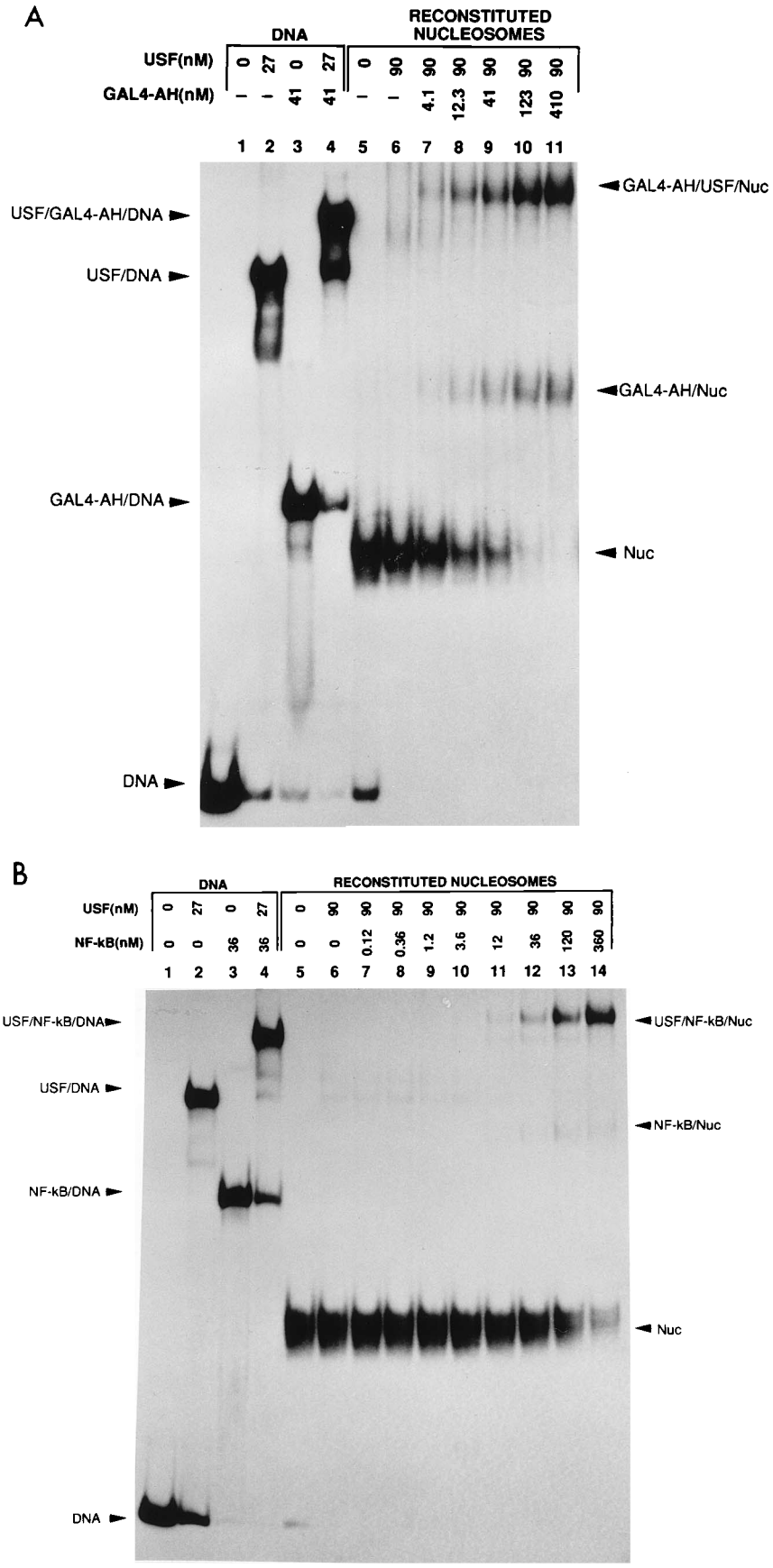
GAL4-AH/Nuc
Nuc


A
DNA
RECONSTITUTED NUCLEOSOMES
USF(nM)
GAL4-AH(nM)
0 27 0 27 0 90 90 90 90 90 90
– – 41 41 – – 4.1 12.3 41 123 410
1 2 3 4 5 6 7 8 9 10 11
USF/GAL4-AH/DNA
USF/DNA
GAL4-AH/DNA
DNA
GAL4-AH/USF/Nuc
GAL4-AH/Nuc
Nuc
B
DNA
RECONSTITUTED NUCLEOSOMES
USF(nM)
NF-kB(nM)
0 27 0 27 0 90 90 90 90 90 90 90 90 90
0 0 36 36 0 0 0.12 0.36 1.2 3.6 12 36 120 360
1 2 3 4 5 6 7 8 9 10 11 12 13 14
USF/NF-kB/DNA
USF/DNA
NF-kB/DNA
DNA
USF/NF-kB/Nuc
NF-kB/Nuc
Nuc

level of USF binding, even though the concentration of USF was constant at 90 nM. However, at all concentrations of GAL4-AH tested, only slightly more than half of the GAL4-bound nucleosomes are shifted by USF. This finding indicates that the concentration of USF is close to its K_d for GAL4-bound nucleosomes and that USF binding is not reciprocally stimulating GAL4-AH binding to the outside site. Thus, while the affinity of USF for the inside site on the nucleosome DNA is increased by the presence of GAL4-AH, the affinity of GAL4-AH for the outside site is not significantly affected by the presence of USF. Conversely, the results in Fig. 5B indicate that there is reciprocal stimulation between USF and NF-κB when binding probe NUB reconstituted into nucleosome cores. As the concentration of NF-κB in the binding reaction mixtures was increased, from 0.12 to 360 nM, the nucleosomal population shifted to the ternary complex consisting of both activators bound to the nucleosomes (lanes 7 to 14, USF/NF-κB/Nuc), while binding of NF-κB alone to the outside site was barely detectable (NF-κB/Nuc). The difference in the responses of GAL4-AH and NF-κB to USF binding at a more internal position appears to be due to the differences between the affinities that GAL4-AH and NF-κB have for their sites at the outside positions on these nucleosomes. GAL4-AH binding to the edge of nucleosomes reconstituted on probe GUB is not as influenced by USF binding because it has a higher intrinsic affinity by itself (Fig. 5A, GAL4-AH/Nuc) for its site at this location than does NF-κB (Fig. 5B, NF-κB/Nuc). Taken together, these results provide evidence that the level of cooperativity that occurs between disparate transcription factors when binding nucleosomes is influenced by the affinities that they have for their individual sites on the nucleosomes that they are binding.

Cooperative binding to nucleosomes is dependent on the distance between transcription factor-binding sites. To investigate whether there were distance requirements for cooperative transcription factor binding, we tested for cooperative binding between GAL4-AH and USF on probe GNUB containing an additional 20-bp insertion (which includes an NF-κB site) between the GAL4 and USF sites (Fig. 4A; Materials and Methods). This insertion increased the distance between the centers of the GAL4 and USF sites from 23 to 43 bp, or essentially two full helical turns of DNA (compare probe GUB [Fig. 1A] with probe GNUB [Fig. 4A]). We then isolated a nucleosome-length fragment of DNA containing the GAL4 site at the same location as in Fig. 1C and a USF site now positioned 20 bp closer to the nucleosome dyad axis, centered 64 bp from the GAL4 end. Following reconstitution of this radiolabeled probe into nucleosome cores, binding of GAL4-AH and USF was analyzed by DNase I footprinting (Fig. 6). As shown in lanes 1 to 4, individual and simultaneous binding of both USF and GAL4 proteins to mock-reconstituted probe DNA occurred, evidenced by the complete footprints over their respective binding sites, while USF binding to nucleosomes was not seen under any conditions. One of the cleavage sites within the nucleosome core (lane 5), consisting of two to three nucleotides, lies within the USF-binding site and remained accessible to DNase I cleavage even in the presence of high concentrations of USF and irrespective of whether GAL4-AH was bound (lanes 6 to 16). This lack of USF binding under these conditions was also confirmed by EMSA as in Fig. 1C (data not shown). This result indicates that cooperative binding between GAL4-AH and USF was dependent on the distance between the binding sites, although an alternative explanation could be that USF is simply unable to bind to its cognate site because of its proximity to the nucleosome dyad axis (40).

To clarify this result, we tested whether the addition of NF-κB protein to binding reactions, to bridge the gap between GAL4-AH and USF, could restore binding of USF. As discussed above, the GAL4 and USF sites in probe GUBend (Fig. 1A) were separated by inserting an oligonucleotide containing a 12-bp palindromic NF-κB-binding site, placing the center of the site 46 bp from the end (probe GNUB; Fig. 4A). After reconstitution of this probe DNA into nucleosome cores, we analyzed the binding of all three proteins, GAL4-AH, NF-κB, and USF, by DNase I footprinting (Fig. 7). Once again, as demonstrated in Fig. 6, USF protein was unable to bind its site on these nucleosomes, and no evidence for a footprint was detected (lanes 7 to 10). Similarly, NF-κB was also unable to bind, as evidenced by the lack of any detectable footprint over its binding site (lanes 11 to 14). However, when GAL4-AH was bound to its site on the edge of the nucleosomes, NF-κB binding to its site was greatly enhanced (lanes 15 to 19). This result confirmed earlier EMSA data in Fig. 4C demonstrating stimulation of NF-κB binding by GAL4-AH to this same probe reconstituted into nucleosomes. In addition, in the presence of both GAL4-AH and NF-κB binding, USF binding was enhanced approximately 300-fold. These results indicate that there is an inherent cooperativity between transcription factors when binding adjacent sites on nucleosomes and further indicate that cooperative binding can proceed over a distance by the binding of multiple factors.

Cooperative nucleosome binding does not require transcription factor-induced sliding of the histone octamer. Inherent cooperative binding to nucleosomal DNA by disparate transcription factors could be explained by two distinct mechanisms: (i) localized disruption of repressive histone-DNA contacts by the binding of the first factor and (ii) factor-induced sliding of the histone octamer. Either of these events could result from the binding of the first transcription factor to the more accessible position nearer the end of the nucleosome core and conceivably facilitate the binding of the second factor. In an attempt to differentiate between these two possibilities, we tested whether binding of GAL4-AH to its cognate site on the edge of a nucleosome (as in Fig. 1C and 4C) resulted in the sliding of the underlying histone octamer.

To test for sliding of the histone octamer, we analyzed the abilities of three different restriction enzymes (*Stu*I, *Pvu*II, and *Xho*I) to cleave their respective sites at the end of a nucleosome-reconstituted DNA fragment, opposite the GAL4-binding site (Fig. 8A), in the presence or absence of GAL4-AH binding. As shown in Fig. 8B, the accessibility of these restriction sites was reduced on a nucleosome reconstituted on a fragment constituting position B (*Bst*EII-to-*Bam*HI fragment)

FIG. 5. The level of cooperativity between two transcription factors can vary. (A) EMSA (4% PAGE) analysis of USF binding to probe GUB (155 bp), in the absence (−) or presence (+) of increasing concentrations of GAL4-AH. GAL4-AH was titrated into binding reaction mixtures containing radiolabeled probe DNA that had been mock reconstituted (DNA; lanes 1 to 4) or reconstituted into nucleosomes (lanes 5 to 11). The concentrations of USF and NF-κB in each binding reaction are given. (B) EMSA (4% PAGE) analysis of USF binding to probe NUB (147 bp) in the presence of increasing concentrations of NF-κB. NF-κB was titrated into binding reaction mixtures containing radiolabeled probe DNA that had been mock reconstituted (DNA; lanes 1 to 4) or reconstituted into nucleosomes (lanes 5 to 14). The concentrations of NF-κB and USF in each binding reaction are given. Complexes representing proteins bound to free DNA (DNA) are labeled on the left, while complexes resulting from proteins bound to nucleosome cores (Nuc) are labeled on the right.

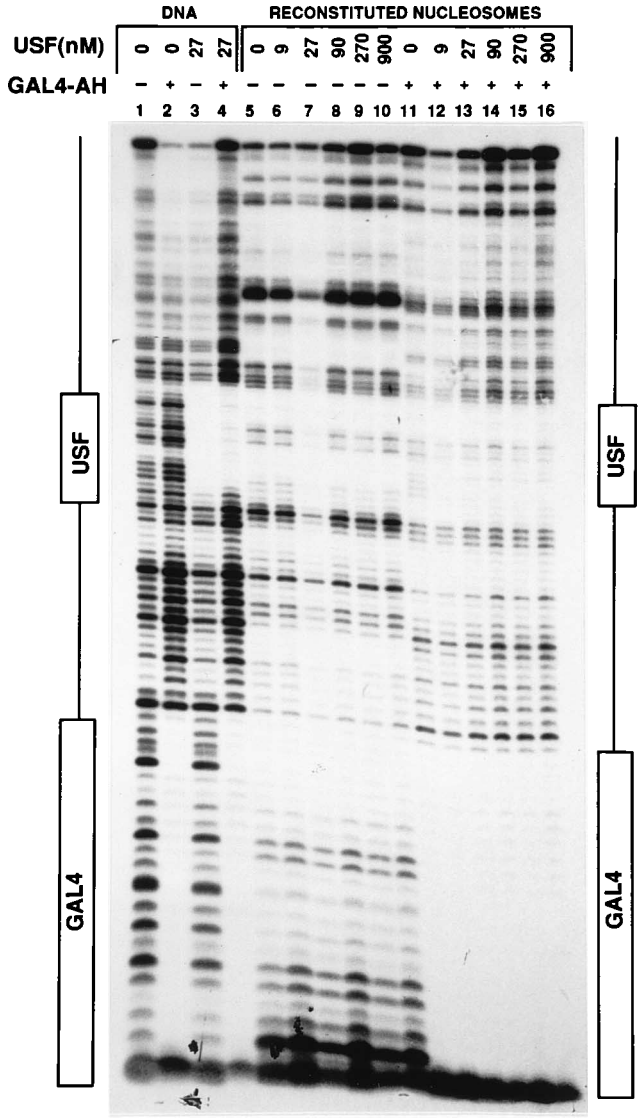


FIG. 6. Cooperative binding of USF and GAL4 to nucleosomes is dependent on the distance between their binding sites. DNase I footprinting of USF binding to probe GNUB as free DNA or nucleosomes in the absence (−) or presence (+) of GAL4-AH binding. Probe DNA was labeled at the *Bam*HI site by Klenow fill-in of the recessed end. USF was titrated into binding reaction mixtures containing radiolabeled probe DNA that had been mock reconstituted (DNA; lanes 1 to 4) or reconstituted into nucleosomes (lanes 5 to 16). The concentration of USF used in each binding reaction is given. The concentrations of GAL4-AH were 123 nM for samples with naked DNA (lanes 2 and 4) and 2.1 μM for samples with nucleosomes (lanes 11 to 16). Following DNase I digestion, the DNA was purified, resolved on an 8% sequencing gel, transferred to blotting paper, and subjected to autoradiography. DNase I cleavage sites on the lower strand of probe GNUB are visualized. The region of the DNA fragment corresponding to the USF-binding site, footprinted in the naked DNA samples, is illustrated. The region of the DNA fragment corresponding to the GAL4-binding site, footprinted in both the naked DNA and nucleosome samples, is also indicated.

(where the sites are more internal to the nucleosome core) than a nucleosome reconstituted on a fragment constituting position A (*Bam*HI-to-*Ssp*I fragment). If GAL4-AH binding were able to push the histone octamer off its binding sites, it would move from position A toward position B and the accessibility of these restriction enzymes to their sites on nucleosome A would be reduced by GAL4-AH binding. As shown in Fig. 8B, this is not the case. GAL4-AH binding does not influence the cleavage by these distal restriction enzymes on either naked DNA or nucleosome cores. While there is a drastic difference between the cutting by both *Stu*I and *Pvu*II on nucleosome A compared with nucleosome B, GAL4-AH binding had no effect on the level of cleavage of nucleosome A, even though all of these nucleosomes were bound by GAL4-AH (Fig. 8C and data not shown). Even on a longer DNA fragment capable of being packaged into a nucleosome in position A or position B (*Bam*HI-to-*Bam*HI fragment), no reduction in *Stu*I or *Pvu*II cleavage was observed in the presence of GAL4-AH binding (position A/B; Fig. 8B). The level of cleavage detected on the longer fragment, nucleosome A/B, is between the levels seen for nucleosome A and nucleosome B, suggesting that the positioning of the histone octamer on this fragment is heterogeneous. However, this fragment is long enough (179 bp) to encompass both a bound GAL4-AH dimer and a nucleosome core with 146 bp of DNA in position B. Importantly, GAL4-AH binding does not push these nucleosomes into position B, as indicated by the lack of a reduction of enzyme cleavage to that of position B alone. These results indicate that the binding of GAL4-AH to the outside site on a nucleosome core does not result in sliding of the histone octamer under these binding conditions; thus, nucleosome sliding was not required for GAL4-AH stimulation of USF or NF-κB binding to the internal sites. Instead, cooperative nucleosome binding is likely a result of modest alterations in core particle structure due to the binding of the first activator protein (i.e., loosening of histone-DNA contacts in the vicinity of the bound factor), thus facilitating the binding of additional activators to adjacent sites on the same nucleosome.

DISCUSSION

Often, transcription factor-binding sites are clustered together within gene regulatory regions, for example, in many viral enhancers and in the regulatory regions of cellular genes activated by steroid hormones (16, 61). Transcription factor-binding sites also commonly reside within nucleosome-free regions typically detected as nuclease-hypersensitive sites in chromatin (reviewed in reference 21). This observation raises the possibility that there is a connection between the presence of multiple, closely spaced activator-binding sites and the ability of activators to bind and disrupt or displace the underlying nucleosomes. Previous studies have demonstrated that chromatin remodeling can occur at promoters and enhancers in the absence of replication, initiated by the binding of transcription factors. For example, the RU5 region of the human immunodeficiency virus type 1 long terminal repeat is packaged by a sequence-positioned nucleosome that is disrupted or displaced, creating a nuclease-hypersensitive site, immediately following induction of the promoter by the phorbol ester tetradecanoyl phorbol acetate. One proposed mechanism for this structural transition is the binding of three AP-1 transcription factors to closely spaced binding sites on the DNA packaged by this nucleosome (71). Similarly, the binding of multiple steroid receptors has been shown to remodel the chromatin structure at gene promoters including the rat tyrosine aminotransferase gene promoter and the mouse mammary tumor virus promoter, leading to the appearance of nuclease hypersensitivity (3, 27, 55). Also consistent with this idea is the observation of Taylor et al. (67) that multiple GAL4 derivatives bind cooperatively to adjacent sites on a nucleosome core in vitro, which destabilizes the nucleosome core and thus allows its displacement (12, 78). Cooperative binding of GAL4 derivatives to nucleosomes contributes to the increased transcriptional syn-

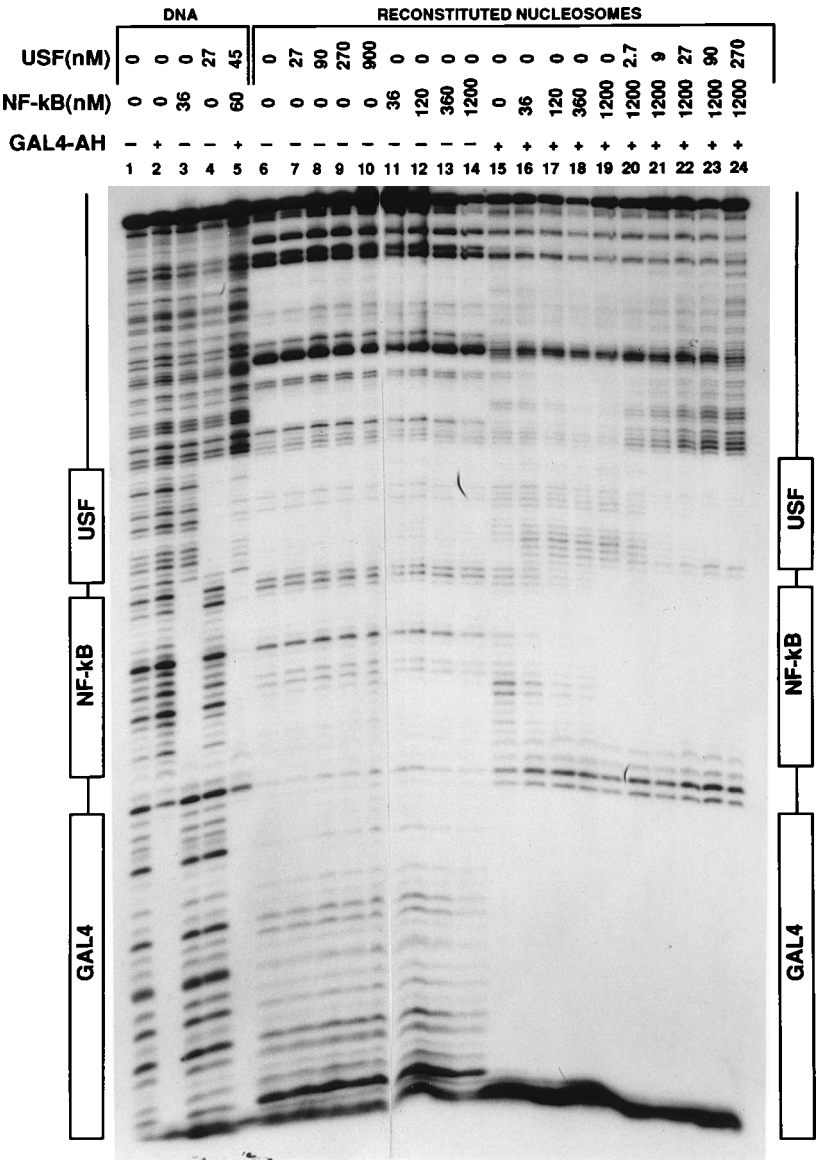
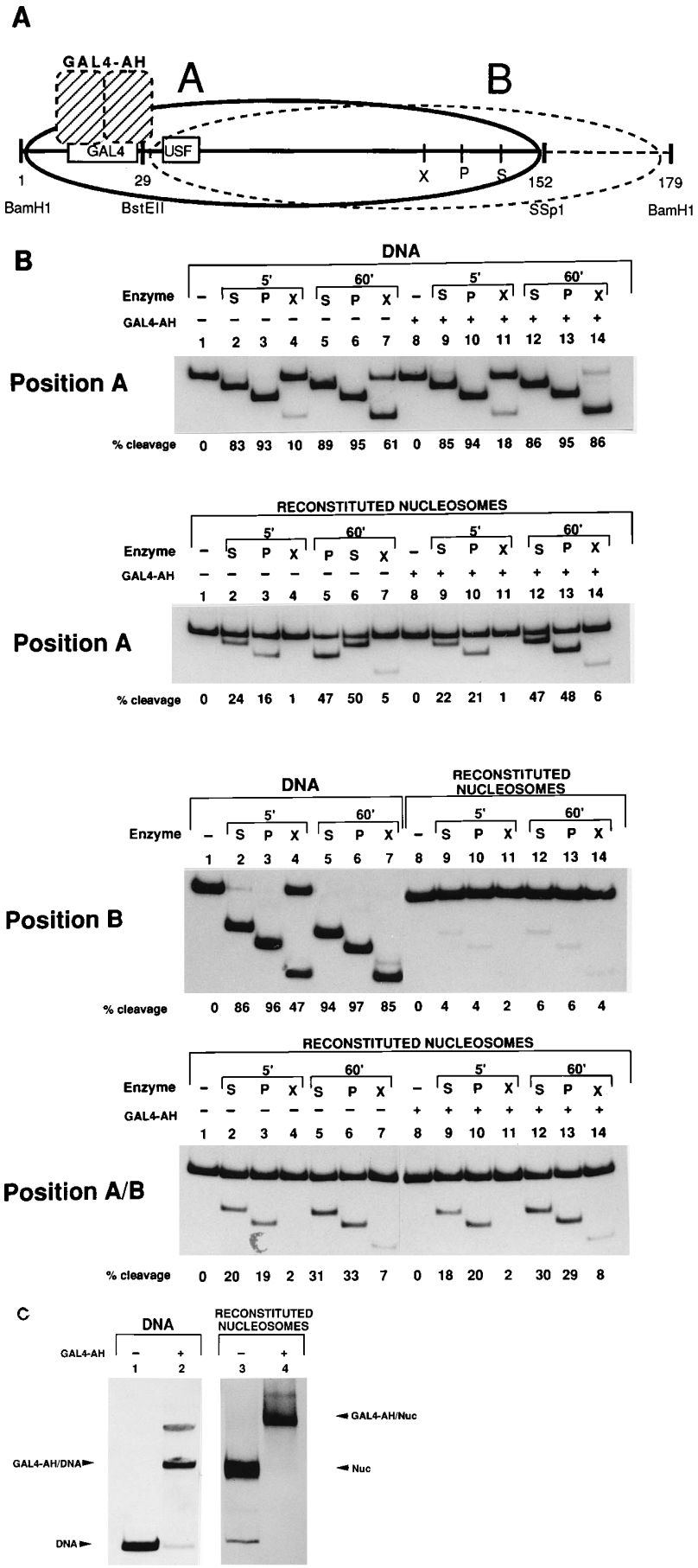


FIG. 7. Cooperative binding of three unrelated transcription factors, GAL4-AH, NF-κB, and USF, to adjacent sites on a nucleosome core; DNase I footprinting of binding of transcription factors GAL4-AH, NF-κB, and USF to probe GNUB as free DNA or as nucleosomes. Probe DNA was labeled by Klenow fill-in of the recessed *Bam*HI end and mock reconstituted (DNA; lanes 1 to 5) or reconstituted into nucleosome cores (lanes 6 to 24). NF-κB was titrated into binding reaction mixtures containing nucleosomes in the absence (−; lanes 11 to 14) or presence (+; lanes 16 to 19) of 2.1 μM GAL4-AH. USF was titrated into binding reaction mixtures containing reconstituted nucleosomes in the absence of any other transcription factors (lanes 7 to 10) or in the presence of 2.1 μM GAL4-AH and 1,200 nM NF-κB (lanes 20 to 24). The concentrations of both USF and NF-κB in all samples are given. The concentration of GAL4-AH in the binding reactions with free DNA (lanes 2 and 5) was 123 nM. Following incubation for transcription factor binding, samples were digested with DNase I, and the DNA was purified, resolved on an 8% sequencing gel, transferred to blotting paper, and subjected to autoradiography. DNase I cleavage sites on the lower strand of probe GNUB are visualized. The regions of the DNA fragment corresponding to the GAL4-, NF-κB-, and USF-binding sites, footprinted in the naked DNA samples and on the nucleosomes, are illustrated.

ergy of multiple GAL4-VP16 activators on chromatin versus DNA templates in vitro (11). These observations suggest that multiple transcription factors, either the same or different factors, may function together to gain occupancy of adjacent sites on DNA packaged into nucleosomes, eventually leading to promoter opening and transcriptional activation. Here, we investigated mechanisms responsible for regulating the initial binding of multiple activators to nucleosomal DNA and provide evidence that inherent cooperativity exists between disparate transcription factors when binding closely spaced sites on the surface of a nucleosome.

We have shown that any two of the three transcription factors, GAL4-AH, NF-κB, and USF, can bind cooperatively to adjacent sites on a nucleosome, such that binding of the first factor enhances binding of the second factor by up to 2 orders of magnitude. This cooperativity is independent of the orientation of the binding sites within the nucleosome core. Thus, the mechanism responsible for this cooperativity is not dependent on specific protein-protein interactions or a specific DNA-binding motif interacting with a distinct region of the histone octamer. The binuclear Zn clusters of GAL4 (45), helix-loop-helix/basic domain of USF (17), and *rel* domain of NF-κB (19, 30) all participated in cooperative binding. Binding of any of these transcription factors to sites closer to the edge


A
GAL4-AH
A
B
GAL4
USF
1
29
X
P
S
152
179
BamH1
BstEII
SSp1
BamH1
B
DNA
Enzyme
5'
60'
5'
60'
– S P X S P X – S P X S P X
GAL4-AH
– – – – – – – + + + + + + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Position A
% cleavage 0 83 93 10 89 95 61 0 85 94 18 86 95 86
RECONSTITUTED NUCLEOSOMES
Enzyme
5'
60'
5'
60'
– S P X P S X – S P X S P X
GAL4-AH
– – – – – – – + + + + + + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Position A
% cleavage 0 24 16 1 47 50 5 0 22 21 1 47 48 6
DNA
RECONSTITUTED NUCLEOSOMES
Enzyme
5'
60'
5'
60'
– S P X S P X – S P X S P X
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Position B
% cleavage 0 86 96 47 94 97 85 0 4 4 2 6 6 4
RECONSTITUTED NUCLEOSOMES
Enzyme
5'
60'
5'
60'
– S P X S P X S – P X S P X
GAL4-AH
– – – – – – – + + + + + + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Position A/B
% cleavage 0 20 19 2 31 33 7 0 18 20 2 30 29 8
C
DNA
RECONSTITUTED NUCLEOSOMES
GAL4-AH
– + – +
1 2 3 4
GAL4-AH/Nuc
GAL4-AH/DNA
Nuc
DNA

of a nucleosome (usually the higher-affinity site) potentiates the binding of the second factor to more difficult internal sites. However, reciprocal stimulation also occurs, and the affinity of each transcription factor can be enhanced as a result of binding by the other. Furthermore, cooperative binding to nucleosomes did not require the sliding of the underlying histone octamer, and the order of addition of the transcription factors to the binding reaction mixtures was found to be irrelevant for cooperative nucleosome binding to occur (data not shown). This finding indicates that structural alterations in the nucleosome core particle are responsible for their increased accessibility to transcription factor binding and that this alteration occurs immediately upon binding of the first transcription factor.

These data illustrate an important new concept with regard to the accessibility of nucleosomal DNA to transcription factors. While the nucleosome core can create a significant impediment to transcription factor binding (1), a nucleosome core previously bound by one factor is a substantially better substrate for the binding of additional factors. This leads to a level of cooperativity in factor binding not observed in binding to naked DNA. Hence, disruption of nucleosome structure by initial factor binding results in enhanced affinity of adjacent binding sites. The mechanism by which this occurs is undoubtedly linked to the manner in which nucleosomes inhibit transcription factor binding. The alterations in nucleosome structure associated with the binding of the first transcription factor could range from a localized disruption of histone DNA contacts to partial dissolution of the histone octamer (i.e., loss of one or both H2A-H2B dimers, etc.). Support for the former possibility arises from the fact that proteolytic removal of the histone amino termini both stimulates GAL4 derivative binding to nucleosomal DNA and reduces the apparent cooperativity of GAL4-AH binding (72). Therefore, the cooperativity of GAL4 derivative binding appears to be in response to these basic histone domains which have a high affinity for DNA (23). Together, these data support a mechanism whereby initial factor binding disrupts interactions of the core histone amino termini with nucleosomal DNA, leading to a localized enhanced accessibility of surrounding DNA sequences. We also observed a strong distance dependence on cooperativity of binding. GAL4-AH greatly stimulated USF binding to a site 20 bp away but had less effect on a site 40 bp distant (Fig. 6). This distance dependence may reflect a modular nature of repression by the histone amino termini. Each histone amino termini may interact with a primary region of nucleosomal DNA (reviewed in reference 70).

What is the importance of cooperative binding of transcription factors to nucleosomes and what is its relationship to transcriptional activation in vivo? Most significantly, it is known that cooperative binding can contribute to transcriptional synergy often observed for multiple binding elements in vivo (47). For example, estrogen response elements from the *Xenopus* vitellogenin B1 and B2 and chicken VTG II genes act synergistically to activate transcription in response to estrogen; however, hormone responsiveness and synergistic activation absolutely require close spacing of at least two elements (10, 31) and require only the estrogen receptor DNA-binding domain (5, 31). In addition, transcriptional synergy between steroid receptors and a plethora of other disparate activators in chimeric promoter transfection studies supports the positive role of cooperative binding to nucleosomal DNA, simply because of the tremendous diversity in the binding sites and activator proteins used (61, 63). In some instances where transcriptional synergy between adjacent binding elements was observed in vivo, cooperative binding by the corresponding activator proteins to deproteinized DNA was also observed (5, 8, 15, 49, 68). Alternatively, in other instances, cooperative binding to deproteinized DNA was not observed and therefore was not considered a probable mechanism responsible for the observed transcriptional synergy (31, 52). However, the data presented here indicate an inherent cooperativity of transcription factor binding to nucleosomal DNA, suggesting that in chromatin, cooperative binding may be a major contributor to transcriptional synergism even when cooperative binding to naked DNA is not observed. It is important to note, however, that the participation of GAL4(1-94) in cooperative nucleosome binding suggests that this binding is not dependent on activation domains and thus cannot alone bring about synergistic transcription activation. Clearly some of the resulting bound activators would need to contain activation domains which might also function synergistically in stimulation of transcription initiation complex formation (9, 24, 42) and might participate in further chromatin disruption (4, 65). Indeed, it is easy to envisage ways in which both of these activities could contribute to dramatic threshold responses in vivo. Cooperative binding may lead to loading of an enhancer or promoter with numerous different factors which would allow synergistic functions of their various activation domains.

These observations raise intriguing possibilities regarding the function of nucleosomes at enhancer or promoter elements which bind multiple disparate factors. Indeed, nucleosomes might modulate the function of these elements by increasing their dependence on the simultaneous availability of numerous transcription factors to which they bind. Cooperative nucleosome binding could bring about the occupancy of regulatory elements with numerous ubiquitous factors which may potentiate subsequent binding of inducible factors (reviewed in reference 77). Alternatively, cooperative nucleosome binding may result in the inducible or tissue-specific binding of ubiquitous factors to some tissue-specific or inducible promoters. The in vivo binding of ubiquitous factors to a regulatory element can

FIG. 8. Cooperative nucleosome binding does not require sliding of the histone octamer. (A) Diagram of nucleosome positions A and B on DNA probe GUB and derivative fragments. Nucleosome position A corresponds to the primary location of the histone octamer on the *Bam*HI-*SSp*I DNA fragment (probe GUB as in Fig. 1A) reconstituted into nucleosomes, while position B corresponds to the histone octamer location on the reconstituted *Bst*EII-*Bam*HI fragment. The larger *Bam*HI-*Bam*HI fragment (179 bp) contains enough DNA for the histone octamer to adopt position A or position B. S, *Stu*I; P, *Pvu*II; X, *Xho*I. All nucleotides are numbered from the 5′ *Bam*HI site (+1). (B) Restriction enzyme digestion analysis of nucleosomal DNA in the presence or absence of GAL4-AH binding. Three different DNA probe fragments were excised from pGALUSFBend (Fig. 1A). Position A corresponds to the *Bam*HI-*SSp*I fragment, position B corresponds to the *Bst*EII-*Bam*HI fragment, and position A/B corresponds to the *Bam*HI-*Bam*HI fragment (see panel A). Radiolabeled DNA was mock reconstituted (DNA) or reconstituted into nucleosome cores (RECONSTITUTED NUCLEOSOMES), added to binding reaction mixtures with (+) or without (−) 2.1 μM GAL4-AH, and digested with 10 U of the appropriate restriction enzyme for either 5 or 60 min at 37°C. S, *Stu*I; P, *Pvu*II; X, *Xho*I. The efficiency of restriction enzyme cleavage was determined by quantitating the specific activity of each band with a PhosphorImager (Molecular Dynamics). The average of two independent experiments is given (% cleavage). (C) EMSA of GAL4 binding prior to restriction enzyme digestion. To demonstrate efficient binding of GAL4 to DNA templates prior to restriction enzyme digestions, duplicate samples containing either mock-reconstituted or reconstituted DNA were incubated with or without saturating concentrations of GAL4-AH and then analyzed by 4% PAGE instead of by restriction enzyme digestion. The concentrations of GAL4-AH were 410 nM for mock-reconstituted DNA and 2.1 μM for reconstituted nucleosomes.

depend on the presence of tissue-specific or inducible factors (6). In such instances, the presence of a tissue-specific or inducible transcription factor may be required to initiate occupancy of all transcription factors via cooperative nucleosome binding.

Cooperative nucleosome binding is not the only mechanism which enhances transcription factor binding to nucleosomal DNA. Transcription factor binding is also stimulated by the ATP-dependent disruption of nucleosomes by the SWI/SNF complex (13, 25, 36) and other ATP-dependent activities (69). At present, it is not clear to what extent and under what circumstances these mechanisms function additively or redundantly. However, these different pathways present the cell with numerous opportunities to regulate transcription factor access via combinations of regulatory factors and ATP-dependent protein complexes.

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