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Nucleosome Core Displacement in Vitro via a Metastable Transcription Factor–Nucleosome Complex

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In order to function, transcription factors must compete for DNA binding with structural components of chromatin, including nucleosomes. Mechanisms that could be used in this competition have been characterized with the use of the DNA binding domain of the yeast GAL4 protein. The binding of GAL4 to a nucleosome core resulted in a ternary complex containing GAL4, the core histone proteins, and DNA. This ternary complex was unstable; upon the addition of nonspecific competitor DNA, it dissociated into either the original nucleosome core particle or GAL4 bound to naked DNA. Nucleosome core destabilization by GAL4 did not require a transcriptional activation domain. These data demonstrate the displacement of nucleosome cores as a direct result of binding by a regulatory factor. Similar mechanisms might affect the establishment of factor occupancy of promoters and enhancers in vivo.

An initial hurdle in activating gene expression involves the ability of regulatory factors to access their binding sites in chromatin. For numerous genes, activation results in the interruption of nucleosome arrays at enhancer and promoter sequences and the binding of specific regulatory factors to these sequences (*1*). For most genes, it is not clear whether binding by transcription factors causes the observed disruptions in chromatin structure or if chromatin structure must first be perturbed (by DNA replication, for example) to subsequently allow factor binding (*2*, *3*). However, an example of the former mechanism is provided by studies of the mouse mammary tumor virus (MMTV) promoter that have demonstrated that the glucocorticoid receptor can bind to a nucleosome associated with this promoter (*4*, *5*). It has been proposed that this binding alters the nucleosome so that the additional factors nuclear factor 1 (NF1) and TATA box binding factor (TFIID) can bind to this promoter and activate transcription (*5*). Similar mechanisms that involve replication-independent nucleosome displacement are illustrated in the activation of the PHO5 promoter in *Saccharomyces cerevisiae* (*6*), and indeed manipulation of nucleosome density in vivo alters the regulation of PHO5 and additional promoters (*7*). These studies and others have led to the proposal that transcription factors and nucleosomes are involved in a dynamic competition for occupancy of regulatory DNA sequences (*3*). We tested the hypothesis that binding by a regulatory factor can directly destabilize nucleosome cores. This mechanism would allow the initiation of occupancy of DNA sequences by regulatory factors in chromatin.

We have previously shown that derivatives of the yeast regulatory protein GAL4 can bind to nucleosomal DNA and transcriptionally activate nucleosomal templates in vitro (*8*, *9*). The binding of GAL4 to nucleosomes is facilitated by the presence of multiple GAL4 sites, which perhaps is relevant to the occurrence of multiple GAL4 sites in naturally found promoters (*9*). To determine the effect of this binding on nucleosome stability, we developed a protocol using purified components so that the protein composition of the resulting complexes could be determined directly. We used polymerase chain reaction (PCR) amplification to generate homogeneous DNA fragments of nucleosome core length (150 bp). The central portion of these fragments contained five GAL4 binding sites that covered 95 bp. These fragments were reconstituted into nucleosome core particles by salt-gradient dialysis with purified HeLa core histones and purified as 11S particles by sedimentation (*10*). Gradient-purified nucleosome core particles reconstituted by this protocol contain equal stoichiometries of the four core histones as illustrated by Coomassie blue–stained gels of the purified reconstituted particles (Fig. 1A) (*11*). Nucleosomes reconstituted by this method also demonstrate a kinetic barrier to micrococcal nuclease digestion at approximately 146 bp and demonstrate the 10-bp deoxyribonuclease (DNase I) digestion ladder characteristic of native nucleosome core particles (*11*).

We first verified that the GAL4 protein could bind to these purified nucleosome cores. We used GAL4-AH, a fusion protein that contains the DNA binding and dimerization domains of GAL4 and an artificial activation domain (*12*). This protein binds to the 150-bp PCR fragment that contains the five GAL4 sites and also binds to the same fragment reconstituted into nucleosome cores as measured by electrophoretic mobility-shift assay (EMSA) (Fig. 1B). The purified DNA fragment was shifted through a series of complexes, which represents the binding of an increasing number of GAL4-AH dimers (from one to five dimers). Similarly, a series of complexes was generated upon GAL4-AH binding to the reconstituted nucleosome cores. The specificity of this interaction was demonstrated by DNase I footprinting of templates under similar conditions (9) and by the lack of formation of these complexes when the same amounts of protein were incubated with PCR-raised templates that did not contain GAL4 binding sites. GAL4 binding to the purified nucleosome cores, relative to binding naked DNA, was inhibited even less than the tenfold reported previously (9) in this assay. This lowered inhibition was a result of the high concentration (above the dissociation constant) of nucleosome cores used to facilitate the protein analysis. The intermediate complexes with increasing numbers of GAL4 dimers bound to the nucleosome cores were decreased in mobility relative to the analogous complexes formed with the naked DNA probe. Although this "supershift" became more subtle with an increasing number of bound GAL4 dimers, it was still apparent when five dimers were bound (9). These data suggest that components of the original nucleosome core (core histones) could still be present in the complexes with bound GAL4 dimers.

To address this question, we took advantage of the fact that there were only five

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proteins (GAL4-AH, H2A, H2B, H3, and H4) in the binding reactions. Reactions similar to those in lanes 5 and 8 of Fig. 1B (nucleosome cores with or without GAL4-AH) were scaled up approximately 40-fold, and the resulting complexes were separated on native acrylamide gels as in Fig. 1B. We determined the protein composition of each complex by excising the entire lane from the mobility-shift gel, running a second dimension of SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (15%), and visualizing the proteins by silver staining (Fig. 1C) (*13*). Protein markers were loaded adjacent to the bottom of the excised lane of the mobility-shift gel in the SDS-PAGE analysis (Fig. 1C). In the absence of GAL4, the nucleosome core particles were detected near the bottom of the mobility shift by the appearance of the four core histones in the second dimension SDS gel. When the nucleosome cores were incubated with GAL4-AH to form five bound GAL4 dimers, the core histones were shifted to a slower mobility in the native gel dimension (Fig. 1C). All four core histones comigrated with GAL4-AH in this complex. This comigration was not fortuitous because further experiments with GAL4 derivatives of varying sizes demonstrated that the nucleosome cores comigrated with the GAL4 protein regardless of the mobility of the ternary complex in the EMSA. The two minor bands flanking H4 (Fig. 1C, bottom panel) were apparently breakdown products of GAL4-AH that retained DNA binding activity. Thus, the binding of GAL4-AH to nucleosome cores resulted in a mobility shift of both the nucleosomal DNA (Fig. 1B) and the histone octamer (Fig. 1C).

These results illustrate that the histone octamer is capable of occupying the same 150 bp of DNA with five dimers of GAL4 derivatives. To investigate whether the binding of GAL4-AH destabilized the nucleosome core, we challenged these complexes with an excess of nonspecific competitor DNA (*14*). The addition of up to 4 μg of plasmid DNA had no effect on the stability of the reconstituted nucleosome cores in the absence of GAL4 (Fig. 2A). In contrast, the GAL4-AH–nucleosome ternary complex (Fig. 2B) dissociated into two complexes upon the addition of increasing concentrations of competitor. One of these (the lower complex) migrated at the position of the original nucleosome cores, which suggests that the GAL4-AH dimers had dissociated. The other complex migrated at the position of GAL4-AH–DNA complexes (lanes 1 and 6 to 8), which suggests that the histone octamer had dissociated.

We analyzed the protein composition of these complexes with a second dimension of SDS-PAGE (*13*) (Fig. 2C). The binding of GAL4-AH to nucleosome cores in the absence of competing DNA resulted in a ternary-shifted complex that contained all four core histones as well as GAL4-AH. However, after the addition of nonspecific competitor DNA, all four of the core histones migrated in the mobility-shift gel to a position that corresponded to the migration of nucleosome core particles. Thus, the addition of competitor DNA resulted in the removal of GAL4-AH and the regeneration of the original nucleosome cores from some of the metastable ternary complexes. In addition, only GAL4 protein was found at the position corresponding to the GAL4-DNA complex on the mobility-shift gel (Fig. 2C). There were no detectable histone proteins comigrating with this GAL4-AH–DNA complex. Thus, the GAL4-AH–DNA complexes were released from some of the GAL4-AH–nucleosome core complexes by the dissociation of all of the histone proteins. The GAL4-AH and histones that were released from the labeled DNA and bound to the nonspecific competing plasmid DNA migrated at the top of the mobility-shift gel (Fig. 2C). We conclude that five GAL4 dimers can occupy the same 150-bp fragment of DNA as the four core histones, but the resulting complex was metastable and dissociated into either GAL4-DNA or nucleosome cores in the presence of nonspecific competing DNA. The generation of GAL4-DNA complexes in these reactions demonstrates the displacement of nucleosome cores as a result of GAL4 binding. At the protein concentrations used in these

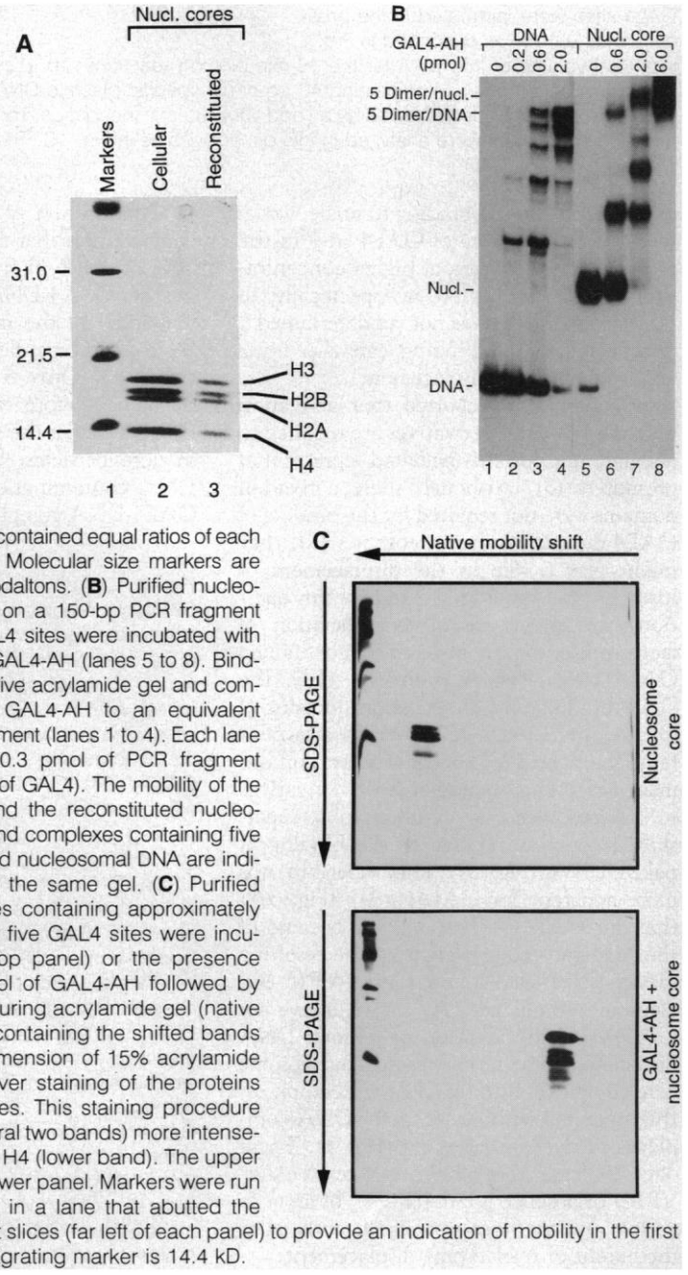

Fig. 1. Binding of GAL4-AH to homogeneous nucleosome cores reconstituted with purified components. (**A**) The protein composition of the gradient-purified nucleosome cores (Nucl. cores) was verified by Coomassie blue staining of SDS-polyacrylamide gels (*11*). Lane 3 shows the histone composition of purified reconstituted nucleosome cores (containing approximately 0.9 μg of DNA). For comparison, 5 μg of cellular nucleosome cores is shown in lane 2. Both the reconstituted and cellular nucleosome cores contained equal ratios of each of the four core histones. Molecular size markers are indicated to the left in kilodaltons. (**B**) Purified nucleosome cores reconstituted on a 150-bp PCR fragment bearing five centered GAL4 sites were incubated with the indicated amounts of GAL4-AH (lanes 5 to 8). Binding was analyzed on a native acrylamide gel and compared to the binding of GAL4-AH to an equivalent amount of naked PCR fragment (lanes 1 to 4). Each lane contained approximately 0.3 pmol of PCR fragment (enough to bind 3.0 pmol of GAL4). The mobility of the naked fragment (DNA) and the reconstituted nucleosome cores (Nucl. core) and complexes containing five GAL4 dimers on naked and nucleosomal DNA are indicated. All lanes are from the same gel. (**C**) Purified nucleosome core particles containing approximately 12 pmol of fragment with five GAL4 sites were incubated in the absence (top panel) or the presence (lower panel) of 180 pmol of GAL4-AH followed by separation on a nondenaturing acrylamide gel (native mobility shift). The lanes containing the shifted bands were run on a second dimension of 15% acrylamide SDS gels followed by silver staining of the proteins from the shifted complexes. This staining procedure stains H2A and H2B (central two bands) more intensely than H3 (top band) and H4 (lower band). The upper band is GAL4-AH in the lower panel. Markers were run in the second dimension in a lane that abutted the bottom of the mobility-shift slices (far left of each panel) to provide an indication of mobility in the first dimension. The fastest migrating marker is 14.4 kD.

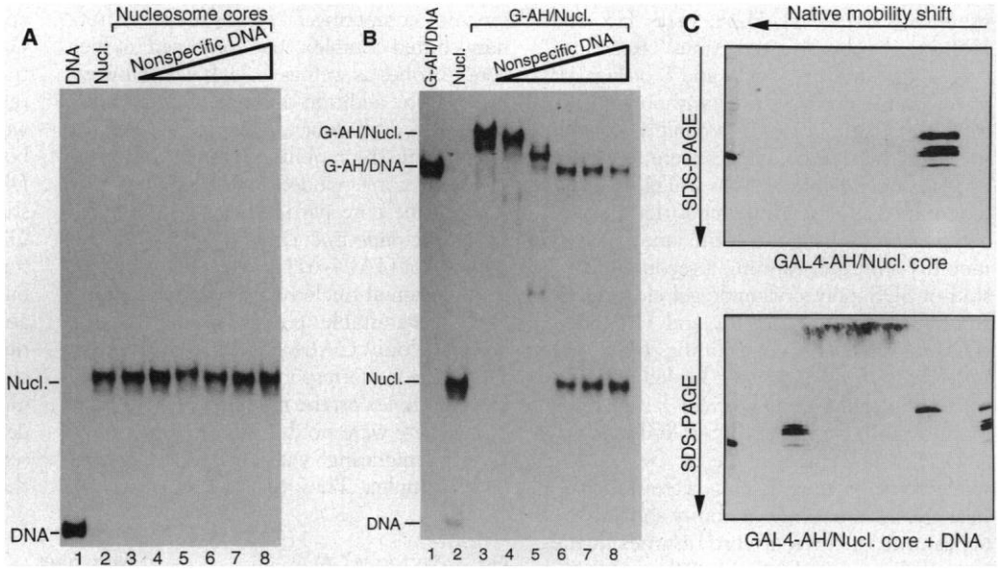

Fig. 2. The dissociation of GAL4–nucleosome core complexes in the presence of nonspecific competitor DNA. Nucleosome cores (approximately 0.3 pmol containing 30 ng of DNA) with five GAL4 sites were incubated in the absence (**A**) or presence (**B**) of 6 pmol of GAL4-AH (G-AH) as in Fig. 1. After the binding reactions, increasing amounts of nonspecific plasmid DNA were added followed by a 30-min incubation. The resulting complexes were analyzed on nondenaturing gels. The amounts of competitor DNA added in each panel were: lane 3, buffer control; lane 4, 40 ng; lane 5, 120 ng; lane 6, 400 ng; lane 7, 1.2 μg; and lane 8, 4 μg. Lane 1, migration of the DNA fragment (A) or the fragment bound to five GAL4-AH dimers (B); lane 2, mobility of the original nucleosome cores in each panel. (**C**) Purified nucleosome core particles with five GAL4 sites were incubated in the presence of GAL4-AH as described in Fig. 1 in a total volume of 203 μl. After the 30-min binding reactions, 10 μl of buffer (top panel) or 10 μl containing 40 μg of nonspecific plasmid DNA (lower panel) was added followed by an additional 30-min incubation. The resulting complexes were analyzed in two dimensions as in Fig. 1C. The prominent band at the far left of each panel is a 14.4-kD marker run adjacent to the bottom of the mobility-shift slice. In the lower panel, the proteins observed indicate (from left to right) nucleosome cores, GAL4-DNA complexes, and proteins competed onto plasmid DNA.

experiments, GAL4 binding to nucleosomes required the presence of GAL4 sites in the probe DNA. However, at higher concentrations GAL4 will bind nonspecifically to nucleosomes. We have not yet determined if nonspecific GAL4 binding can also bring about nucleosome displacement.

We previously reported that activation domains of GAL4 derivatives are required to alleviate nucleosome-mediated repression of promoters (*8*). Although these activation domains were not required for the binding of GAL4 derivatives to nucleosomes (*9*), they might play a role in the displacement of histones after binding. To address this question, we investigated the dissociation of factor-nucleosome complexes that contained GAL4(1–94), which contains only the DNA binding and dimerization domains of GAL4, and GAL4-VP16, which also contains the potent VP16 acidic activation domain (*15*). The binding of these derivatives to nucleosomes also resulted in a supershifted complex relative to the binding of naked DNA (Fig. 3). This supershift was more apparent for GAL4(1–94) (Fig. 3A) than for GAL4-VP16 (Fig. 3B) because of the high molecular weight of the resulting GAL4-VP16–DNA and GAL4-VP16–nucleosome complexes. As shown above for GAL4-AH, the addition of plasmid DNA dissociated these ternary factor–nucleosome core complexes into two different complexes that migrated with either factor-DNA complexes or nucleosome cores (Fig. 3). These data indicate that the presence (GAL4-VP16) or absence [GAL4(1–94)] of an activation domain does not significantly alter the extent of nucleosome displacement.

Quantitation of these experiments (*14*) demonstrates that after competitions, 40 to 50% of the PCR fragments were contained in the GAL4-DNA complexes with the remainder of the fragments in the nucleosome cores regardless of the GAL4 derivative used. Only 5 to 10% of the initial fragment (before factor binding) was free DNA, whereas the remainder was contained in core particles. Subtraction of this free DNA contamination from the fraction of GAL4-DNA complexes apparent after competition indicates that 35 to 45% of the fragment that began in nucleosome cores ended in GAL4-DNA complexes. Thus, approximately 40% of the original nucleosome cores are displaced by factor binding when nonspecific competing DNA is added to the reaction.

The observation that the transcriptional activation domain was not required for destabilization implies that this destabilization is independent of the mechanism used by GAL4 derivatives to prevent nucleosome-

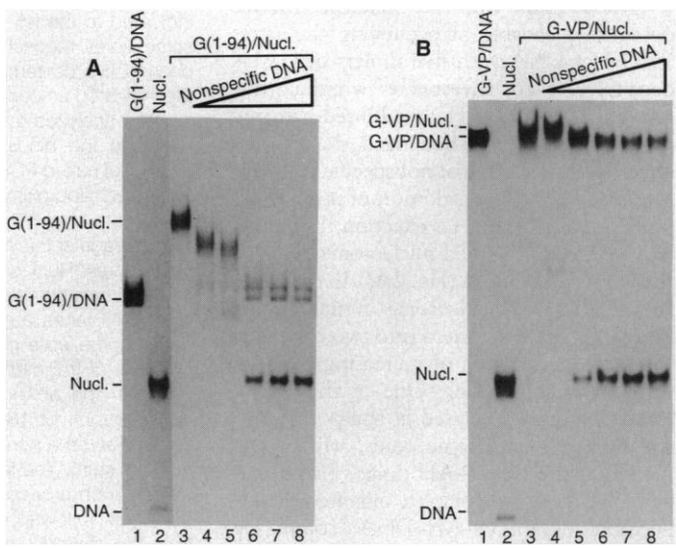

Fig. 3. Dissociation of GAL4–nucleosome core complexes does not require an activation domain. Nucleosome cores with five GAL4 sites were incubated in the presence of GAL4(1–94) [G(1–94)], which contains only the binding and dimerization domains (**A**), or GAL4-VP16 (G-VP), which also contains the potent VP16 acidic activation domain (**B**), followed by the addition of nonspecific plasmid DNA as in Fig. 2. The resulting complexes were analyzed on nondenaturing gels. Both GAL4(1–94)–nucleosome core complexes (A) and GAL4-VP16–nucleosome core complexes (B) dissociated into either GAL4-DNA complexes or nucleosome cores. All the lanes in (A) were from the same gel but the order was changed in the figure to correspond to that in (B) and in Fig. 2, A and B. Lane 1, migration of the GAL4(1–94)–DNA (A) or the GAL4-VP16–DNA (B) complexes; all other lanes are as in Fig. 2, A and B.

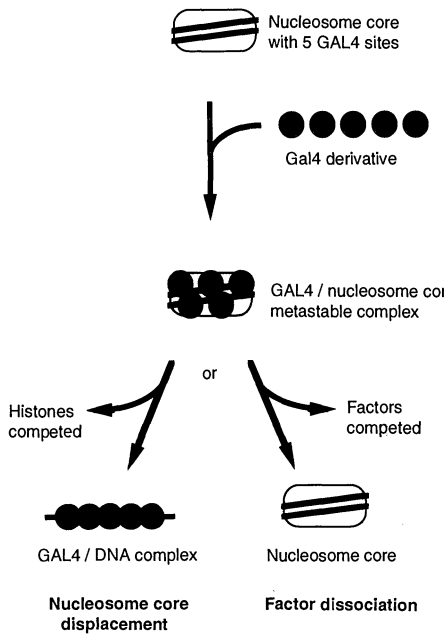

Fig. 4. Diagram of the function of GAL4 derivatives in nucleosome displacement. Up to five dimers of GAL4 derivatives (each dark circle represents a dimer of GAL4) were able to bind to GAL4 sites contained in a nucleosome core particle (rectangular shapes in which the cross stripes represent DNA). The binding of GAL4 dimers generated a ternary complex that contains the GAL4 proteins, the histone octamer, and the nucleosomal DNA. The ternary complex was metastable and readily dissociated in the presence of competitor. When the GAL4 derivatives were competed, the original nucleosome core, including all four core histones, was released. When the histones were competed, a GAL4-DNA complex was generated.

mediated repression of the basal transcription complex, as that function absolutely requires an activation domain (*8*). Rather, our data indicate that this displacement is mediated primarily by the nature of the DNA binding domain as is the initial ability of factors to bind nucleosomes (*9*). Thus, the structure and function of different DNA binding motifs may be particularly relevant to mechanisms used to initiate occupancy of promoter and enhancer sequences by regulatory factors in chromatin. After binding, the activation domains of regulatory factors play a crucial role in a second competition for occupancy of the core promoter (the TATA box and transcription start site) between nucleosomes and general transcription factors and RNA polymerase II (*8*).

Our data demonstrate a destabilization of the core nucleosome as a direct consequence of DNA binding by GAL4 (Fig. 4). It is important to note that we have identified that destabilization by observing a GAL4-enhanced displacement of the nucleosomal core histones onto nonspecific plasmid DNA. It is unclear what mechanistic role the nonspecific DNA plays in histone displacement. One possibility is that the dissociation rates for both GAL4 and the nucleosomal core histones are increased in the ternary complex and that the nonspecific DNA simply traps molecules in solution that have dissociated. A second possibility is that the nonspecific DNA plays an active role in directly removing histones from the ternary complex. In this latter instance, the dissociation rates for the core histones to solution might remain unchanged upon GAL4 binding; however, the accessibility of core histones to direct transfer onto competing DNA would be enhanced (by a change in conformation, for example). This raises the possibility that in the nucleus other factors might be involved in removing histones from factor-nucleosome complexes. For example, such a role might be played by acidic chromatin assembly factors that bind histones [nucleoplasmin, N1, or CAF-1 (*16*)]. Other candidate proteins that might provide such a function include certain of the SWI and SNF proteins in *S. cerevisiae* and the Brahma protein in *Drosophila* (*17*). These proteins are required for the activation of numerous genes, do not contain readily apparent DNA binding motifs, but do contain acidic domains that might interact with basic histones. Moreover, some *swi*$^-$ mutations can be suppressed by mutations in histone genes (*17*).

The binding of histone H1 to nucleosome core particles provides a second layer of transcription repression in vitro (*18*) and might be expected to further modulate the interactions of factors with nucleosome cores. Preliminary experiments suggest that H1 binding to nucleosome cores does not dramatically change the ability of GAL4 derivatives to bind sites within the core particle (*19*). However, it remains to be determined if H1 reduces the displacement of histones from factor-nucleosome complexes.

Processes such as hormonal induction, cell differentiation, and development all require the function of promoters and enhancers that were previously complexed with nucleosome cores and histone H1 (*1–3*, *18*, *20*). Factor binding and consequent nucleosome core destabilization and displacement represent initial steps in the establishment of occupancy of these regulatory sequences in chromatin in the absence of replication and chromatin reassembly (dynamic competition) (*3*). Assuming that the capabilities we have characterized here are not unique to GAL4, our data indicate that one class of regulatory factors can directly participate in the displacement of underlying nucleosome cores by means of a function of their DNA binding domain. Other regulatory factors, such as NF1 and heat shock factor, appear to be incapable of binding their sites on nucleosomal DNA (*5*, *9*). The destabilization of nucleosomal cores by factors such as GAL4 might be an important step in the restructuring of nucleosomal DNA in enhancers and promoters to allow occupancy by additional factors, the binding of which would otherwise be inhibited by nucleosome structures.

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10. HeLa core histones were purified by hydroxylapatite chromatography [A. Stein and M. Mitchell, *J. Mol. Biol.* **203**, 1029 (1988); J. L. Workman, I. C. A. Taylor, R. E. Kingston, R. G. Roeder, *Methods Cell Biol.* **35**, 419 (1991)]. The PCR fragment used for reconstitution was amplified from plasmid pG5H (*8*) in Boehringer Mannheim buffer with the following primers: UG5H, 5′-CCAAGCTTGCATGCCTGCAG and DG5H, 5′-CCCCTGGGCTTTTATAAGTCG. The products of 20 100-μl amplification reactions (which included 3 μCi of [α-^{32}P]deoxycytidine triphosphate each) were pooled, extracted with phenol and chloroform, and precipitated with ethanol. The DNA was resuspended in 50 μl of 10 mM tris (pH 8.0) and 1 mM EDTA. For nucleosome reconstitution, 50 μl of concentrated PCR fragment (approximately 150 μg) was mixed with core histones (100 μg) in a total volume of 100 μl with final concentrations of 2 M NaCl, 10 mM Hepes (pH 7.5), 5 mM tris, 2 mM 2-mercaptoethanol, 1 mM EDTA, and bovine serum albumin (BSA) (1 mg/ml). This mix was dialyzed for 2 hours against 200 ml of a solution of 2 M NaCl, 10 mM Hepes (pH 7.5), 2 mM 2-mercaptoethanol, and 1 mM EDTA. The dialysis buffer was diluted by the slow dropwise addition of 600 ml of the same buffer without NaCl over 15 hours at 4°C with a peristaltic pump. This lowered the salt concentration to 0.5 M. The reconstituted solutions were then dialyzed for 2 hours against the same buffer with 250 mM NaCl. To purify the reconstituted core particles from free and precipitated DNA, we loaded the samples onto 5 to 20% linear sucrose gradients that contained 100 mM KCl, 10 mM Hepes (pH 7.5), and 1 mM EDTA and centrifuged them for 20 hours at 85,000*g* in a Beckman SW55 rotor. The 11*S* peak fractions were collected from the gradients, divided into small aliquots, frozen in liquid nitrogen, and stored at −80°C. Aliquots were thawed only once before use.
11. The silver-staining procedure used in the two-dimensional gel analysis (*13*) often stains H2A and H2B more intensely than H3 and H4 at subsaturating histone concentrations [for example, see A. Shimamura, D. Tremethick, A. Worcel, *Mol. Cell. Biol.* **8**, 4257 (1988); P. B. Becker and C. Wu, *ibid.* **12**, 2241 (1992)]. To verify that the gradient-purified nucleosome cores contained equal stoichiometries of each core histone, we ran 150 μl of the gradient-purified material (approximately 0.9 μg) on a 15% acrylamide SDS-protein gel, adjacent to H1-depleted cellular nucleosome cores, and stained the material with Coomassie blue. Both the reconstituted and cellular nucleo-

some cores contained equal amounts of the four core histones (Fig. 1A). We prepared cellular H1-depleted nucleosome cores by solubilizing nucleosomes from cellular chromatin by micrococcal nuclease digestion followed by gel filtration chromatography in the presence of 0.6 M NaCl. Gradient-purified nucleosome cores reconstituted by salt dialysis also resemble cellular nucleosome cores with regard to nuclease digestions. Digestion of the 150-bp PCR fragment nucleosomes, which were labeled throughout during amplification, with DNase I showed a 10-bp ladder between cutting sites. DNase I digestion of end-labeled restriction fragments (which contained the same sequences) after similar reconstitution into nucleosome cores showed a strong 10-bp digestion pattern not apparent on naked DNA, which demonstrates rotational phasing of the DNA on the histone octamer surface (H. Chen and J. L. Workman, unpublished data). The PCR fragments used (150 bp) were close to nucleosome core length (146 bp), which made micrococcal nuclease digestion analysis of these nucleosome cores difficult. However, nucleosome cores reconstituted with 180-bp PCR fragments (from the same plasmid but extended 15 bp on each end) showed a digestion intermediate with micrococcal nuclease of approximately 146 bp (*19*). Thus, the reconstituted nucleosomes resemble cellular nucleosome cores by several criteria.

12. GAL4 derivatives were purified according to Chasman *et al.* (*15*). For the binding reactions shown in Fig. 1B, nucleosome cores that contained approximately 0.3 pmol of DNA (with five GAL4 sites) or the unreconstituted PCR fragment (diluted in the appropriate mixture of the sucrose gradient solutions) in 5 μl was mixed with the indicated amounts of GAL4-AH, which were in 3 μl of BC100 buffer [20% glycerol, 100 mM KCl, 20 mM Hepes (pH 7.9), and 0.2 mM EDTA] with BSA (1 mg/ml), 10 mM 2-mercaptoethanol, and 10 μM $ZnCl_2$ (*8*). Reactions (8 μl total volume) were incubated for 30 min at 30°C and then analyzed on 4% acrylamide (29:1, acrylamide:bis), 0.5× TBE [45 mM tris-borate (pH 8.0) and 1 mM EDTA] gels.
13. For the two-dimensional gels to analyze protein composition (Figs. 1C and 2C), 200 μl of reconstituted nucleosome cores (12 pmol) were incubated for 30 min at 30°C with 180 pmol of GAL4-AH (added as undiluted protein in 3 μl). In the experiment shown in Fig. 2C, 40 μg of nonspecific plasmid DNA was added followed by an additional 30-min incubation at 30°C. After the binding and competition reactions, the samples were separated on native acrylamide gels (4%) as described above. The full-length lanes that contained the samples were cut out of the gel and placed over a short stacking gel (3 cm) above a SDS-polyacrylamide separating gel (15%). Low molecular weight markers (Bio-Rad) were loaded in a marker lane placed directly against the bottom of the native gel slices. The gels were electrophoresed until the bromphenol blue dye (Sigma) ran off the bottom. After electrophoresis, the stacking gels were cut off, and the separating gels were stained with silver [W. Wray, T. Boulikas, V. P. Wray, R. Hancock, *Anal. Biochem.* **118**, 197 (1981)] with the following modifications. The gels were stained in solution C for 30 min and developed until the entire gel became very dark to ensure staining and development of protein in the center of the gels. The gels were cleared (destained) in Kodak Rapid Fix (undiluted) until the background staining was completely removed, allowing visualization of the stained proteins.
14. The experiments shown in Fig. 2, A and B, and in Fig. 3, A and B, were similar to that in lane 8 of Fig. 1B. However, after the binding reactions the indicated amounts of nonspecific competitor DNA or buffer [10 mM tris and 1 mM EDTA (pH 8.0)] were added in a volume of 3 μl. After an additional 30-min incubation at 30°C, the samples were analyzed on acrylamide gels (4%) as described above. The nonspecific plasmid DNA was pNOTM, a plasmid bearing the SV40 replication origin, the major-late promoter of adenovirus, and the tetracycline resistance gene of pBR322 in pUC13. Other nonspecific plasmid DNAs functioned identically. Mobility-shift gels were dried and exposed to film at −80°C. Dried gels were also counted on a Betascope blot analyzer (Betagen) followed by quantitation of the bands.
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Identification of a Second Pseudoautosomal Region Near the Xq and Yq Telomeres

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The telomeres of Xq and Yq have been observed to associate during meiosis, and in rare cases a short synaptonemal complex is present. Molecular cloning of loci from Xqter and Yqter has revealed that their sequence homology extends over 400 kilobases, which suggests the possibility of genetic exchange. This hypothesis was tested by the development of two highly informative microsatellite markers from yeast artificial chromosome clones that carried Xqter sequences and the following of their inheritance in a set of reference pedigrees from the Centre d'Etude du Polymorphisme Humain in Paris, France. From a total of 195 informative male meioses, four recombination events between these loci were observed. In three cases, paternal X alleles were inherited by male offspring, and in one case a female offspring inherited her father's Y allele. These data support the existence of genetic exchange at Xq-Yq, which defines a second pseudoautosomal region between the sex chromosomes.

The human Y chromosome serves two known primary functions. It carries sequences on the short arm coding for a testis-determining factor (*1–3*), which is required to initiate male development. It also provides a pairing partner for the X chromosome during meiosis. Koller and Darlington (*4*) first proposed the existence of differential (sex-specific) and common [pairing, later termed pseudoautosomal (*5*)] segments between the sex chromosomes to explain their cytogenetic association in rat spermatocytes. During the early stages of prophase I, the sex chromosomes condense and associate to form a heterochromatic mass known as the XY body (*6*). The formation of a synaptonemal complex is restricted to the short arm pseudoautosomal region, but the extent of nonhomologous synapsis between the X and the Y chromosomes is variable and could extend into Yq (*7*). A secondary end-to-end association between Xq and Yq is occasionally observed in meiotic spreads, and in rare cases a short synaptonemal complex is present (*8*). The sex chromosomal region that undergoes homologous pairing in humans occupies the most distal segments of the X and Y short arms. The cloning of sequences from the pairing region provided molecular evidence for recombination between distal Xp and Yp (*9*). A gradient of recombination can be observed in males between pseudoautosomal markers and the sex-determining locus, with the most telomeric marker segregating independently of sex (*10*).

In addition to the pseudoautosomal segment, the X and Y chromosomes share several regions of homology that are not known to undergo legitimate recombination during male meiosis (*2*, *11*). The isolation of DNA segments corresponding to loci DXYS61 (isolated from a flow-sorted Y library) (*12*) and DXYS64 (iden-

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