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Remodeling the chromatin structure of a nucleosome array by transcription factor-targeted *trans*-displacement of histones

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To investigate mechanisms of chromatin remodeling, we have examined the fate of a single nucleosome core within a spaced nucleosome array upon the binding of transcription factors. GAL4 binding to this nucleosome within an array resulted in the establishment of DNase I hypersensitivity adjacent to the bound factors mimicking *in vivo* hypersensitive sites. The positions of adjacent nucleosomes were unchanged upon GAL4 binding, suggesting that histone octamer sliding did not occur. In addition, novel assays were used to determine whether the histones remained present during factor binding. GAL4 binding alone did not independently dislodge or move the underlying histones, which remained in a ternary complex with the bound GAL4. GAL4 binding did, however, specifically predispose the histones contained in this nucleosome to displacement *in trans*. Addition of the histone binding protein, nucleoplasmin, mediated the displacement of the core histones in the GAL4-bound nucleosome, resulting in the formation of a nucleosome-free region. These data illustrate *trans*-displacement of histones as one mechanism for transcription factor-targeted generation of a nucleosome-free region in chromatin. They also illustrate the limitations of nuclease digestions in analyzing changes in chromatin structure and provide important mechanistic details beyond the basic phenomenon of DNase I hypersensitivity.

Keywords: chromatin/DNase hypersensitive sites/ histones/nucleosome/transcription factors

Introduction

Transcriptional regulation requires the coordinated binding of a range of specific and basal transcription factors to their target sites. However, *in vivo*, the bulk of the genome is assembled into chromatin which creates a barrier to factor binding. It has become apparent that the remodeling of chromatin structure in such a way as to increase the ability of transcription factors to recognize the appropriate regulatory elements is a key step in the regulation of expression from chromosomal genes. In eukaryotic genomes, remodeled chromatin has been detected classically as sites of DNase I hypersensitivity (DHS sites) (Elgin, 1988; Gross and Garrard, 1988). The formation of these hypersensitive sites can either precede the activation of a gene (persistent or preset DHSs) or they can form upon the inducible binding of transcription factors (reviewed in Svaren and Horz, 1993; Workman and Buchman, 1993; Wallrath *et al.*, 1994). Persistent or preset DHSs might result from transcription factor binding their target sites prior to chromatin assembly during DNA replication. Indeed, such a mechanism is supported by *in vitro* experiments illustrating replication-dependent activation of the developmentally regulated globin genes in synthetic nuclei (Barton and Emerson, 1994). However, it is also clear that in other instances, chromatin remodeling can occur independently of DNA replication. *In vivo*, changes in the chromatin structure of the PHO5 promoter occur independently of DNA replication (Schmid *et al.*, 1992) and *in vitro* the binding of constitutive factors subsequent to chromatin assembly is able to generate hypersensitive sites in arrays of nucleosomes (reviewed in Becker, 1994; Tsukiyama *et al.*, 1994; Varga-Weisz *et al.*, 1995). Thus, it is also possible that persistent or preset DHSs may form in a manner similar to that achieved by many inducible factors where transcription factor binding promotes direct remodeling of pre-assembled chromatin structures.

The appearance of DNase I hypersensitive sites in chromatin indicates a region in which canonical nucleosomes are absent. In principle there are at least three mechanisms by which the binding of transcription factors might generate these sites. (i) The binding of transcription factors to a nucleosome might disrupt a nucleosome and alter the sensitivity of the nucleosomal DNA to DNase I, but might not otherwise move the underlying histones. (ii) Transcription factor binding might induce sliding of the histone octamer along the DNA *in cis* to move the octamer off the recognition elements. (iii) Transcription factor binding might induce displacement of the histones from the DNA *in trans*. Each of these mechanisms could apply in certain circumstances and there are experimental observations consistent with each of these possibilities.

Transcription factor binding is not inconsistent with the retention of histones on the same region of DNA. *In vivo* cross-linking studies of the mouse mammary tumor virus (MMTV) promoter indicate that when a nucleosome is disrupted by glucocorticoid receptor (GR) binding, the core histones appear to remain present (Bresnick *et al.*, 1992). Moreover, *in vivo* footprinting studies suggest that numerous factors can bind the MMTV promoter in the continued presence of the underlying nucleosome (Truss *et al.*, 1995). These observations are consistent with numerous reports illustrating that the binding of many different transcription factors (i.e. GR, GAL4-derivatives, Sp1, USF, NF-κB, Myc/Max, TFIIIA) to nucleosomal DNA in purified *in vitro* systems results in the formation of ternary complexes containing transcription factors, DNA and histones (reviewed in Owen-Hughes and Workman, 1994). While the nuclease sensitivity of these complexes may be altered, histones clearly can occupy the same sequence of DNA as bound transcription factors.

However, in the presence of multiple simultaneously bound factors, these ternary complexes are unstable, favoring the loss of histones from the factor-bound sequences (Workman and Kingston, 1992). In principle, histones could be removed from such ternary complexes by sliding *in cis* or displacement *in trans*.

Sliding of histone octamers on DNA is suggested by several *in vitro* experiments. Early experiments illustrated that histone octamers are able to relocate onto other regions of DNA *in cis*; an observation consistent with octamer sliding (Beard, 1978). An inherent localized mobility of the histone octamer on DNA has been demonstrated *in vitro* (Pennings *et al.*, 1991; Meersseman *et al.*, 1992; Ura *et al.*, 1995) which may result in multiple frames of nucleosome positioning (Fragoso *et al.*, 1995; Roberts *et al.*, 1995). In addition, Becker and colleagues have described repositioning of nucleosomes in the presence of activities in *Drosophila* embryo extracts which enhances accessibility to GAGA factor or even restriction enzymes (Varga-Weisz *et al.*, 1995; Wall *et al.*, 1995). The most straightforward interpretation of these data is that the GAGA factor and restriction enzymes take advantage of histone octamer mobility *in cis* to gain access to their recognition sites.

Transcription factor-induced displacement of histones from single nucleosomes has been demonstrated *in vitro*. Binding of multiple GAL4 derivatives to a nucleosome core results in displacement of the histones onto competing DNA or histone binding proteins (Workman and Kingston, 1992; Chen *et al.*, 1994; Walter *et al.*, 1995). While these studies illustrate the loss of histones from a transcription factor-bound fragment of DNA, they do not rule out the possibility that histone displacement might have occurred by the histone octamer (or the H3/H4 tetramer) sliding off the end of the nucleosome-length fragment of DNA. Within a continuous linear array of nucleosomes, such a mechanism would not lead to *trans*-displacement of histones but would instead lead to octamer sliding. Thus, it is crucial to establish whether *trans*-displacement of histones is possible within the more physiological context of a nucleosomal array.

To study the fate of the histone octamer during transcription factor-induced structural remodeling of nucleosome arrays, we have developed novel assays of chromatin structure using a system in which all the components are highly purified. We find that transcription factor binding occurs similarly within the context of a nucleosomal array and on single nucleosomes. Furthermore, in both contexts GAL4-derivative binding predisposes the factor bound nucleosome to histone displacement *in trans*.

Results

Reconstitution of a nucleosome array containing transcription factor binding sites with purified components

We sought to analyze transcription factor binding and histone loss from nucleosome arrays in a purified system so that observed activities could be attributed to defined components. To reconstitute nucleosome arrays in such a system, tandem repeats of the Sea Urchin 5S rRNA gene were employed. These repeating sequences have been demonstrated previously to position nucleosomes within a continuous array after nucleosome reconstitution with purified components (Simpson *et al.*, 1985). Tandem repeats of the sea urchin 5S RNA gene nucleosome positioning sequence were cloned on either side of a DNA sequence containing transcription factor binding sites. This construct is illustrated schematically in Figure 1. It consists of five 208 bp nucleosome positioning sequences on either side of a DNA fragment containing five GAL4 sites. The 2343 bp DNA insert containing the ten 5S repeats and the GAL4 sites was excised from pG5-208-10 by digestion with *Mlu*I and *Pvu*II, end-labeled and reconstituted into a nucleosome array by histone octamer transfer (Côté *et al.*, 1995).

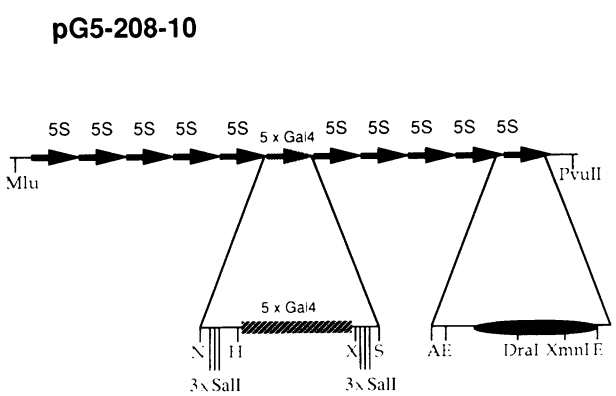

Fig. 1. The nucleosome array construct. Diagram of the DNA fragment that was used to reconstitute an 11 nucleosome array. The 2343 bp *Mlu*I–*Pvu*II fragment excised from pG5-208-10 contains 2×5 direct repeats of the sea urchin 5S rDNA nucleosome spacing sequences. Between the two repeats is the nucleosome-length fragment bearing five GAL4 binding sites. This fragment is flanked by triple *Sal*I restriction endonuclease sites. Other restriction endonuclease sites indicated are *Dra*I, *Xmn*I, *Eco*RI (E) and *Ava*I (A) in the 5S rDNA and *Nhe*I (N), *Hin*dIII (H), *Xba*I (X) and *Spe*I (S) in the GAL4-site nucleosome fragment.

To test the level of reconstitution, the assembled array was subjected to micrococcal nuclease (MNase) digestion and agarose gel electrophoresis. The results are shown in Figure 2A. There is a repeating pattern of cleavage and protection on the reconstituted array (lanes 5–8) that is distinct from that observed on naked DNA (lanes 1–4). Furthermore, the regions protected align well with the positions that nucleosome cores have previously been reported to adopt on tandem repeats of the 208 bp nucleosome positioning sequences (Dong *et al.*, 1990; Pennings *et al.*, 1991; Simpson *et al.*, 1985). A similar result was observed when the reconstituted nucleosome array was digested with DNase I (Figure 2B). A nucleosome repeat was observed following DNase I digestion of the reconstituted array (lane 2) which was not apparent upon digestion of the naked DNA fragment (lane 3). Nucleosome protection from DNase I cleavage was also observed over the region of the GAL4 sites (lane 2), the location of which is illustrated by the GAL4-AH footprint on the naked DNA (lane 4). Both MNase and DNase I digestion reveal a region of ~165 bp that includes the GAL4 binding sites and is protected after assembly with nucleosomes. This suggests that a positioned nucleosome is assembled onto this DNA. This was confirmed by releasing this central DNA fragment from the array by digestion with *Sal*I and studying the mobility of this DNA during native gel shifts (for example see Figure 6). Thus,

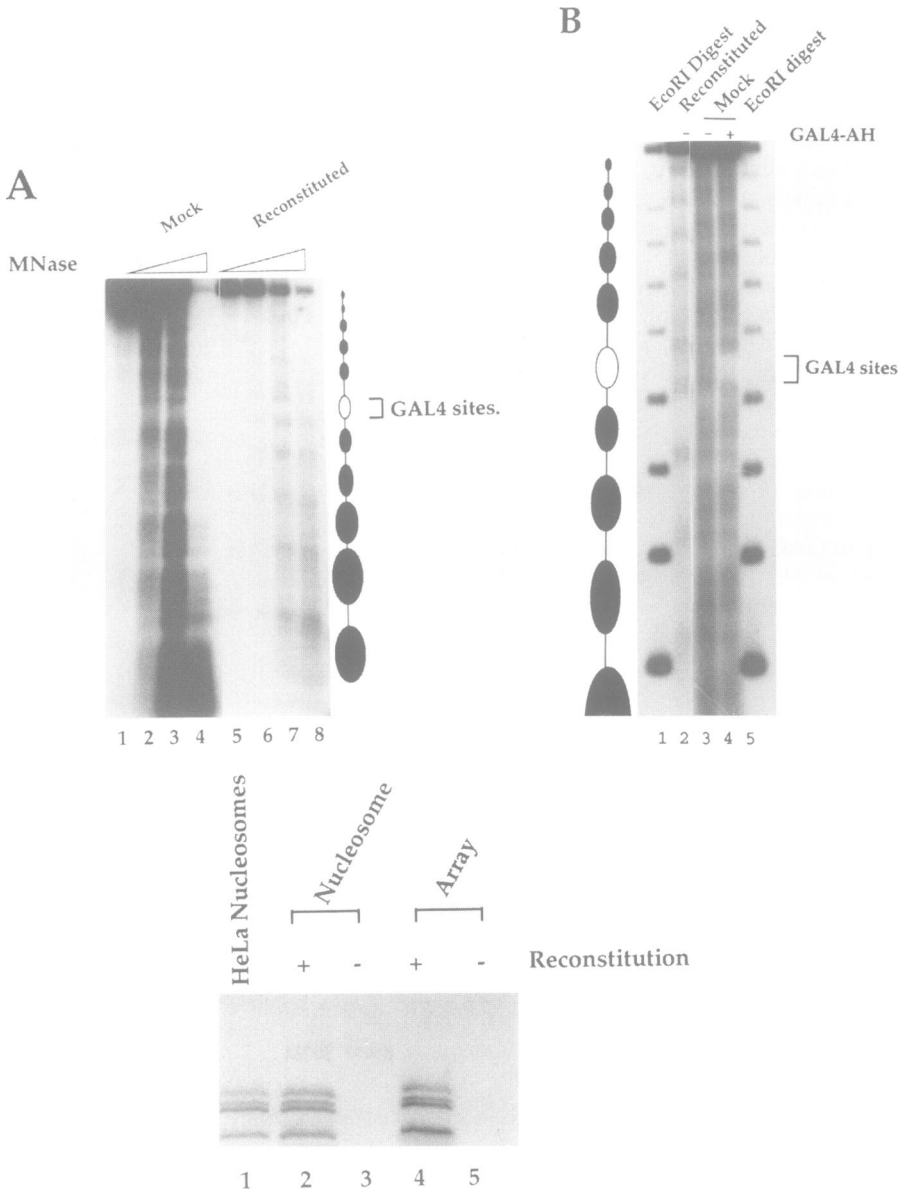

Fig. 2. Analysis of the reconstituted nucleosome arrays. (**A**) Micrococcal nuclease digestion of mock reconstituted naked DNA (lanes 1–4) and nucleosome reconstituted (lanes 5–8) 2343 bp array fragment. The positions of the 5S rDNA nucleosomes on the array construct are indicated on the right as well as the location of the nucleosome bearing the five GAL4 sites. The amounts of MNase (Sigma) used in each lane are as follows: (lane 1, 5×10^{-4} U; lanes 2 and 5, 5×10^{-3} U; lanes 3 and 6, 5×10^{-2} U; lanes 4 and 7, 5×10^{-1} U; lane 8, 5 U). (**B**) DNase I digestion of nucleosome reconstituted (lane 2) and mock reconstituted (lanes 3 and 4) 2343 bp array fragments. The locations of nucleosomes indicated by the digestion are illustrated on the left and agree with those in A. The 5S rDNA repeats are indicated by the partial *Eco*RI digest of the array fragment (lanes 1 and 5). The location of the GAL4 sites is indicated by the footprint in lane 4 where the mock reconstituted sample was bound by GAL4-AH. (**C**) Protein analysis of nucleosome-reconstituted samples by SDS–PAGE. The nucleosome array fragment (lanes 4 and 5) and a mononucleosome-length DNA fragment (lanes 2 and 3) bearing the five GAL4 sites was either reconstituted into nucleosome cores (+) or mock reconstituted (–), purified from donor nucleosomes using paramagnetic beads and analyzed on a 15% polyacrylamide gel. All four core histones were present on each fragment after nucleosome reconstitution (lanes 2 and 4) but not following mock reconstitution (lanes 3 and 5). Lane 1 illustrates the histone composition of the donor HeLa nucleosome cores used for nucleosome reconstitution which was identical to that of the reconstituted nucleosome cores and nucleosome arrays.

both MNase and DNase I digestions revealed the presence of an array of nucleosomes formed on the pG5-208-10 fragment.

To confirm that the nucleosome array was reconstituted with all four core histones, we purified the reconstituted array and analyzed its protein composition (Figure 2C). Lane 1 of this silver-stained SDS gel illustrates the histone composition of the donor HeLa nucleosome cores used as a source of histone octamers for reconstitution. All four core histones were apparent in the donor nucleosomes. A mononucleosome-length fragment, bearing the five GAL4 sites, and the entire 2343 bp array fragment were reconstituted into nucleosome cores, purified from the donor nucleosomes and analyzed for protein composition. These reconstituted DNA fragments contained equal amounts of the four core histones (lanes 2 and 4). Moreover, the presence of the core histones on the reconstituted mononucleosome and array fragments was dependent upon the assembly of nucleosomes by dilution transfer. When these DNA fragments were mixed with the donor nucleosome

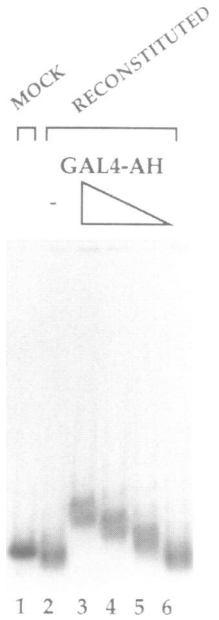

Fig. 3. Gel-shift analysis of GAL4-AH binding to the reconstituted nucleosome arrays. The 2343 bp array fragment was mock reconstituted (naked DNA) (lane 1) or reconstituted into nucleosome cores (lane 2) and run on a 1% native agarose gel. Note that nucleosome reconstitution increases electrophoretic mobility of the array fragment (compare lanes 1 and 2). The nucleosome reconstituted arrays were also incubated in the presence, on increasing concentrations, of GAL4-AH prior to resolution on the agarose gel (lane 3, 150 nM; lane 4, 50 nM; lane 5, 17 nM; lane 6, 5 nM). GAL4-AH binding to the array is detected by the reduction of electrophoretic mobility in the presence of GAL4-AH (i.e. compare lanes 2 and 3).

cores subsequent to dilution (mock reconstitutions), histones did not purify with these DNA fragments (lanes 3 and 5). Thus, in this purified system histones did not transfer between DNA fragments at physiological ionic strength in the absence of bound transcription factors.

Binding of transcription factors to a nucleosome within an array

To test the ability of GAL4-AH to bind the GAL4 sites contained within the nucleosome array, we first performed mobility shift assays with the entire nucleosome array (Figure 3). When the reconstituted nucleosomal array was subject to native agarose gel electrophoresis it was found to have a slightly faster mobility than the same fragment as free DNA (lanes 1 and 2). This is consistent with previous observations where the compaction occurring upon assembly into chromatin was found to be sufficient to counter the increase in molecular weight attributable to the histones (Simpson *et al.*, 1985). Importantly, the reconstituted array migrated as a discrete species, suggesting that most molecules contain a similar number of nucleosomes. Figure 3 also shows the effect of GAL4-AH upon the mobility of the reconstituted nucleosomal array (lanes 3–6). In the presence of increasing concentrations of GAL4-AH, the mobility of the array was reduced, indicating that GAL4-AH was able to bind the array of nucleosomes. The concentrations of GAL4-AH required to bind the array were similar to those required for binding to single nucleosomes bearing five GAL4 sites, suggesting that GAL4-AH binding occurred similarly in both contexts.

The mobility shift gel shown in Figure 3 also illustrates that all of the nucleosome arrays were efficiently bound by GAL4-AH, demonstrating that binding was not limited to a small subfraction of reconstituted samples. However, in order to confirm that GAL4-AH binding occurred via its recognition sites, DNase I digestion assays were again employed.

Figure 4A shows the effect of GAL4-AH binding upon the DNase I digestion pattern of the entire array construct. In the absence of GAL4-AH (lanes 1–3) a nucleosome repeat pattern was observed as in Figure 2B. However, in the presence of GAL4-AH, a region of protection at the GAL4 sites flanked by regions of hypersensitivity was observed (lanes 4–9). This low resolution footprint illustrates sequence-specific binding of GAL4-AH to its sites within the context of the nucleosomal array. It is also notable that while the protection and hypersensitivity occurs at the location of the nucleosome core bearing the GAL4 sites, there are no major changes in the digestion pattern outside the region occupied by the central nucleosome. Thus, the binding of GAL4-AH did not disrupt or effect the position of the adjacent nucleosomes in the array. The same DNase I digestion assay of GAL4-AH binding was performed in the presence of the histone binding protein, nucleoplasmin (Figure 4A; lanes 10–18). This protein has been shown to facilitate histone displacement upon transcription factor binding (Chen *et al.*, 1994; Walter *et al.*, 1995), thus we reasoned that it might have a more dramatic effect on the chromatin structure of the array during GAL4-AH binding. The presence of nucleoplasmin was found to cause a small increase in the overall digestion of the array (Figure 4A; compare lanes 3 and 12). Moreover, only a small increase in the hypersensitivity flanking the occupied GAL4 sites was observed (Figure 4A; compare lanes 5, 6, 8 and 9 with 13, 14, 16 and 17, but see below).

Alteration of the chromatin structure of a nucleosome array as a consequence of transcription factor binding

The protection of the GAL4 sites and adjacent hypersensitivity to DNase I seen upon GAL4-AH binding to the nucleosome array, is reminiscent of DNase I digestion patterns observed earlier upon GAL4 derivative binding to five GAL4 sites in nucleosome reconstituted DNA (Workman *et al.*, 1991a; Pazin *et al.*, 1994). This pattern of protection and hypersensitivity at the GAL4 sites has been interpreted as reconfiguration of nucleosomes to move them to positions adjacent to the GAL4 sites (Pazin *et al.*, 1994). However, interpretation of these patterns is hampered by the fact that similar protection and hypersensitivity was observed upon GAL4-derivative binding to naked DNA (Pazin *et al.*, 1994). Thus, detection and interpretation of potential changes in nucleosome structure or location by DNase I digestion is limited by the changes in DNase I cleavage (protection and hypersensitivity) resulting from the interaction of the transcription factor with the DNA. Thus, to investigate the fate of the nucleosome containing the GAL4 sites upon GAL4-AH binding to the nucleosome array, we have used an oligonucleotide competition assay. GAL4-AH was first bound to the array as in Figure 2A, but subsequently removed by competition with a double stranded oligo-

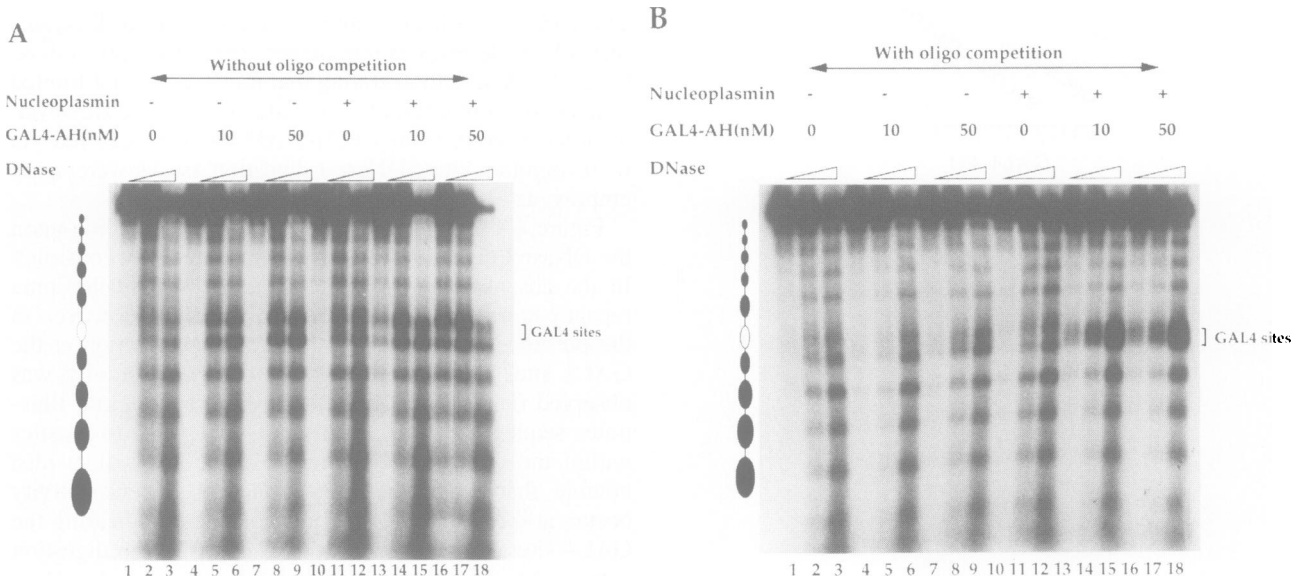

Fig. 4. DNase I analysis of GAL4-AH binding and chromatin remodeling of the nucleosome array. (**A**) GAL4-AH binding to the reconstituted nucleosome array in the absence (lanes 1–9) or in the presence (lanes 10–18) of nucleoplasmin was analyzed by DNase I digestion followed by agarose gel electrophoresis. The concentrations of GAL4-AH included in each binding reaction are indicated. The location of nucleosomes on the array as indicated by DNase I digestion in the absence of GAL4-AH are indicated on the left and the position of the GAL4 sites in the array on the right. Note that the location of adjacent nucleosomes has not changed upon GAL4-AH binding in the presence or absence of nucleoplasmin. Also note the similarity of protection at the GAL4 sites and the adjacent hypersensitivity upon GAL4 binding in the absence or presence of nucleoplasmin. The amounts of DNase I were 0.1 U for lanes 1, 4, 7, 10, 13 and 16; 0.3 U for lanes 2, 5, 8, 11, 14 and 17; 1 U for lanes 3, 6, 9, 12, 15 and 18. (**B**) The perturbation of the chromatin structure of the nucleosome array by GAL4-AH binding was analyzed by DNase I digestion after competing off the bound GAL4-AH with double-stranded GAL4-site oligo. The presence or absence of nucleoplasmin and the GAL4-AH concentrations are indicated. The DNase I titrations are as in A. Note the dramatic difference in the extent of DNase I sensitivity resulting from GAL4-AH binding in the presence (lanes 14–18) versus the absence (lanes 4–9) of nucleoplasmin which was revealed subsequent to oligo competition.

nucleotide containing a GAL4 binding site. By removing GAL4-AH from the array prior to DNase I digestion, it is possible to detect structural changes in the underlying nucleosome resulting from GAL4-AH binding that are otherwise masked by the presence of the transcription factor. The results of such an experiment are shown in Figure 4B.

While GAL4-AH binding to the nucleosome array led to DNase I hypersensitivity adjacent to the GAL4 binding sites in the presence or absence of nucleoplasmin (Figure 4A), in the absence of nucleoplasmin, once the GAL4-AH was removed by the oligo, most of the hypersensitivity was lost (Figure 4B lanes 4–9). Removal of GAL4-AH subsequent to its binding to the array resulted in the template reverting back to a conformation most closely resembling that observed in the absence of GAL4-AH (lanes 1–3). This suggests that on most of the templates, GAL4-AH was in fact bound on top of a nucleosome core, which was again apparent after oligo competition. Conversely, in the presence of the histone binding protein, nucleoplasmin, removal of the bound GAL4-AH by oligo competition resulted in the appearance of a dramatic DNase I hypersensitive site (black hole) at the location of the GAL4 sites (lanes 13–18). This change in chromatin structure was also dependent on GAL4-AH binding as it was not observed in the absence of GAL4-AH (lanes 10–12). Thus, while DNase I digestion was unable to detect the loss of nucleosomes in the presence of bound factors (Figure 4A), the oligo competition assay indicated that the most dramatic structural changes occurred in the presence of both histone binding protein and bound transcription factors.

From Figures 4A and 4B it is important to note that under all of the conditions tested there was no change in the DNase I digestion patterns outside of the location of the GAL4 site nucleosome. The location and stability of the neighboring nucleosomes, as well as those more distal on the array, were not perturbed by GAL4-AH binding and/or the presence of nucleoplasmin. This observation indicates that the DNase I hypersensitive site resulting from GAL4-AH binding in the presence of nucleoplasmin (as detected following subsequent removal of the GAL4-AH) did not result from the sliding of histone octamers to new positions on the DNA fragment. Thus, GAL4-AH binding to the nucleosome array in the presence of nucleoplasmin led to a dramatic change in chromatin structure which was targeted to the GAL4 site nucleosome.

Transcription factor binding predisposes the bound nucleosome to histone displacement in trans

The simplest interpretation of the data in Figure 4 is that GAL4-AH binding to the nucleosome array in the absence of nucleoplasmin resulted in the formation of a ternary complex in which GAL4-AH was bound concurrently with the histone octamer. In the presence of nucleoplasmin these histones were displaced *in trans*, resulting in a nucleosome-free gap in the array at the location of the GAL4 sites. However, it is also possible that the central nucleosome remained in contact with the DNA in a partially disrupted and nuclease-sensitive state. To distinguish between these possibilities, an assay for the presence of a nucleosome that did not depend on nuclease sensitivity

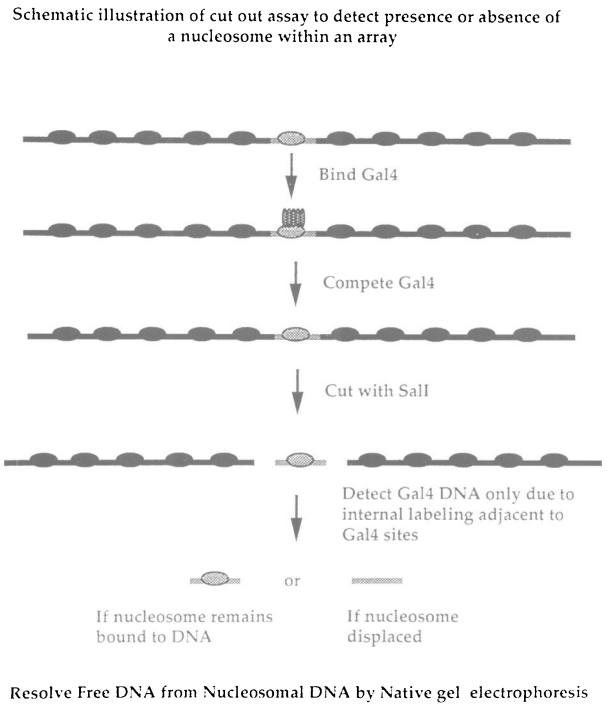

Fig. 5. The cut out assay to detect the displacement of nucleosomes (histones) as a consequence of transcription factor binding.The presence or absence of nucleosomal histones at the GAL4 binding sites was tested directly. Subsequent to GAL4 oligo competition the GAL4 binding sites were excised from the array with *Sal*I followed by native acrylamide gel electrophoresis to distinguish free DNA from nucleosome cores. See text and Materials and methods for details.

was required. Thus, we devised a novel assay in which the oligonucleotide competition assay was combined with *Sal*I digestion of the array to release the central DNA fragment containing the GAL4 sites. The experimental design is illustrated schematically in Figure 5. GAL4-AH was again first bound to the array in the presence or absence of nucleoplasmin and then removed by competition with the GAL4 oligonucleotide. The array was then digested with *Sal*I, releasing the central DNA fragment containing the GAL4 sites (cut out assay). This DNA was then subjected to native gel electrophoresis to determine whether it had the mobility of nucleosomal or naked DNA.

The results of the cut out assay from the nucleosome arrays are shown in Figure 6A. In the absence of GAL4-AH, the GAL4-site DNA fragment was released from the array as a species with the same mobility as the *Sal*I digestion product assembled as a mononucleosome (data not shown). Thus, in the absence of GAL4-AH, a nucleosome was present on this central DNA fragment regardless of the presence or absence of nucleoplasmin (lanes 1, 3, 5 and 7). When GAL4-AH was present and not removed by oligo competition, the fragment was released by *Sal*1 digestion as a GAL4-AH bound complex (lanes 2 and 4). In order to determine whether the histone octamer was contained in these complexes, the GAL4-AH was removed by oligo competition prior to releasing the fragment from the array by *Sal*1 digestion. In the absence of nucleoplasmin, removal of the bound GAL4-AH resulted in the release of the original nucleosome core from the array, indicating that in this instance the histone octamer was retained in the GAL4-AH bound complex (lane 6). Conversely, when nucleoplasmin was present, removal of bound GAL4-AH from the array resulted in the subsequent release of the GAL4-site fragment by *Sal*1 digestion as naked DNA (lane 8). Thus, in the presence of bound GAL4-AH and nucleoplasmin, the core histones were displaced from the GAL4-site sequences leaving GAL4-AH bound to a region of naked DNA in the middle of the nucleosome array. Histone displacement from within the array occurred similarly to histone displacement from single nucleosomes (Figure 6B). In both instances, GAL4-AH binding and the presence of nucleoplasmin were required for the *trans*-displacement of the histones from the DNA. Moreover, while the experiments shown utilized nucleoplasmin as a histone acceptor, experiments which instead used naked DNA as a histone sink led to similar results (data not shown).

The results in Figure 6 confirm the interpretation of the DNase I digestion experiments in Figure 4, namely that GAL4-AH binding results in the formation of a transcription factor/nucleosome ternary complex in which the histones are predisposed to displacement *in trans* onto histone binding components, generating a nucleosome-free gap within the nucleosome array. As there were no major changes to the adjacent nucleosome positions upon GAL4-AH binding in the presence or absence of nucleoplasmin (Figure 4), repositioning of nucleosomes to accommodate movement of the central nucleosome *in cis* did not occur. In fact, the only way this data could be explained by histone movement *in cis* was if a series of repositioning events occurred, moving nucleosomes that were not bound by GAL4-AH. This would require that one nucleosome be displaced from the end of the array and that the intervening five nucleosomes all shift one position towards that end, only in the presence of nucleoplasmin. To address this formal possibility we have ligated the end-labeled 2.3 kb nucleosome array fragment into high molecular weight multimers (average mol. wt of ~23 kb), to remove the vast majority of ends, and reconstituted these into extended nucleosome arrays (see Materials and methods). After GAL4-AH binding in the presence or absence of nucleoplasmin, the GAL4-AH was competed off, followed by DNase I digestion as in Figure 4B. Analysis of the DNase I digestion products on agarose gels (following restriction endonuclease digestion to regenerate the end label) revealed that GAL4-AH and nucleoplasmin generated the DNase hypersensitive nucleosome-free gap in these extended arrays without altering the positions of surrounding nucleosomes (data not shown) as observed in the 11 nucleosome array (Figure 4B). Thus, the ends of the array fragment were not required for histone displacement, confirming that this model system provides an example of transcription factor-induced histone displacement *in trans*.

Discussion

In this report, we have described a mechanism for the generation of nucleosome-free sites within a nucleosome array. This was found to proceed via the formation of a ternary complex in which both the core histones and transcription factors were bound to the same region of DNA. The histones present in this complex were, however, predisposed to displacement. Thus, these data demonstrate

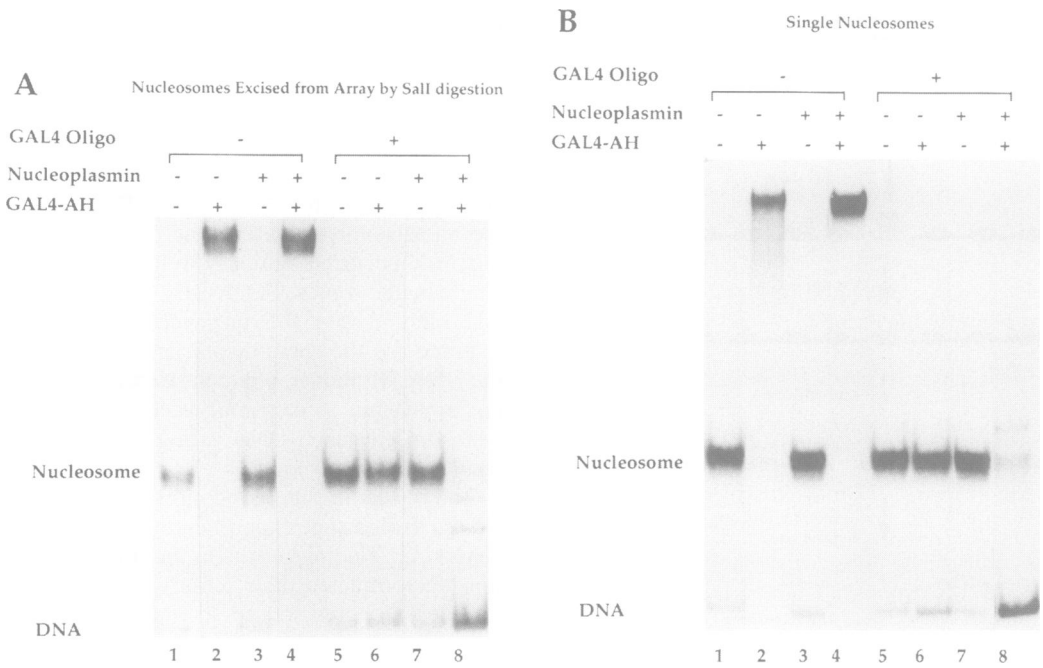

Fig. 6. Analysis of GAL4-targeted histone displacement from the nucleosome array by the cut out assay. (**A**) The internally radiolabeled DNA fragment bearing the GAL4 sites was excised from the nucleosome array by *Sal*I digestion and released as a mononucleosome core in the presence or absence of nucleoplasmin (lanes 1 and 3). Upon GAL4-AH binding this fragment was released as a low mobility GAL4-AH-bound complex (lanes 2 and 4). Following oligo competition, the GAL4-site fragment was released from the array as a nucleosome core after binding by GAL4-AH (lane 6) or exposure to nucleoplasmin (lane 7). However, following exposure to nucleoplasmin during GAL4-AH binding, the GAL4-site fragment was released as naked DNA (lane 8). (**B**) The oligo-competition assay was applied to a mononucleosome bearing the five GAL4 sites under identical reaction conditions to those used in A. Note that displacement of histones from mononucleosomes occurred under the same set of conditions (i.e. upon GAL4-AH binding in the presence of nucleoplasmin) with the mononucleosome core (lane 8) as within the nucleosome array. In both A and B, GAL4-AH is at 50 nM where present.

transcription factor-targeted *trans*-displacement of histones as a mechanism for generating nucleosome-free regions within a nucleosome array.

Transcription factor binding and histone displacement from nucleosome arrays

The affinity of GAL4-AH for its binding site does not appear to be greatly affected whether its binding site is present on a single nucleosome or within the context of an array. We have also observed similar affinities of USF, Sp1 and NF-κB for nucleosomes within arrays and individual mononucleosomes (D.Steger, T.Owen-Hughes and J.L.Workman, unpublished). Thus, previous studies of transcription factor binding to single nucleosomes (reviewed in Owen-Hughes and Workman, 1994) do provide a fair indication of how a given factor will bind in the more physiological context of a nucleosomal array. Furthermore, the mechanism of transcription factor binding and subsequent histone displacement within the array appear similar to that occurring on single nucleosomes of the same sequence (Workman and Kingston, 1992; Chen *et al.*, 1994; Walter *et al.*, 1995). This eliminates the possibility that the formation of a metastable ternary complex containing both the core histones and transcription factor bound to DNA is an artifact attributable to the use of single nucleosome cores. As is the case with single nucleosomes, both factor binding and the ability to displace nucleosomes appears to be independent of the activation domain present on the transcription factor (T.Owen-Hughes and J.L.Workman, unpublished results).

The histones in the nucleosome bound by GAL4-AH were clearly displaced in the presence of nucleoplasmin as indicated by the dramatic hypersensitivity which was retained even after subsequent GAL4-AH removal and confirmed by the cut out assay which releases this fragment as naked DNA. Conversely, prior to GAL4-AH competition the pattern of DNase I hypersensitivity was identical regardless of whether GAL4-AH was bound in a ternary complex with the nucleosome core or if the histones had been displaced. Thus, in this system, DNase I digestion alone was unable to dectect the displacement of the underlying histones during factor binding. This illustrates the limitations of assays of chromatin structure that rely solely upon nuclease sensitivity in distinguishing between mechanisms of chromatin remodeling.

A similar pattern of hypersensitivity was observed flanking five GAL4 sites upon GAL4 binding to templates assembled with spaced nucleosomes using a *Drosophila* embryo extract (Pazin *et al.*, 1994). This is consistent with the possibility that nucleosome 'reconfiguration' in this system may also reflect the formation of ternary complexes and perhaps *trans*-displacement of histones. Moreover, hypersensitivity flanking factor binding sites was also observed when sequences derived from the HIV LTR were bound by the appropriate transcription factors, suggesting a similar effect of factors binding to this natural enhancer (Pazin *et al.*, 1996). The use of alternative techniques such as the oligo competition and the cut out assays described here will enable the role of histone

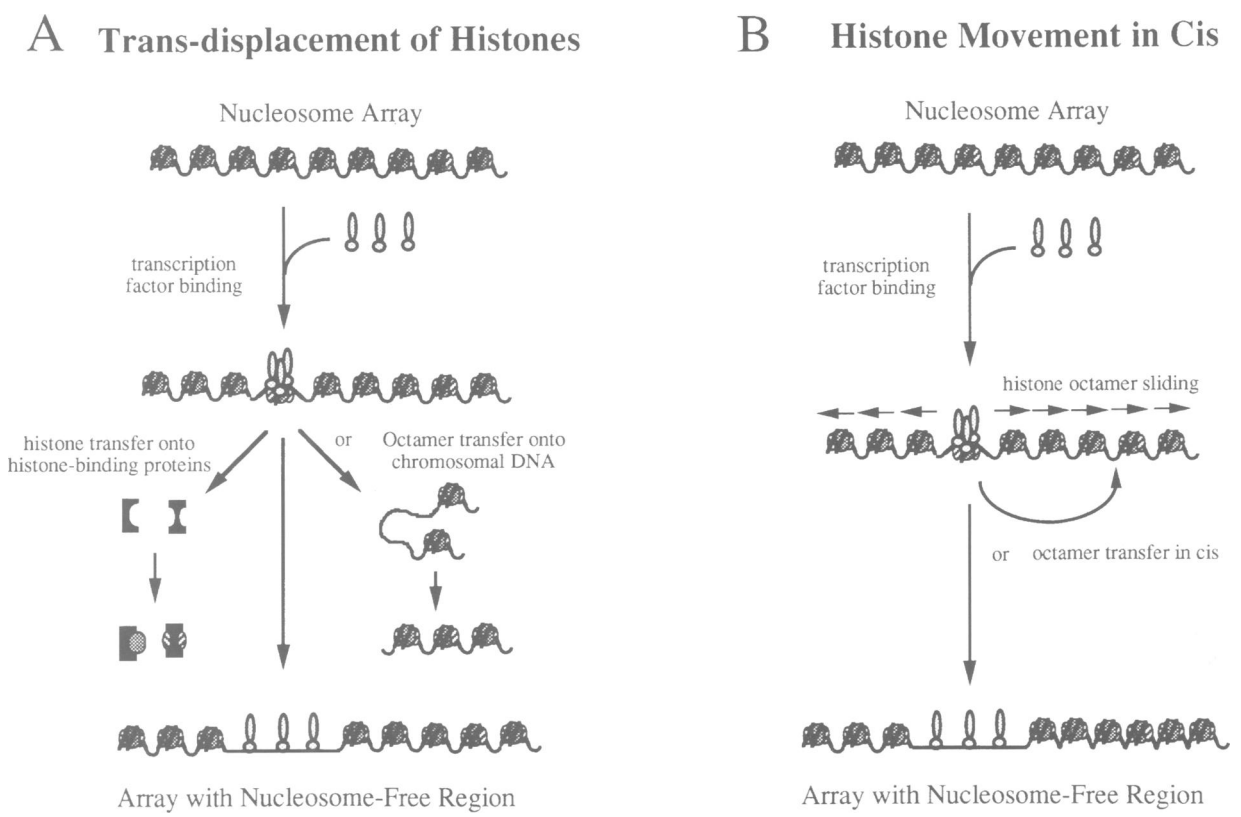

Fig. 7. Schematic comparison of potential steps involved in *trans*- and *cis*-displacement of histones. (**A**) Transcription factor targeted *trans*-displacement of histones from a nucleosome array. Binding of transcription factors to a nucleosome bearing its recognition elements within a nucleosome array results in the formation of a metastable ternary complex in which the same sequences are simultaneously occupied by transcription factors and histones. Histones could be lost from this ternary complex *in trans* by two distinct mechanisms. (i) The histone octamer could disassemble, first losing H2A/H2B dimers onto histone binding proteins followed by loss of the H3/H4 tetramer. Each of these sequential steps of histone loss increases the affinity of the transcription factor for its binding sites. (ii) Intact histone octamers could transfer from the transcription factor-bound sequences onto other segments of chromosomal DNA through direct transfer, avoiding dissociation into solution. Either of these mechanisms results in the formation of a nucleosome-free region occupied by transcription factors at high affinity. (**B**) Transcription factor targeted generation of a nucleosome-free region via histone movement *in cis*. The binding of transcription factors to their recognition elements in a nucleosome could induce sliding of the bound and adjacent histone octamers, repositioning nucleosomes to generate a nucleosome-free region. Alternatively, transcription factor binding might take advantage of inherent nucleosome mobility or mobility caused by separate activities. Factors might bind during the transient exposure of their recognition sequences in linker DNA. In addition, histone movement *in cis* might occur over longer distances. Intact histone octamers might transfer from transcription factor-bound ternary complexes by direct transfer onto another region of the same strand of chromosomal DNA. This pathway is mechanistically identical to octamer transfer onto other strands of chromosomal DNA *in trans* described in (A), but might be favored in some instances by the inherently high local concentration of the *cis* DNA strand.

displacement in transcriptional regulation to be studied in more detail.

Trans-displacement of histones versus histone movement in cis

A comparison of the requisite steps for *trans*-displacement of histones versus histone octamer sliding is shown in Figure 7. The limiting step for *trans*-displacement of histones might be expected to be the availability of other components onto which the histones can transfer once destabilized by the binding of transcription factors (Figure 7A). In this regard it is important to note that transcription factor-induced *trans*-displacement of histones has been shown to be mediated by the nucleosome assembly proteins, nucleoplasmin and nucleosome assembly protein 1 (NAP-1) (Chen *et al.*, 1994; Walter *et al.*, 1995). The same proteins which mediate nucleosome assembly can therefore participate in chromatin remodeling via histone displacement from transcription factor-bound nucleosomes. In addition, *trans*-displacement of histones from transcription factor-bound nucleosomes can also occur onto other pieces of DNA (Workman and Kingston, 1992) and apparently onto other regions of chromatin (see Walter *et al.*, 1995), a possibility supported by the fact that a second histone octamer will readily bind an existing nucleosome core (Voordouw and Eisenberg, 1978; Stein, 1979). Thus, there would appear to be several potential histone acceptors which could mediate *trans*-displacement of histones in the eukaryotic nucleus.

For nucleosome-free regions to form by histone octamer sliding, nucleosomes up and down the array would have to also slide and reposition to generate the nucleosome-free space (Figure 7B). Consequently, this seems like a difficult task where repositioning of several surrounding nucleosomes onto a limited amount of linker DNA is required. However, Becker and colleagues have illustrated that nucleosome arrays assembled on plasmids in *Drosophila* embryo extracts are extremely dynamic due to the presence of ATP-dependent activities in the embryo extract (Varga-Weisz *et al.*, 1995; Wall *et al.*,1995). In this system it seems quite feasible that arrays of nucleosomes can reposition to generate a nucleosome-free space. On the

other hand, histone octamer sliding might occur less readily in situations where surrounding nucleosomes are predisposed to unique positions as in the system described here. Further experiments are required to demonstrate histone octamer sliding and to determine in which instances sliding or *trans*-displacement is the chosen pathway to generate a nucleosome-free gap. In this respect, it should be noted that the detection of changes in the positions of nucleosomes surrounding factor binding sites is not necessarily diagnostic of nucleosome displacement *in cis*. *Trans*-displacement of a histone octamer from a template on which nucleosomes are not uniquely positioned could also affect the positions of a subset of the surrounding nucleosomes.

Potential pathways of transcription factor-induced histone movement *in cis*- and *trans*-displacement might be mechanistically related. For example, histone octamer movement *in cis* might occur through jumps of the histone octamer from one region of DNA to another (octamer transfer *in cis*, Figure 7B). Such a mechanism might be similar to the spooling mechanism proposed by Felsenfeld and colleagues for the transfer of intact histone octamers around elongating prokaryotic RNA polymerases. This model proposes the direct transfer of histone octamers from one region of DNA to another via an intermediate state, where both segments of DNA are simultaneously in contact with the histone octamer (Clark and Felsenfeld, 1992; Studitsky *et al.*, 1994, 1995). Interestingly, intact histone octamers can also be induced to transfer onto another piece of DNA as a result of transcription factor binding (Walter *et al.*, 1995). Thus, in this instance histone octamer movement *in cis* (Figure 7B) or *in trans* (Figure 7A) might occur by a similar mechanism, differing only in the identity of the recipient DNA.

The mechanisms of chromatin remodeling activities

Biochemical and genetic analysis are revealing further activities implicated in chromatin remodeling both *in vivo* and *in vitro*. These include the SWI/SNF complex (Hirschhorn *et al.*, 1992, 1995; Côté *et al.*, 1994; Imbalzano *et al.*, 1994; Kwon *et al.*, 1994), additional distinct activities in *Drosophila* including the SWI-related NURF complex (Pazin *et al.*, 1994; Tsukiyama *et al.*, 1995; Varga-Weisz *et al.*, 1995; Wall *et al.*, 1995) and activities in human cells implicated in histone displacement or transfer (Heggeler-Bordier *et al.*, 1995; T.Owen-Hughes and J.L.Workman, unpublished). Biochemically, SWI/SNF and NURF have been shown to stimulate factor binding and to alter nuclease digestion patterns of nucleosomal DNA but there is currently no evidence available regarding their mechanism of action or the fate of core histones. One possibility is that the ATPase activity of these complexes serves to break histone DNA contacts, facilitating histone displacement as observed for the DNA helicase, SV40 large T-antigen (Ramsperger and Stahl, 1995). In this regard it is interesting to note that the ATP-dependent action of SWI/SNF in stimulating transcription factor binding to nucleosomal DNA is enhanced by the presence of nucleoplasmin (Côté *et al.*, 1994) suggesting that the chromatin remodeling activities of SWI/SNF-like ATPase complexes and histone binding proteins may be complimentary. Well defined *in vitro* model systems such as that described here, will enable mechanistic analysis of these activities. These approaches will render important mechanistic details beyond the basic phenomenon of DNase I hypersensitivity.

Materials and methods

Plasmid construction

pG5-208-10 was constructed as follows: A *Bam*HI deletion was made in the polylinker region of the plasmid pBend (Kim *et al.*, 1989) to create pBendΔ. Five copies of the sea urchin 5S RNA nucleosome positioning sequence were then isolated as a tandem repeat from a partial *Ava*I digest of p5S207-12 (Simpson *et al.*, 1985). After filling in the ends with klenow this DNA fragment was subcloned into *Eco*RV cut pBendΔ. A clone in which the 5S repeats were oriented such that the *Ava*I sites were closest to the *Bam*HI end of the pBendΔ polylinker was named p208-5@RVrev. The insert in p208-5@RVrev was excised with *Xho*I and *Pvu*II and the 5′ overhangs filled in with klenow. p208-5@*Bgl*IIrev contains this DNA fragment, with the 5S sequences in the same orientation as for p208-5@RVrev, subcloned into the *Bgl*II site of pBendΔ. The primers, CTAGCTAGCGTCGACGTCGACGTCGACAA-GCTTGCATGCCTGC and TCAACTAGTCGACGTCGACGTCGACA-ATCTTTTTGTTGTCAAGCTG, were used to amplify five GAL4 sites from the plasmid pG5I160E4T (Lin *et al.*, 1988). The product was cut with *Nhe*I and *Spe*I and ligated into *Nhe*I, *Spe*I cut p208-5@*Bgl*IIrev in the orientation that maintains the *Nhe*I and *Spe*I sites to form p208-5@*Bgl*IIrevG_5. Finally, the *Nhe*I to MluI fragment from p208-5@*Bgl*IIrevG$_5$ was subcloned into *Mlu*I, *Nhe*I cut p208-5@*Bgl*IIrev to create pG5-208-10. The *Mlu*I–*Pvu*II fragment from pG5-208-10 is illustrated in Figure 1.

Protein purification

GAL4-AH was prepared from bacterial strains as described by Lin *et al.* (1988). H1 depleted oligonucleosomes were prepared from HeLa nuclear pellets (Workman *et al.*, 1991b) as described by Côté *et al.* (1995). Nucleoplasmin was prepared as described by Walter *et al.* (1995).

Preparation of probe DNA

In studies using the end-labeled array construct, the insert from plasmid pG5-208-10 was excised using the enzymes *Mlu*I and *Pvu*II. The digestion products were end-labeled with [α-^{32}P]dCTP using Klenow and the 2343 bp fragment bearing the GAL4 sites gel purified using β-agarase (New England Biolabs) prior to reconstitution. To prepare internally labeled array construct, plasmid pG5-208-10 was cut with *Xba*I and then dephosphorylated using alkaline phosphatase. This material was then gel purified, and end-labeled using polynucleotide kinase and [γ-^{32}P]ATP. Twenty microliters kinase reactions were heat inactivated at 75°C and then diluted to 40 μl with ligation buffer containing 1 U ligase (Boehringer Mannheim). Ligations were performed overnight at 16°C. Ligation products were digested with *Mlu*I and *Pvu*II and the GAL4 site containing insert DNA isolated by gel purification. The central 165 bp GAL4 site containing probe was prepared by *Sal*I digestion of pG5-208-10, followed by end-labeling with [α-^{32}P]dCTP and [α-^{32}P]dATP using Klenow.

Nucleosome reconstitution

Nucleosome reconstitution was performed essentially as described by Côté *et al.* (1995). Briefly, 12.5 pmol HeLa nucleosomes were mixed with ~0.2 pmol of probe DNA in 50 mM HEPES pH 7.5, 1 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF in a final volume of 10 μl. This was diluted with 1.8, 3.5, 4.7, 13, 17 μl 50 mM HEPES pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM PMSF with 15 min incubations at 30°C at each step. Finally the reaction was brought to 0.1 M NaCl by the addition of 50 μl 10 mM Tris–HCl pH 8, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1% Nonidet P-40, 20% glycerol, 100 μg/ml BSA. Reconstitutions were aliquoted and stored at –80°C until required.

Binding reactions and gel shift assays

Binding reactions (20 μl) contained 2 μl 10× binding buffer (200 mM HEPES pH 7.5, 500 mM KCl, 50% glycerol, 20 mM DTT, 10 μM $ZnCl_2$, 2 mM PMSF, BSA 1 mg/ml). Typically they contained 1 fmole reconstituted probe DNA together with donor nucleosomes. GAL4-AH dilutions (2 μl) in G4D buffer (10 mM HEPES pH 7.5, 100 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 10 μM $ZnCl_2$, 20% glycerol, 1 mg/ml BSA) were included in the binding reactions where indicated.

Nucleoplasmin was diluted with NPD buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 0.4 mM EDTA, 0.1 mM PMSF, 0.1% NP40, 0.7 mg/ml BSA) and added to binding reactions in 1 µl to give a final concentration of 2 nM. Where either nucleoplasmin or GAL4-AH were not present, the appropriate volumes of dilution buffer were included in the binding reaction. Binding reactions were performed at 30°C for 60 min. Where GAL4-AH was to be removed by oligo competition, a duplex GAL4 site containing DNA was created by annealing the oligonucleotides CTAGACCGGACGACAGTACTCCGACT and CTAGAGTCGGAGTACTGTCGTCCGGT. 1 µg of duplex oligonucleotide was added to binding reactions in 2 µl of CB (0.5 mM Tris pH 7.5, 0.5 mM EDTA, 1 M NaCl, 50 mM $MgCl_2$) and the competition performed for 60 min at 37°C.

The binding/competition reactions were then subject to analysis by native gel shift or DNase I digestion. Where the central nucleosome of the array was to be studied by native gel shift, the internally labeled array was digested with *Sal*I (20 U) in the presence of 150 mM NaCl, 5 mM $MgCl_2$ for 10 min at 37°C subsequent to binding/competition reactions. Gel shifts were performed in 4% polyacrylamide (acrylamide:bisacrylamide = 29:1) 0.5× TBE gels. Probe DNA was detected by autoradiography.

Where the array was to be studied by DNase I digestion, end-labeled template DNA was used in reconstitutions. 1 µg GAL4 oligo in 2 µl CB was added to all binding reactions that did not already contain it, followed by the immediate addition of DNase I (1 µl) (Boehringer Mannheim) for 4 min at room temperature. Reactions were stopped by the addition of 20 µl DSB (3% SDS, 50 mM Tris–HCl pH 8, 0.1 M EDTA, 25% glycerol, 0.002% bromophenol blue, 0.002% xylene cyanol). Proteinase K digestion was performed at 55°C for 1 h, before samples were resolved on 1% agarose gels. After drying the gels, digestion products were detected by autoradiography.

For the control experiment in which multimers of the array construct were ligated together, probe DNA was prepared from *Stu*I and *Mlu*I cut pG5-208-10. This was end-labeled and gel purified as described above for the *Mlu*I–*Pvu*II fragment. This array fragment was then ligated at high DNA concentrations to form multimers. Multimers containing an average of 10 repeats of the array construct were gel purified from the ligation reaction. This DNA was reconstituted, subject to binding reactions and DNase I digestion as described above, except that subsequent to DNase I and proteinase digestion, the DNA was ethanol precipitated and digested with *Pvu*II prior to electrophoresis to regenerate the end label.

MNase digestions were performed similarly to DNase digestions except that 1 mM $CaCl_2$ was added to binding reactions prior to digestion. MNase dilutions were made in MNase dilution buffer (10 mM HEPES pH 7.5, 50% glycerol, 100 mM NaCl, 1 mM $CaCl_2$).

Confirmation of histone content

To analyze the histone content of the reconstituted array, paramagnetic beads were used to purify the template from donor nucleosomes. This was done by end-labeling probe DNA with Biotin-14-CTP (Gibco BRL) using Klenow. Biotinylated DNA was then gel purified, and attached to Streptavidin linked Dynabeads (Dynal) according to the manufacturers instructions. DNA linked to the beads could be reconstituted with nucleosomes using the same procedure described above except that agitation is required to prevent the beads sedimenting.

After reconstitution, the assembled probe DNA could be purified from donor nucleosomes by MPC (Magnetic Particle Concentration), washed with 20 mM HEPES pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 0.2 mM PMSF and then resuspended in 1× loading buffer prior to SDS–PAGE analysis on a 15% acrylamide gel. Histones were detected by silver staining as described by Wray *et al.* (1981).

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