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A positive role for nucleosome mobility in the transcriptional activity of chromatin templates: restriction by linker histones

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Nucleosome mobility facilitates the transcription of chromatin templates containing only histone octamers. Inclusion of linker histones in chromatin inhibits nucleosome mobility, directs nucleosome positioning and represses transcription. Transcriptional repression by linker histone occurs preferentially on templates associated with histone octamers relative to naked DNA. Mobile nucleosomes and the restriction of mobility by linker histones might be expected to exert a major influence on the accessibility of chromatin to regulatory molecules.

Keywords: histones/linker/nucleosomal templates/repression/transcription

Introduction

The histones and the chromatin structures they assemble have an essential role in transcriptional regulation (Felsenfeld, 1992; Grunstein *et al.*, 1992). Although the assembly of nucleosomes can restrict the access of *trans*-acting factors to DNA, molecular mechanisms clearly exist that modulate histone–DNA interactions and facilitate transcription within chromatin (Owen-Hughes and Workman, 1994; Wolffe, 1994a). Nevertheless, the structural features of the nucleosome that might contribute to transcriptional regulation are incompletely defined.

Nucleosome positioning has been shown to influence *trans*-acting factor access to DNA (Simpson, 1991; Lee *et al.*, 1993; Li and Wrange, 1994). Intrinsic DNA structure and *trans*-acting factors determine where histones associate with DNA (Drew and Travers, 1985; Fedor *et al.*, 1988). Not all histone octamers adopt a unique stable position with respect to DNA sequence (Dong *et al.*, 1990; Meersseman *et al.*, 1991). Bradbury and colleagues have proposed that nucleosome mobility is a general behavior (Meersseman *et al.*, 1992). Although this remains to be conclusively established, mobile nucleosomes might facilitate transcription factor access to DNA. Thus, mobile nucleosomes might be a feature of transcriptionally competent chromatin. Linker histones restrict nucleosome mobility (Pennings *et al.*, 1994); this restriction might influence transcriptional efficiency.

Linker histones (e.g. H1, H5) have been proposed to act as transcriptional repressors (Brown, 1984; Weintraub, 1984, 1985). Substantial evidence supports this proposal. Histone H1 selectively represses the transcription of specific genes *in vivo* (Schlissel and Brown, 1984; Bouvet *et al.*, 1994; Kandolf, 1994). The activation of specific promoters *in vivo* correlates with depletion of histone H1 (Bresnick *et al.*, 1992). The reconstitution of histone H1 into chromatin *in vitro* can lead to transcriptional inactivity (Shimamura *et al.*, 1989; Wolffe, 1989; Laybourn and Kadonaga, 1991; Kamakaka *et al.*, 1993; but see Sandaltzopoulos *et al.*, 1994). Nevertheless, the molecular mechanisms determining linker histone-mediated transcriptional repression are unknown. Linker histones and isomorphous transcription factors (e.g. HNF-3) may influence nucleosome position (Meersseman *et al.*, 1991; Chipev and Wolffe, 1992; McPherson *et al.*, 1993). The formation or stabilization of specific histone–DNA contacts with promoters following incorporation of linker histones into chromatin may lead to steric occlusion of *trans*-acting factor access. Alternatively, the assembly of higher-order chromatin structures dependent on linker histones (Graziano *et al.*, 1994), or the aggregation and precipitation of the template under transcription conditions following addition of linker histones (Widom, 1985), might also influence transcriptional activity.

In this work, we establish a model system for investigation of the selective repression of transcription from nucleosomal templates by linker histones. We make use of physiologically spaced dinucleosomal templates in which it is possible to assay both chromatin structural and transcriptional characteristics. We find that nucleosome mobility is a characteristic of transcriptionally competent chromatin templates. Reconstitution of chromatin with linker histones restricts nucleosome mobility and locks the nucleosome into a unique position. This fixation of histone–DNA contacts is concomitant with transcriptional repression. Thus, stable states of gene repression can be established at the nucleosomal level.

Results

A dinucleosomal template for transcription

We reconstituted a 424 bp template containing two *Xenopus* somatic 5S RNA genes with histone octamers (Figure 1). We wished to make use of strong nucleosome positioning signals in the 5S RNA gene to separate nucleosomes (Simpson, 1991) and the capacity to have very efficient *in vitro* transcription of these genes as short linear DNA fragments *in vitro* (Wolffe *et al.*, 1986). The reconstituted chromatin was fractionated on a sucrose gradient and each fraction was analyzed by nucleoprotein gel electrophoresis. Free DNA was resolved from mono-, di- and trinucleosomal complexes (Figure 2A). Dinucleosomal complexes were pooled and refractionated on a second sucrose gradient. Controls in which dinucleosomes were mixed with unlabeled chicken erythrocyte oligonucleosomes established that we had achieved at least a >90% enrichment in dinucleosomes with no naked DNA

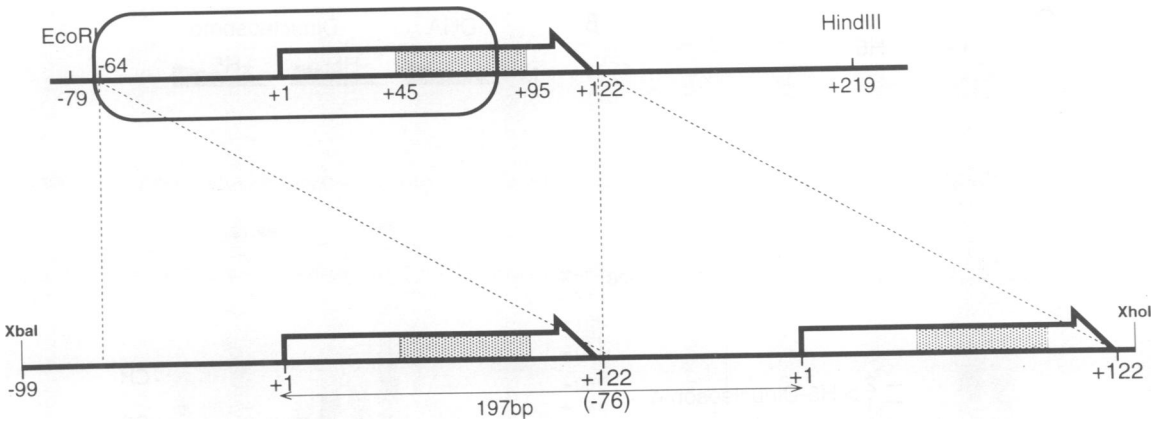


Fig. 1. Structure of the X5S197-2 dinucleosome construct. The 424 bp template contains two 197 bp two tandem repeats of a *X.borealis* somatic 5S RNA gene and associated upstream sequences (–64 to +122 relative to the start site of transcription, +1) encompassing the entire nucleosome positioning element within this DNA. Arrows show the location and orientation of the 120 bp 5S RNA, while dotted boxes show the location of 45 bp internal control region (ICR). The oval (–70 to +79) indicates the location of the nucleosome core region upon *in vitro* reconstitution of the monomer construct (Hayes and Wolffe, 1993).

contamination (Figure 2B). This chromatin template was used in subsequent experiments.

We wished to reconstitute linker histones into the dinucleosomal templates. Earlier work established that linker histones (H1 and H5) prefer to interact with DNA wrapped around histone octamers rather than with naked DNA (Hayes and Wolffe, 1993). Importantly, reconstitution with linker histones occurs with a precise stoichiometry at a precise site within the 5S nucleosome (Hayes and Wolffe, 1993; Hayes *et al.*, 1994). We mixed a dinucleosome fraction with naked DNA (Figure 3A, lane 3). The addition of one molecule of H5 per dinucleosome leads to no change in the free DNA band, yet leads to the assembly of two new core histone-associated complexes resolved by electrophoresis on agarose gels (Figure 3A, lane 1). Note that these gels do not distinguish between different nucleosome positions (but see Figure 4). An increase in the amount of H5 to two molecules per dinucleosome leads to the almost complete assembly of the upper complex (Figure 3A, lane 2). Control experiments indicate that free DNA is also bound at higher excesses of H5, appearing as a heterogeneous distribution of complexes migrating below those containing core histones (Hayes and Wolffe, 1993, not shown). An additional assay for the stable inclusion of linker histones into chromatin is the appearance of DNA fragments of distinct size, during micrococcal nuclease digestion (Simpson, 1978; Allan *et al.*, 1980; Hayes and Wolffe, 1993). Core particles containing 146 bp of DNA accumulate in the presence of histone octamers alone. In the presence of histone octamers and linker histones, chromatosome particles accumulate containing all of the histones and >166 bp of DNA. Naked DNA is rapidly digested with micrococcal nuclease (Figure 3B, lanes 1–4). Using the dinucleosome template reconstituted with the core histones alone, a stable kinetic intermediate of micrococcal nuclease digestion accumulates with the size of core particle DNA (CP; Figure 3B, lanes 5–8). In the presence of core histones and one molecule of H5 per dinucleosome, an intermediate of chromatosome size accumulates (CH; Figure 3B, lanes 9–12). Similar results were obtained at ratios of two molecules of H5 per dinucleosome and with histone H1

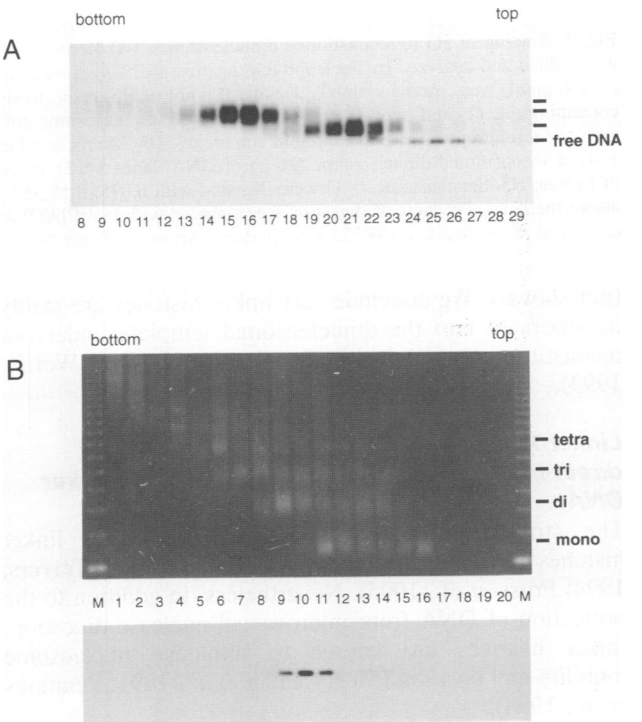


Fig. 2. Preparation and characterization of reconstituted 5S dinucleosome cores. (**A**) Radiolabeled DNA templates were reconstituted at a molar ratio of core histones to DNA of 1.0 with purified core histone proteins. The products were fractionated on 5–20% sucrose gradients and each fraction was analyzed by nucleoprotein gel electrophoresis (left is bottom fraction). The reconstituted nucleosomes were separated into four bands. The bottom band was free DNA. (**B**) Purified 5S dinucleosome core reconstitutes co-sediment with native dinucleosome complexes. Putative dinucleosome fractions 13–17 were combined and refractionated on sucrose gradient with unlabeled chicken erythrocyte oligonucleosomes (upper panel). DNA from each fraction was run on a 1.5% agarose gel and stained with ethidium bromide. M, 123 bp ladder as DNA size markers. Lane 1 is the bottom fraction. Location after sedimentation of native mono-, di-, tri- and tetranucleosomes is indicated. (Lower panel) Autoradiograph of the gel in (B) showing the location of reconstituted 5S dinucleosomes in the gradient.

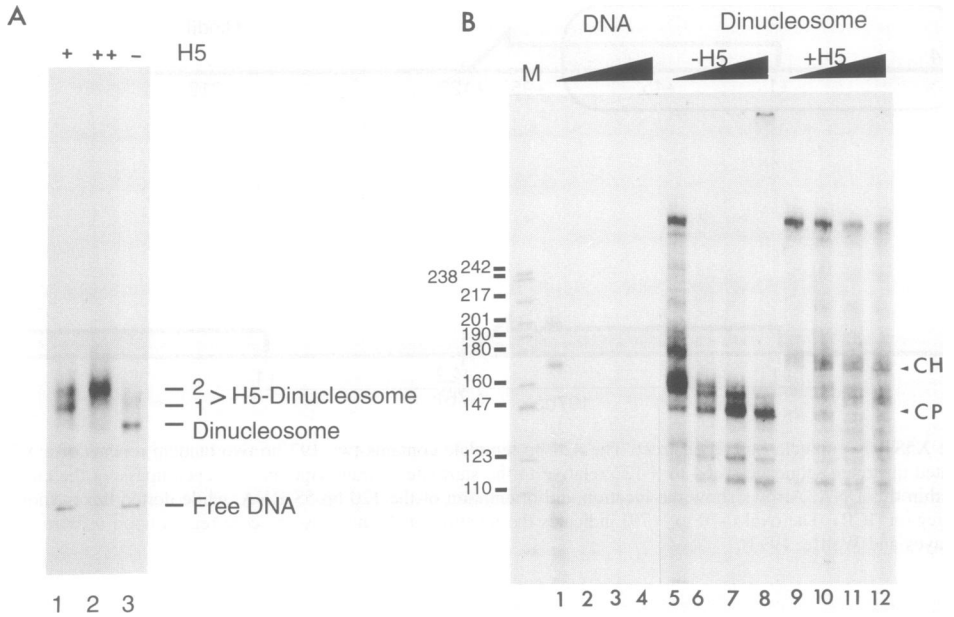


Fig. 3. Binding of H5 to reconstituted dinucleosomes. (**A**) Reconstituted dinucleosomes were mixed with free DNA before various amounts of H5 were added and analyzed by nucleoprotein agarose (0.7%) gel electrophoresis. Lanes 1–3, 100 ng (DNA content) of reconstituted nucleosome cores (= 0.6 pmol) were mixed with 0.6, 1.2 and 0 pmol of H5, respectively. The positions of Free DNA, Dinucleosomes and H5-Dinucleosomes containing one (1) and two (2) molecules of H5 per dinucleosome are indicated. Note that nucleosome mobility (Meersseman *et al.*, 1992) interferes with the resolution of the nucleoprotein complexes. (**B**) Micrococcal nuclease digestion of reconstituted dinucleosome. Naked DNA (500 ng, lanes 1–4) or reconstituted dinucleosome (80 ng of DNA, lanes 5–12), in the absence (lanes 5–8) or presence (lanes 9–12) of 16 ng of histone H5 (moles of histone H5/dinucleosome = 1) were digested with 0.075, 0.15, 0.3 and 0.6 U of micrococcal nuclease (5 min, 22°C) as indicated by the triangles above the lanes. Products of digestion were labeled with [γ-^{32}P]ATP and analyzed by native polyacrylamide (6%) gel electrophoresis. Lane 1 contained *Msp*I-digested pBR322 size markers. Arrows indicate the core particle (CP) and chromatosome (CH) products of digestion, respectively.

(not shown). We conclude that linker histones are stably incorporated into the dinucleosomal template under our reconstitution conditions (see also Hayes and Wolffe, 1993).

Linker histones restrict nucleosome mobility, direct nucleosome position and constrain linker DNA

The structural consequences of incorporating linker histones into a nucleosome are poorly understood (Travers, 1994; Pruss *et al.*, 1995). Nevertheless, in addition to the protection of DNA from micrococcal nuclease digestion, linker histones are known to influence nucleosome mobility and position (Meersseman *et al.*, 1991; Pennings *et al.*, 1994).

Histone octamers alone can adopt a number of translational positions along a DNA sequence spaced by helical turns of DNA (i.e. 10–11 bp intervals) (Dong *et al.*, 1990; Meersseman *et al.*, 1991). Bradbury and colleagues have determined, using two-dimensional nucleoprotein electrophoresis, that this spacing is indicative of mobile nucleosomes (Meersseman *et al.*, 1992). We examined whether mobile nucleosomes assemble on the dinucleosomal template. To assess nucleosome mobility, we made use of electrophoresis on non-denaturing polyacrylamide gels (Meersseman *et al.*, 1992; Pennings *et al.*, 1994). In comparison with non-denaturing agarose gel electrophoresis (Figure 3A), resolution of nucleoprotein complexes on polyacrylamide gels is sensitive to the conformation of the histone–DNA complex (Meersseman *et al.*, 1992). Thus, a single histone octamer associated with a 424 bp DNA fragment can be resolved into multiple complexes on a single dimension of electrophoresis (Figure 4A, lane 1) dependent on the translational position of the histone octamer along the DNA fragment (Meersseman *et al.*, 1992). Addition of linker histones H5 (Figure 4A, lanes 2–4) leads to a change in the position and number of nucleoprotein complexes resolved. Evidence for the mobility of a histone octamer comes from carrying out a second dimension of electrophoresis (Meersseman *et al.*, 1992). If the octamer changes position during a 1 h incubation at 4 or 37°C prior to the second dimension, then this will be detected by the appearance of a nucleoprotein complex that migrates at a position away from a simple diagonal. Nucleosome mobility is temperature dependent; using the 424 bp DNA fragment reconstituted with a single histone octamer, more nucleoprotein complexes migrate off the diagonal at 37 than at 4°C (Figure 4B, compare 37 with 4°C, –H5). Nevertheless, histone octamers are mobile at 4°C, and hence a redistribution of positions will occur under transcription conditions at 22°C (see below). Reconstitution of histone H5 into the mononucleosome partially restricts octamer mobility (Figure 4B, compare +H5 with –H5); this is visualized as fewer nucleoprotein complexes migrating off the diagonal, consistent with earlier observations (Pennings *et al.*, 1994).

We next investigated the mobility of histone octamers when a dinucleosomal template was reconstituted. Mono-, di- and trinucleosomes fractionated after sucrose gradient centrifugation were resolved on one-dimensional non-denaturing gels (Figure 4C). Increasing numbers of histone octamers limit the number of discrete nucleoprotein species resolved through this analysis. Nevertheless, two-dimensional analysis of dinucleosomal templates

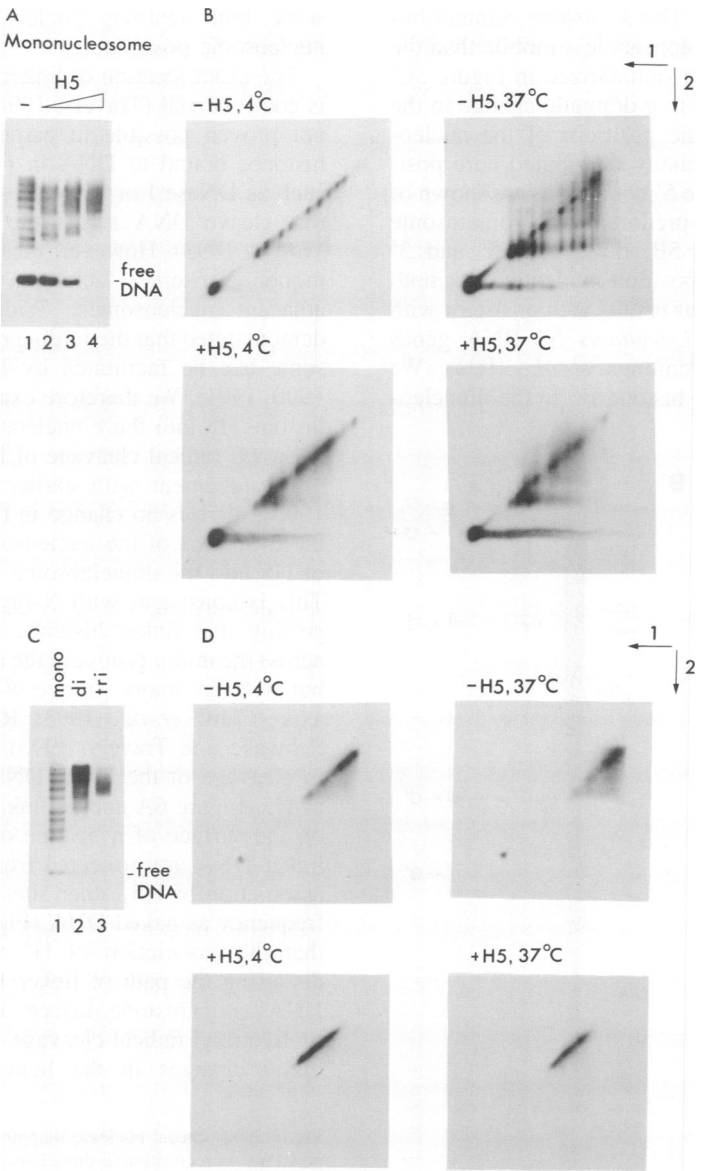


Fig. 4. The influence of histone H5 on nucleosome mobility. **(A)** Mononucleosomes were resolved on a non-denaturing 4% polyacrylamide gel in the absence (lane 1) or presence of increasing molar ratios of histone H5 per histone octamer (lanes 2–4 contained 1, 2 and 4 mol of H5/mol of octamer). The position of free DNA is indicated. **(B)** Mononucleosomes either not containing (–H5) or containing (+H5) a single H5 molecule per octamer were resolved on a 4% polyacrylamide gel at 4°C before excision of the gel lane and incubation at 4 or 37°C for 1 h as indicated, followed by a second dimension of electrophoresis (Materials and methods). The directions of electrophoresis are indicated. **(C)** Non-denaturing 4% polyacrylamide gel electrophoresis of mono- (lane 1), di- (lane 2) and trinucleosomes (lane 3) fractionated on sucrose gradients (Figure 2). The position of free DNA is indicated. **(D)** As in (B), except that dinucleosomes were used.

reconstituted with two histone octamers reveals the octamers to be mobile, dependent on temperature (Figure 4D, –H5, 4 and 37°C). Once again, a substantial fraction of the radioactive nucleoprotein complexes migrate off the diagonal at 4°C (Figure 4D, upper left panel). This implies that octamers will be mobile on the transcription template at 22°C under transcription conditions. Importantly, reconstitution of linker histone H5 into the dinucleosomal template restricts octamer mobility (Figure 4D, compare –H5 and +H5 panels). We conclude that like the mononucleosomal templates (Figure 4B; Pennings *et al.*, 1994), inclusion of linker histones restricts the mobility of histone octamers on dinucleosomal templates (Figure 4D).

We next used an independent nuclease mapping methodology (Meersseman *et al.*, 1991) to investigate nucleosome mobility and the associated variation in nucleosome position. Micrococcal nuclease digestion was followed by restriction endonuclease mapping of the translational positioning of both of the histone octamers relative to the 5S RNA gene sequences on the dinucleosome template (Figure 5). We find that the 5′ histone octamer adopts at least five distinct translational positions spaced by 10–11 bp intervals (seen by *Eco*RV cleavage; Figure 5A, lane 4, dots; only the 5′ boundaries of the octamer are shown). These multiple translational positions indicate nucleosome mobility (Meersseman *et al.*, 1991, 1992). A unique restriction site (*Fnu*4HI; Figure 5C, marked by an asterisk) allows discrimination between the boundaries of DNA contact made by the 5′ and 3′ histone

octamers (Figure 5A, lane 7). The 3′ histone octamer has fewer positions and therefore appears less mobile than the 5′ octamer. Core positions are summarized in Figure 5C. Inclusion of histone H5 leads to a dramatic change in the number and distribution of the positions of the nucleosomes. Instead of multiple equally distributed core positions (Figure 5A, dots; only the 5′ boundaries are shown of the 5′ octamer), only a single predominant chromatosome position is apparent (Figure 5B, dots; both 5′ and 3′ boundaries are shown). This position is identical for both the 5′ and 3′ nucleosomes. Our results are consistent with earlier observations on the *Lytechinus* 5S RNA genes (Meerseman *et al.*, 1991; Pennings *et al.*, 1994). We conclude that the inclusion of histone H5 in the dinucleosome both restricts nucleosome mobility and directs nucleosome positioning.

The exact location of linker histones in the nucleosome is controversial (Travers, 1994; Pruss *et al.*, 1995). It has not proven possible in earlier work to footprint linker histones bound to DNA in nucleosomes using enzymes such as DNase I or reagents such as the hydroxyl radical that cleave DNA across the minor groove (Hayes and Wolffe, 1993). However, these experiments made use of mononucleosomes lacking a true linker DNA between adjacent nucleosomes. Widom and colleagues have demonstrated that the folding of linker DNA in a dinucleosome can be facilitated by linker histones (Yao *et al.*, 1990, 1991). We therefore examined whether inclusion of histone H5 into the dinucleosome influenced DNase I or hydroxyl radical cleavage of linker DNA.

In agreement with earlier work (Hayes and Wolffe, 1993), there is no change in the cleavage of DNA across the dyad axis of the nucleosome following incorporation of H5 into the dinucleosome (Figure 6A and B, arrows). This is consistent with X-ray crystallographic data suggesting that linker histones do not interact with DNA across the minor groove at the dyad axis of the nucleosome, but with the major groove of DNA away from the dyad axis (Clark *et al.*, 1993; Ramakrishnan *et al.*, 1993; Schwabe and Travers, 1993). There is a marked change in cleavage of the linker DNA following the association of H5 (Figure 6A and B, linker DNA). Like DNA wound on the surface of a histone octamer, certain sites within linker DNA are protected from DNase I cleavage by the association of H5, other sites are cleaved with the same frequency as naked DNA (Figure 7). This result suggests that the association of H5 with linker DNA is either distorting the path of linker DNA or constraining linker DNA onto a histone surface. This analysis is substantiated by hydroxyl radical cleavage (Figure 8) which reveals no major changes in the helical periodicity or cleavage

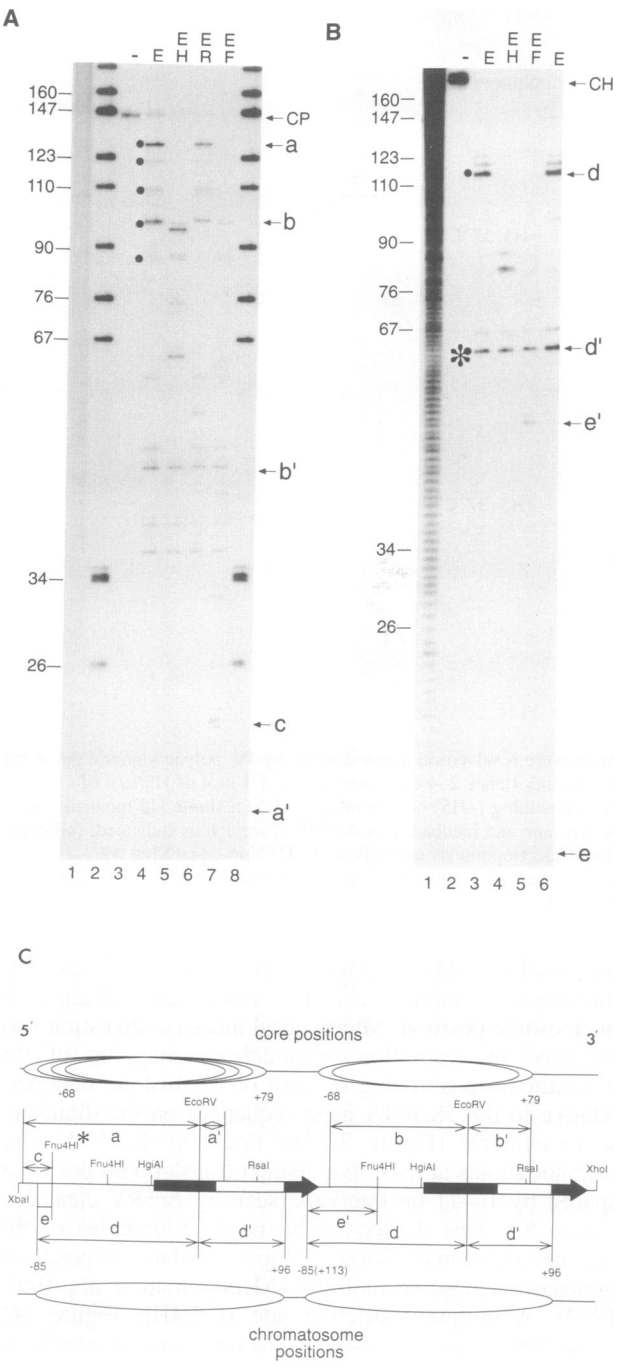
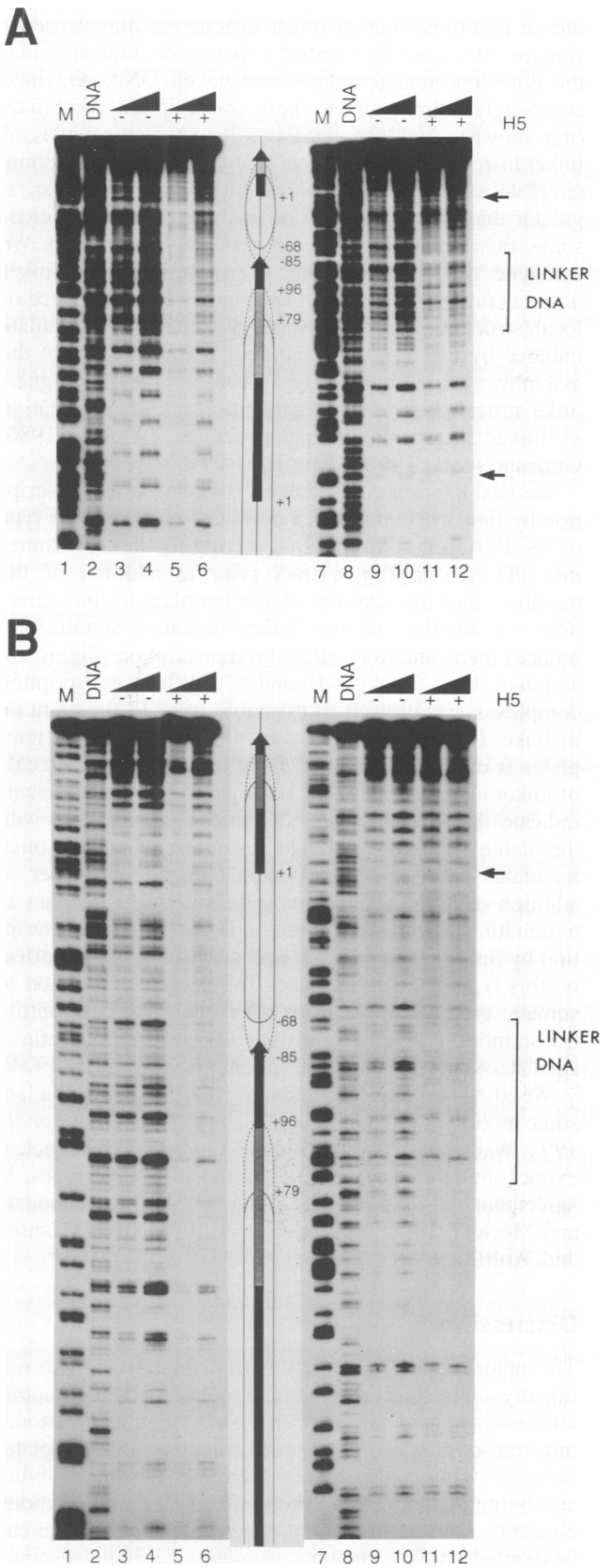


Fig. 5. Micrococcal nuclease mapping of core and chromatosome positions on reconstituted dinucleosome complexes. DNA from the nucleosome core particle and the chromatosome was recovered from an acrylamide gel (see Figure 3B) and digested with restriction enzymes to determine the positions of the boundaries of histone–DNA complexes. (**A**) Core particle positions. Predominant products of *Eco*RV (E) digestion of core particle DNA were a 129, a 100, a 46 and a 17 bp fragment, labeled a, b, b′ and a′, respectively (lane 4). However, a ladder of bands is visible below the 129 bp a band separated by 10–11 bp (dots). These represent the multiple positions occupied by the histone octamer on the 5′ 5S RNA gene repeat. Cleavage with *Eco*RV plus *Fnu*4HI (lane 7) allows resolution of 5′ boundaries of the 3′ octamer and leads to the accumulation of a novel 23 bp fragment, labeled c (lane 7). The upper bands represent the boundaries of the octamers on the 3′ 5S RNA gene repeat and the novel fragment the DNA sequence (c) from the 5′ *Fnu*4HI site to the *Xba*I site in the 5′ 5S RNA gene repeat. Band lengths were determined using *Msp*I-digested pBR322 size markers (lanes 2 and 8) and DNA fragments from a hydroxyl radical cleavage reaction (lane 1), which provide a more accurate indication of fragment sizes intermediate to the size markers. (**B**) Chromatosome positions. *Eco*RV digestion of chromatosome DNA produced a 117 and 63 bp fragment, labeled d (marked with a dot) and d′ (marked with an asterisk), respectively (lanes 3 and 6). Cleavage with *Eco*RV plus *Fnu*4HI led to a 51 and 10 bp fragment, e and e′, respectively (lane 4). Lane 1, DNA fragments from a hydroxyl radical cleavage reaction. (**C**) Location of restriction fragments in (A) and (B). Thick arrows are the 5S RNA genes. Open boxes are the internal control regions which are the binding sites for TFIIIA. Major core and chromatosome positions are indicated.

pattern following H5 association (Figure 8A). However, densitometric analysis of H5-bound dinucleosomes demonstrates an enhanced 10–11 bp modulation of cleavage in the linker DNA (Figure 8B). The DNase I and hydroxyl radical cleavage patterns, taken together, are strongly suggestive of a continual coiling of linker DNA between contacts with the histone octamers (see also McGhee *et al.*, 1983; Yao *et al.*, 1990, 1991; see Figure 11). We conclude that H5 association does not influence histone–DNA interactions across the dyad axis of the nucleosome, but modulates the path of DNA where it enters and exits wrapping around the histone octamer.



Linker histones selectively repress transcription of dinucleosome templates

We have established that dinucleosomes contain mobile nucleosomes and that inclusion of linker histones into the dinucleosomal template restricts nucleosome mobility, fixes nucleosomal position and constrains linker DNA. We next examined the functional consequences of these structural alterations.

In earlier work, we restricted nucleosome mobility on the 5S RNA gene in the absence of linker histones by using short DNA fragments (~200 bp; Hayes *et al.*, 1990, 1991). We found that contacts between core histones and DNA alone were sufficient to restrict the association of transcription factor TFIIIA with the 5S RNA gene (Hayes and Wolffe, 1992, 1993; Lee *et al.*, 1993). These results are consistent with the repression of transcription obtained when core histones are assembled into close-packed nucleosomal arrays on plasmid DNAs containing 5S RNA genes (Clark and Wolffe, 1991). Removal of histones H2A and H2B from these positioned nucleosomes allowed TFIIIA to bind to its recognition site within the 5S RNA gene and facilitated transcription of the gene (Clark and Wolffe, 1991; Hayes and Wolffe, 1992; Lee *et al.*, 1993). Our mapping of the boundaries of the histone octamer associated with the 5′ 5S RNA gene of the dinucleosome (Figure 5C) reveals that key TFIIIA binding sites (Hayes and Tullius, 1992) will be completely accessible in at least 50% of the templates. In the presence of linker histones, the TFIIIA binding site will not be accessible (see Figure 11). Thus, we first examined whether the dinucleosome template is transcriptionally competent in the absence of linker histones.

Transcription of naked DNA, mono-, di- and trinucleosome templates in extracts of *Xenopus* oocyte nuclei (germinal vesicles, GV) under efficient conditions (Birkenmeier *et al.*, 1978; Wolffe *et al.*, 1986) reveals a

Fig. 6. DNase I footprinting of dinucleosomes containing histone H5. Bound or unbound H5 dinucleosome were prepared and digested with DNase I. Individual complexes were isolated by nucleoprotein agarose (0.7%) gel electrophoresis. DNA from these complexes was isolated and analyzed by denaturing polyacrylamide (6%) gel electrophoresis. (**A**) Lanes 1–6, the 3′-end-radiolabeled coding strand of the 5S RNA gene is used as template; lanes 7–12, the 5′-end-radiolabeled non-coding strand of the 5S RNA gene is used as template. Lanes 1 and 7 show G-specific cleavage reaction used as markers. Digestion of naked DNA (lanes 2 and 8), of dinucleosomes (lanes 3, 4, 9 and 10) and of dinucleosomes containing H5 (lanes 5, 6, 11 and 12) is shown, as indicated at the top. Filled triangles indicate increasing DNase I digestion. Small arrows indicate the position of the axis of dyad symmetry of nucleosome. The large vertical arrows show the location and orientation of the 5S RNA gene. Gray boxes show the internal control region (ICR). Solid and dotted ovals indicate the predominant regions contacted by the nucleosome cores and chromatosome, respectively. The position of linker DNA is indicated. (**B**) The same set of samples was electrophoresed for a longer time to highlight the linker region of the dinucleosome.

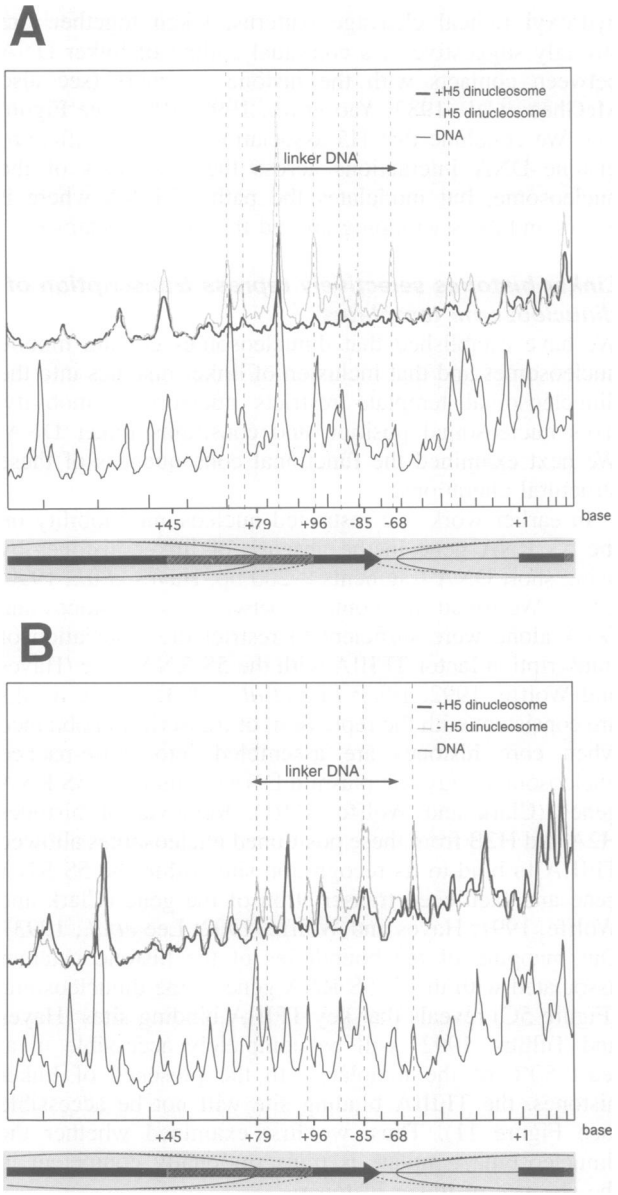


Fig. 7. Densitometric scans of DNase I cleavage of 5S dinucleosome complexes before and after histone H5 association. (**A**) Scans of the coding strand (Figure 6, lanes 2, 3 and 6); (**B**) scans of the non-coding strand (Figure 6, lanes 3, 9 and 12). The position of linker DNA is indicated. Vertical dotted lines over the peaks of DNase I cleavage of naked DNA are to facilitate comparison. The key to symbols is as in Figure 6.

progressive reduction in transcriptional activity concomitant with the increase in the number of histone octamers bound (Figure 9). In agreement with earlier work (Clark and Wolffe, 1991), close-packed histone octamers (one per 141 bp) almost completely repress transcription. However, the dinucleosome template in which histone octamers are spaced by a 50 bp linker (see Figure 1) retains significant transcriptional activity. We suggest that the accessibility of the TFIIIA binding site due to nucleosomal mobility facilitates the transcription of these templates.

Addition of linker histones to the dinucleosome templates prior to addition of oocyte nuclear extract (GV) represses transcription almost completely when one molecule is present per nucleosome (Figure 10A, lane 8). The addition of an excess of linker histones (>1 per nucleosome) does not alter the chromatosome boundaries or mobility of dinucleosomes in the extract, nor does the extract influence these parameters or nucleosome stability (Clark and Wolffe, 1991; data not shown). The transcription of naked DNA is inhibited to a much lower extent than the dinucleosome template following the addition of one or two molecules of linker histone per dinucleosome (Figure 10A, lane 3). Control experiments indicated that the dinucleosomal templates and naked DNA remained completely soluble under these transcription conditions (not shown). At higher excesses than two molecules of linker histone per nucleosome, >50% of the dinucleosome template and 'naked' DNA remain soluble. However, at greater than four molecules of linker histone per nucleosome, their solubility is greatly reduced (not shown). We conclude that the repression of transcription by linker histones on a dinucleosomal template is a consequence of local structural changes in the dinucleosomal template induced by the linker histone, and is independent of the assembly of contiguous arrays of nucleosomes into higher-order structures and of the aggregation and/or precipitation of linker histone-containing structures (Widom, 1986; Graziano *et al.*, 1994).

We next examined whether the repression of transcription by linker histones was a dominant effect on this type of 5S RNA gene. We find that mixing the linker histones into the transcription extract prior to addition of the template, and the addition of the template to the extract prior to addition of the linker histones, significantly reduces their inhibitory effect on transcription (Figure 10, compare lane 8 in A, B and C). When transcription complexes are allowed to assemble prior to the addition of linker histones, transcription from dinucleosomal templates is only reduced by 20% at a ratio of one molecule of linker histone per histone octamer. Control experiments indicate that the efficiency of repression correlates with the stable inclusion of linker histones into nucleosomal templates (not shown). We conclude that the order of addition of transcription factors versus linker histones to chromatin structures is important. Repression of transcription by linker histones is not necessarily a dominant effect on this type of 5S RNA gene. This lack of repression of somatic type 5S RNA genes after prior assembly into a transcription complex by histone H1 within chromatin is consistent with *in vivo* observations (Bouvet *et al.*, 1994).

We also explored the potential role of ATP in nucleosome mobility (Tsukiyama *et al.*, 1994; Varga-Weisz *et al.*, 1995; Wall *et al.*, 1995) within the *Xenopus* oocyte nuclear extract, but found no destabilization of nucleosomes, in agreement with earlier data (data not shown; Almouzni and Méchali, 1988; Almouzni *et al.*, 1990, 1991; Hansen and Wolffe, 1992).

Discussion

The major conclusions from this work are (i) that transcriptionally competent chromatin templates contain mobile nucleosomes and (ii) that inclusion of linker histones into transcriptionally competent dinucleosomal templates represses transcription by restricting nucleosome mobility and fixing histone–DNA contacts over essential promoter elements. Transcriptional repression by linker histones can be exerted within structures as small as a dinucleosome.

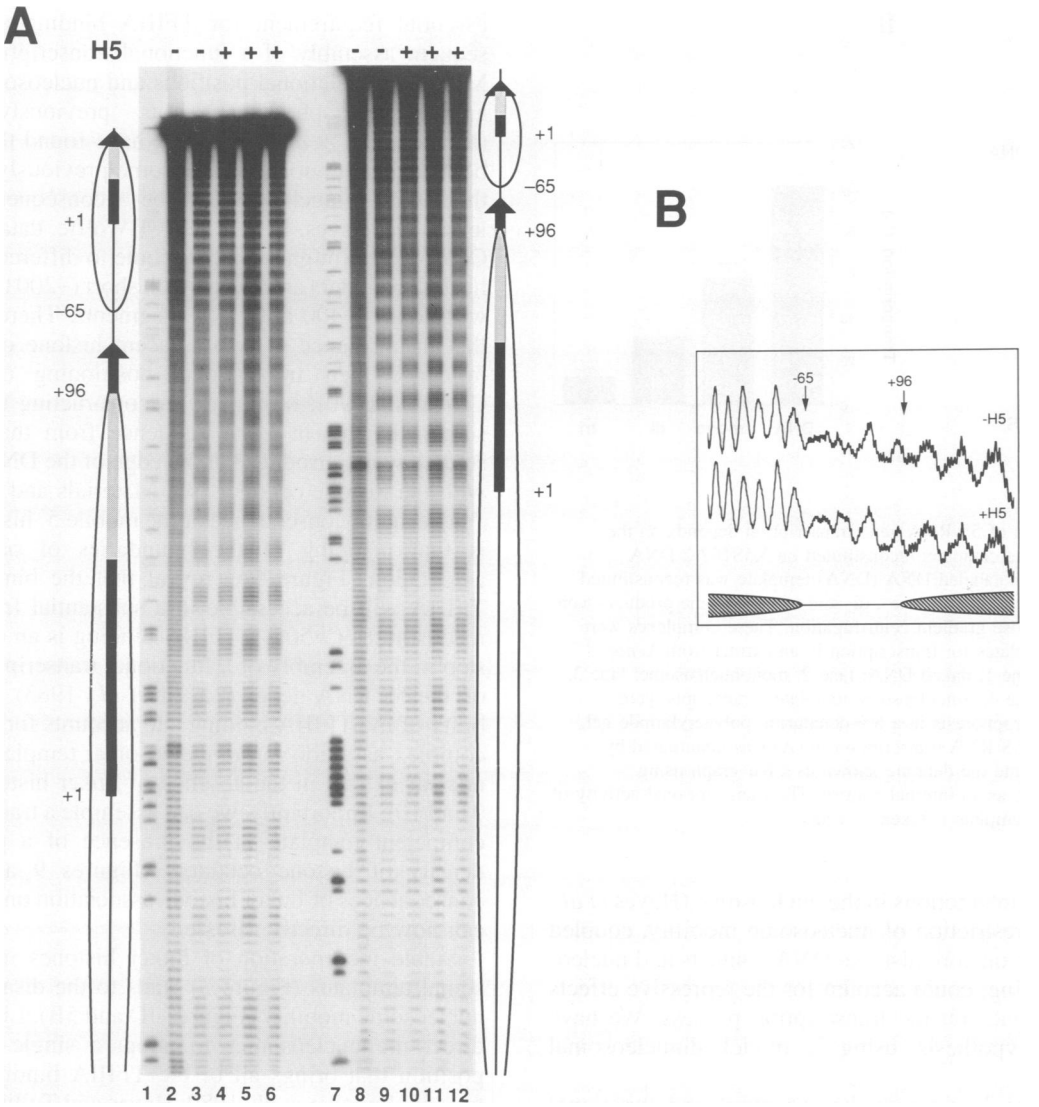


Fig. 8. Hydroxyl radical footprinting of dinucleosomes. (**A**) Hydroxyl radical footprinting of dinucleosomes. Lanes 1 and 7, Maxam–Gilbert G-specific reaction; lanes 2 and 8, cleavage pattern of naked 5S tandem repeat; lanes 3 and 9, cleavage pattern of 5S dinucleosome core complex; lanes 4 and 10, 5 and 11, and 6 and 12, footprint of dinucleosome core complex with one, two or three molar equivalents of histone H5, respectively. Lanes 1–6 and 7–12 show identical samples run for longer or shorter times, respectively, to highlight different regions of the cleavage pattern. Complexes were labeled on the coding strand. (**B**) Densitometer scans of the hydroxyl radical cleavage pattern of the linker region in the presence and absence of H5, from lanes 3 and 6, respectively. The positions of the chromatosome boundaries are indicated. The key to symbols is as in Figure 6.

Nucleosome mobility and transcriptional activity

5S RNA genes are useful templates for investigating the influence of chromatin structure on transcription. Nucleosome positioning on *Lytechinus* 5S RNA genes (Simpson and Stafford, 1983) is dependent on both core histones and linker histones (Dong *et al.*, 1990; Dong and van Holde, 1991; Hansen *et al.*, 1991; Meersseman *et al.*, 1991). When assembled into arrays (Simpson *et al.*, 1985), the folding of adjacent nucleosomes inhibits the transcription of these genes (Hansen and Wolffe, 1992, 1994). A feature of these positioned nucleosomes within arrays is the presence of multiple translational positions for the histone octamer spaced by 10–11 bp (Dong *et al.*, 1990; Meersseman *et al.*, 1991). Bradbury and colleagues have demonstrated that this multiplicity of position is due to the histone octamer being mobile, such that it has an equilibrium interaction with several different overlapping DNA sequences (Meersseman *et al.*, 1992; Pennings *et al.*, 1994). This mobility would allow a histone octamer to transiently interact with an extended DNA sequence over 146 bp in length, it would also transiently expose DNA sequences that would be stably sequestered by the histones if the octamer was fixed. This nucleosome mobility may provide a general mechanism for transcription factors to gain access to DNA. Importantly, linker histones severely inhibit nucleosome mobility (Pennings *et al.*, 1994). This restriction might limit transcription factor access to DNA.

Linker histones also influence nucleosome position (Meersseman *et al.*, 1991; Chipev and Wolffe, 1992). The basis for this may involve sequence selectivity in linker histone binding to DNA (Satchwell and Travers, 1989), it might also reflect structural selectivity (Clark *et al.*, 1993; Pruss *et al.*, 1995). Linker histones have intimate contacts with core histones (Boulikas *et al.*, 1980) and alter core

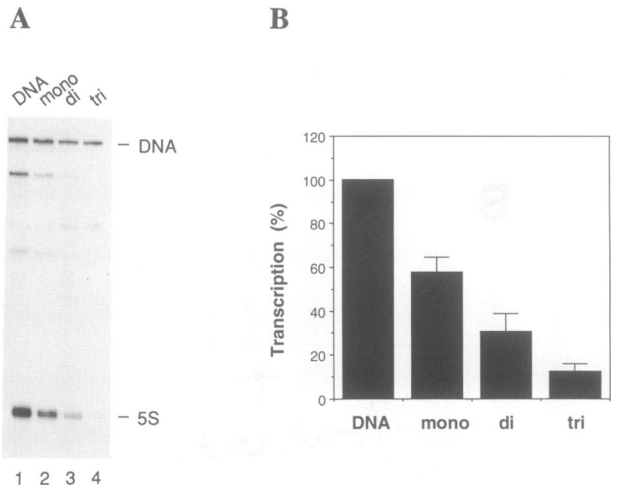


Fig. 9. Repression of 5S RNA gene transcription depends on the numbers of histone octamers reconstituted on X5S197-2 DNA template. (**A**) Radiolabeled DNA (DNA) template was reconstituted with histone octamers and mono-, di- and trinucleosome products were separated by sucrose gradient centrifugation. These complexes were then used as templates for transcription in an extract from *Xenopus* oocyte nuclei. Lane 1, naked DNA; lane 2, mononucleosome; lane 3, dinucleosome; lane 4, trinucleosome template. Transcripts were analyzed by electrophoresis in a 6% denaturing polyacrylamide gel. (**B**) The level of 5S RNA transcription in (A) was quantitated by PhosphorImager and the data are shown as a bar graph using radiolabeled DNA as an internal control. The transcriptional activity of the naked DNA template is taken as 100%.

histone–DNA interactions in the nucleosome (Hayes *et al.*, 1994). Thus, restriction of nucleosome mobility, coupled to an influence on core histone–DNA contacts and nucleosome positioning, could account for the repressive effects of linker histones on the transcription process. We have tested this hypothesis using a model dinucleosomal template.

We chose to investigate the structural and functional properties of a dinucleosome containing *Xenopus borealis* somatic 5S RNA genes for several reasons. DNA fragments of dinucleosomal size (~400 bp) can be efficiently transcribed *in vitro* using a homologous extract of *Xenopus* oocyte nuclei (Wolffe *et al.*, 1986). These 5S RNA genes position histone octamers uniquely on shorter (~200 bp) DNA fragments (Hayes *et al.*, 1990, 1991; Hayes and Wolffe, 1993). Alterations of nucleosome position and histone modification had been found to influence transcription factor access to their recognition sites (Lee *et al.*, 1993). Unlike the extended *Lytechinus* 5S nucleosomal arrays (Hansen *et al.*, 1989), dinucleosomes will not form higher-order structures that might influence the transcription process (Hansen and Wolffe, 1992, 1994). Thus, we can distinguish local effects of nucleosome structure on transcription from the consequences of the assembly of higher-order structures.

Surprisingly, unlike the mononucleosomes we have previously studied (Hayes and Wolffe, 1993), the 5′ histone octamer in the dinucleosome are mobile and show multiple translational positions (Figures 4 and 5). Since TFIIIA will not bind to an unmodified histone octamer assembled on a shorter (211 bp) DNA fragment (Hayes and Wolffe, 1992; Lee *et al.*, 1993), we interpret octamer mobility on the longer DNA fragment (424 bp) as an essential requirement for TFIIIA binding and the subsequent assembly of a functional transcription complex. Multiple translational positions and nucleosomal mobility are related phenomena, as previously described (Meersseman *et al.*, 1992). We have found the difference between the immobile nucleosome previously studied and the mobile dinucleosome to be a consequence of DNA length (J.J.Hayes, K.Ura and A.P.Wolffe, data not shown). Other factors might also contribute to differences between histone octamers associated with short (~200 bp) compared with long (>400 bp) DNA fragments. There is evidence that the presence of two adjacent histone octamers will influence their translational positioning (Shrader and Crothers, 1990). Moreover, in constructing the dinucleosome, changes in DNA sequence from that previously studied were introduced at the edge of the DNA associated with the histone octamer (see Materials and methods).

The major consequence of the mobile 5′ histone octamer is that mapping of the boundaries of octamer–DNA association (Figure 5A) reveal that the binding site for TFIIIA will be accessible in a substantial fraction of the reconstitutes (>50%). TFIIIA binding is an essential first step in the assembly of a functional transcription complex on the 5S RNA genes (Lassar *et al.*, 1983). The accessibility of the TFIIIA binding site accounts for transcription complex assembly on dinucleosomal templates in oocyte nuclear extract in the absence of linker histones (Figures 9 and 10). Importantly, we can assemble a transcriptionally competent template in the presence of a physiological density of histone octamers (Figures 9 and 10). The consequences of linker histone association on transcription can now be directly assessed.

Stable incorporation of linker histones into dinucleosomal templates (Figure 3) leads to the disappearance of nucleosome mobility (Figures 4D and 5B). Linker histones direct the nucleosomes to adopt a single predominant position that brings all of the TFIIIA binding site (+45 to +95; Engelke *et al.*, 1980; Hayes and Tullius, 1992) into contact with the histones (Figure 5C). A dinucleosomal template with the nucleosomes immobile and positioned over the TFIIIA binding site is transcriptionally silent (Figure 10). We conclude that nucleosome mobility is an important characteristic of transcriptionally competent templates. Fixation of core histone DNA contacts through incorporation of linker histones can determine transcriptional repression at a dinucleosomal level.

Recent work by Becker and colleagues (Varga-Weisz *et al.*, 1995) has established nucleosome mobility as an important aspect of transcriptional competence within chromatin templates assembled in *Drosophila* embryonic extracts. In their studies, ATP hydrolysis is necessary to generate mobile nucleosomes and addition of histone H1 is without effect. Differences in our results might be related to chromatin composition, embryonic chromatin might contain unusual histone variants and be enriched in non-histone HMG proteins that differ from the somatic core histones and linker histones used in this study (Dimitrov *et al.*, 1993, 1994; Dimitrov and Wolffe, 1995).

Linker histones and the transcription of naked versus nucleosomal DNA

Kadonaga and colleagues have examined the transcriptional repression of templates associated with H1 in the

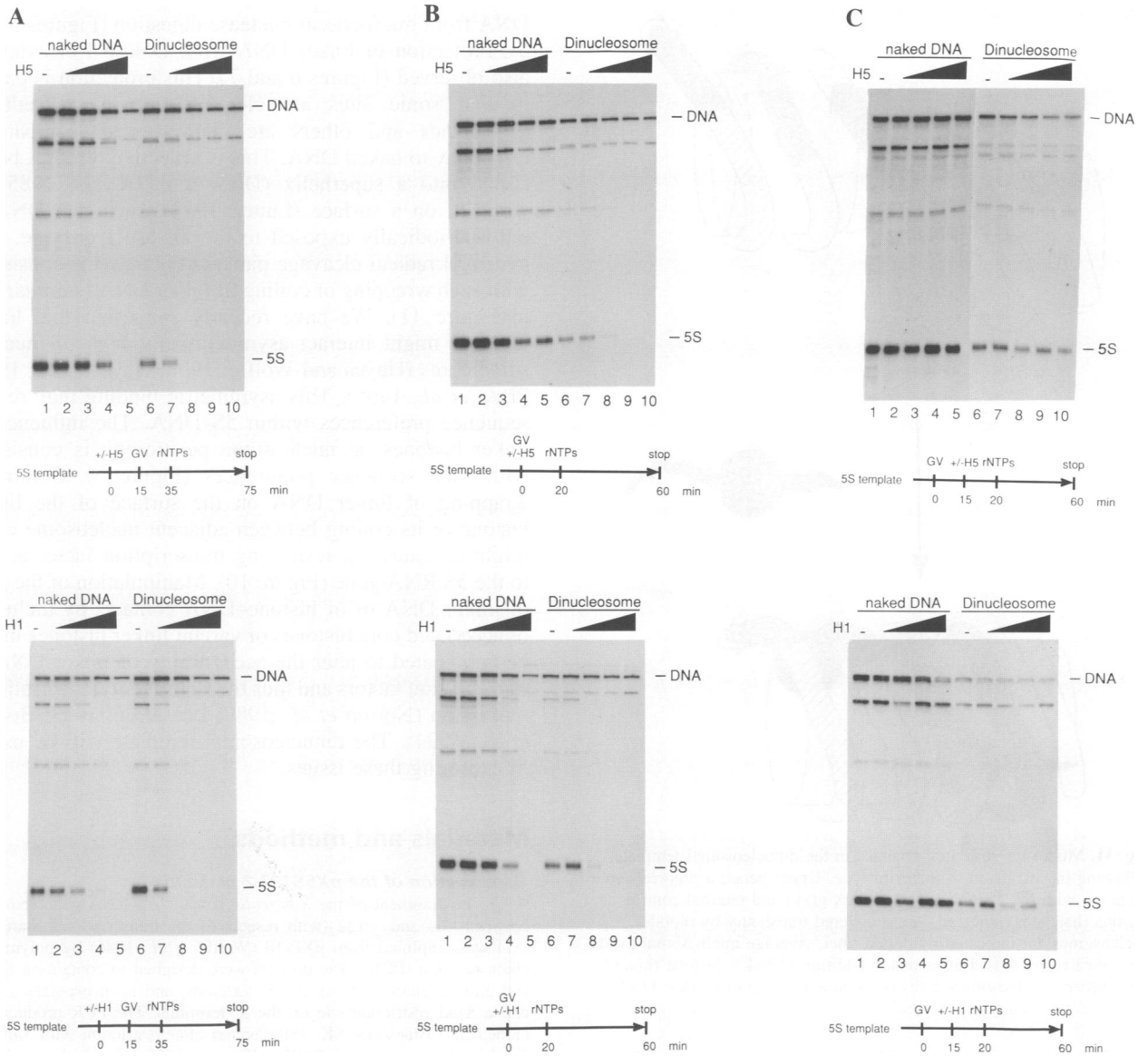


Fig. 10. Preferential repression of transcription from 5S dinucleosome templates upon binding of histone H5 (upper panel) or H1 (lower panel). Upper panels: (**A**) dinucleosome reconstituted or naked radiolabeled X5S197-2 DNA fragment (DNA) were incubated with various amounts of histone H5 (0, 0.5, 1.0, 2.0 and 4.0 molecules/nucleosome core) in 50 mM KCl at 22°C for 15 min, then transcribed *in vitro* as designated in the scheme. Lanes 1–5, 10 ng of naked DNA or lanes 6–10, 10 ng of reconstituted dinucleosomes in 50 mM KCl with 0, 1, 2, 4 and 8 ng/pmol of histone H5, respectively. GV, *Xenopus* nuclear extract. (**B**) Dinucleosome or naked DNA template was incubated with various amounts of histone H5 in an extract from *Xenopus* oocyte nuclei and then transcribed *in vitro* as shown in the scheme. Each lane was the same as with (A).
(**C**) Dinucleosome or naked DNA template was incubated with *Xenopus* oocyte nuclear extract before the addition of various amounts of histone H5. Each lane was the same as with (A). Lower panels: as above, except that histone H1 replaces H5. In each case, the position of 5S rRNA is indicated (5S). Radioactive bands that are above 5S rRNA are from radiolabeled DNA (DNA) if they are constant in all lanes; if they are diminished as linker histones are reconstituted, they represent transcripts initiated at DNA ends or that 'read-through' the 5S rRNA gene terminator.

presence or absence of core histones (Croston *et al.*, 1991; Laybourn and Kadonaga, 1991; Kamakaka *et al.*, 1993). A possible interpretation of these results is that the association of linker histones with core histones in the presence of linker DNA is more repressive than either linker histone association with close-packed nucleosomes or naked DNA (Kamakaka *et al.*, 1993; Wolffe, 1994b). Our results demonstrating the selective repression of transcription from templates associated with spaced histone octamers compared with naked DNA (Figure 10) are consistent with this conclusion. This functional result reflects the structural preference of linker histones for association with octamer templates containing linker DNA rather than with naked DNA alone (Figure 3; Hayes and Wolffe, 1993).

The order of addition of transcription factors relative to linker histones is also important in determining transcriptional activity. Prior assembly of stable transcription complexes on the *X.borealis* somatic 5S RNA gene (Bogenhagen *et al.*, 1992; Lassar *et al.*, 1983) prevents transcriptional repression by linker histones (Figure 10). However, when linker histones are mixed with the transcription extract prior to addition of the dinucleosomal template, transcription is still efficiently repressed, albeit at a higher excess of linker histone molecules per nucleosome (Figure 10B). Thus, the final stage of nucleosome assembly

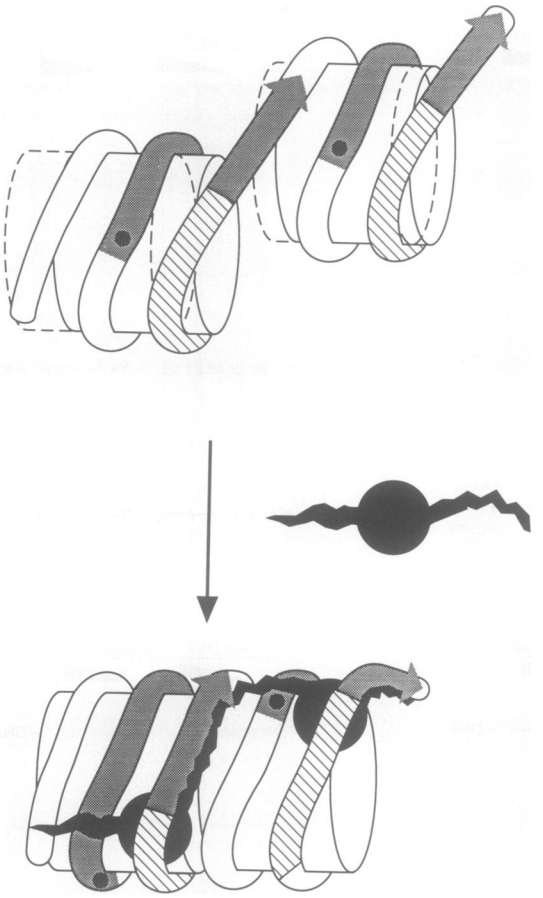


Fig. 11. Model for structural changes in the dinucleosomal template following the inclusion of linker histone. Upper panel: a dinucleosome is shown with the 5S rRNA genes (dark gray) and internal control regions (hatched) indicated. Areas covered transiently by mobile nucleosomes are indicated by dotted lines. Average nucleosomal dyads are indicated by dots. Lower panel: addition of linker histone (black) immobilizes nucleosomes, fixes position and constrains linker DNA.

involving incorporation of linker histones can establish a repressive chromatin structure before transcription complex assembly can occur in the oocyte nuclear extract. *In vivo*, assembly of the histone octamer itself occurs in stages (Worcel *et al.*, 1978; Smith and Stillman, 1991). Since linker histones do not associate with DNA bound to the histone tetramer (Hayes *et al.*, 1994), more time will presumably be available for transcription complex assembly under physiological assembly conditions before linker histone incorporation into chromatin. The failure of histone H1 to repress transcription of this type of somatic 5S RNA gene *in vitro* is consistent with *in vivo* results (Bouvet *et al.*, 1994; Kandolf, 1994).

Structural consequences of linker histone incorporation into a dinucleosome

Widom and colleagues have demonstrated that linker histones facilitate the compaction of dinucleosomes (Yao *et al.*, 1990, 1991). These observations imply that linker DNA between adjacent nucleosome cores can be constrained in a similar manner to that found in higher-order structures. In our experiments using the model dinucleosome, we find that stable incorporation of linker histones (Figure 3) leads to increased protection of linker DNA from micrococcal nuclease digestion (Figures 3 and 5). Protection of linker DNA from DNase I cleavage is also observed (Figures 6 and 7). This protection is partial in that some sites are cleaved with much reduced efficiencies and others are cleaved with comparable efficiency to naked DNA. This is reflective of DNA being coiled into a superhelix (Drew and Travers, 1985) or wrapped on a surface (Lutter, 1978), such that DNA is only periodically exposed to the DNase I enzyme. The hydroxyl radical cleavage pattern (Figure 8) is consistent with such wrapping or coiling of linker DNA (summarized in Figure 11). We have recently suggested that linker histones might interact asymmetrically with 5S nucleosome cores (Hayes and Wolffe, 1993; Hayes *et al.*, 1994; Pruss *et al.*, 1995). This asymmetric binding may reflect sequence preferences within 5S DNA. The influence of linker histones on nucleosome positioning is consistent with such sequence preferences (Figure 5). Continued wrapping of linker DNA on the surface of the linker histone or its coiling between adjacent nucleosome cores might be causal in restricting transcription factor access to the 5S RNA gene (Figure 10). Manipulation of the path of linker DNA or of histone–DNA contacts by inclusion of acetylated core histones or variant linker histones might be anticipated to alter the accessibility of linker DNA to transcription factors and thus the stability of transcriptional repression (Norton *et al.*, 1989; Lee *et al.*, 1993; Bouvet *et al.*, 1994). The dinucleosomal template will be useful in exploring these issues.

Materials and methods

Construction of the pX5S197-2 plasmid

A 185 bp fragment of the *X.borealis* somatic 5S RNA gene sequence between –64 and +122 (with respect to the transcriptional start site, +1) was amplified from pXP-10 (Wolffe *et al.*, 1986) by polymerase chain reaction (PCR). The primers were designed to contain an *Eco*RI and *Sal*I restriction site on the 5′ terminus, and to incorporate a *Xho*I and a *Kpn*I restriction site on the 3′ terminus. The PCR product was cloned into Bluescript SK- (Stratagene) after restriction with *Sal*I and *Kpn*I, and sequenced (pX5S197). Plasmid pX5S197 was digested with *Eco*RI and self-ligated (pX5S197′). An 888 bp *Sal*I–*Kpn*I fragment derived from plasmid pX5S197 was cloned into the *Xho*I–*Kpn*I site of pX5S1974 to make two tandem repeats of the 5S RNA gene. The transcription start sites of each 5S RNA gene are 197 bp away from each other (Figure 1). This plasmid was named pX5S197-2. A 424 bp *Xba*I–*Xho*I fragment derived from plasmid pX5S197-2 was isolated from non-denaturing acrylamide gels for nucleosome reconstitution after end-radiolabeling at the *Xba*I site with T4 polynucleotide kinase or Klenow fragment (New England BioLabs). This fragment was reconstituted into nucleosomes.

Purification of nucleosome core particles, histone octamers and histone H5

Histone octamers were obtained from Hela cells. HeLa nuclei and histone octamers were prepared as described by O'Neill *et al.* (1992). Nucleosome core particles (nucleosome monomer) were prepared from chicken erythrocyte nuclei (Tatchell and van Holde, 1977; Reeves and Nissen, 1993). Histone H5 was prepared from chicken erythrocytes by means of 5% perchloric acid extraction of nuclei and acetone precipitation (Hayes *et al.*, 1994).

Nucleosome reconstitution

Nucleosome cores were reconstituted onto radiolabeled DNA fragments either by exchange from chicken erythrocyte core particles or by dialysis from high salt with purified HeLa core histones (Tatchell and van Holde, 1977; Hansen *et al.*, 1991). In no circumstances did we detect differences in results due to the reconstitution methodology employed or the source of histones. In the histone exchange method, an ~15-fold template mass

excess of core particles was mixed with radiolabeled DNA in tubes followed by slow adjustment of NaCl concentration (to 1 M). Tubes were incubated at 37°C for 15 min. Samples were transferred to a dialysis bag (with a molecular size limit of 6–8 kDa) and dialyzed against 1.0 M NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM 2-mercaptoethanol for 4 h at 4°C, then dialyzed in 0.75 M NaCl buffer for 4 h, followed by a final dialysis against 10 mM Tris–HCl (pH 7.5), 0.1 mM EDTA and 1 mM 2-mercaptoethanol overnight. In this case, almost all products were dinucleosome cores and no naked DNA fragment or mononucleosome cores were detectable by nucleoprotein agarose electrophoresis.

For the salt dialysis method using purified histone octamers, radiolabeled DNA (500 ng) and unlabeled DNA (4.5 μg) were mixed with histone octamers in 2.0 M NaCl. The final DNA concentration was 0.1 mg/ml, and the histone octamer concentration was 1.0 mol of octamer/mol of DNA repeat. Samples were then dialyzed at 4°C against 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1 mM PMSF, 1 mM 2-mercaptoethanol and NaCl as follows: 2.0 M NaCl, 1 h; 1.5 M NaCl, 4 h; 1 M NaCl, 4 h; 0.75 M NaCl, 4 h. The final dialysis was overnight into 10 mM Tris–HCl (pH 7.5), 1 mM EDTA and 1 mM 2-mercaptoethanol at 4°C. The products contained naked DNA, mono-, di- and trinuclesome cores.

After reconstitution, the oligonucleosome cores were loaded on 5–20% sucrose gradients containing 10 mM Tris–HCl (pH 7.5) 1 mM EDTA and 0.1 mM PMSF, and then centrifuged for 16 h at 35 000 r.p.m. at 4°C in a Beckman SW41 rotor. Fractions were collected and analyzed by nucleoprotein agarose (0.7%) gel in 0.5× TBE (1× TBE is 90 mM Tris base/90 mM boric acid/2.5 mM EDTA) (Figure 2A). Fractions containing mono-, di- or trinucleosomes were pooled separately, concentrated to ~2.5 μg/ml using microcon-30 (amicon), and dialyzed against 10 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 1 mM 2-mercaptoethanol overnight at 4°C. Samples were stored on ice until use. Chicken erythrocyte oligonucleosomes (chromatin lengths of 1–30 nucleosomes) were prepared, after removal of linker histones (Lee *et al.*, 1993), and used as a control for isolation of the native dinucleosome complex (Figure 2B).

H5 binding experiments

A total of 100 ng (DNA content) of reconstituted nucleosome cores (0.6 pmol) were incubated with various amounts of histone H5 in 10 μl of binding buffer [10 mM Tris–HCl (pH 8.0)/50 mM NaCl/0.1 mM EDTA/5% (v/v) glycerol] at room temperature for 15–30 min (Hayes and Wolffe, 1993). Samples were loaded directly onto running 0.7% agarose gel in 0.5× TBE. After electrophoresis, the gels were dried and autoradiographed.

Two-dimensional gel experiments

Two-dimensional gel experiments to show redistribution of nucleosome cores were performed following the procedure of Meersseman *et al.* (1992) with slight modifications. Reconstitutes with or without histone H5 were loaded onto non-denaturing 4% polyacrylamide (29:1 acrylamide: bisacrylamide) gels at 4°C in 0.5× TBE. The gels were run at a maximum of 10 V/cm. Each lane was cut in half lengthwise. One half of each lane was left at 4°C, and the other was sealed and immersed at 37°C for 1 h. The gel strips were then arranged on top of a second non-denaturing gel in the cold, and the second dimension was electrophoresed at 4°C under the same conditions as the first dimension.

DNase I and hydroxyl radical footprinting

Reconstitutes with or without histone H5 were treated with either DNase I or hydroxyl radicals prior to resolving nucleoprotein complexes on preparative 0.7% agarose gels (Hayes and Wolffe, 1992). Samples contained labeled dinucleosome (60 ng of DNA) and chicken erythrocyte core particles (~1 μg), and were incubated with or without 200 ng histone H5 (1 molecule/nucleosome core) as described above. Mg^{2+} was adjusted to 4 mM concomitantly with addition of DNase I. Naked DNA was digested with 12 ng of DNase I (Gibco BRL), nucleosome cores without H5 were digested with 30–60 ng enzyme, and nucleosome cores with H5 were digested with 480–960 ng enzyme. DNase I reaction were carried out at room temperature for 1 min and terminated by addition of EDTA (5 mM). Glycerol (5%, v/v) was added to the sample and the entire reaction volume was transferred directly into a preparative gel. The hydroxyl radical reaction were carried out as described by Hayes *et al.* (1990). Free radical reactions were quenched with the addition of glycerol to a concentration of 5%, and the entire volume was applied to a gel, as described above. After electrophoresis, bound or unbound H5 dinucleosome complexes were excised from the gel. DNA from these complexes was isolated and analyzed by denaturing polyacrylamide (6%) gel electrophoresis. Specific DNA markers were produced by Maxam and Gilbert cleavage at G residues.

Micrococcal nuclease mapping

Dinucleosome (80 ng of DNA) in the absence or presence of 16 ng of histone H5 (molar ratio of histone to DNA = 1) were digested with 0.075–0.6 U of micrococcal nuclease (Pharmacia) for 5 min at 22°C. Incubation with H5 was as described above. Ca^{2+} was adjusted to 0.5 mM concomitantly with addition of micrococcal nuclease. Digestions were terminated with addition of EDTA (5 mM), SDS (0.25%, w/v) and proteinase K (Gibco BRL) (1 mg/ml). The DNA was recovered and 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and the end-labeled DNA fragments were separated by electrophoresis in non-denaturing 6% polyacrylamide gels. DNA fragments of nucleosome core and chromatosome products were recovered and digested with restriction endonucleases to determine micrococcal nuclease cleavage sites (Hayes and Wolffe, 1993).

Transcription reactions

Mono-, di- or trinucleosome complexes previously resolved and separated by sucrose gradient centrifugation or naked DNA were used as templates for transcription in an extract from *Xenopus* oocyte nuclei. Oocyte nuclear extract was prepared as described previously (Birkenmeier *et al.*, 1978). Transcription reaction conditions were as follows: 10 ng radiolabeled template were added to a 10 μl of reaction mixture containing 5 μl of nuclear extract in J buffer [10 mM HEPES (pH 7.4), 50 mM KCl, 7 mM $MgCl_2$, 2.5 mM dithiothreitol (DTT), 0.25 U/μl RNasin (Gibco BRL) and 0.1 mM EDTA] and pre-incubated for 20 min before addition of exogenous triphosphates to 250 μM ATP, CTP and GTP, 50 μM UTP with 2.5 μCi added [α-^{32}P]UTP. The reaction temperature was 22°C. Labeling was continued for 40 min after pre-incubation. Radiolabeled transcripts were extracted with phenol, precipitated with ethanol and analyzed by electrophoresis in a 6% denaturing polyacrylamide gel. The level of 5S RNA transcription was quantitated with a Molecular Dynamics PhosphorImager. The radiolabeled 5S DNA template served as an internal control for recovery.

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