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Differential association of HMG1 and linker histones B4 and H1 with dinucleosomal DNA: structural transitions and transcriptional repression

Kiyoe Ura, Karl Nightingale and Alan P.Wolffe

Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, NIH, Bldg 6, Room B1A-13, Bethesda, MD 20892-2710, USA

We examined the structural and functional consequences of incorporating either histone H1, histone B4 or HMG1 into a synthetic dinucleosome containing two 5S rRNA genes. We found that all three proteins bind to linker DNA, stabilizing an additional 20 bp from micrococcal nuclease digestion and restrict nucleosome mobility. Histone H1 has the highest-affinity interaction with the dinucleosome; histone B4 and HMG1 associate with significantly reduced affinities. We found that histone H1 binds to the dinucleosome template with a dissociation constant (K_D) of 7.4 nM, whereas the K_D is 45 nM for histone B4 and 300 nM for HMG1. The K_Ds for the interaction of these proteins with naked DNA are 18 nM for H1, 80 nM for B4 and 300 nM for HMG1. The differences in association of these proteins with the dinucleosome are reflected in the efficiency with which the different proteins repress transcription from the 5S rRNA genes. Thus, although all three proteins can contribute to the organization of chromatin, the stability of the structures they assemble will vary. Our results provide a molecular explanation for the transcriptional promiscuity of *Xenopus* early embryonic chromatin, which is enriched in HMG1 and linker histone B4, but deficient in histone H1.

Keywords: dinucleosomes/DNA/histone B4/histone H1/HMG1

Introduction

During early embryogenesis in *Xenopus*, there are major changes in chromosomal composition that correlate with alterations in nuclear activity (Newport and Kirschner 1982; Rupp and Weintraub, 1991; Dimitrov *et al.*, 1993, 1994; Hock *et al.*, 1993; Bouvet *et al.*, 1994; Dworkin-Rastl *et al.*, 1994; Hyrien *et al.*, 1995). Early embryonic chromatin prior to the mid-blastula transition (MBT) is enriched in HMG1 and linker histone B4 (Dimitrov *et al.*, 1993, 1994; Dworkin-Rastl *et al.*, 1994; Nightingale *et al.*, 1996a). After the MBT, the developmental stage at which transcription is first activated (Newport and Kirschner, 1982), HMG1 and histone B4 are progressively diluted as histone H1 accumulates in chromatin (Dimitrov *et al.*, 1993; Hock *et al.*, 1993; Dworkin-Rastl *et al.*, 1994). This replacement has a causal role in the repression of oocyte 5S rRNA genes (Bouvet *et al.*, 1994; Kandolf, 1994). The exact molecular mechanisms that lead to these changes in chromatin composition and structure, thereby establishing a more repressive environment for transcription, have not been resolved.

A useful approach to interrelate chromatin structure with transcription has been to make use of short DNA fragments that are long enough to be competent for transcription, but short enough to allow aspects of their nucleoprotein organization to be determined (Wolffe *et al.*, 1986; Lorch *et al.*, 1987; Losa and Brown, 1987; Schild *et al.*, 1993; Studitsky *et al.*, 1994; Ura *et al.*, 1995). A synthetic dinucleosome containing two 5S rRNA genes was used to demonstrate that physiologically spaced histone octamers would partially repress transcription, but only by ~70% relative to naked DNA (Ura *et al.*, 1995). The addition of one molecule of histone H1 or H5 per histone octamer allowed the establishment of a completely repressive chromatin structure. Histone octamers are mobile on an extended (>200 bp) DNA molecule, but the addition of histone H1 or H5 restricts the mobility of histone–DNA contacts and fixes nucleosome position (Pennings *et al.*, 1994; Ura *et al.*, 1995). This provides a potential explanation for the transcriptional competence of templates assembled only with histone octamers and for the repressive influence of histone H1 on transcription (Schlissel and Brown, 1984; Wolffe, 1989; Meersseman *et al.*, 1991, 1992; Chipev and Wolffe, 1992; Bouvet *et al.*, 1994; Pennings *et al.*, 1994; Ura *et al.*, 1995).

We have recently shown using mononucleosomes that both HMG1 and histone B4 can associate with linker DNA, and stabilize it against digestion with micrococcal nuclease (Nightingale *et al.*, 1996a). These properties are similar to those of histone H1 (Noll and Kornberg, 1977; Simpson, 1978). We next wished to explore whether histone B4 and HMG1 would organize a true linker DNA between two adjacent nucleosomes (Ura *et al.*, 1995), and whether they would also restrict nucleosome mobility, fix nucleosome position and potentially repress transcription. We find that incorporation of histone B4 and HMG1 into dinucleosomal templates will lead to structural transitions comparable with those obtained with histone H1, and that these correlate with transcriptional repression. However, histone B4 and HMG1 associate more weakly with dinucleosomes than histone H1. This instability provides a molecular explanation for the functional properties of early embryonic chromatin.

Results

The association of histone H1, histone B4 and HMG1 with dinucleosomal templates

We reconstituted a dinucleosome using two *Xenopus borealis* 5S rRNA genes within a 424 bp DNA fragment (Ura *et al.*, 1995). We wished to make use of strong nucleosome positioning signals in the 5S RNA genes to

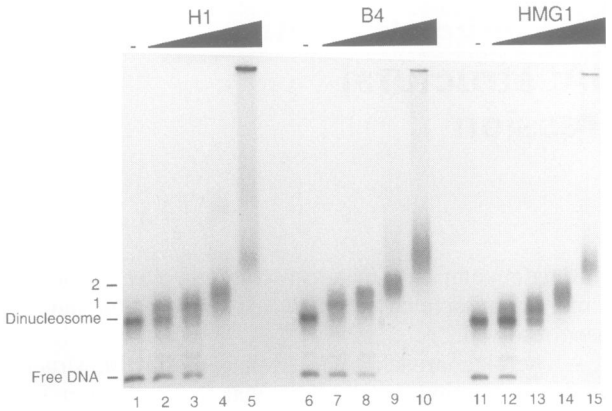

Fig. 1. Binding of histone H1, B4 and HMG1 to dinucleosome cores. Reconstituted dinucleosomes were mixed with free DNA before various amounts of histone H1, B4 and HMG1 were added. Complexes were analyzed by nucleoprotein agarose (0.7%) gel electrophoresis. Dinucleosome cores (7.5 ng DNA content) were mixed with 0, 1.25, 2.5, 5.0 and 10.0 ng of histone H1 (lanes 1–5); 0, 12.5, 25, 50 and 100 ng of B4 (lanes 6–10); or 0, 50, 100, 200 and 400 ng of HMG1 (lanes 11–15). The positions of free DNA, dinucleosomes and retarded complexes containing one (1) and two (2) molecules of H1, B4 or HMG1 per dinucleosome are indicated.

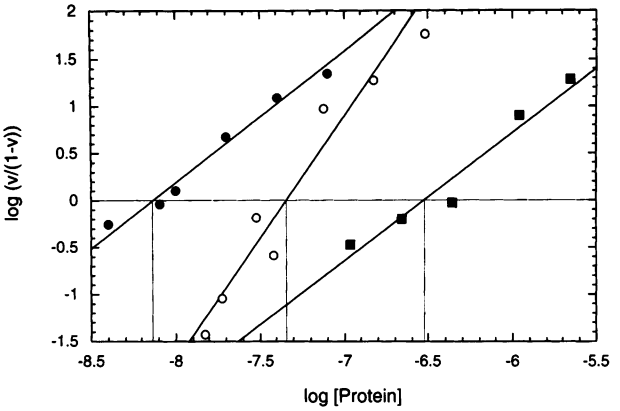

Fig. 2. Hill plot to show the binding affinity of dinucleosome cores for either histone H1, B4 or HMG1. Dinucleosome cores (1.5 nM) were mixed with a series of concentrations of histone H1 (●), B4 (○) or HMG1 (■). The fraction of bound dinucleosome (v) was quantitated and plotted against the protein concentration ([Protein]) reciprocally. Dissociation constants (K_D) were 7.4 nM for histone H1, 45 nM for histone B4 and 300 nM for HMG1.

separate nucleosomes along the DNA fragment (Simpson *et al.*, 1985; Simpson, 1991) and the capacity to have very efficient *in vitro* transcription of these genes as short linear DNA fragments (Wolffe *et al.*, 1986). The reconstituted chromatin was fractionated on a sucrose gradient and each fraction analyzed by nucleoprotein gel electrophoresis. Dinucleosomal complexes were pooled and used in subsequent experiments (Ura *et al.*, 1995).

To examine the reconstitution of histone H1, histone B4 and HMG1 into dinucleosomes, we first made use of a gel-retardation assay. 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; Hayes *et al.*, 1994; Ura *et al.*, 1995; Nightingale *et al.*, 1996a,b). Histone H1, histone B4 and HMG1 were added to a mixture of dinucleosomes and naked DNA (Figure 1). Increasing concentrations of histone H1 revealed the selective association of one molecule of histone H1 with the dinucleosome template before significant binding of histone H1 to naked DNA occurred (Figure 1, lane 3). The association of a second molecule of histone H1 with the dinucleosome then occurs concomitant with the binding of H1 to naked DNA (Figure 1, lane 4). Higher excesses of H1 lead to aggregation of both dinucleosomes and naked DNA templates (Figure 1, lane 5). With histone B4, two molecules of linker histone can bind to the dinucleosome template before significant association with free DNA occurs (Figure 1, lane 8). Aggregation of histone B4 bound to dinucleosomes and to naked DNA templates occurs at higher histone B4 concentrations (Figure 1, lanes 9 and 10). HMG1 shows less selectivity for nucleosomal DNA compared with naked DNA (Figure 1, lanes 11–15). Although two discrete complexes are obtained on reconstitution of HMG1 into dinucleosomes (Figure 1, lanes 13 and 14), this association occurs under conditions such that naked DNA is also bound by HMG1. We conclude that specific complexes containing one or two molecules of either histone H1, histone B4 or HMG1 can be reconstituted using dinucleosomes. However, the selectivity of the interaction varies, with histones H1 and B4 favoring nucleosomal compared with naked DNA, whereas HMG1 shows relatively little preference for nucleosomal DNA compared with naked templates.

Our next experiments quantitated the affinity with which the first molecule of histone H1, histone B4 and HMG1 was incorporated into a dinucleosomal template (Figure 2). We found that histone H1 binds to the dinucleosome template with a dissociation constant (K_D) of 7.4 nM, whereas the K_D is 45 nM for histone B4 and 300 nM for HMG1. Thus, histone B4 binds to dinucleosomes with a 6-fold reduction in affinity compared with histone H1, and HMG1 with a 40-fold reduction in affinity. Naked DNA is not readily resolved into a discrete complex with HMG1, B4 or H1 in these gel-retardation assays. This is because linker histones and HMG1 do not bind at a single site relative to DNA sequence and multiple proteins readily associate with 'naked' DNA, eventually forming non-physiological aggregates. Thus, binding constants for proteins with naked DNA have to be determined in separate experiments to those using dinucleosomes. The dissociation constants for the interaction of these proteins with naked DNA are 18 nM for H1, 80 nM for B4 and 300 nM for HMG1 (data not shown). In our subsequent experiments, we normalized the excess of histone H1, histone B4 and HMG1 to ensure that one to two molecules bind per dinucleosomal template. This stoichiometry of linker histones to core histones within the dinucleosome approximates to that found in the chromatin of somatic cells (Bates and Thomas, 1981). However, the stoichiometry of HMG1 to core histones within the dinucleosome greatly exceeds that found in normal somatic chromatin, but approximates to that found in the chromatin of paternal pronuclei in *Xenopus* (Dimitrov *et al.*, 1994; Nightingale *et al.*, 1996a). We next examined the structural transitions within the dinucleosomal template that occur following inclusion of these different proteins.

The nucleoprotein organization of dinucleosomes containing either histone H1, histone B4 or HMG1

A standard assay for the stable inclusion of linker histones or HMG1 into chromatin is the appearance of new DNA

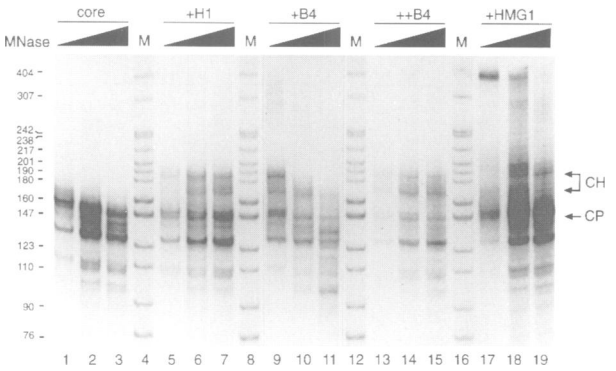

Fig. 3. Micrococcal nuclease digestion of reconstituted dinucleosome. Reconstituted dinucleosomes (20 ng of DNA) were digested with increasing amounts of micrococcal nuclease in the absence of linker histone (lanes 1–3) or the presence of either 4 ng of histone H1 (lanes 5–7), 12.5 ng of B4 (lanes 9–11), 25 ng of B4 (lanes 13–15) or 200 ng of HMG1 (lanes 17–19). Products of digestion were labeled with [γ-^{32}P]ATP and analyzed by native PAGE. Lanes 4, 8, 12 and 16 contained *Msp*I-digested pBR322 size makers. Arrows indicate the core particle (CP) and chromatosome (CH) products of digestion. Note that this figure shows results from two different sample digestions and gels; data from one experiment are shown in lanes 1–12 and the second in lanes 13–19.

fragments of distinct size during micrococcal nuclease digestion compared with those generated in the presence of core histones alone (Simpson, 1978; Allan *et al.*, 1980; Hayes *et al.*, 1993; Nightingale *et al.*, 1996a). Using mononucleosomal particles, in the presence of histone octamers and either linker histones or HMG1, core particles containing 148 bp of DNA and chromatosome particles containing 168 bp of DNA accumulate (Nightingale *et al.*, 1996a). However, during the digestion of native chromatin consisting of nucleosomal arrays by micrococcal nuclease, a series of digestion intermediates accumulates: the core particle containing 146 bp of DNA, and H1 containing particles of 165, 175 and 185 bp (Bavykin *et al.*, 1990). The varying extent of DNA protection on micrococcal nuclease digestion of nucleosomal arrays containing H1 has been interpreted as reflecting the restricted access of the enzyme to DNA when coiled as a continual superhelix within the higher-order structures found in native chromatin (Bavykin *et al.*, 1990; Ura *et al.*, 1995; see Felsenfeld and McGhee, 1986). Micrococcal nuclease gains access to DNA much more frequently when the double helix is exposed towards solution, this will occur once per helical turn within a putative superhelix between two adjacent nucleosomes (i.e. every 10–11 bp). Note that micrococcal nuclease can only be used to examine kinetic intermediates in the digestion of nucleoprotein complexes. The enzyme disrupts protein–DNA interactions and can only provide a quantitative assay for complex assembly. The quantitative assay for the assembly of a nucleoprotein complex is the gel-retardation assay.

Analysis of the kinetic intermediates during micrococcal nuclease digestion of dinucleosomal particles reconstituted with histone octamers alone reveals that the longest DNA fragments accumulating are 146 bp DNA in length (Figure 3, lanes 1–3). This corresponds to the length of DNA in a nucleosome core particle (CP) (van Holde, 1988). In the presence of histone H1, longer DNA fragments of 167 and 184 bp accumulate, together with core particle size DNA (146 bp) (Figure 3, lanes 5–7). The longer DNA fragments reflect the assembly of H1 into the nucleosome and the protection of additional linker DNA within the dinucleosome in chromatosome-like particles (CH) (Simpson, 1978; Bavykin *et al.*, 1990). Micrococcal nuclease digestion of the 5S mononucleosome only yields chromatosome particles containing DNA of 168 bp in length (Nightingale *et al.*, 1996a); thus, the presence of two adjacent nucleosomes is necessary to generate the chromatosome particles containing DNA that is 184 bp in length. This presumably reflects additional constraints on the path of linker DNA in the dinucleosome. Using the dinucleosomal template, longer DNA fragments of 167 and 184 bp accumulate in the presence of histone B4 (Figure 3, lanes 9–15), and following the reconstitution of HMG1 into the dinucleosome (Figure 3, lanes 17–19). However, the relative proportions of the two intermediates in the digestion of 184 and 167 bp differ for HMG1 compared with dinucleosomes containing histones H1 or B4 (Figure 3, compare lanes 17–19 with lanes 5–7 and 9–15). A significantly smaller proportion of the 167 bp fragment accumulates during digestion compared with the 184 bp fragment in dinucleosomes containing HMG1 (see Discussion). Thus, although all three proteins (histone H1, histone B4 and HMG1) protect linker DNA from digestion, the exact pattern of digestion by micrococcal nuclease differs. This presumably reflects variation in the constraint of linker DNA in the dinucleosome by these very different proteins. We next investigated whether the inclusion of histone H1, histone B4 or HMG1 into dinucleosomes would influence the positioning of nucleosomes.

Micrococcal nuclease digestion of the various dinucleosome reconstitutes was followed by restriction endonuclease mapping of the boundaries of histone–DNA complexes relative to the 5S RNA gene sequences (Dong *et al.*, 1990; Meersseman *et al.*, 1991). We determined translational positioning using both the 184 and 167 bp DNA fragments (Figure 4, summarized in Figure 5). We find that the histone octamers alone occupy four predominant translational positions spaced by 9–11 bp intervals (seen by *Eco*RV cleavage in Figure 4A, lane 4 dots, only the 5′ boundaries of the octamer are shown). These multiple translational positions separated by integral helical turns of DNA (summarized in Figure 5, core positions) suggest that nucleosome mobility exists with respect to DNA sequence (Meersseman *et al.*, 1991; Ura *et al.*, 1995; see Figure 6 later). Recovery of the DNA fragments 167 bp in length from chromatosomes containing histone B4, histone H1 and HMG1 reveals changes in the position and stability of the boundaries of nucleosomes. Multiple translational positions exist for the histone–DNA complexes containing histone B4 (Figure 4A, lanes 8–11); however, a single predominant translational position exists for those containing histone H1 (Figure 4A, lanes 12–15) or HMG1 (Figure 4A, lanes 17–20). These positions are shown in Figure 5 (compare B4, H1 and HMG1, chromatosome 167 bp positions). Thus, histone B4 appears to exert less constraint on the position of histone–DNA contacts than histone H1 or HMG1. Surprisingly, extension of this analysis to kinetic intermediates in micrococcal nuclease digestion of 184 bp in length demonstrates that histone H1, histone B4 and HMG1 all confer a stable nucleosome position (Figure 4B, two boundaries separated by 4 bp

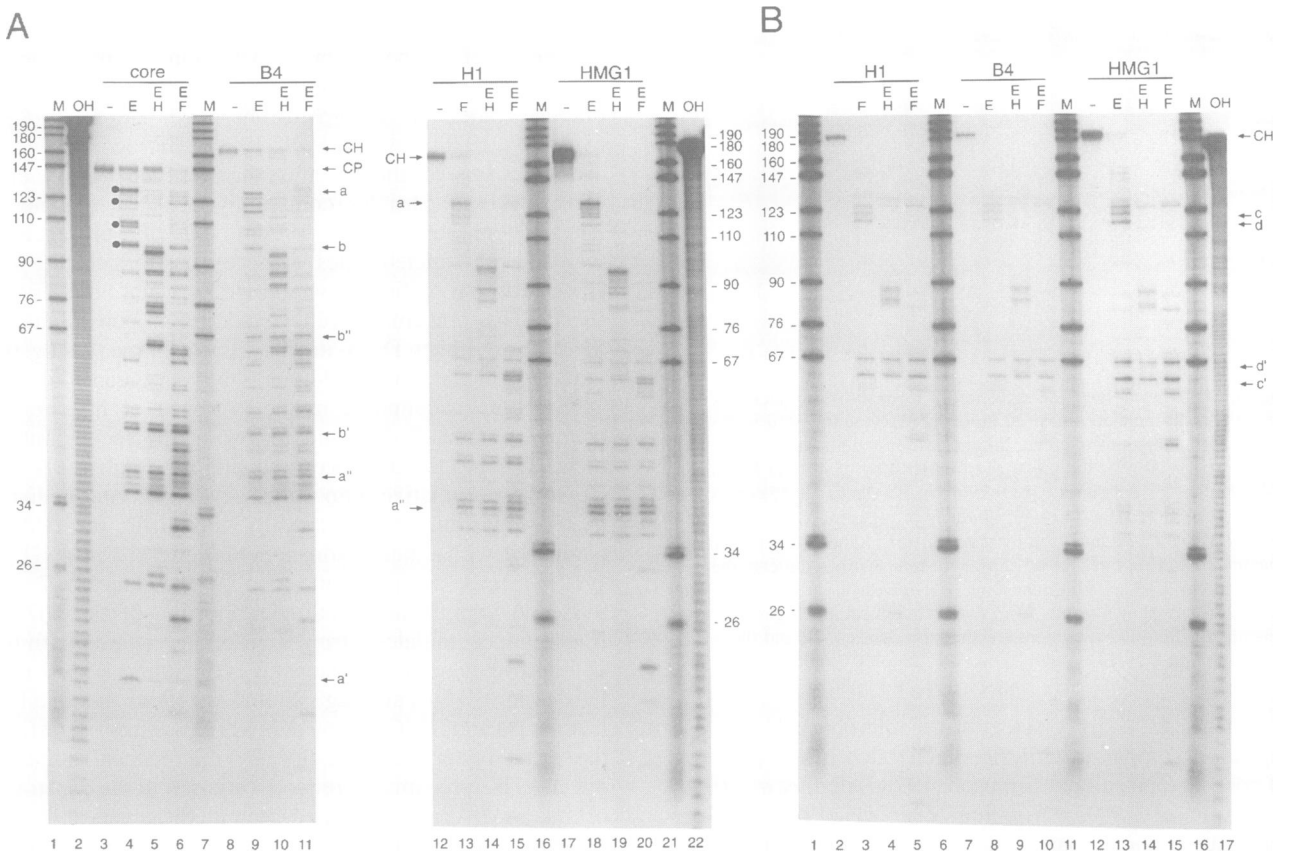

Fig. 4. Micrococcal nuclease mapping of core particle (CP) and chromatosome (CH) positions on dinucleosome complexes. DNA from the nucleosome CP and CH was recovered from an acrylamide gel (see Figure 3) and digested with *Eco*RV (E) and additional restriction enzymes, *Hgi*AI (H) and *Fnu*4HI (F) to determine the positions of the boundaries of histone–DNA complexes. (**A**) Micrococcal nuclease mapping of positions of the core particle (lanes 3–6) and the chromatosomes containing 167 bp DNA and either histone B4 (lanes 8–11), H1 (lanes 12–15) or HMG1 (lanes 17–20). DNA fragments of core particle (lane 3) and chromatosome (lanes 8, 12 and 17) were digested with restriction enzymes, as indicated. Predominant products of *Eco*RV digestion of core particle DNA are labeled a, b, b′ and a′, respectively (lane 4); those from chromatosomes are labeled a and a″, respectively (lanes 9, 13 and 18). Dots between lanes 3 and 4 indicate the 5′ boundaries of DNA contacts made by the histone octamer in reconstitutes only containing the four core histones. (**B**) Micrococcal nuclease mapping of positions of the chromatosome containing 184 bp DNA and either histone H1 (lanes 2–5), B4 (lanes 7–10), or HMG1 (lanes 12–15). DNA fragments from chromatosomes (lanes 2, 7 and 12) were digested with restriction enzymes, as indicated. Predominant products of *Eco*RV digestion of chromatosome are labeled c, d, d′ and c′, respectively (lane 3, 8 and 13). Fragment lengths were determined using *Msp*I-digested pBR322 size makers (lane M) and DNA fragments from a hydroxyl radical cleavage reaction (lane OH).

within a single helical turn of DNA are delineated). We favor two possible interpretations of this difference in positioning for nucleosomes containing B4 that are dependent on linker length: either a mixture of two different complexes exists in our reconstitutes reflecting distinct modes of linker histone B4 incorporation into the nucleosome, or the positioning of histone–DNA complexes containing B4 is destabilized as the length of DNA in the complex is reduced from 184 to 167 bp. Both of these possibilities have been previously suggested by Mirzabekov and colleagues (Bavykin *et al.*, 1990; Mirzabekov *et al.*, 1990). An alternate possibility is that two molecules of linker histone interact with a single histone octamer in a fraction of our templates generating a stable 184 bp digestion intermediate. This appears unlikely due to the reduced affinity of a second linker histone molecule for a mononucleosome already containing a linker histone molecule (Nightingale *et al.*, 1996b).

Our next experiments examined the influence of the incorporation of histone H1, histone B4 or HMG1 on the mobility of histone octamers within the dinucleosome (Meersseman *et al.*, 1991, 1992; Pennings *et al.*, 1994; Ura *et al.*, 1995). The assay for nucleosome mobility makes use of two dimensions of non-denaturing PAGE (Meersseman *et al.*, 1992; Ura *et al.*, 1995). Movement of the histone octamer between two different translational positions along the DNA molecule, during incubation of the gel slice isolated after the first dimension of electrophoresis, is reflected in nucleoprotein complexes that migrate away from a simple diagonal. Consistent with earlier work, we find that histone octamers alone are mobile with respect to DNA sequence and that mobility increases from 4 to 37°C (Figure 6A, compare 4°C with 37°C, the arrowheads indicate discrete complexes migrating away from the diagonal) (Pennings *et al.*, 1994; Ura *et al.*, 1995). Reconstitution of histone H1 into the dinucleosome restricts nucleosome mobility (compare 37°C panels between Figure 6A and B). Likewise, reconstitution of histone B4 and HMG1 into the dinucleosome restricts nucleosome mobility, albeit to a lesser extent than histone H1 (Figure 6C and D). It should be noted that a 5-fold larger excess of B4 and a 40-fold higher excess of HMG1 are required to restrict nucleosome mobility to

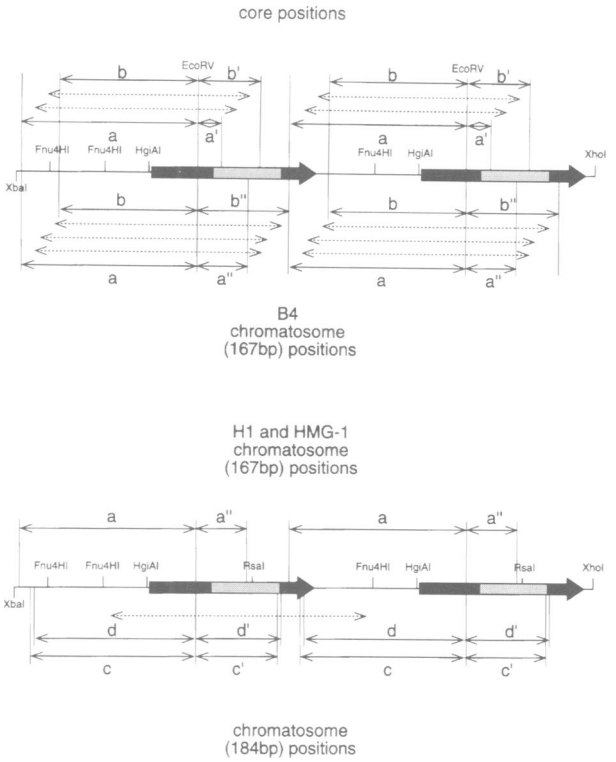

Fig. 5. Location of restriction fragments described in Figure 4. Thick arrows, 5S RNA gene; dotted boxes, internal control region. Dotted lines indicate minor nucleosome positions which are not labeled in Figure 4.

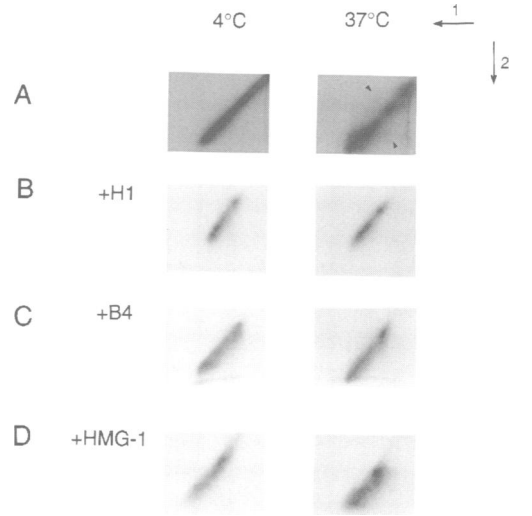

Fig. 6. The influence of histone H1, B4 and HMG1 on nucleosome mobility. Dinucleosomes (100 ng DNA content) either in the absence (**A**) or presence of 20 ng of histone H1 (**B**), 100 ng of B4 (**C**) or 800 ng of HMG1 (**D**) were resolved on a native 4% polyacrylamide gel at 4°C before incubation of the gel lane 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. In (A), the arrowheads indicate nucleoprotein complexes that migrate away from the diagonal and are therefore mobile.

approximately the same extent as H1 (Figure 6). We conclude that consistent with their interaction with linker DNA (Figures 3–5), histones H1 and B4, and HMG1 constrain the mobility of the histone octamer with respect to DNA sequence (Figure 6).

In earlier work, we were able to demonstrate the selective constraint of the path of linker DNA following incorporation of histone H5 into a dinucleosome (Ura *et al.*, 1995). This result is in marked contrast to the lack of any H5 footprint obtained on mononucleosomal reconstitutes (Hayes and Wolffe, 1993; Hayes *et al.*, 1994). Likewise, reconstitution of histone B4 and HMG1 into mononucleosomes did not lead to any change in hydroxyl radical or DNase I cleavage pattern (Nightingale *et al.*, 1996a). In agreement with earlier data using histone H5 (Ura *et al.*, 1995), inclusion of histone H1 into dinucleosomes leads to a general reduction in the efficiency of DNase I cleavage and to specific alterations in the cleavage pattern of linker DNA between the two nucleosomes (Figure 7A, lanes 3–5). Some DNase I cleavages remain, others are eliminated in linker DNA (small arrowheads within linker DNA), consistent with the coiling of the double helix between adjacent nucleosomes. Very similar changes in the DNase I cleavage pattern of linker DNA occur when histone B4 is reconstituted into dinucleosomes (Figure 7B, lanes 3–5, small arrowheads within linker DNA). In contrast to the general protection of DNA within the dinucleosomes from DNase I following reconstitution of H1 or B4, reconstitution with HMG1 does not lead to significant changes in overall cleavage; however, there are minor changes in the cleavage of linker DNA that resemble those obtained with the linker histones (Figure 7C, lanes 3–5, small arrowheads within linker DNA). We conclude that the exact constraint of linker DNA within the dinucleosome varies dependent on reconstitution with histone H1, histone B4 or HMG1.

Transcriptional properties of reconstituted dinucleosomes

We have established that the assembly of histone octamers onto a 424 bp DNA fragment leads to nucleosome mobility and that inclusion of linker histones H1 or B4, or HMG1 restricts this mobility, constrains the path of linker DNA and fixes nucleosome position (Figures 3–7). Histones H1 and B4, and HMG1 all direct these structural transitions once incorporated into the dinucleosome, with some qualitative variation (Figures 3–7); however, they bind to the dinucleosome with very different affinities. Histone H1 binds 6-fold more stably than B4 and 40 times more stably than HMG1 (Figures 1 and 2). We next examined whether stable inclusion of these different proteins into the dinucleosome would lead not only to similar structural transitions, but also to similar functional consequences. In earlier work, we established that a dinucleosomal template containing histone octamers alone was competent for transcription in a *Xenopus* oocyte nuclear extract (Ura *et al.*, 1995). Reconstitution of histone H1 into the dinucleosome begins to direct the repression of transcription at a stoichiometry of less than one molecule of linker histone per dinucleosome (Ura *et al.*, 1995; Figure 8A, lanes 7–11 and Figure 9A). A comparable repression of transcription requires more than two molecules of histone B4 per dinucleosome (Figure 8B, lanes 7–11 and Figure 9B) and for HMG1 at a stoichiometry of less than one molecule of HMG1 per dinucleosome (Figure 8C, lanes 7–12 and Figure 9C). Inclusion of one or two molecules of histone H1 or HMG1 per histone octamer within the

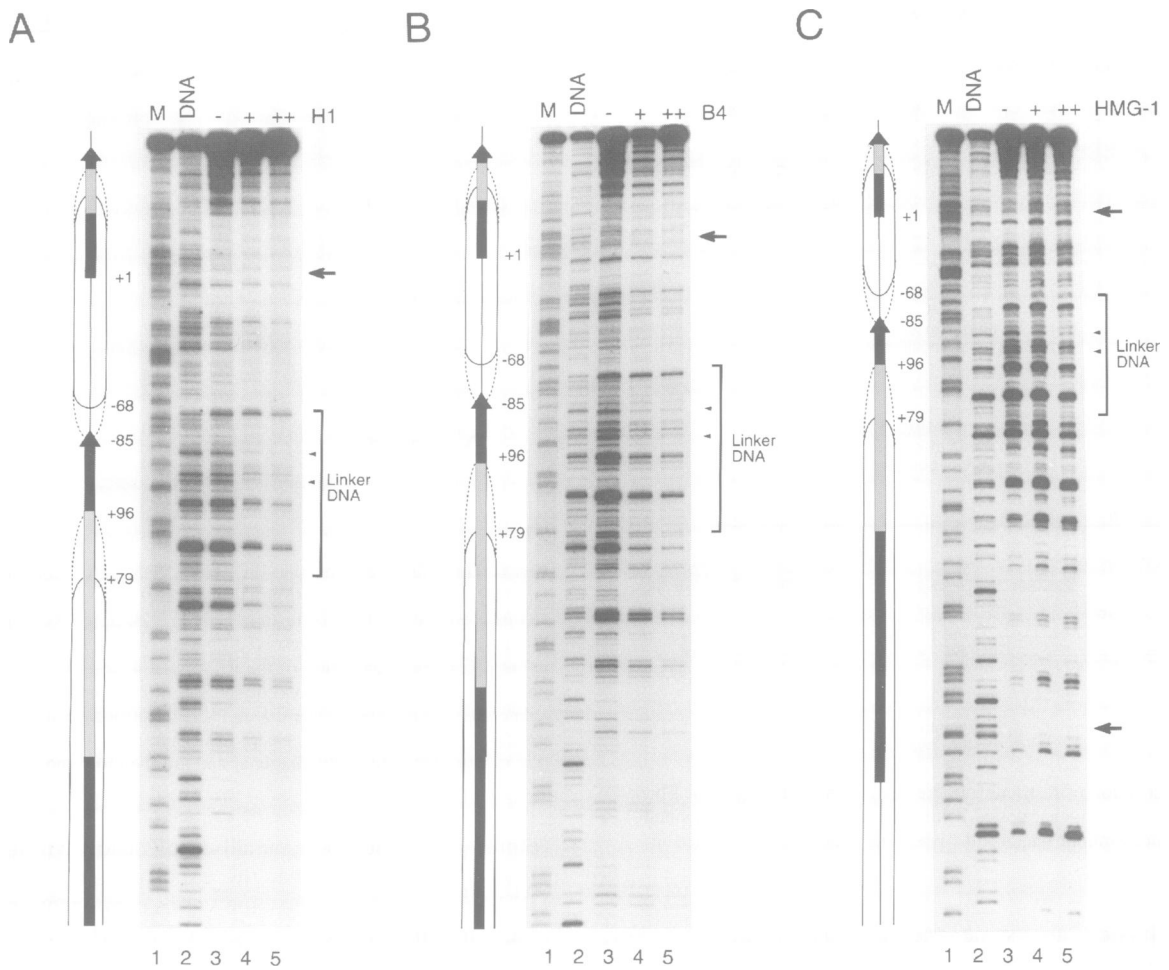

Fig. 7. DNase I footprinting of dinucleosomes containing histone H1 and B4 and HMG1. The coding strand of the 5S RNA genes within a 424 bp *Xba*I–*Xho*I fragment was 3′ end radiolabeled at the *Xba*I site with Klenow fragment. Dinucleosomes were incubated with either histone H1 (**A**), B4 (**B**) or HMG1 (**C**) and digested by DNase I. DNA from unbound or bound protein dinucleosome complexes was isolated by nucleoprotein agarose gel electrophoresis (Wolffe and Hayes, 1993). DNA from the native gel was purified and analyzed by denaturing PAGE. Lane 1 shows the G-specific cleavage reaction used as markers. Digestion of naked DNA (lane 2), of dinucleosomes (lane 3), and of dinucleosomes containing one molecule of protein (lanes 4) or two molecule of protein (lane 5) is shown, as indicated at the top. The arrows indicate the position of the axis of dyad symmetry of the nucleosome as derived from earlier studies using mononucleosomes (Hayes and Wolffe, 1993). The large vertical arrows show the location and orientation of the 5S RNA gene. Dotted boxes show the internal control region (ICR). The positions of the nucleosome cores (solid line) and chromatosomes (dashed line) are indicated by ellipsoids. The position of linker DNA is indicated. The small arrowheads within the linker DNA indicate changes in DNase I cleavage apparent on reconstitution of H1, B4 or HMG1 into nucleosomes.

dinucleosome directs transcriptional repression of the dinucleosomal template without significant effects on the transcription of naked DNA (Figure 8, lanes 1–5 and Figure 9). The repression of transcription of these chromatin templates occurs while the template is free in solution (data not shown). Higher excesses (4-fold) lead to transcriptional repression of naked DNA by H1 (Figure 8A, lane 6) and HMG1 (Figure 8C, lane 6), but not for B4 (Figure 8B, lane 6). Under these circumstances, the H1 or HMG1 proteins form aggregates with DNA that can be easily removed from solution (data not shown). These variations in the efficiency of repression suggest that histone H1 and HMG1, once reconstituted into chromatin, are more effective transcriptional repressors than histone B4 (Figures 8 and 9). However, the most significant result is that for every concentration of H1 in the reaction necessary to allow two molecules to bind to the dinucleosome and fully repress transcription, it takes at least a 6-fold higher concentration of either histone B4 or HMG1 to elicit the same functional changes (Figures 2, 8 and 9).

Discussion

We made use of a reconstituted dinucleosome capable of transcription *in vitro* to examine the structural and functional consequences of changes in nucleosomal composition. Three different proteins (histone H1, histone B4 and HMG1) exert similar constraints on the structure of the dinucleosome. They protect linker DNA from digestion with micrococcal nuclease (Figure 3), direct the positioning of nucleosomes (Figures 4 and 5), restrict nucleosome mobility (Figure 6) and constrain the path of linker DNA (Figure 7). They all repress transcription from the dinucleosome template (Figures 8 and 9). An important difference between the association of histone H1, histone B4 and HMG1 with the dinucleosome is the affinity with which these different proteins are incorporated into the structure (Figures 1 and 2). Histone B4 and HMG1 associate with the dinucleosome with significantly reduced affinities compared with histone H1. Thus, although all three proteins can contribute to the organization of chro-

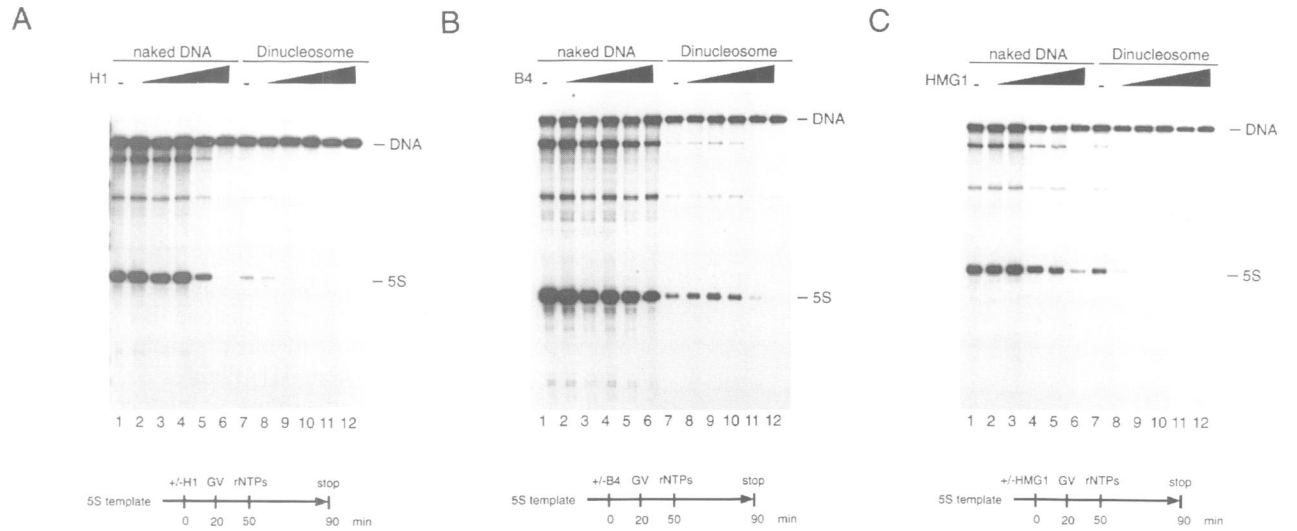

Fig. 8. Repression of transcription from 5S dinucleosome templates depend on binding of histone H1, B4 and HMG1. (**A**) 6.25 ng of naked radiolabeled pX5S192-2 DNA fragments (lanes 1–6) or reconstituted dinucleosome templates (lanes 7–12) were incubated with 0, 0.4, 0.8, 1.6, 3.2 and 6.4 ng of histone H1, respectively, then transcribed *in vitro* as designated in the scheme. GV is *Xenopus* nuclear extract (Birkenmeier *et al.*, 1978). (**B**) As above, except that templates were incubated with 0, 2, 4, 8, 16 and 32 ng of histone B4, respectively. (**C**) As above, except that templates were incubated with 0, 17, 34, 68, 136 and 272 ng of HMG1, respectively. The position of 5S rRNA is indicated (5S). Radiolabeled DNA 5S DNA (DNA) served as an internal control for recovery of nucleic acid.

matin, the stability of the structures they assemble will vary. Our results with the model chromatin template are consistent with the hypothesis that early embryonic chromatin enriched in HMG1 and histone B4 is less stable than chromatin containing histone H1. This instability might account for the transcriptional promiscuity of embryonic chromatin at the mid-blastula transition (Newport and Kirschner, 1982; Wormington and Brown, 1983; Rupp and Weintraub, 1991; Bouvet *et al.*, 1994; Kandolf, 1994).

The structural role of HMG1 and histone B4 in chromatin: comparison with histone H1

HMG1 is an abundant and highly conserved chromosomal protein with unknown structural and functional roles in somatic cells (van Holde, 1988; Bustin *et al.*, 1990; Bianchi, 1994). Within a normal somatic cell, there is approximately one molecule of HMG1 per 20 nucleosomes (Goodwin *et al.*, 1977; Isackson *et al.*, 1980). In contrast, *Xenopus* paternal pronuclei accumulate large quantities of HMG1 during their assembly in *Xenopus* egg cytoplasm, approximate stoichiometries suggest one molecule of HMG1 for every two nucleosomes (Dimitrov *et al.*, 1994; Nightingale *et al.*, 1996a). This protein is incorporated into chromatin from large amounts stored in the *Xenopus* oocyte (Kleinschmidt *et al.*, 1983). A comparable enrichment in chromatin of the highly related protein, HMG-D, is found within *Drosophila* nuclei during the cleavage divisions of early embryogenesis (Ner and Travers, 1994). This abundance, which is comparable with that of the linker histones in normal somatic cells, leads to the hypothesis that HMG1 and HMG-D have a structural role in organizing chromatin in these embryonic nuclei (Dimitrov *et al.*, 1994; Ner and Travers, 1994).

Early experiments suggested that HMG1 might replace histone H1 in the nucleosome (Jackson *et al.*, 1979; Jackson and Rill, 1981). Several properties are shared between histone H1 and HMG1: both proteins require linker DNA for stable incorporation into chromatin (Varshavsky *et al.*, 1976; Schröter and Bode, 1982; Nightingale *et al.*, 1996a), they protect linker DNA from nuclease digestion (Simpson, 1978; Nightingale *et al.*, 1996a), they can have selective interactions with the core histones (Glotov *et al.*, 1978; Boulikas *et al.*, 1980; Bernues *et al.*, 1983), and they both bind four-way junction DNA (Lilley, 1992; Varga-Weisz *et al.*, 1994). Nevertheless, HMG1 and H1 are very different proteins and recognize DNA through distinct mechanisms. The winged helix class of DNA binding proteins (e.g. linker histones) interact with DNA through the major groove, bending the double helix towards the protein (Clark *et al.*, 1993; Ramakrishnan *et al.*, 1993; Pierrou *et al.*, 1994). In contrast, HMG1 contains two DNA binding domains known as HMG boxes. The tertiary fold of a HMG box has the form of a V-shaped arrowhead (Read *et al.*, 1993; Weir *et al.*, 1993). The HMG box binds DNA through a concave surface on the inside of the V-shape (Werner *et al.*, 1995). Sequence selectivity might also arise from contacts mediated by the minor wing of the HMG box (Read *et al.*, 1994). In spite of these differences, histone H1 and HMG1 elicit similar structural changes in the dinucleosome (Figures 3–7).

Both H1 and HMG1 interact with the dinucleosome to assemble discrete nucleoprotein complexes (Figure 1); however, the affinity with which histone H1 binds to nucleosomal DNA is 40-fold higher than that of HMG1 (Figure 2). This difference might reflect preferred interactions of histone H1 with DNA and core histones within the nucleosome (Pruss *et al.*, 1995). However, a large contribution apparently derives from differences in binding to DNA: histone H1 binds to naked DNA with a 17-fold higher affinity than HMG1. Our binding constants for HMG1 association with naked DNA are similar to those previously reported by Churchill *et al.* (1995). Once bound, HMG1 protects linker DNA from digestion with micrococcal nuclease (Figure 3); however, HMG1 tends to protect a more extended linker DNA than H1. This

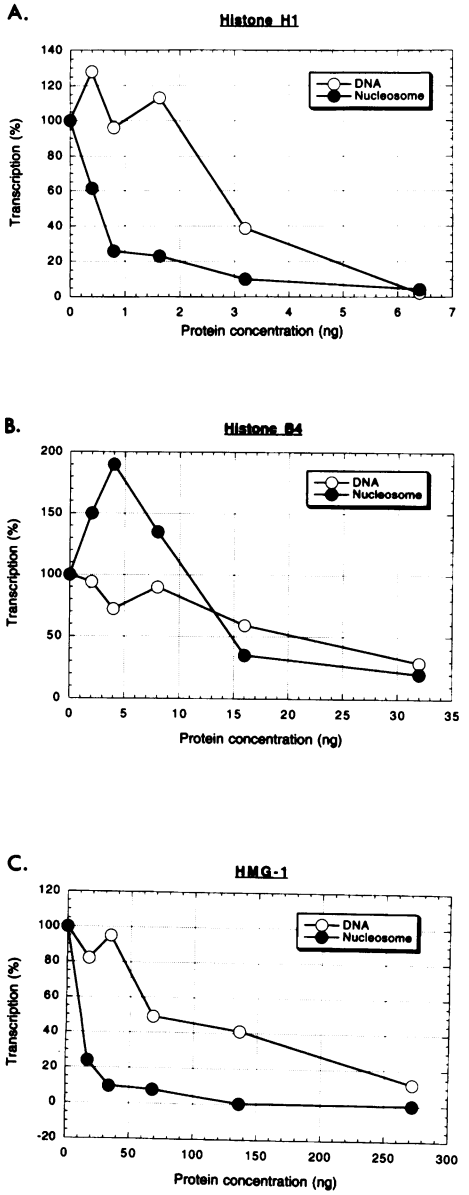

Fig. 9. Quantitation of transcriptional repression of dinucleosome templates dependent on the binding of histone H1, B4 and HMG1. Transcription was quantitated using a Molecular Dynamics Phosphoimager and is shown relative to the activity (100%) obtained in the absence of linker histones for DNA (○) or nucleosomal (●) templates. (**A**) Histone H1; (**B**) histone B4; (**C**) HMG1.

might reflect the more elongated structure of the two HMG boxes that bind to DNA in comparison to the winged helix DNA binding domain of histone H1. Both HMG1 and H1 very effectively fix nucleosome position with respect to DNA sequence (Figures 4A and B, and 5). Moreover, both proteins also restrict nucleosome mobility. Our hypothesis is that both histone H1 and HMG1 elicit these latter structural transitions by binding to linker DNA and stabilizing the path of linker DNA in the dinucleosome. Contacts made by H1 and HMG1 with the core histones might also contribute to these constraints (Glotov *et al.*, 1978; Boulikas *et al.*, 1980; Bernues *et al.*, 1983). Although similarities exist in the DNase I cleavage pattern of dinucleosomes reconstituted with HMG1 and H1 (Figure 7), there are differences in the overall protection of the templates from the enzyme. The greater protection of DNA within nucleosomes containing H1 might reflect the consequences of the association of the extended basic amino- and carboxyl-terminal tails of histone H1 (Hill *et al.*, 1991). These extended basic tails are lacking in HMG1.

Histone B4 has 30% sequence identity with a group of invertebrate linker histones, and rather less identity with a typical vertebrate somatic histone H1 (Smith *et al.*, 1988; Cho and Wolffe, 1994; Nightingale *et al.*, 1996a). Nevertheless, incorporation of histone B4 into the dinucleosome elicits almost identical structural transitions to those obtained for histone H1 (Figures 2–7). The major difference is the reduced affinity of histone B4 for the dinucleosome compared with histone H1. This difference is also reflected in the reduced affinity of histone B4 for naked DNA compared with histone H1, this may reflect the reduced basicity of histone B4 compared with histone H1 (Smith *et al.*, 1988; Dimitrov *et al.*, 1993). A second difference between histone B4 and histone H1 lies in the failure of histone B4 to fix nucleosome boundaries during protracted digestion with micrococcal nuclease to generate chromatosomes containing DNA fragments of 167 bp (Figure 4A, lanes 8–11). Since the isolation of chromatosomes containing longer DNA fragments of 184 bp reveals more discrete boundaries of histone–DNA interaction (Figure 4B, lanes 7–10), we suggest that these results reflect the destabilization of histone–DNA contacts during extended digestion with micrococcal nuclease. This again might reflect a weaker interaction of histone B4 with DNA in the nucleosome compared with histone H1.

We conclude that the only major difference between dinucleosomes containing histone H1, histone B4 and HMG1 lies in the stability with which these proteins are incorporated into chromatin. Our results lend strong support to the hypothesis that HMG1 has a structural role in organizing chromatin in a nucleosomal context (Dimitrov *et al.*, 1994; Ner and Travers, 1994). This hypothesis might also explain how both mitotic chromosomes and replication-competent nuclei assemble in *Xenopus* egg extracts deficient in linker histones (Ohsumi *et al.*, 1993; Dasso *et al.*, 1994). We suggest that abundant HMG1 substitutes structurally and functionally for linker histones in these chromosomes and nuclei.

Biological consequences of nucleosome variation with respect to content of HMG1, histone B4 and H1

Several functions have been proposed for proteins of the HMG1 family. These include a role in nucleosome assembly (Bonne-Andrea *et al.*, 1984) and disassembly (Waga *et al.*, 1989), the stimulation of transcription (Tremethick and Molloy, 1986; Shykind *et al.*, 1995) and transcriptional inhibition (Ge and Roeder, 1994). HMG1 has been proposed to function both as a stable component of nucleoprotein complexes (Paull *et al.*, 1993; Dimitrov *et al.*, 1994; Ner and Travers, 1994) and transiently as a DNA chaperone in their assembly (Travers *et al.*, 1994). Our results indicate that HMG1 can inhibit transcription, but that is does so most effectively as part of a nucleosomal array (Figure 8). We interpret the inhibitory effects of HMG1 on transcription as being a consequence of the constraint of linker DNA and the restriction of nucleosome

mobility (Ura *et al.*, 1995). Nucleosome mobility has been proposed as an important variable in regulating the activity of chromatin templates (Ura *et al.*, 1995; Varga-Weisz *et al.*, 1995; Wall *et al.*, 1995). Thus, the high content of HMG1 in the *Xenopus* egg and embryo (Kleinschmidt *et al.*, 1983; Dimitrov *et al.*, 1994) might contribute to the transcriptional quiescence of early embryonic chromatin (Newport and Kirschner, 1982; Prioleau *et al.*, 1994; Almouzni and Wolffe, 1995). Nevertheless, HMG1 binds to nucleosomal DNA with a reduced affinity compared with linker histones B4 and H1 (Figure 2); thus, chromatin enriched in this protein might be intrinsically less stable (Dimitrov *et al.*, 1994; Ner and Travers, 1994). This might provide less impediment to replication fork progression, or to the reorganization of chromosome structure associated with the nuclear cell cycle. The continued presence of HMG1 in chromatin at the mid-blastula transition, when transcription in first activated (although stores of the protein are depleted at this stage; Dimitrov *et al.*, 1993, 1994), might account for the pleiotropic activation of the transcription of many genes (Wormington and Brown, 1983; Rupp and Weintraub, 1991; Bouvet *et al.*, 1994). This might reflect easier access of transcriptional machinery to the HMG1-containing chromatin.

Histone B4 is also abundant in the *Xenopus* egg and remains in embryonic chromatin throughout cleavage, through the mid-blastula transition and is only substantially diluted during gastrulation (Dimitrov *et al.*, 1993). Histone B4 can substitute for histone H1 in chromatin, but has a reduced affinity for nucleosomal DNA (Figure 2). This will lead to the assembly of less stable chromatin structures and thus might also contribute to the assembly of embryonic chromatin structures that are more easily replicated and transcribed (Figures 8 and 9). It is the progressive replacement of HMG1 and histone B4 with histone H1 as embryogenesis progresses that can drive the dominant and specific repression of the oocyte 5S rRNA genes (Bouvet *et al.*, 1994; Kandolf, 1994). One hypothesis to explain this phenomenon is to propose that these genes are regulated through an equilibrium mechanism in which final transcriptional activity depends not only on the abundance and affinity of transcription factors, but also on the abundance and affinity of chromatin proteins for the genes (Bouvet *et al.*, 1994). The replacement of HMG1 and histone B4 by histone H1 would stabilize chromatin structure at lower linker histone concentrations and potentially contribute to the displacement of the transcriptional machinery from DNA. Future experiments will explore this possibility.

Materials and methods

DNA fragments

A 424 bp *Xba*I–*Xho*I fragment derived from plasmid pX5S197-2, which contains two tandem repeats of *X.borealis* somatic 5S RNA gene (Ura *et al.*, 1995), 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 DNA fragment was reconstituted into nucleosomes.

Purification of nucleosome core particles, histone H1, B4 and HMG1

Nucleosome core particles were prepared from chicken erythrocyte nuclei (Tatchell and van Holde, 1977). Histone H1 was prepared from calf thymus as described previously (Hayes and Wolffe, 1993). HMG1 protein was purified from calf thymus by means of 5% perchloric acid extraction of nuclei (Nightingale *et al.*, 1996a). Recombinant histone B4 was prepared as in Nightingale *et al.* (1996a) with minor modification. The histone B4 cDNA was subcloned into pGEX, transformed into *Escherichia coli* strain BL21(DE3) pLysS, and expressed as a glutathione-*S*-transferase (GST) fusion protein. The fusion protein was purified using glutathione–Sepharose-4B affinity resin (Pharmacia) and cleaved by thrombin (Sigma). The released B4 protein was recovered by washing with 0.5 M NaCl. All proteins, whether salt or acid extracted, isolated from natural sources or in recombinant form, had comparable DNA binding properties. Several different preparations were used in each experiment with very similar results (data not shown).

Nucleosome reconstitution

Nucleosome cores were reconstituted onto radiolabeled DNA fragments by exchange from chicken erythrocyte core particles (Tatchell and van Holde, 1977). In the histone exchange method, ~2-fold template mass excess of core particle was mixed with 15 μg radiolabeled DNA in 100 μl of reconstitution buffer [10 mM Tris–HCl pH 8.0, 1 mM EDTA, 1 mM 2-mercaptoethanol], followed by slow adjustment of NaCl concentration (to 1 M). Samples were incubated at 37°C for 15 min and 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 phenylmethylsulfonyl fluoride (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, 1 mM EDTA and 1 mM 2-mercaptoethanol overnight.

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 electrophoresis in 0.5× TBE. Fractions containing dinucleosome cores were pooled and stored on ice until use. The concentration of the dinucleosome preparation was ~3 μg/ml. The preparations were assayed for the stability of histone–DNA interactions before any experiments were initiated (Godde and Wolffe, 1995).

Gel-shift experiments

Reconstituted dinucleosomes (7.5 ng DNA content) were incubated with various amounts of histone H1, B4 or HMG1 protein in 10 μl of binding buffer [10 mM Tris–HCl, pH 8.0/50 mM NaCl/0.1 mM EDTA/3% (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 gel was dried and autoradiographed.

The titration experiments to determine the binding affinities of histone H1, B4 and HMG1 were performed in 8 μl reactions that contained 1.5 nM dinucleosome in binding buffer. The fraction of bound dinucleosome was quantitated with a Molecular Dynamics PhosphorImager and plotted against the histone H1, B4 or HMG1 concentration used.

Micrococcal nuclease mapping

Dinucleosome (20 ng of DNA) in the absence or presence of either 4 ng of histone H1, 12.5 or 25 ng of B4, or 200 ng HMG1 were digested with 0.035, 0.075 and 0.15 U of micrococcal nuclease (Worthington Biochemical Corporation) for 5 min at 22°C. Incubation with either H1, B4 or HMG1 was as described above except the binding reactions contained 150 μg/ml of bovine serum albumin (BSA). 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 μg/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 a 6% polyacrylamide gel. DNA fragments of nucleosome core and chromatosome products were recovered and digested with restriction endonuclease. Restriction fragments were analyzed by electrophoresis in a 8% denaturing polyacrylamide gel to determine micrococcal nuclease cleavage sites (Meerssemann *et al.*, 1991).

Two-dimensional gel experiments

Two-dimensional gel experiments to show the redistribution of histone–DNA complexes were performed as described previously (Meersseman *et al.*, 1992) with slight modifications. Approximately 15-fold template mass excess of core particle was mixed with 500 ng radiolabeled DNA in 100 μl of reconstitution buffer to reconstitute dinucleosomes. In this case, almost all products were dinucleosomes and no naked DNA fragment or mononucleosomes were detectable by nucleoprotein agarose

electrophoresis. Reconstitutes were incubated with or without histone H1, B4 or HMG1 as described above and loaded onto non-denaturing 4% polyacrylamide (29:1, acrylamide:bisacrylamide) gels at 4°C in 0.5× TBE. The gels were run at 10 or 2.5 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 electrophresed at 4°C under the same condition as the first dimension.

DNase I footprinting

Samples containing labeled dinucleosome (200 ng of DNA) were incubated with or without histone H1 (100 or 200 ng), B4 (500 ng or 1 μg) or HMG1 (4 or 8 μg) in 100 μl of binding buffer as described above. Reconstitutes were treated with 25 U of DNase I (Boehringer Mannheim) prior to resolving nucleoprotein complexes on preparative 0.7% agarose gels (Hayes and Wolffe, 1992). Mg^{2+} was adjusted to 2.5 mM concomitantly with addition of DNase I. DNase I reactions were carried out at room temperature for 4 min and terminated by addition of EDTA (5 mM). Glycerol (3% v/v) was added to the sample and the entire reaction volume was transferred directly into a preparative gel. After electrophoresis, first or second shifted or unbound dinucleosome complexes were excised from the gel. DNA from these complexes was isolated and analyzed by denaturing polyacrylamide (6%) gel electrophoresis (Wolffe and Hayes, 1993). Specific DNA makers were produced by Maxam and Gilbert cleavage at G residues.

Transcription reactions

Dinucleosome complexes separated by sucrose gradients or naked DNA were used as templates for transcription in an extract from *Xenopus* oocyte nuclei. Reconstituted dinucleosome templates or naked radiolabeled pX5S192-2 DNA fragments (6.25 ng) were incubated with various amounts of histone H1, B4 or HMG1 in 50 mM KCl at 22°C for 20 min, then transcribed in an extract from *Xenopus* oocyte nuclei. Oocyte nuclear extract was prepared as described previously (Birkenmeier *et al.*, 1978). Transcription reaction conditions were as described (Ura *et al.*, 1995). Radiolabeled transcripts were extracted with phenol, precipitated with ethanol, and analyzed by electrophoresis in 6% denaturing polyacrylamide gel. The radiolabeled 5S DNA template served as an internal control for recovery.

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