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Incorporation of chromosomal proteins HMG-14/HMG-17 into nascent nucleosomes induces an extended chromatin conformation and enhances the utilization of active transcription complexes

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The role of chromosomal proteins HMG-14 and HMG-17 in the generation of transcriptionally active chromatin was studied in a *Xenopus laevis* egg extract which supports complementary DNA strand synthesis and chromatin assembly. Chromosomal proteins HMG-14/HMG-17 enhanced transcription from a chromatin template carrying a 5S rRNA gene, but not from a DNA template. The transcriptional potential of chromatin was enhanced only when these proteins were incorporated into the template during, but not after, chromatin assembly. HMG-14 and HMG-17 stimulate transcription by increasing the activity, and not the number, of transcribed templates. They unfold the chromatin template without affecting the nucleosomal repeat or decreasing the content of histone B4. We suggest that HMG-14/HMG-17 enhance transcription by inducing an extended conformation in the chromatin fiber, perhaps due to interactions with histone tails in nucleosomes. By disrupting the higher order chromatin structure HMG-14/HMG-17 increase the accessibility of target sequences to components of the transcriptional apparatus.

Key words: active chromatin/chromatin assembly/chromosomal proteins/transcription/*Xenopus*

Introduction

The molecular mechanisms involved in the generation of the chromatin structure of transcriptionally active genes are not fully understood. A variety of experimental evidence suggests that this process is, at least in part, determined during chromatin assembly (Svaren and Chalkley, 1990; Wolffe, 1993). The first evidence related to this point was obtained from *in vitro* reconstitution experiments which indicated that the order of addition of histones and TFIIIA to the assembly mixture affects the transcription of the 5S gene (Bogenhagen *et al.*, 1982; Gottesfeld and Bloomer, 1982) and that the order of addition of histones and a nuclear extract affects the generation of a DNase I hypersensitive site in the chicken adult β-globin gene (Emerson and Felsenfeld, 1984). Likewise, the position of a nucleosome with respect to initiation of transcription may either inhibit or facilitate the binding of regulatory factors to their target (Simpson, 1991). Competition between the binding of transcription factors and histones during chromatin assembly on replicating DNA has been shown to affect the transcription potential of the resulting chromatin (Almouzni and Wolffe, 1993; Kamakaka *et al.*, 1993; Wolffe, 1993). Thus, in some cases, the transcriptional potential of a template may be dependent on the kinetics of the assembly of the various components into the final chromatin structure. Most of the studies on the effect of chromatin assembly on transcriptional potential have focused on the kinetics of assembly of histones and DNA into nucleosomes. Non-histone proteins which are associated with the chromatin fiber may also affect the transcription potential of chromatin.

The high mobility group (HMG) chromosomal proteins are among the most abundant and ubiquitous non-histones found in nuclei of all higher eukaryotes (reviewed in Bustin *et al.*, 1990). Members of this protein group have profound effects on DNA structure (Ferrari *et al.*, 1992; Paul *et al.*, 1993) and have been shown to be part of the transcription initiation complex of defined genes (Du *et al.*, 1993; Thanos and Maniatis, 1992). A variety of experimental results suggests that two related proteins, HMG-14 and HMG-17, may be involved in the generation or maintenance of structural features which are unique to active chromatin (reviewed in Bustin *et al.*, 1990). In immunofractionation experiments these proteins associate preferentially with transcriptionally active sequences (Druckmann *et al.*, 1986; Postnikov *et al.*, 1991). Evidence for a possible involvement of these proteins in gene expression comes from experiments in which myoblasts were transfected with plasmids expressing HMG-14 under the control of the MMTV promoter. In this system, aberrant expression of HMG-14 protein inhibits the myogenic process, suggesting that cellular differentiation requires regulated expression of HMG-14 protein (Pash *et al.*, 1993). In SV40 minichromosomes, elevated levels of HMG-14 increase the rate of transcriptional elongation (Ding *et al.*, 1994). Thus, this protein may facilitate the passage of RNA polymerase through nucleosome arrays.

Studies with cell free extracts allow investigations of the molecular mechanisms involved in transcriptional potentiation of chromatin structures. In *Xenopus* egg extracts chromatin assembly is coupled to DNA replication and the transcription potential of defined genes is related to their chromatin structure (Almouzni and Wolffe, 1993b). Recently we demonstrated that this system is suitable for studies involving the assembly of a non-histone protein into chromatin. Incorporation of recombinant human HMG-17 into chromatin during replication resulted in a 5-fold enhancement of the transcriptional potential of the

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assembled chromatin template (Crippa *et al.*, 1993). The study suggested that the generation of active chromatin may be linked to the kinetics of assembly of non-histone proteins into chromatin during DNA replication. In addition, the ability of HMG-17 to enhance the transcriptional potential of a chromatin template provided a functional assay for this structural protein.

In the present investigation we studied the mechanism whereby HMG-14 and HMG-17 increase the transcriptional potential of chromatin. The results indicate that proper deposition of these proteins into chromatin increases the turnover of active 5S rRNA gene transcription complexes formed in the chromatin assembly mixtures. The presence of these HMG proteins reduces the compactness of the minichromosomes and therefore we postulate that the proteins facilitate access of RNA polymerase to the template. The results are relevant to the understanding of the cellular function of the HMG-14 and HMG-17 proteins and their putative involvement in the generation of the chromatin structure of transcriptionally active genes.

Results

Deposition of either HMG-14 or HMG-17 into nascent, but not into preassembled, chromatin enhances the transcriptional potential of class III genes

Since *Xenopus laevis* egg extracts do not contain structural homologs of the HMG-14/HMG-17 chromosomal protein family it is possible to use these extracts to study the effects of exogenously added HMG-14/HMG-17 on chromatin structure and transcription (Crippa *et al.*, 1993). Using this system we demonstrated that incorporation of exogenous HMG-17 into nascent nucleosomes enhances the transcriptional potential of chromatin. Approximately 30% of the sequence of the various members of the HMG-14/HMG-17 family is absolutely conserved (Bustin *et al.*, 1990). Indeed, a variety of studies have indicated that HMG-14 and HMG-17 are similar in many aspects, suggesting that they are involved in similar cellular functions (Bustin *et al.*, 1990; Einck and Bustin, 1985). However, every eukaryotic cell which contains HMG-14 contains also HMG-17, raising the possibility that both proteins may be required for proper cellular function. We used the transcription enhancement assay as a way to test for possible functional differences between HMG-14 and HMG-17. Minichromosomes carrying a somatic 5S rRNA gene of *Xenopus borealis* were assembled in the egg extract in either the presence or absence of exogenously added HMG-14. After completion of DNA synthesis and chromatin assembly, ^{32}P-labeled CTP and a double stranded plasmid carrying satellite I DNA from *X.laevis* were added, and the incubation continued for another 30 min. The double stranded DNA acts as a control to ensure that addition of HMG-14 does not have non-specific effects on the transcriptional process. In this system, most of the single stranded plasmid DNA replicates and assembles into chromatin within 2 h. The assembly of histones on double stranded DNA is much slower (Almouzni and Wolffe, 1993b) and therefore most of the double stranded DNA is not assembled into chromatin. Thus, the templates for transcription are 5S chromatin and satellite I DNA. The autoradiogram of the RNA extracted from these reaction mixtures (Figure 1A) indicates that the presence of HMG-14 in the assembly mixture increases the amount of RNA produced by the chromatin template (5S rRNA genes) but not that transcribed from the naked DNA (satellite I DNA). An incremental increase in the amount of HMG-14 brings about a concomitant increase in the amount of RNA synthesized from the 5S rRNA gene chromatin template. High concentrations of HMG-14 are inhibitory (compare lanes 4 and 5). The effects of HMG-14 on the transcription of the satellite I DNA are negligible. Quantitative analysis indicates that HMG-14 increases the transcription of 5S chromatin 7.6-fold while the maximum increase with naked DNA is 1.1-fold (Figure 1B). From these results we conclude that the HMG-14/HMG-17-dependent increase in transcription reflects changes occurring in the structure of the chromatin template, rather than non-specific effects on other components of the transcriptional machinery.

Chromosomal proteins HMG-14 and HMG-17 will bind to isolated nucleosome core particles and also to preassembled chromatin. Studies with HMG-17 suggested that its binding to preassembled chromatin differs from that seen when it is incorporated into chromatin during nucleosome assembly (Crippa *et al.*, 1993). To test whether the same situation applies to HMG-14, transcription was measured from templates to which HMG-14 was added either during or after chromatin assembly. Addition of HMG-14 to preassembled chromatin did not affect the transcription of the template (Figure 1C). The effects of HMG-14 and HMG-17 are indistinguishable (Figure 1D and Crippa, *et al.*, 1993). Thus, the transcription enhancement assay did not indicate differences between HMG-14 and HMG-17, suggesting that they have similar effects on chromatin structure. Both proteins have to be incorporated into chromatin in a specific way since transcriptional potentiation is observed only when the proteins are deposited into chromatin during, and not after, nucleosome assembly.

Inhibition of transcriptional activation by the nucleosomal binding domain of HMG-14/HMG-17

In isolated chromatin subunits HMG-14/HMG-17 proteins contact the DNA in the two major grooves flanking the nucleosomal dyad axis and near the entry/exit points of the DNA in the nucleosome core particle (Alfonso *et al.*, 1994). Thus, the proteins bridge two adjacent DNA strands on the surface of the nucleosomal core particle and stabilize its structure. A 30 amino acid long peptide (named 'peptide 2'), corresponding to the putative nucleosomal binding domain of HMG-17, retains many of the properties of the intact protein (Crippa *et al.*, 1992). This peptide specifically shifts the mobility of cores, elevates the t_m of the cores, and protects from DNase I digestion the same nucleosomal sites as the intact protein. Furthermore, hydroxyl radical and DNase I footprinting indicate that the placement of the peptide on the nucleosome core particle is very similar to that of the intact protein (not shown). We wished to examine whether the nucleosomal binding domain of the protein can, by itself, enhance the transcriptional potential of chromatin.

The results presented in Figure 2 indicate that the nucleosomal binding domain by itself did not enhance the transcription potential of chromatin (compare lanes 1 and

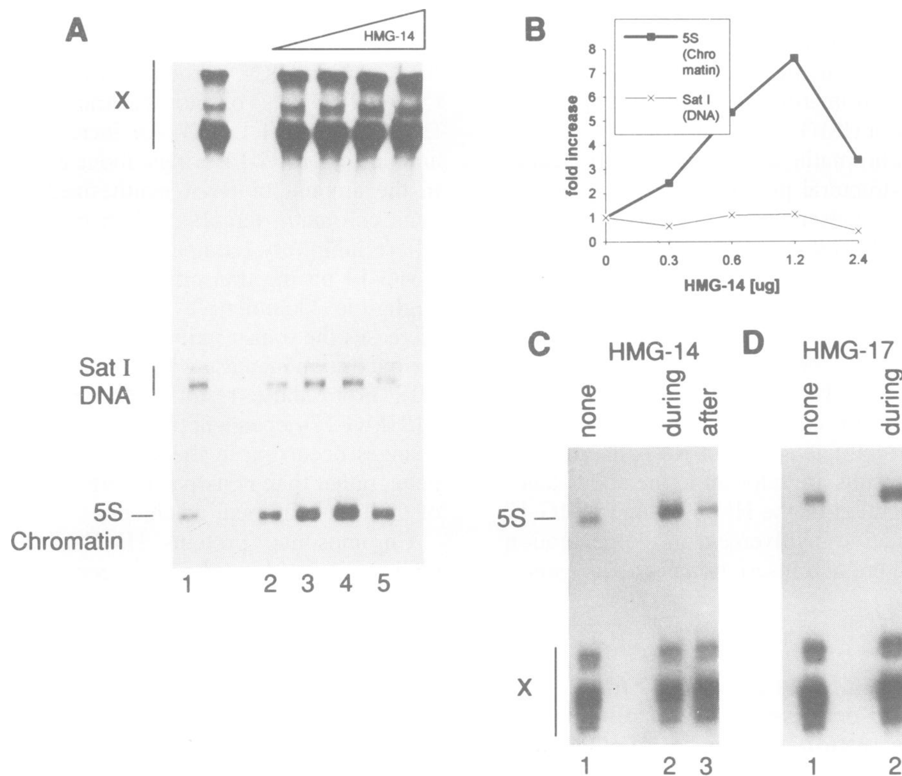

Fig. 1. Dose-dependent specific transcriptional potentiation of chromatin by HMG-14. (**A**) HMG-14 enhances the transcription potential of chromatin. Single stranded M13 containing the 5S rRNA gene was replicated and assembled into chromatin in the presence of various amounts of HMG-14. After 2 h [^{32}P]CTP and a double stranded plasmid carrying the satellite I gene were added. After 30 min of transcription the products were analyzed as described in Materials and methods. The unidentified bands in the extract (marked X) indicated that equal amounts of material were applied to the gels. Note that HMG-14 affects significantly only the transcription of the 5S gene. The amounts of HMG-14 added in lanes 1–5 were respectively: 0, 0.3 μg, 0.6 μg, 1.2 μg, 2.4 μg. (**B**) Quantitation of the effect of HMG-14 on the amount of RNA transcribed either from chromatinized 5S rRNA gene or from satellite I DNA. (**C**) HMG-14 enhances the transcriptional potential of chromatin only when assembled into chromatin during replication. Autoradiograms of RNA transcribed from minichromosomes, containing the 5S rRNA gene. (1) Minichromosomes assembled in the absence of HMG-14; (2) Minichromosomes assembled in the presence of 1.2 μg HMG-14; (3) HMG-14 (1.2 μg) added after completion of DNA replication and chromatin assembly. (**D**) Effect of HMG-17. 1.2 μg protein added to lane 2.

5 with 2 and 3). Western analysis indicated that the peptide is present on the nucleosome cores (not shown). On the other hand, the peptide competitively inhibited the ability of the protein to enhance transcription (lanes 7 and 8). Neither a control peptide with exactly the same amino acid composition but a different sequence, nor peptides from other regions of the protein inhibited the transcription activation capability of the protein (not shown). Therefore, we conclude that the proper binding of the peptide to nucleosome cores interferes with the binding of the intact protein. Similar results were obtained with protein HMG-14 and its nucleosomal binding domain (not shown).

These results demonstrate that transcriptional potentiation of chromatin by HMG-14/HMG-17 is specific and requires correct interaction between the proteins and the chromatin template. Furthermore, the data suggest that protein regions beyond the nucleosomal binding domain are required for transcriptional activation. We suggested that the negatively charged C-terminal domain of the proteins may be involved in transcriptional activation (Landsman and Bustin, 1991).

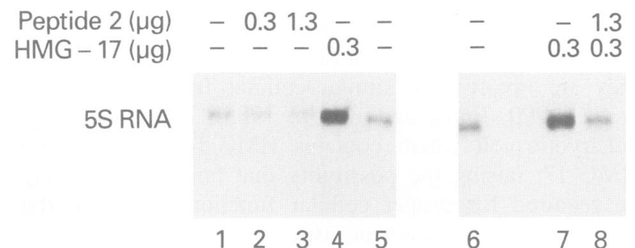

Fig. 2. Specific inhibition of the transcriptional activation by the HMG-17 nucleosomal binding domain. Single stranded M13 (150 ng) containing the 5S rRNA gene was replicated and assembled into chromatin in the presence of various combinations of HMG-17 and a 30 amino acid long peptide (peptide 2) corresponding to the nucleosomal binding domain of the protein. ^{32}P-labeled RNA was extracted and analyzed as described in Materials and methods. Note that the peptide neither enhances or represses the transcription (lanes 2 and 3), that HMG-17 protein enhances transcription (lanes 4 and 7) and that the peptide inhibits the HMG-17 mediated transcriptional activation (lane 8).

HMG-14/HMG-17 increase the turnover and not the number of transcription complexes

During chromatin assembly histones compete with transcription factors for binding to the 5S rRNA gene sequences. In ~90% of the minichromosomes assembled in the egg extract, a nucleosome forms over the 5S rRNA gene region, thereby rendering it transcriptionally inactive (Almouzni *et al.*, 1991). A possible way to account for the 5- to 7-fold increase in transcription upon HMG addition would be to assume a proportional increase in the number of active templates. Such an increase would

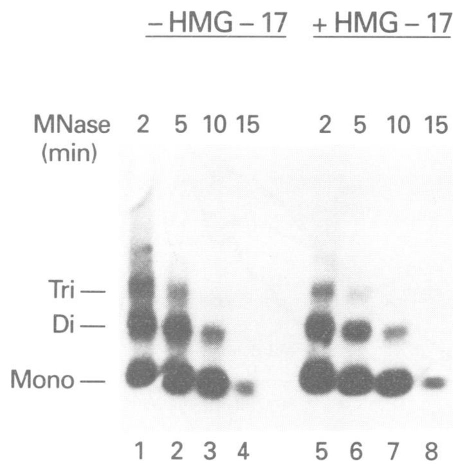

Fig. 3. Southern analysis of the 5S rRNA gene in a micrococcal nuclease digest of assembled minichromosomes. Chromatin assembled either with or without HMG-17 was digested with micrococcal nuclease for the time indicated. The purified DNA was fractionated on a 2% agarose gels and analyzed by Southern hybridization with a 5S rRNA gene probe. The relative amounts of mono-, di- and trinucleosomes, as well as the smear above the trinucleosome region, indicate that the DNA in lane 5 is digested to a greater extent than the DNA in lane 1.

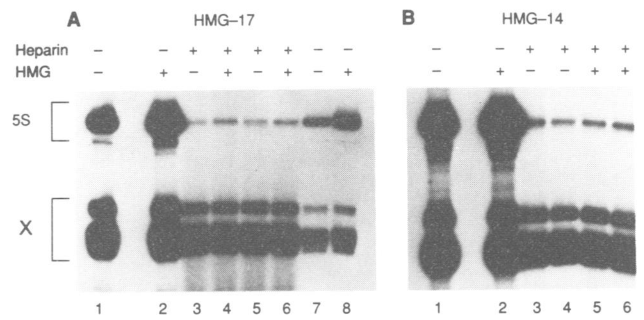

Fig. 4. Single-round transcription assays. Chromatin assembly and transcription analysis were performed exactly as before except that where indicated heparin was added at the same time as the ^{32}P-labeled CTP. In (**A**) lanes 5 and 6 are duplicates of lanes 3 and 4. In (**B**) lanes 4 and 6 are duplicates of 3 and 5. Lanes 7 and 8 in (A) are an aliquot of lanes 1 and 2 taken after 5 min. In the presence of heparin, addition of HMG increased the amount of RNA transcribed by <1.4-fold. In lanes 1 and 2 the transcription was continued for 30 min while in lanes 3–6 it was continued for only 5 min.

conceivably disrupt the nucleosome on the 5S rRNA gene in a significant fraction of the minichromosomes. However, Southern hybridization analysis of micrococcal nuclease digests of minichromosomes, using the 5S rRNA gene as a probe, indicated that the presence of HMG-17 (Figure 3) or HMG-14 (not shown) did not significantly change the nucleosomal repeat over the 5S rRNA gene region. This result indicates that both in the presence and in the absence of HMG-14 most of the 5S rRNA genes are assembled into nucleosomes and are transcriptionally inactive.

Southern analysis is not a sufficiently sensitive method to detect a small change in the number of transcriptionally active 5S rRNA genes. We therefore used single-round transcription assays (Almouzni *et al.*, 1991; Kassavetis *et al.*, 1990) to compare directly the effect of HMG-14 or HMG-17 proteins on the relative number of active transcription complexes. In these assays, heparin was added to the reaction mixtures at the same time as the radioactive nucleotide used to measure the transcription from the assembled chromatin templates. Heparin prevents reinitiation of transcription by RNA polymerase and therefore each template undergoes only a single round of transcription. The amount of RNA transcribed in the absence of chromatin was significantly higher than that transcribed in the presence of heparin (Figure 4, compare lanes 1 and 2 with lanes 3–6) indicating that, as expected, heparin prevents reinitiation. In this assay HMG-14/HMG-17 did not significantly increase the amount of RNA produced. In the absence of heparin, under conditions where multiple initiation events can occur, both HMG-17 (Figure 4A, lanes 1, 2, 7 and 8) and HMG-14 (Figure 4B, lanes 1 and 2) increased the transcription potential of the assembled chromatin. In contrast, when heparin was added to the preassembled complexes and multiple initiation events did not occur, HMG-14/HMG-17 had only a marginal effect on the amount of RNA produced (compare lanes 3 with 4 or 5 with 6 in Figure 4A and lanes 3 or 4 with 5 or 6 in Figure 4B). Quantitative analysis indicates that the maximum stimulation of RNA production by HMG-14 or HMG-17 in the presence of heparin is 1.4-fold. In the absence of heparin these proteins increased the amount of RNA produced >5-fold. We conclude, therefore, that the presence of HMG-14/HMG-17 on the assembled templates increases the utilization of the active templates without significantly increasing their number.

We have recently demonstrated that addition of HMG-14 stimulates the rate of polymerase II elongation, rather than initiation, from an SV40 minichromosome template (Ding *et al.*, 1994). Thus, in both systems, HMG-14/HMG-17 increase the utilization, rather than the number, of the transcribed genes.

HMG-14/HMG-17 decondense and decrease the compaction of the assembled minichromosomes

Transcriptionally active 5S rRNA genes are complexed with transcription factors and are not organized in a nucleosomal conformation (Wolffe and Morse, 1990). Because HMG-14/HMG-17 bind preferentially to nucleosome cores, it was surprising to find that these proteins enhance the transcription of a nucleosome-free 5S rRNA gene. It has been demonstrated that the degree of compaction of the chromatin fiber has a major effect on the transcription potential of a chromatin template (Hansen and Wolffe, 1992). We therefore tested whether HMG-14/HMG-17 decrease the ability of the nucleosome arrays to fold into a condensed fiber and enhance transcription by rendering the 5S rRNA gene region more accessible to RNA polymerase or other components of the transcription complex. To address this possibility, we tested the accessibility of the linker regions in the minichromosomes to digestion by micrococcal nuclease. Minichromosomes were assembled either in the presence or absence of HMG-14/HMG-17, digested with micrococcal nuclease and the digestion products fractionated on agarose gels (Figure 5).

The results indicate that minichromosomes assembled in the presence of HMG-14/HMG-17 are digested faster than those assembled in the absence of HMG-14/HMG-17, suggesting that the linker regions in these minichromo-

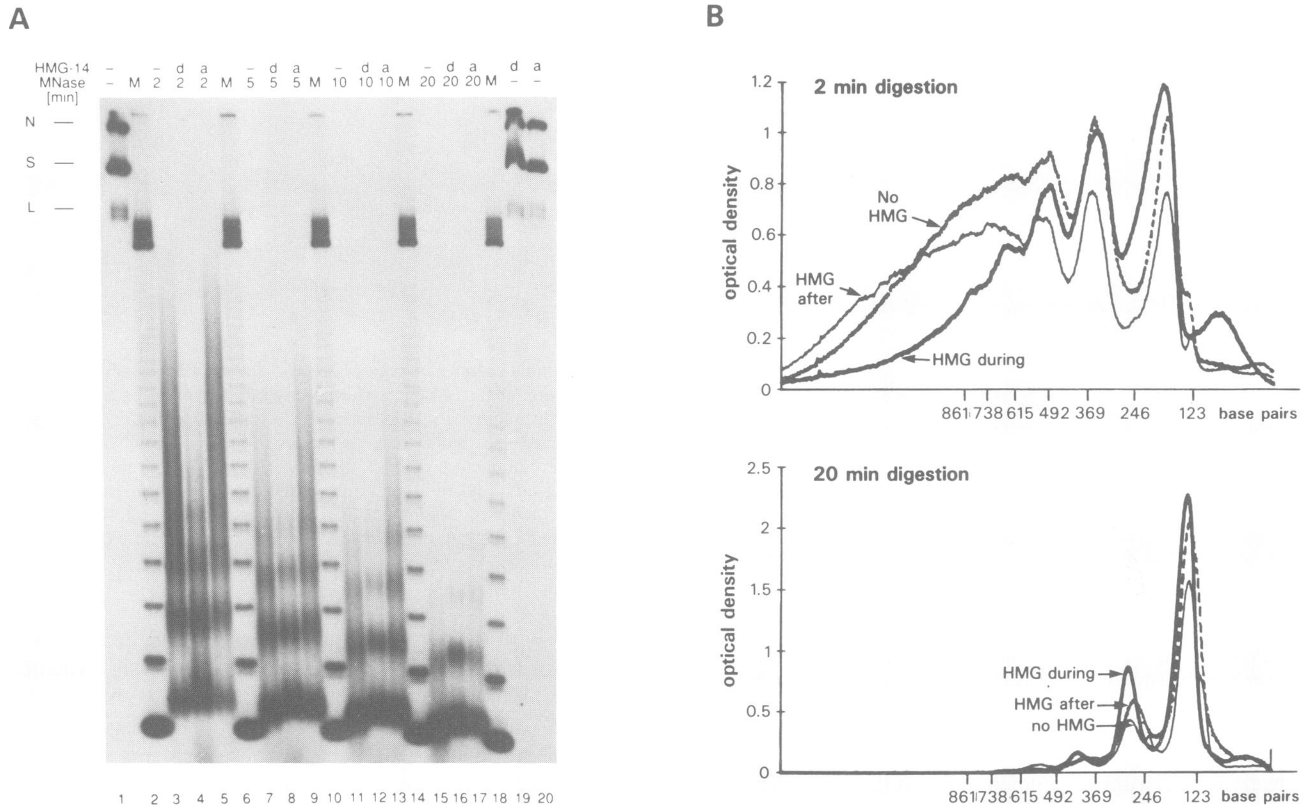

Fig. 5. Effect of HMG-14 on micrococcal nuclease digestion of chromatin. (**A**) Chromatin assembled either in the presence or absence of HMG-14 (molar ratio of HMG to core = 70) was digested in the extract with 0.7 U/μl micrococcal nuclease for the time indicated, the DNA purified, fractionated on long 0.8% agarose gels and exposed for autoradiography. M, 123 bp ladder molecular weight marker; d, HMG-14 present during assembly; a, HMG-14 added after assembly. The mobility of the supercoiled (S), linear (L) and nicked (N) DNAs is indicated on the left. (**B**) Densitometric scans of the 2 min and 20 min digestion points. Note that at short digestion times incorporation of HMG-14 into chromatin does not affect the nucleosomal repeat (lanes 3 and 4).

somes are more accessible to the enzyme (in Figure 5A compare the extent of the large molecular weight smears in lane 3 with that in lane 4, or compare lane 7 with lane 8). Likewise, analysis of early digestion points (Figure 5A, lanes 3–5) also indicates that the relative amount of mono- and dinucleosomes generated by micrococcal nuclease digestion of chromatin assembled in the presence of HMG-14 is higher than that generated from digestion of chromatin assembled in the absence of HMG-14. The increased rate of generation of mono- and dinucleosomes is consistent with a faster rate of digestion in the linker region. These results are in agreement with the analysis of the nucleosomal organization of the 5S gene region (Figure 3) which also indicates that incorporation of HMG-17 into nucleosomes during chromatin assembly increases the rate of chromatin digestion by micrococcal nuclease (compare the relative amounts of mono-, di- and trinucleosomes and the extent of the smear above the trinucleosomes in lane 1 with that in lane 5). Addition of HMG-14 to preassembled minichromosomes does not enhance the rate of digestion. In fact, the extent of the large molecular weight DNA smears shown in Figure 5A (for example compare lanes 4 and 5) suggests that the late addition of HMG-14 slows the rate of micrococcal nuclease digestion, perhaps due to non-specific binding of the protein to linker regions. These results are in agreement with the transcription enhancement assays which also indicate that HMG-14/HMG-17 enhance transcription only when incorporated into chromatin during nucleosome assembly.

The assembly conditions do not affect the repeat distance between the various multimers. This is most evident from the points taken at early digestion times (Figure 5A, lanes 3, 4 and 5 and 5B), when the mobility of the oligonucleosomes obtained from chromatin assembled in the presence of HMG-14 is the same as that obtained from chromatin assembled in the absence of HMG-14. However, during the course of digestion, oligonucleosomes derived from chromatin assembled in the presence of HMG-14 seem to migrate more slowly than those produced from chromatin assembled in the absence HMG-14. These results could be interpreted to suggest that HMG-14 affects nucleosomal spacing. However, we believe that this interpretation is incorrect and that the short digestion times (lanes 3–5) which indicate that HMG-14 does not affect spacing, reflect the correct situation in chromatin. Since in Figure 5A every fourth lane contains a molecular weight marker it is easy to see that the size of the oligonucleosome multimers decreases during the course of digestion. This decrease in the size of the nucleosome monomers during the course of digestion has been previously observed by others (Lohr *et al.*, 1977) and results from exonucleolytic attack on the DNA. In addition, nucleosomes slide during the course of digestion, resulting in a time-dependent decrease in the length of the nucleo-

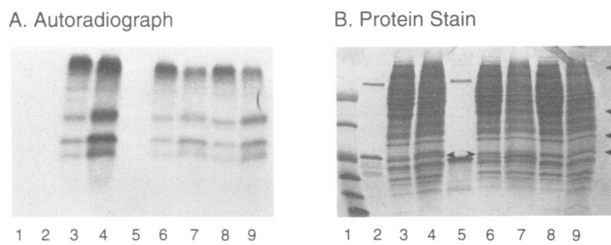

Fig. 6. HMG-14/HMG-17 are not phosphorylated in the egg extract. Proteins were phosphorylated in the extract as indicated in Materials and methods. In lanes 3, 6 and 8, 3 μl of the reaction mixture were loaded on the gel. In lanes 4, 7 and 9 the proteins were first precipitated with 25% trichloroacetic acid. Note that the same four proteins (arrows) were phosphorylated in the extract supplemented with HMG-14 (lanes 3 and 4) or HMG-17 (lanes 6 and 7) and in the control mixtures which were not supplemented with HMG proteins (lanes 8 and 9). Thus HMG-14/HMG-17 are not phosphorylated in the extract. Lane 1, molecular weight markers; lane 2, marker HMG-14; lane 5, marker HMG-17. Note that the electrophoretic mobility of one of the phosphorylated bands is faster than that of HMG-14 but slower than that of HMG-17 (arrows in lane 5).

some repeat (Godde and Widom, 1992). HMG-14 and HMG-17 bind at the entry/exit point of the nucleosomal DNA (Alfonso *et al.*, 1994). By virtue of their location in chromatin these proteins stabilize the position of the nucleosome, minimize sliding and decrease the rate of exonucleolytic attack. Thus, during the course of digestion the oligonucleosomes derived from chromatin assembled in the presence of HMG-14 or HMG-17 will be somewhat longer than those assembled in the absence of these proteins. As described, this is a result of the effect of these HMGs on the kinetics of digestion and not an effect on nucleosome spacing. The true situation is reflected in the gels depicting the products of short digestion times where the effects of exonucleolytic attack and nucleosome sliding are minimized.

It has been reported that only phosphorylated HMG-14/HMG-17 can serve as nucleosomal spacing factors (Drew, 1993; Tremethick and Drew, 1993). Although we believe that HMG-14/HMG-17 affect the kinetics of micrococcal nuclease digestion rather than nucleosomal spacing we still tested whether they are phosphorylated in the *Xenopus* egg extract. HMG-14 and HMG-17 were added to an extract containing [γ-^{32}P]ATP and after 2 h of incubation the protein extracts were fractionated on polyacrylamide gels. Autoradiography of the gels indicated that during the incubation four proteins, one of which had a mobility close to that of HMG-14/HMG-17, were phosphorylated. However, these four phosphorylated proteins were also present in the control extracts to which HMG-14 or HMG-17 was not added (Figure 6). To test for minor phosphorylated components and examine the possibility that a small fraction of HMG-14 or HMG-17 is transiently phosphorylated additional autoradiographs, which were exposed for a long period, were examined. In a separate experiment we examined the phosphorylation state of proteins extractable by 5% perchloric acid (not shown). All the data clearly indicate that chromosomal proteins HMG-14 and HMG-17 are not phosphorylated by the extract. Thus, during chromatin assembly in the egg extract unphosphorylated HMG-14/HMG-17 are incorporated into the minichromosomes without affecting the nucleosomal spacing. The presence of the proteins

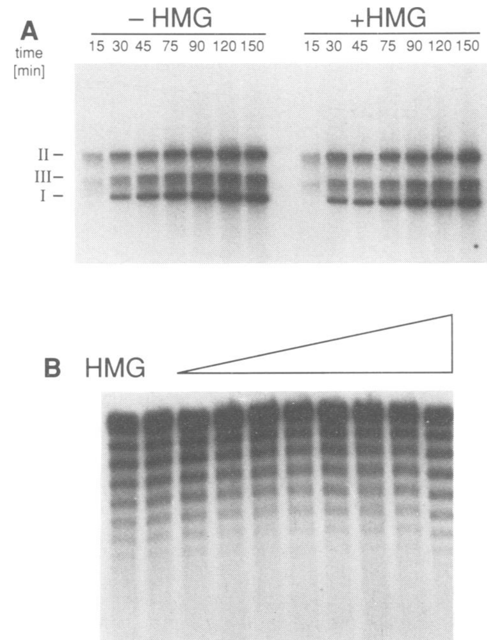

Fig. 7. Effect of HMG-14 on chromatin assembly. (**A**) HMG-14 does not affect the rate of chromatin assembly. Chromatin was assembled either in the presence or absence of HMG-14 (molar ratio of HMG to core = 70). [^{32}P]DNA in aliquots taken from the assembly mixtures at various times was fractionated on 0.8% agarose gels. The mobility of the supercoiled (form I), linear (form III) and nicked (form II) DNAs is indicated. (**B**) Analysis of DNA topoisomers. Radiolabeled DNA was extracted from chromatin assembled in the presence of various amounts of HMG-14 and fractionated on an 0.8% (w/v) agarose gel in 1× TPE buffer, containing chloroquine. The left lane contained no protein, the second lane contained bovine serum albumin, the following lanes containing bovine serum albumin and HMG-14 at the following HMG-14 to core particle ratio: 2, 5, 10, 15, 20, 50, 100, 500.

facilitates the accessibility of the micrococcal nuclease to the linker region thereby increasing the rate at which the minichromosomes are digested into smaller chromatin subunits.

We also tested whether HMG-14/HMG-17 affect the kinetics of DNA replication and chromatin assembly. Single stranded DNA and radioactive dCTP were added to *Xenopus* egg extracts in either the presence or absence of HMG-17 and the rate of supercoil formation was monitored by electrophoresis on agarose gels. Throughout the course of DNA replication and chromatin assembly the relative ratios of the supercoiled, linear and relaxed DNA were not affected by HMG. Likewise, HMG-17 did not change the number of topoisomers resolved on agarose gels containing chloroquine (Figure 7), providing further support that HMG-17 does not affect the nucleosomal spacing.

To test further whether HMG-14 or HMG-17 could unfold the chromatin template and promote access to a specific sequence, the minichromosomes were probed with either *Eco*RV or *Sca*I which produce a single cut within the 5S rRNA gene region. The purified DNA was then cut with *Bgl*II, resulting either in a 840 bp *Eco*RV−*Bgl*II fragment or a 800 bp *Sca*I−*Bgl*II fragment. From the intensity of these specific bands (Figure 8), it is clear that both restriction sites were more accessible in the minichromosomes assembled in the presence of HMG-14

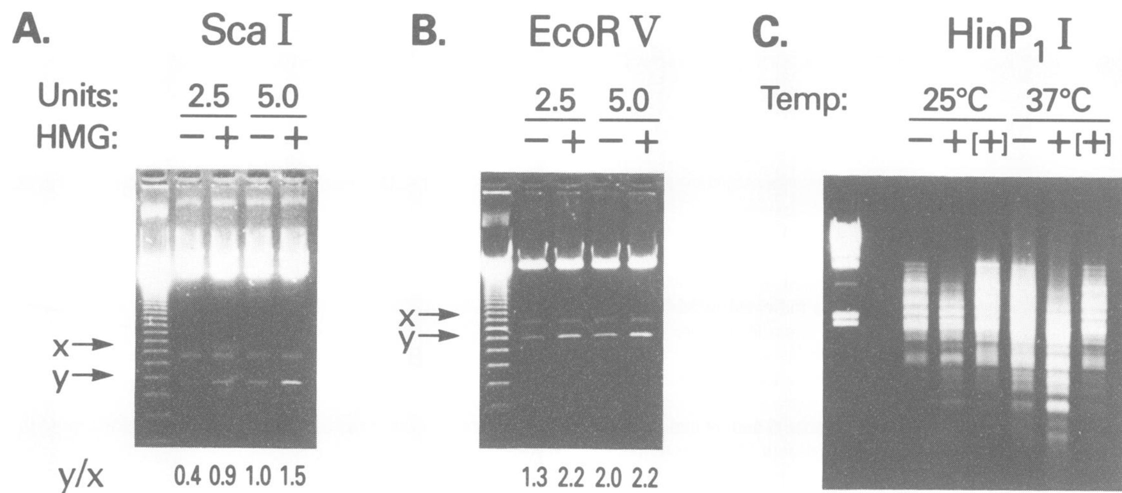

Fig. 8. Susceptibility of chromatin to digestion by restriction endonucleases. Minichromosomes assembled either in the presence or absence of HMG-14 were digested with various endonucleases. The enzyme units used are indicated on top of the lanes. −, no HMG; +, with HMG during assembly; [+] HMG added to preassembled chromatin; Y, DNA fragment resulting from endonuclease digestion; X, non-specific band which was used to normalize the intensity of the specific band; Y/X, ratio of specific to non-specific band. The value is proportional to the extent of nuclease digestion. Note that the presence of HMG during assembly invariably increases this value. (**A**) Digestion with *Sca*I; (**B**) digestion with *Eco*RV. (**C**) digestion with *Hin*P1I at two temperatures. Note that addition of HMG to preassembled minichromosomes did not enhance the rate of restriction nuclease digestion.

than in those assembled without HMG-14. An approximation of the increase in the cutting at a specific site was made by normalizing the intensity of the specific bands resulting from the restriction nuclease digestions (marked Y in Figure 8A and B), to that of an unidentified DNA fragment present in the DNA preparation (marked X in Figure 8A and B). The ratios (given in each lane under the bands) indicate that the presence of HMG-14 increased the rate of digestion at these unique restriction site. For example, the intensity of the 800 bp *Sca*I−*Bgl*III fragment after 1 h digestion with *Sca*I, was 2.2-fold higher in digests of minichromosomes containing HMG-14 than in those assembled in the absence of this protein. Likewise, digestion with a low concentration of *Eco*RV revealed that the presence of HMG-14 increased the rate of digestion of this unique restriction site in the chromatinized plasmid. As expected from kinetic considerations, the apparent effect of HMG-14 was less pronounced when higher enzyme concentrations were used.

The effect of HMG is not restricted to the vicinity of the 5S rRNA gene. Digestion of the minichromosomes with *Hin*P1I, which in this plasmid has 26 cutting sites, indicates that incorporation of HMG-14 into chromatin during DNA replication renders the entire minichromosome more accessible to the restriction enzyme, compared with minichromosomes assembled without HMG. Addition of HMG-14 to preassembled minichromosomes did not affect the rate of digestion by *Hin*P1I (Figure 8C).

We conclude therefore that incorporation of HMG-14 into nucleosomes during chromatin assembly on replicating DNA renders the entire minichromosomes more accessible to digestion by nucleases. The addition of HMG-14 to preassembled chromatin has no effect or may even result in inhibition of nuclease digestion. These results suggest that correct assembly of HMG-14/HMG-17 into nucleosomes reduces the compaction of the minichromosomes.

As an additional test for the effect of HMG-14/HMG-17 on chromatin structure, minichromosomes assembled either in the presence or absence of these proteins were sedimented through a sucrose gradient. In >10 experiments the minichromosomes assembled in the presence of either HMG-14 or HMG-17 invariably sedimented more slowly than those assembled in the absence of the protein (Figure 9). Thus, minichromosomes containing HMG-14 or HMG-17 seem to have a more open, i.e. less compact, conformation than those lacking HMG-14/HMG-17.

Protein composition of assembled minichromosomes

Next we tested whether HMG-14/HMG-17 unfold chromatin and enhance the transcriptional potential of chromatin by relieving structural constraints and transcriptional repression caused by a chromosomal component. A possible transcriptional inhibitor in *Xenopus* egg extracts is histone B4 which has considerable sequence homology with the linker histone H1 (Smith *et al.*, 1988; Wolffe, 1989). Histone H1 can act as a global repressor of transcription (Laybourn and Kadonaga, 1991). Although the binding of HMG-14/HMG-17 and linker histones to nucleosomes is not mutually exclusive (Ring and Cole, 1979; Albright *et al.*, 1980; Alfonso *et al.*, 1994), interactions between these proteins may affect the transcriptional potential of chromatin. Therefore we examined whether HMG-14/HMG-17 change the amount of B4, or any other protein, associated with the assembled chromatin.

Minichromosomes assembled either in the presence or absence of HMG-14 or HMG-17 were first purified on sucrose gradients (Figure 9) and then sedimented through a sucrose cushion. Analysis of the protein content of the purified minichromosomes by electrophoresis on polyacrylamide gels (Figure 10A) indicated that the stoichiometry of the core histones was unaffected by the

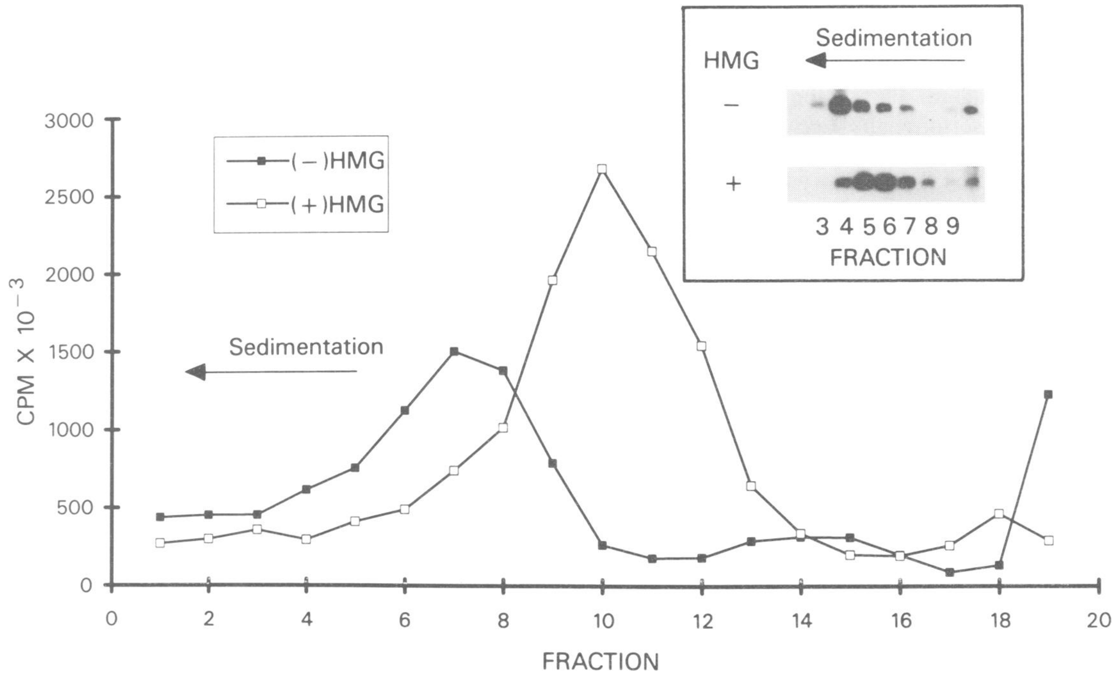

Fig. 9. Sucrose gradient sedimentation analysis of assembled minichromosomes: 0.5 ml fractions were collected. Inset: Analysis of the content of supercoiled plasmid DNA in various fractions of another sucrose gradient: 1.0 ml fractions collected.

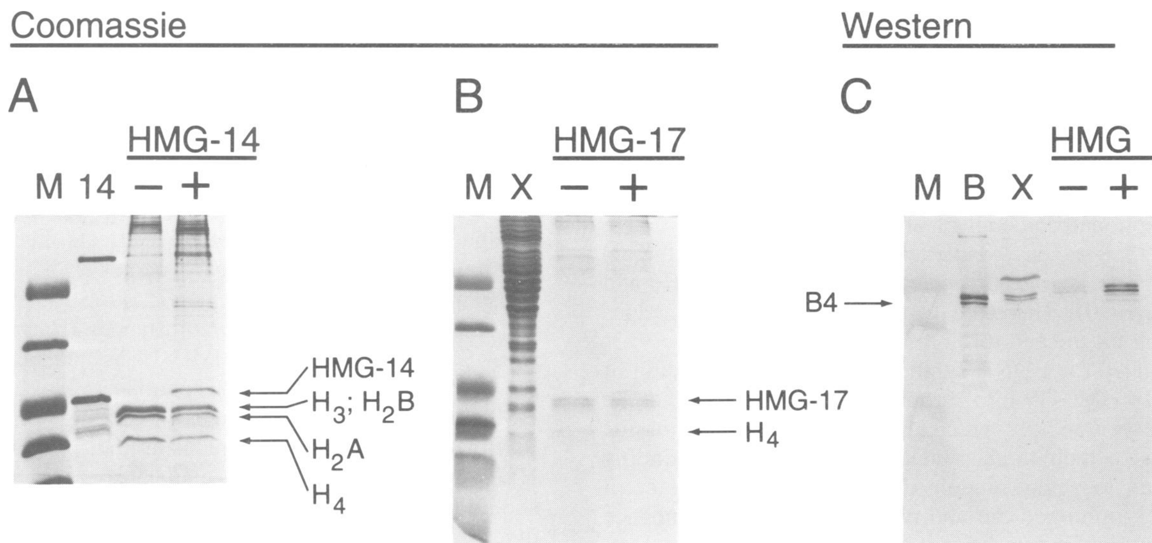

Fig. 10. Analysis of proteins in purified minichromosomes. (**A**) Coomassie Blue stain of assembly with or without HMG-14. (**B**) Coomassie Blue stain of assembly with or without HMG-17. (**C**) Western analysis of the proteins shown in (B) with antibodies to B4. M, molecular weight markers; X, 1 μl egg extract; B, partially purified recombinant B4; −, + minichromosomes assembled either in the absence or presence of 70-fold molar excess of HMG-17.

presence of HMG. The amount of HMG-14/HMG-17 present in minichromosomes was similar to that of the core histones. Thus, most, and perhaps all, of the nucleosomes in this chromatin contained HMG-14 or HMG-17. In addition, to core histones the minichromosomes contained a heterogeneous complement of high molecular weight proteins. It is not clear whether these proteins are associated with the minichromosomes or are complexed in macromolecular structures which co-sediment with the minichromosomes. Histone B4 was not detectable by Coomassie Blue stain. However, Western analysis revealed the presence of this protein in all minichromosome preparations. The content of B4 was ~3-fold higher in the minichromosomes assembled in the presence of HMG-17 (Figure 10C). Conceivably, unfolding of the minichromosomes rendered an increased number of binding sites accessible to B4. It is important to note, however, that the amount of B4 was significantly lower than that of core histones or HMG-17 protein, and could not be detected by Coomassie Blue staining.

Discussion

Our major finding is that HMG-14/HMG-17 enhance the transcriptional potential of chromatin by increasing the turnover of the transcriptionally active templates. The turnover of minichromosomes containing HMG-14/HMG-17 is increased because the proteins reduce the overall compactness of the minichromosomes. As a result, these HMGs presumably increase the accessibility of the 5S gene region to RNA polymerase and perhaps also other components of the transcriptional apparatus.

Effect of HMG-14/HMG-17 on chromatin structure

In spite of numerous studies, the exact organization of HMG-14/HMG-17 in the chromatin fiber is not fully understood. Upon micrococcal nuclease digestion, under conditions where the linker DNA is digested and histone H1 and HMG-1/HMG-2 are released from nucleosome cores, HMG-14/HMG-17 remain associated with these chromatin particles (Tahourdin *et al.*, 1981). Thus, these proteins are the only nuclear proteins known to associate specifically with the nucleosome core particle. *In vitro* reassociation studies indicate that at physiological ionic strength two molecules of HMG-14/HMG-17 bind to nucleosome cores in a co-operative fashion (Paton *et al.*, 1983; Sandeen *et al.*, 1980; Schroter and Bode, 1982). The bound HMGs contact the DNA 25 bp from its entry/exit point around the histone octamer, and in the two major grooves flanking the dyad axis (Alfonso *et al.*, 1994). In this fashion the proteins bridge two adjacent DNA strands on the surface of the cores and stabilize the structure of the nucleosome core, perhaps by inhibiting the unraveling of the DNA from the histone octamer. An increase in the stability of the cores does not provide an explanation for the HMG-dependent enhancement of the transcriptional potential of chromatin. In fact, the active 5S rRNA gene is complexed with transcription factors and may be devoid, or depleted, of histones (Wolffe and Morse, 1990). Therefore in this case transcription does not have to proceed through nucleosomes.

The need for a DNA synthesis-dependent deposition of HMG-14/HMG-17 into chromatin is an indication that the effects seen require precise and correct binding of the proteins to their target. Indeed, a peptide corresponding to the nucleosomal binding domain of the proteins competitively inhibited the ability of the proteins to enhance transcription (Figure 2).

HMG-14 and HMG-17 proteins also bind to preassembled chromatin. However, the effects on the kinetics of nuclease digestion of chromatin brought about by the binding of HMG to preassembled chromatin is opposite to the effects of HMG incorporation into nascent chromatin. When incorporated into nascent chromatin HMG-14/HMG-17 enhance the rate of digestion by nucleases; when added to preassembled chromatin HMGs reduce the rate of chromatin digestion by nucleases (compare lanes 4 and 5, 8 and 9 and 12 and 13 in Figure 5A). Detailed analysis of the micrococcal nuclease digestion patterns (see Figures 3 and 5 and Crippa *et al.*, 1993) indicate that the proteins affect the kinetics of digestion rather than the nucleosomal spacing (Drew, 1993; Tremethick and Drew, 1993). As discussed in Results, nucleosome repeat length has previously been shown to decrease during micrococcal nuclease digestion as a consequence of nucleosome sliding (Godde and Widom, 1992) and the exonucleolytic DNase activity of micrococcal nuclease (Lohr *et al.*, 1977). Because HMG-14 and HMG-17 are located at the entry/exit point of the nucleosomal DNA (Alfonso *et al.*, 1994) and stabilize the structure of nucleosome subunits (for review see Bustin *et al.*, 1990) they minimize sliding and reduce the rate of the exonucleolytic attack. Therefore, at the later stages of micrococcal nuclease digestion, the DNA in the oligonucleosomes generated from chromatin containing HMG-14/HMG-17 will be somewhat longer than that obtained from chromatin assembled in the absence of these proteins. Thus the correct effect of HMG-14/HMG-17 on the nucleosomal repeat in chromatin is depicted in the analysis of the DNA extracted at short micrococcal nuclease digestion times, where artifacts due to sliding and exonucleolytic attack are minimized (lanes 3–5, Figure 5A). Our data clearly show that under these conditions HMG-14/HMG-17 do not affect the nucleosomal spacing. It is relevant that studies with intact cells suggest that the formation of physiologically spaced nucleosomes is established during the assembly of the core histones on DNA and does not require the presence of HMGs or even histone H1 (Dimitrov *et al.*, 1994; Ohsumi *et al.*, 1993).

In view of previous reports that only phosphorylated HMG-14/HMG-17 affect nucleosomal spacing (Drew, 1993; Tremethick and Drew, 1993; Tremethick, 1994) we examined whether the exogenously added HMG-14/HMG-17 became phosphorylated during incubation in the egg extract. Our results (Figure 6) clearly indicate that they did not, in agreement with previous findings indicating that unphosphorylated HMG-14/HMG-17 proteins do not affect nucleosomal spacing (Drew, 1993; Tremethick and Drew, 1993; Tremethick, 1994). It is conceivable that only phosphorylated HMG-14/HMG-17 affects nucleosomal spacing; however, it is not clear whether HMG-17 is ever phosphorylated (Espel *et al.*, 1987; Barratt *et al.*, 1994) and except under unusual circumstances (Barratt *et al.*, 1994) only a fraction of HMG-14 is phosphorylated. Furthermore, phosphorylation abolishes the ability of HMG-14 to bind to nucleosomes (Spaulding *et al.*, 1991). In view of the above, the conclusion that phosphorylated HMG-14 or HMG-17 can space nucleosomes in chromatin should be re-examined. Analysis of micrococcal nuclease digestion products have to take into account the ability of HMG-14/HMG-17 to minimize nucleosome sliding and exonucleolytic attack during the digestion. We suggest that HMG-14/HMG-17 proteins affect the kinetics of chromatin digestion by nucleases. When incorporated into nascent chromatin the proteins bind to the end of the nucleosomal DNA, stabilize the structure of the nucleosome cores, prevent nucleosomal sliding and pose a barrier to the exonucleolytic activity of micrococcal nuclease. It is important to note that although HMG-14/HMG-17 stabilize the structure of the nucleosome subunit they relax and unfold the higher order chromatin structure. The resulting chromatin sediments slower and is digested faster by various nucleases. When HMG-14 or HMG-17 is added to preassembled chromatin the chromatin is more resistant to digestion, presumably because some of the protein incorrectly binds to the linker DNA and hinders the

accessibility of the micrococcal nuclease or the restriction enzymes to these regions.

The present studies establish that the orderly deposition of HMG-14/HMG-17 into nucleosomes reduces the compactness of the assembled minichromosomes. Incorporation of HMG-14/HMG-17 into minichromosomes reduces the rate of sedimentation in sucrose gradients and increases the rate of digestion by several restriction endonucleases and by micrococcal nuclease. Addition of HMG-14/HMG-17 to preassembled chromatin does not enhance the rate of nuclease digestion. Previous reports from several laboratories established that chromatin regions enriched in HMG-14 and HMG-17 are more susceptible to digestion by either DNase I or micrococcal nuclease (reviewed in Bustin *et al.*, 1990). Likewise, neutron scattering experiments suggest that HMG-14 may reduce the compactness of chromatin fibers (Graziano and Ramakrishnan, 1990). Thus, the structural effect of HMG-14/HMG-17 seen in the replication–assembly system in *Xenopus* egg extracts seems to mirror the situation observed in nuclei and chromatin isolated from higher eukaryotes.

In summary, changes in higher order chromatin structure may be the main mechanism whereby HMG-14/HMG-17 enhance the transcriptional potential of chromatin.

Transcriptional potentiation of chromatin by HMG-14/HMG-17

Traditionally, transcriptionally active chromatin is thought to have a less condensed conformation than transcriptionally inert chromatin (for reviews see van Holde, 1989; Wolffe, 1992). Hansen and Wolffe (1992) demonstrated that salt-dependent compaction of minichromosomes containing the *X.laevis* 5S rRNA gene represses the ability of polymerase III to transcribe this gene. Thus, transcriptional potentiation of chromatin by HMG-14/HMG-17 could simply be due to the ability of these proteins to reduce the compactness of the minichromosome superstructure. Obviously, a reduction in compactness would facilitate access of the various components of the transcriptional machinery to their target.

What is the mechanism whereby HMG-14/HMG-17 reduces the compactness of the minichromosomes? One possibility is that these proteins increase nucleosomal spacing and decrease the number of nucleosomes on the minichromosomes. Our results (Figures 3 and 5, and Crippa *et al.*, 1993) showing that HMG-14/HMG-17 do not affect the nucleosomal repeat are not compatible with this possibility. A second possibility is that HMG-14/HMG-17 decrease the content of H2A and H2B thereby decreasing chromatin folding (Hansen and Wolffe, 1994). We did not find that these HMGs reduced the amount of these histones in chromatin (Figure 10A and B). A third possibility is that HMG-14/HMG-17 decrease the amount of protein B4 in the minichromosomes. Protein B4 is a homolog of histone H1, a linker protein associated with chromatin compaction. The results shown in Figure 10C are not compatible with this possibility. In fact the presence of HMG-14 or HMG-17 during assembly increased the amount of B4 in the minichromosomes, raising the possibility that B4 is a part of the mechanism whereby HMG-14/HMG-17 enhance transcription.

We favor a fourth possibility, that HMG-14/HMG-17 interact with histone tails in the nucleosomes, and that alterations in the organization of histone tails bring about changes in chromatin folding. HMG-17 does not bind to trypsinzed core particles (Crippa *et al.*, 1992) and histone tails play an important role in the stabilization of higher order chromatin structure (Allan *et al.*, 1982; Garcia-Ramirez *et al.*, 1992). Because of their placement in isolated chromatin subunits (Alfonso *et al.*, 1994) we suggest that these HMGs may affect the binding of histone tails near the nucleosomal dyad axis or near the entry/exit point of the linker DNA.

The recent finding that HMG-14 increases the rate of RNA polymerase II elongation on an SV40 minichromosome template (Ding *et al.*, 1994) is compatible with the suggestion that this protein reduces the compactness of the minichromosome. Obviously the RNA polymerase can negotiate through an array of nucleosomes on an extended fiber faster than through an array of nucleosomes in a folded, compact fiber. However, the stimulation of polymerase II elongation by HMG-14 was not linked to replication. We note that the two systems are different in many aspects. Transcription by RNA polymerase II proceeds through nucleosomes while transcription by polymerase III does not. In the RNA polymerase III systems used here transcription is measured in the extract without purifying the template. In the SV40 minichromosome system transcription is measured by the addition of an exogenous transcription extract to purified minichromosomes. This extract may contain inhibitors, such as H1, whose effect is competed out by HMG-14. Furthermore, the SV40 minichromosomes have been assembled in the presence of the HMG-14/HMG-17 proteins present in the CV1 cells while the *Xenopus* egg extract does not contain these HMGs. The exogenously added HMG-14 could bind to regions which have been transiently associated with the cellular proteins. Indeed, the DNase I sensitivity of transcribable regions in chromatin can be reversibly reconstituted with 0.35 M NaCl extracts which contain these, as well as other, HMG proteins (Weisbrod and Weintraub, 1979). Likewise, several reconstitution experiments with isolated nucleosome cores revealed a preference of HMG-14/HMG-17 for particles enriched in sequences from transcribed genes (Sandeen *et al.*, 1980; Brotherton and Ginder, 1986; Brotherton *et al.*, 1990). Thus, HMG-containing nucleosomes may have unique features which are preserved even when the proteins have been removed. Previous studies suggest that these HMGs may recognize cores particles enriched in modified histones (Goodwin *et al.*, 1982; Malik *et al.*, 1984) or with an increased length of DNA (Albright *et al.*, 1980).

It is important to note that in both experimental systems HMG-14 stimulates transcription, that this stimulation is not due to an increase in the number of transcribed molecules and that the protein enhances transcription from a chromatin template but not from 'naked' DNA. Furthermore, the results obtained in both experimental systems are compatible with the suggestion that HMG-14/HMG-17 enhance the transcriptional potential of chromatin by unfolding the template.

Materials and methods

Xenopus laevis egg extract

X.laevis unfertilized egg extract was prepared as described previously (Almouzni *et al.*, 1990a,b).

DNA replication, chromatin assembly and transcription

All reactions were carried out as described (Almouzni et al., 1990a,b) with single stranded M13 DNA, prepared by buoyant density centrifugation, carrying the somatic 5S rRNA gene of X.borealis (Peterson et al., 1980). Routinely, the reactions contained 150 ng DNA and 15 µl extract in a final volume of 20 µl. When the X.borealis 5S rRNA gene was used in transcription reactions, 1 µl of an extract enriched in class III transcription factors TFIIIA and TFIIIC [prepared according to Smith et al. (1984) and Wolffe (1988)] at ~0.3 µg/µl was added to the extract prior to the addition of the DNA. DNA replication and chromatin assembly reactions mixtures were complete after 2 h incubation at 2°C. Transcription reactions were monitored by addition of radiolabeled CTP, ribonucleotides and RNasin and routinely were carried out for 30 min. DNA and RNA purifications and analysis on agarose or sequencing gels were performed as previously described (Almouzni et al., 1990a,b).

Single round transcription assay

The nucleotide mixture used to monitor transcription contained 1 µl of 4 mg/ml heparin. The final heparin concentration during the transcription assay was 200 µg/ml.

Micrococcal nuclease digestion and analysis of digestion products

Micrococcal nuclease (Worthington) digestion of minichromosomes in the extract was initiated by adding $CaCl_2$ to a final concentration of 1 mM. The digestions used either 1.5 U/µl or 0.7 U/µl at room temperature for the times indicated in the figures. The digestions were stopped with 20 mM Tris−HCl pH 8, 20 mM EDTA pH 8, 0.5% (w/v) lithium dodecyl sulfate, followed by proteinase K digestion (500 µg/ml, 37°C, 1 h), phenol−chloroform extraction and electrophoretic separation of the DNA on agarose gels. Southern blots of the digests were probed with the BamHI fragment from pXb10 which contains a somatic X.borealis 5S rRNA gene (Wolffe et al., 1986).

Restriction endonuclease digestion

The minichromosomes were digested in the extract with EcoRV, ScaI or HinP1I. After chromatin assembly, appropriate restriction buffers were added. After 1 h digestion the DNA was purified by proteinase K digestion, phenol−chloroform extraction and ethanol precipitation. The EcoRV and ScaI digests were redigested with BglII and the purified DNA was analyzed on 1.5% agarose gels. The HinP1I digest was analyzed by electrophoresis on 0.8% agarose gels.

Purification and analysis of assembled minichromosomes

Radiolabeled minichromosomes assembled in the extract were purified on a sucrose gradient with an SW40 rotor for 2.5 h at 40 000 r.p.m., using a 10–30% (w/v) linear sucrose gradient in 70 mM KCl, 20 mM HEPES−KOH, pH 7.5, 1 mM EDTA, 0.25% Triton. Minichromosomes recovered from the gradient were pelleted through a 30% (w/w) sucrose cushion in the same buffer (Wolffe and Schild, 1991; Crippa et al., 1993). The samples were dissolved in SDS loading buffer and analyzed on 15% polyacrylamide−0.1% SDS gels (Laemmli, 1970). Western analysis with anti-B4 (Dimitrov et al., 1994) was performed as described. For topoisomer analysis radiolabeled DNA was extracted from chromatin assembled in the presence of various amounts of HMG and fractionated on an 0.8% (w/v) agarose gel in 1× TPE buffer containing chloroquine.

Recombinant HMG-14/HMG-17 proteins and control peptide

Recombinant HMG-14 or HMG-17 (Bustin et al., 1991) was dissolved in 0.1% (w/v) bovine serum albumin (BSA) and added to the extract at the concentrations indicated in the figures. The control peptide used in the inhibition studies had exactly the same amino acid composition as peptide 2, which is the conserved nucleosomal binding domain of HMG-14/HMG-17 (Crippa et al., 1993). The sequence of the control peptide is RAKPAKLPKAAPSPKADKERSRPKPQPKEP.

Phosphorylation assay

The phosphorylation state of the HMG proteins after incubation in the egg extract was analyzed in 15 µl of extract to which 1 µl (10 µCi) of [γ-^{32}P]ATP (specific activity 6000 Ci/mmol) and 1.2 µg of HMG-14 or HMG-17 were added. The reaction mixture was incubated for 2 h at 22°C. An aliquot from this reaction mixture, from components soluble in 5% perchloric acid, or precipitated by 25% trichloroacetic acid was fractionated on SDS−polyacrylamide gels and the gels autoradiographed.

Analysis of autoradiograms

Autoradiograms of gels were analyzed on a Molecular Dynamics computing densitometer with Image Quant and Microsoft Exel 4.0 software.

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