Histone H1 Represses Transcription from Minichromosomes Assembled In Vitro

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We have previously shown that transcription from a *Xenopus* 5S rRNA gene assembled into chromatin in vitro can be repressed in the absence of histone H1 at high nucleosome densities (one nucleosome per 160 base pairs of DNA) (A. Shimamura, D. Tremethick, and A. Worcel, *Mol. Cell. Biol.* 8:4257–4269, 1988). We report here that transcriptional repression may also be achieved at lower nucleosome densities (one nucleosome per 215 base pairs of DNA) when histone H1 is present. Removal of histone H1 from the minichromosomes with Biorex under conditions in which no nucleosome disruption was observed led to transcriptional activation. Transcriptional repression could be restored by adding histone H1 back to the H1-depleted minichromosomes. The levels of histone H1 that repressed the H1-depleted minichromosomes failed to repress transcription from free DNA templates present in *trans*. The assembly of transcription complexes onto the H1-depleted minichromosomes protected the 5S RNA gene from inactivation by histone H1.

Histone H1, the internucleosomal histone of higher eucaryotes, has often been implicated in the establishment of stable and tissue-specific gene repression (6, 42). Structural studies using isolated cellular chromatin have correlated gene repression with the condensed chromatin structures that result from the binding of histone H1 at physiological ionic strength (41). Sedimentation studies suggest that chromatin of inactive genes is more compacted than chromatin of transcriptionally active genes (17). The differential sedimentation of inactive versus active chromatin is abolished at the lower ionic strengths that fail to induce H1-dependent chromatin compaction (10). Histone H1-dependent chromatin condensation has also been correlated with the relative resistance of transcriptionally inactive genes to nuclease digestion (37). Other studies suggest that histone H1 may be present in active as well as inactive chromatin but that the nature of the binding of histone H1 to these two types of chromatin may differ (42).

The effects of histone H1 on the transcription of chromatin fragments isolated from cells have also been investigated. The addition of histone H1 to H1-depleted chromatin fragments leads to a general inhibition of transcription by *Escherichia coli* RNA polymerase and calf thymus RNA polymerase II (15). Repressed class III genes in *Xenopus* sperm chromatin, which lacks somatic histone H1, can be transcribed upon addition of transcription factors; in contrast, repressed class III genes in *Xenopus* somatic chromatin are not activated by transcription factors (43). Reports that the addition of histone H1 to H1-depleted chromatin represses oocyte-type 5S genes and satellite I DNA more than somatic-type 5S genes and tRNA genes suggest that histone H1 may play a role in differential gene repression (34, 44).

The precise role of histone H1 in gene repression has been difficult to determine because of lack of *in vitro* systems that will assemble H1 together with the four nucleosomal histones into regularly spaced nucleosomes with the periodicities found *in vivo*. A significant obstacle to the development of such systems was the tendency of histones and DNA to precipitate in the presence of histone H1 at physiological

ionic strength (22); at best, only closely packed nucleosomes could be formed on random-sequence DNA by using the five histones (11, 32). In the past, an increase in the spacing of nucleosomes by histone H1 or H5 could be achieved only by assembling chromatin *in vitro* on poly(dA-dT)-poly(dA-dT) DNA in the presence of polyglutamic acid (38, 39).

To begin to address the mechanism of action of histone H1, we have developed and characterized a chromatin assembly system that efficiently packages any DNA into regularly spaced nucleosomes that lack histone H1 (12, 31, 36). Histone H1, when added to this *in vitro* system at the beginning of the chromatin assembly process, binds to the internucleosomal DNA and generates the longer repeat lengths found *in vivo* (30a).

Transcription from circular DNA templates becomes progressively repressed when the DNA molecules are assembled *in vitro* into minichromosomes with increasing nucleosome densities (21, 36). We have previously shown that the 5S rRNA gene can be repressed in the absence of histone H1, provided that high nucleosome densities are attained (36). We now investigate whether such repression can also be obtained at the lower nucleosome densities typical of higher eucaryotes when histone H1 is present.

MATERIALS AND METHODS

Mature female *Xenopus laevis* were obtained from Nasco (LM535MG). Nasco starves the frogs during the summer, so it is essential to set aside frogs for regular feedings at that time. Plasmid pXbsF201 carries a 240-base-pair (bp) insert containing the *Xenopus borealis* somatic 5S rRNA gene in pUC9 (29). The somatic 5S maxigene, pXbs 115/77 in pUC, was the gift of D. Bogenhagen. Topoisomerase I was purified from calf thymus (28). Histone H1 was prepared from *X. laevis* erythrocytes (30a).

Preparation of the S-150 extract and chromatin assembly reactions. The oocyte S-150 extract was prepared as described previously (36). The chromatin assembly reactions (36) were performed at 27°C with the modifications described elsewhere (30a).

Histone H1 was added at the onset of the chromatin assembly reactions (30a). Because the activities of different preparations of histone H1 varied, each preparation was

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titrated in the S-150 extract to determine the amount that, when added at the beginning of the reaction, produced a regular ladder with a 215-bp periodicity upon digestion with micrococcal nuclease (MNase). For the experiments shown in Fig. 1 to 5, the H1-to-nucleosome ratio (H1/n; measured as molecules of H1 per 190 bp of DNA) required to give a 215-bp repeat length was 3. The preparation of histone H1 used in the experiments shown in Fig. 6 and 7 was twice as active, so a 215-bp repeat length was generated with an H1/n ratio of only 1.5. Since the degree of purity was the same in all preparations, the observed variation in H1 specific activity was probably due to different levels of inactivation during purification of the protein.

MNase digestions, minichromosome purification, electron microscopy, and protein analysis on the purified minichromosomes were performed as described previously (36). The nucleosome repeat length was determined either by dividing the size of the DNA plasmid (2.9 kbp) by the number of steps in the regular ladder generated by partial MNase digestion or by dividing the length, in base pairs, of the larger oligonucleosomes by the number of nucleosomes they contain (the 123-bp ladder from Bethesda Research Laboratories, Inc., was used as reference molecular weight marker). These procedures minimize the effects of exonucleolytic trimming by MNase (27).

Biorex treatment of the minichromosomes. Biorex 70 resin (Bio-Rad Laboratories, Richmond, Calif.) was equilibrated first in 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.0) until the pH had stabilized and then in extraction buffer [20 mM HEPES (pH 7.0), 5 mM KCl, 1.5 mM MgCl₂, 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10% glycerol, 10 mM β -glycerophosphate, 0.5 mM dithiothreitol]. The resin was stored for several weeks at 4°C as a slurry of 25% (vol/vol) Biorex in extraction buffer.

To remove histone H1, the slurry was vortexed as the Biorex resin was removed to an Eppendorf tube. After a 15-s spin in a tabletop centrifuge, the extraction buffer supernatant was removed. The chromatin assembly reaction, which contained the minichromosomes that were assembled in the oocyte S-150 after a 6-h incubation under standard conditions, was chilled on ice for 5 min and then added to the pelleted resin. A ratio of 8 to 10 μ l of packed Biorex resin per ml of the chromatin assembly reaction mixture was used. The Biorex resin was then gently mixed by rotation at 4°C for 1 h. The resin was subsequently pelleted with a 15-s spin in a tabletop centrifuge, and the supernatant containing the minichromosomes was removed for further experiments.

We were not able to remove histone H1 from the chromatin when tRNA (16) was added to the chromatin assembly mixture. It is possible that the exogenous tRNA was quickly bound and sequestered by the soluble proteins present in the S-150 extract.

Purification of TFIIA. TFIIA was purified from 7S ribonucleoprotein particles as described elsewhere (7, 47).

Purification of TFIIB and TFIIC. The S-150 extract prepared as described by Glikin et al. (12) was adjusted to 100 mM KCl and applied to a phosphocellulose column (1 ml of packed resin per 10 mg of protein) equilibrated in buffer A–100 mM KCl (buffer A is 20 mM HEPES [pH 7.5], 20% glycerol, 2 mM β -glycerophosphate, 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.1% Triton X-100, 2 μ g of leupeptin per ml, and 2 μ g of pepstatin per ml). The column was washed with 5 column volumes of buffer A–100 mM KCl, followed by a 5-column-volume wash with buffer A–150 mM KCl. TFIIB was eluted with buffer A–270 mM KCl. The column was then

washed with buffer B–350 mM KCl (buffer B is the same as buffer A but without Triton X-100). TFIIC and RNA polymerase III were eluted with 1 M KCl in buffer B (35).

The TFIIB pool of the phosphocellulose column was diluted 1:3 with buffer A and applied to a DE-52 column (1 ml of packed resin per 3 mg of protein) equilibrated in buffer A–100 mM KCl. The column was washed with buffer A–150 mM KCl and eluted with 220 mM KCl in buffer A. The pool was directly loaded onto a hydroxylapatite column equilibrated in buffer A–220 mM KCl (1 ml of packed resin per mg of protein). The column was subsequently washed with buffer A–2 M KCl and then with buffer A–55 mM potassium phosphate (pH 7.5). The TFIIB activity was eluted with buffer A–110 mM potassium phosphate (pH 7.5). The pool was divided into equal samples and kept at –120°C. This led to an approximately 500-fold purification of TFIIB activity with a yield of 70% (the activity after the phosphocellulose column was set as 100%). The protein concentration in the final pool varied between 100 and 240 μ g/ml among preparations.

The TFIIC pool of the phosphocellulose column was dialyzed to buffer B–300 mM KCl. It was then passed over a DEAE-Sephadex column (1 ml of packed resin per 3 mg of protein) equilibrated in dialysis buffer to separate TFIIC from the RNA polymerase III activity (35). The flowthrough, which contained TFIIC, was further purified by precipitation in ammonium sulfate. A saturated ammonium sulfate solution (pH 7.5; supplemented with 0.5 mM dithiothreitol) was added to the TFIIC pool so that the final concentration was 60%. After 30 min on ice, the resulting precipitate was pelleted. The pellet was dissolved in one-half the original volume and dialyzed to buffer B–100 mM KCl. The protein concentration of the final TFIIC pool varied between 0.7 and 1.5 mg/ml among preparations.

RNA polymerase III was eluted from the DEAE-Sephadex column with 1 M KCl in buffer B supplemented with 100 μ g of bovine serum albumin per ml and dialyzed to buffer B–100 mM KCl. The transcription factors were stable over several months when stored at –120°C.

Transcription reactions. The transcription factors TFIIA (20:1 ratio of TFIIA to 5S DNA), TFIIB (2 to 6 μ l/150 ng of DNA), TFIIC (1 to 2 μ l/150 ng of DNA), and RNA polymerase III (2 μ l/150 ng of DNA) were added to the minichromosomes and allowed to form transcription complexes for 1 h at 27°C in the presence of 10 mM MgCl₂. A 1/10 volume of a nucleoside triphosphate (NTP) cocktail containing 6 mM each ATP, UTP, and CTP, 0.2 mM GTP (Pharmacia, Inc.), and 1 μ Ci of [α -³²P]GTP (Amersham Corp.) per μ l was added to the reaction mixture, and transcription was allowed to proceed for 45 min at 27°C. The transcription reactions were stopped with 1 volume of stop solution (20 mM Tris hydrochloride [pH 7.5], 80 mM EDTA, 0.8% sodium dodecyl sulfate, 40 ng of tRNA per ml, and 2 mg of proteinase K per ml) and incubated for 1 h at 37°C or overnight at 22°C. The reaction volume was raised to 250 μ l with urea in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA to yield a final urea concentration of 2.7 M. The reactions were extracted once with phenol-chloroform and once with chloroform and precipitated with ethanol. The RNA was analyzed by electrophoresis in 8% polyacrylamide gels containing 8 M urea (26, 36) and autoradiography.

Transcription of the gradient-purified minichromosomes carrying transcription complexes was performed with 100 μ l of each gradient fraction to which was added 7 mM MgCl₂, 0.5 mM dithiothreitol, 2 μ l of RNA polymerase III, and 1/10 volume of the NTP cocktail. The transcription reactions

FIG. 1. Demonstration that Biorex treatment of minichromosomes containing H1 leads to transcriptional activation without disrupting the nucleosomes. Relaxed 5S DNA plasmid was assembled into chromatin for 6 h at 27°C under standard conditions (30a) in the presence or absence of histone H1 (H1/n ratio of 3:1). The indicated samples were treated with Biorex resin for 1 h, and the control samples were kept on ice. (a) Samples of 30 μ l were removed from each reaction and incubated for either 15 or 30 min at 22°C in the presence of 1 U of DNA topoisomerase I (topoI); control samples (0 min) were incubated for 30 min at 22°C in the topoisomerase buffer but without the exogenously added DNA topoisomerase I. For the protein-free DNA reactions (left three lanes), supercoiled 5S DNA plasmid was incubated, under mock chromatin assembly conditions, in extraction buffer instead of the S-150 extract, and the samples were then treated with DNA topoisomerase I as described above. (b) The chromatin assembled and processed as described above was digested with MNase for 0.5, 1, 2, 4, and 8 min (left to right). (c) The chromatin assembled and processed as described above was assayed for transcription. After a 1-h incubation with transcription factors in the presence of 10 mM $MgCl_2$, transcription was allowed to proceed in the presence of [α - ^{32}P]GTP for

were stopped after 45 min at 27°C and processed as described above.

Transcription was quantitated by excising the bands from the gel for liquid scintillation counting and by densitometric scanning of autoradiograms.

RESULTS

Repression of the 5S RNA gene at lower nucleosome densities in the presence of histone H1. Previous studies have shown that addition of histone H1 to the oocyte S-150 at the onset of chromatin assembly results in minichromosomes that display longer nucleosome repeats and correspondingly lower nucleosome densities than do minichromosomes assembled without H1 (30a). It has also been shown that the transcriptional activity of the assembled minichromosomes is inversely proportional to the nucleosome density (21, 36). Therefore, to investigate the effect of histone H1 on transcription, we had to ensure that the minichromosomes assembled with and without H1 had the same nucleosome densities. The experimental approach used throughout this work was to compare minichromosomes assembled in the presence of histone H1 with those same minichromosomes from which histone H1 had been removed.

The plasmid DNA used in this study was pXbsF201, which contains the somatic 5S rRNA gene of *X. borealis* inserted into pUC 9 (29). 5S DNA plasmids were assembled into chromatin either with or without histone H1 and then treated with Biorex resin, which removes histone H1 from chromatin (24). Care was taken with the levels of Biorex used, since high levels of Biorex also removed the nucleosomal histones from the DNA (A. Shimamura, unpublished observations). To ascertain that the nucleosomes had not been disrupted, the structure of the chromatin before and after treatment with Biorex was analyzed with DNA topoisomerase I and MNase.

Any unconstrained supercoils that might have been generated by nucleosome loss would be relaxed by the addition of DNA topoisomerase I. The endogenous DNA topoisomerase I present in the oocyte S-150 binds to Biorex under these conditions, necessitating supplementation with additional DNA topoisomerase I for this analysis. After incubation with excess DNA topoisomerase I, no DNA relaxation was detected either before or after Biorex treatment of minichromosomes assembled with or without histone H1 (Fig. 1a). Therefore, Biorex treatment of the minichromosomes did not lead to any major loss of nucleosomes, although the possibility that some minichromosomes might have lost one or two nucleosomes cannot be ruled out by this approach.

The effect of Biorex on the nucleosome structure of the minichromosomes assembled with or without histone H1 was next examined by using MNase. After the addition of histone H1 to the chromatin assembly reaction, the nucleosome repeat length was increased from 190 to 215 bp (Fig. 1b; 30a). In all experiments presented here, an amount of

45 min at 27°C. Transcription on the free DNA control was performed on free 5S DNA plasmid immediately after its addition to the S-150. The transcription rate of the free DNA control in this experiment cannot be directly compared with that of the minichromosomes because different protocols were used. Minichromosomes with a 215-bp spacing transcribed with a rate between 10- and 60-fold lower than the free DNA rate, depending on the extract used (see Fig. 6 and 7).

histone H1 sufficient to generate a 215-bp periodicity was added to the chromatin assembly reactions (see Materials and Methods). The minichromosomes assembled in the absence of histone H1 still generated a regular ladder of nucleosomes with a 190-bp periodicity after Biorex treatment. The minichromosomes assembled with histone H1 continued to produce a spaced ladder with a 215-bp periodicity after Biorex treatment, but the bands were broader and the background DNA between nucleosomal bands was slightly higher, indicating that the nucleosomes had some freedom to slide at these lower nucleosome densities (36).

The minichromosomes assembled with histone H1 became about fourfold more sensitive to MNase digestion after Biorex treatment, as would be expected if the internucleosomal DNA were no longer protected by histone H1. The level of MNase digestion at 0.5 min after Biorex treatment was similar to that at 2 min before Biorex treatment (see legend to Fig. 1b; 30a).

Control minichromosomes assembled without H1 became approximately twofold more sensitive to MNase digestion after Biorex treatment (the level of digestion at 0.5 min after Biorex treatment was similar to that at 1 min before Biorex treatment; Fig. 1b). Although we cannot rule out the possibility that a few nucleosomes might have been lost, 15 regular steps could still be counted on the MNase ladder after Biorex treatment of the minichromosomes assembled without H1, and no relaxation by DNA topoisomerase I was observed (the small difference in digestion rate could be due to the removal of other inhibitors of MNase by the Biorex resin [3]).

The effect of Biorex treatment on the transcriptional activities of the minichromosomes is shown in Fig. 1c. Biorex also removed transcription factors from the reaction mixture (see Fig. 6). To ensure that all transcription factors were present, we always supplemented the reactions with the full complement of 5S gene transcription factors (TFIIIA, TFIIB, TFIIC, and RNA polymerase III) before transcription analyses. No significant change in transcription occurred after Biorex treatment of control minichromosomes assembled without histone H1 (Fig. 1c; compare lanes 4 and 5), confirming that the Biorex treatment did not significantly disrupt the nucleosomes. In contrast, Biorex treatment of the minichromosomes assembled with histone H1 resulted in higher levels of transcription.

Transcriptional activation was also observed with Biorex-treated and subsequently purified minichromosomes to which the transcription factors were added after the sucrose gradients. This result excludes the possibility that the removal of components other than H1 might have been responsible for the activation. The transcriptional activation has been consistently observed, although the actual degree of stimulation varies among experiments from 3-fold (Fig. 1c; compare lanes 3 and 2) to approximately 100-fold (see also Fig. 4, 5a, 6, and 7). Possible sources of this variation are considered below.

After Biorex treatment, the transcriptional activity of minichromosomes assembled without H1 (Fig. 1c, lane 5) was lower than that of minichromosomes assembled with H1 (lane 3). Since transcriptional activity falls with increasing nucleosome density (21, 36), this difference probably resulted from the higher nucleosome density of minichromosomes assembled without H1 (one nucleosome per 190 bp) relative to that of minichromosomes assembled with H1 (one nucleosome per 215 bp; Fig. 1b; 30a). Similarly, minichromosomes that had been assembled with histone H1 and then treated with Biorex, which contained an average of one

nucleosome per 215 bp, were less active than 5S DNA templates that were free of any nucleosomes (see also Fig. 6 and 7).

Biorex treatment of the minichromosomes containing histone H1 leads to a more extended chromatin structure. The conformation of the minichromosomes before and after Biorex treatment was analyzed by sucrose gradient sedimentation and electron microscopy. The minichromosomes assembled with histone H1 sedimented at the same rate as did the minichromosomes assembled without H1 (Fig. 2; insets a and d; 30a). Biorex treatment of the minichromosomes containing histone H1 resulted in particles that displayed a slower sedimentation rate (Fig. 2; compare insets b and a). This shift in the sedimentation coefficient, which was consistently observed, could be reversed if histone H1 was added back to the Biorex-treated minichromosomes (Fig. 2, inset c). Whereas minichromosomes assembled without H1 formed a slightly broader peak after treatment with Biorex (Fig. 2, inset e), they failed to undergo the shift in S value observed with minichromosomes containing H1.

Studies using isolated fragments of cellular chromatin have shown that histone H1 binding to chromatin depleted of H1 causes a compaction that is reflected in an increased sedimentation velocity of the chromatin (1). We also observe such an H1-induced increase in the sedimentation velocity of the H1-depleted chromatin (Fig. 2, compare insets c and b). On the other hand, minichromosomes assembled with histone H1 did not sediment at a faster rate than did minichromosomes assembled without H1 (Fig. 2; compare insets a and d; 30a). It is likely that under these conditions, the histone H1-induced compaction of the minichromosomes is canceled out by the decompaction as a result of the lower number of nucleosomes and supercoils that such minichromosomes contain (Fig. 1b; 30a).

The gradient-purified minichromosomes were visualized under the electron microscope. The minichromosomes containing histone H1 appeared compact in size, with little linker DNA visible between the nucleosomes (Fig. 2a). After Biorex treatment, these minichromosomes opened up into more extended circular structures, and the exposed linker DNA was clearly visible (Fig. 2b). The original condensed structure was restored after histone H1 addition to the Biorex-treated minichromosomes (Fig. 2c). Control minichromosomes assembled without histone H1 displayed a structure that was similar to that of the minichromosomes assembled with H1 (30a). No change was observed after Biorex treatment of minichromosomes assembled without H1; therefore, only the electron micrograph of the Biorex-treated minichromosomes is shown in Fig. 2e. The sedimentation profiles of the minichromosomes in the sucrose gradients and the dispersed appearance of the minichromosomes under the electron microscope indicate that under these conditions, histone H1 does not induce aggregation of the minichromosomes (30a).

Removal of histone H1 by Biorex treatment of minichromosomes. Analysis of the DNA-bound proteins on sodium dodecyl sulfate-polyacrylamide gels confirmed that the major effect of the Biorex treatment was the removal of histone H1 from the minichromosomes (Fig. 3; compare lanes 3 and 4). No detectable loss of the core histones was observed after Biorex treatment of the minichromosomes assembled in either the presence or absence of histone H1. Histone H1, when added back to the Biorex-treated minichromosomes, was again bound to the minichromosomes (Fig. 3, lane 5).

Transcription complexes protect the 5S RNA gene from repression by histone H1. We next asked whether the tran-

FIG. 2. Sucrose gradient sedimentation and electron microscope visualization of the minichromosomes before and after Biorex treatment, performed on minichromosomes that had been assembled and treated as follows. (a) Minichromosomes were assembled in the presence of histone H1 ($H1/n = 3$) for 6 h at 27°C . (b) Minichromosomes assembled as in panel a were treated with Biorex resin for 1 h. (c) Minichromosomes treated with Biorex as in panel b were subsequently incubated with histone H1 ($H1/n = 3$) for 30 min at 27°C . (d) Minichromosomes were assembled in the absence of histone H1 for 6 h at 27°C . (e) Minichromosomes assembled as in panel d were treated with Biorex resin for 1 h. (Insets) Samples from each fraction of the sucrose gradients (sedimentation was from right to left) were electrophoresed on agarose gels and stained with ethidium bromide to determine the location of the minichromosomes (the ethidium bromide staining bands at the top of the gradients are the endogenous small RNA molecules present in the S-150 [30a, 36]). Samples from the peak fraction of each gradient were then visualized by electron microscopy (36). Since the electron micrographs of samples d and e were identical, only sample e is shown.

FIG. 3. Protein composition of minichromosomes before and after Biorex treatment. The purified minichromosomes shown in Fig. 2 were electrophoresed on an SDS-polyacrylamide gel and stained with silver. Lanes: 1 and 2, 150 and 300 ng of *Xenopus* histone H1; 3 to 7, samples a to e, respectively, of Fig. 2; 8, all five histones isolated from *Xenopus* erythrocytes. Note that three forms of histone H2A (indicated on the left) are present in *Xenopus* oocytes (8, 36).

scriptional activation resulting from the removal of histone H1 could be abolished by adding histone H1 back to the Biorex-treated minichromosomes. 5S DNA plasmids were assembled into chromatin in the presence of histone H1 under standard conditions, and the minichromosomes containing H1 were then treated with Biorex as before. Once more, the transcriptional activity of the minichromosomes rose after Biorex treatment (Fig. 4; compare lanes 2 and 1). Addition of histone H1 to the H1-depleted minichromosomes restored the transcriptionally repressed state of the minichromosomes (Fig. 4; compare lanes 6 and 2).

When the transcription factors TFIIA, TFIIB, and TFIIC were allowed to assemble onto the H1-depleted minichromosomes before the addition of histone H1, H1 was prevented from repressing transcription (Fig. 4, lane 4). The protection by transcription factors, however, was not complete. Transcription was consistently slightly lower after the addition of histone H1 to the minichromosomes bearing transcription complexes (Fig. 4; compare lanes 4 and 2). This small decrease in transcription was seen within 5 min of the addition of histone H1, and no further repression was seen with increasing time of incubation in the presence of histone H1 (data not shown).

The higher rate of transcription seen when transcription factors were added before H1 was not due to the longer total time of incubation with the factors relative to that when the factors were added after H1 (1.5 h versus 1 h) because transcription complex formation was essentially complete by 1 h (Fig. 4; compare lanes 3 and 2). Because $MgCl_2$ (10 mM final concentration) had to be added to the reactions during transcription complex formation, control reactions were also performed to check the effect of Mg on the action of histone H1. Histone H1 efficiently repressed transcription at the levels of $MgCl_2$ required for transcription complex formation (Fig. 4, lanes 5 and 6); therefore, the failure of H1 to repress

FIG. 4. Protection by transcription complexes of H1-depleted minichromosomes from repression by histone H1. (a) Schematic diagram of the experimental protocol used. 5S DNA plasmid was assembled into chromatin for 6 h at 27°C in the presence of histone H1 (H1/n = 3). Samples were treated with Biorex as indicated, and the reactions were processed as described below. (b) For lane 1, a control sample that had not been treated with Biorex was incubated with transcription factors and 10 mM $MgCl_2$ for 1 h and then transcribed in the presence of [^{32}P]GTP. For lane 2, H1-depleted minichromosomes were incubated with transcription factors and 10 mM $MgCl_2$ for 1 h and then transcribed with [^{32}P]GTP. For lane 3, H1-depleted minichromosomes were incubated with transcription factors and 10 mM $MgCl_2$ for 1.5 h and then transcribed with [^{32}P]GTP. For lane 4, H1-depleted minichromosomes were incubated with transcription factors and 10 mM $MgCl_2$ for 1 h, after which time histone H1 (H1/n = 3) was added for 30 min. The minichromosomes were then transcribed with [^{32}P]GTP. For lane 5, H1-depleted minichromosomes were incubated with histone H1 (H1/n = 3) for 30 min in the presence of 10 mM $MgCl_2$, after which time transcription factors were added for 1 h. The minichromosomes were then transcribed with [^{32}P]GTP. For lane 6, H1-depleted minichromosomes were incubated with histone H1 (H1/n = 3) for 30 min in the presence of 1 mM $MgCl_2$, after which time the level of $MgCl_2$ was raised to 10 mM and transcription factors were added for 1 h. The minichromosomes were then transcribed with [^{32}P]GTP.

transcription when the protein was added after transcription complex formation was not due to the presence of Mg.

Occurrence of transcription on a chromatin template after Biorex treatment. The possibility remained that Biorex treatment of minichromosomes containing H1 may have generated a trace amount of free DNA, which was responsible for the transcriptional activation observed. To address this question, transcription complexes were assembled onto the H1-depleted minichromosomes, and the minichromosomes were then separated from any free DNA by sedimentation through sucrose gradients. The peak positions of the free DNA carrying transcription complexes and of the H1-depleted minichromosomes to which H1 had been restored before the addition of transcription factors were determined in parallel gradients (arrows in Fig. 5b).

Fractions 3 to 18, which spanned the locations of the minichromosomes containing H1, the H1-depleted minichromosomes, and the free DNA, were transcribed in the presence of exogenously added RNA polymerase III. Since no additional transcription factors were added to the fractions,

FIG. 5. Occurrence of transcription on a chromatin template. Relaxed 5S DNA plasmid was assembled into chromatin in the presence of histone H1 ($H1/n = 3$) in duplicate reaction mixtures of 1 ml for 6 h at 27°C. The minichromosomes were then treated with Biorex to remove histone H1. One of the two 1-ml reaction mixtures was subsequently incubated with histone H1 ($H1/n = 3$) for 30 min at 27°C. As a free DNA control, 5S DNA plasmid was incubated in a mock chromatin assembly reaction mixture in which the S-150 was replaced with extraction buffer. The minichromosomes and free DNA were then incubated with TFIIA, TFIIB, TFIIC, and polymerase III in the presence of 10 mM $MgCl_2$ for 1 h at 27°C. (a) Samples (50 μ l) of the unfractionated reactions of the H1-depleted minichromosomes (lane 1) and the minichromosomes to which histone H1 had been restored before the addition of transcription factors (lane 2) were assayed for transcription. Each sample was incubated with 5.5 μ l of NTP cocktail for 45 min at 27°C, and the transcripts were analyzed on a gel. The film was overexposed to detect the faint band in lane 2. (b) The samples of minichromosomes and free DNA, all of which had been incubated with the transcription factors, were loaded onto separate sucrose gradients. After centrifugation and fractionation of the gradients, 15- μ l samples of each gradient fraction were electrophoresed on 1% agarose gels. Sedimentation was from right (fraction 24) to left (fraction 1). Positions of the minichromosomes and free DNA were visualized with ethidium bromide. The gel of the gradient of the H1-depleted minichromosomes is shown; positions of the peak fractions of the H1-containing minichromosomes and of the free DNA are indicated ($\downarrow$). (c) RNA polymerase III was added to fractions 3 to 18 of the gradient containing the H1-depleted minichromosomes, and transcription was assayed as described in Materials and Methods. Lanes in the RNA polyacrylamide gel are aligned with the corresponding fractions in the DNA agarose gel.

all the transcription observed had to originate from bound transcription factors that had been added before the gradient fractionation and that had therefore copurified with the gene template. The peak of transcriptional activity in the free DNA gradient corresponded to the location of the free DNA (arrow in Fig. 5b). Transcription was barely detectable in the unfractionated reaction mixture containing the Biorex-treated minichromosomes to which histone H1 had been restored (Fig. 5a, lane 2), so it was not surprising that no transcription was seen in any of the gradient fractions of this sample (data not shown).

The profile of the transcriptional activity in the gradients of the H1-depleted minichromosomes paralleled the minichromosome DNA profile (Fig. 5c). No transcription was observed in the region of the gradient between the H1-depleted minichromosomes and the location of the free

DNA. We therefore conclude that the transcription detected when transcription factors TFIIA, TFIIB, and TFIIC were added to minichromosomes that were depleted of histone H1 by Biorex was occurring on H1-depleted chromatin templates.

Levels of histone H1 that repress chromatin transcription fail to repress free DNA transcription. To examine whether the repression by histone H1 was specific for a chromatin template or whether it was a general effect of histone H1 on DNA, we tested the effect of histone H1 on H1-depleted minichromosomes that were coincubated with free 5S maxigene DNA. First, a control experiment was performed to ascertain that transcription of the free DNA *in trans* did not affect the transcriptional state of the minichromosomes either before or after Biorex treatment (Fig. 6). No change in transcription of the 5S chromatin template before or after

FIG. 6. Demonstration that transcription of protein-free DNA present in *trans* does not affect the transcription rate of minichromosomes. Relaxed 5S DNA plasmid was assembled into chromatin in the presence of histone H1 for 6 h at 27°C. 5S minichromosomes (150 ng per reaction) were transcribed as follows: lane 1, the minichromosomes were incubated for 1 h with 10 mM MgCl₂ and then transcribed with 1/10 volume of NTP cocktail for 45 min; lane 2, the minichromosomes were treated with Biorex before being processed as for lane 1; lane 3, the minichromosomes were incubated with transcription factors (TFIIIA, TFIIB, TFIIC, and polymerase III) in the presence of 10 mM MgCl₂ for 1 h and then transcribed with 1/10 volume of NTP cocktail for 45 min; lane 4, the minichromosomes were treated with Biorex before being processed as described for lane 3; lanes 5 and 6, the minichromosomes were processed as for lanes 1 and 2, respectively, except that 15 ng of free 5S maxigene DNA was added just before the 1-h incubation with 10 mM MgCl₂; lanes 7 and 8, the minichromosomes were processed as for lanes 3 and 4, respectively, except that 15 ng of free 5S maxigene DNA was added just before the transcription factors.

Biorex treatment was seen after the addition of the protein-free maxigene DNA (Fig. 6; compare the levels of 5S RNA transcription in lanes 1 to 4 with those in lanes 5 to 8, respectively). The same conclusions were reached when the genes were switched so that the 5S maxigene was assembled into chromatin and transcribed in the presence of the protein-free 5S gene (data not shown). It should be noted that the protein-free DNA was added at a 10-fold-lower concentration than the minichromosomes (see legend to Fig. 6) because free DNA competes for transcription factors more efficiently and transcribes at a higher rate than does chromatin (Fig. 1c).

Exogenous transcription factors had to be added to detect the increase in transcriptional activity resulting from the removal of histone H1 because the endogenous factors in the reaction mixture were removed by the Biorex resin (Fig. 6). The transcription seen in lanes 1 and 5 was due to the endogenous transcription factors present in the S-150. No further increase in transcription of either the chromatin or the free DNA was observed after the addition of exogenous transcription factors to the reaction mixtures before Biorex treatment (Fig. 6; compare lanes 3 and 1 and lanes 7 and 5). Without the exogenous transcription factors, the transcription rates on chromatin and on free DNA fell after treatment with Biorex (compare lanes 2 and 1 and lanes 6 and 5). The transcription rates of the Biorex-treated reactions rose after the addition of transcription factors such that the free DNA regained its original level of activity, whereas the H1-depleted chromatin was transcribed at a higher rate than it had been before Biorex treatment (compare lanes 8 and 7 and lanes 4 and 3).

Transcription of both chromatin and free DNA was limited by the low levels of endogenous factors remaining in the

oocyte S-150 after Biorex treatment (Fig. 6). We have occasionally encountered extracts that contain very high levels of endogenous transcription factors. In these cases, the factors are not completely removed by the Biorex treatment, and the transcription rate may increase after the removal of histone H1 with Biorex even in the absence of exogenous factors. In all experiments described here, exogenous factors were added; under these conditions, Biorex treatment of the chromatin-containing histone H1 is consistently accompanied by transcriptional activation of the 5S RNA gene.

In one experiment (Fig. 7), the protein-free maxigene DNA was added at a 10-fold-lower concentration than the 5S minichromosomes, as in the experiment shown in Fig. 6, and additional protein-free pUC DNA was added such that the total amount of protein-free DNA equaled that of chromatin. The preparation of histone H1 used in the experiments of Fig. 6 and 7 was twice as active as H1 preparations used elsewhere in this study (see Materials and Methods). Thus, an H1/n ratio of only 1.5 generated a repeat length of 215 bp per nucleosome (data not shown) and repressed transcription (Fig. 7b; compare lanes 1 and 2 and lanes 6 and 5).

The chromatin template was repressed at low levels of histone H1, whereas the free DNA was not repressed until much higher levels of H1 had been added (Fig. 7b, lanes 9 to 13). Quantitation of the transcription levels by scintillation counting indicated that transcription of chromatin had been repressed by 50% at an H1/n ratio of approximately 0.75, whereas 50% repression of the free DNA required threefold-higher levels of histone H1 (Fig. 7c).

Transcription complexes were also assembled onto H1-depleted chromatin and free DNA before the addition of histone H1 (Fig. 7b, lanes 14 to 18). The preassembly of transcription complexes prevented the repression of chromatin seen at low levels of histone H1. Under these conditions, the effect of histone H1 on chromatin transcription was similar to its effect on free DNA transcription. A 50% repression of transcription from chromatin did not occur until an H1/n ratio of greater than 2.5 was added, and this same level of histone H1 caused a 50% repression of transcription from the free DNA template (Fig. 7d). Thus, prior assembly of transcription complexes onto the minichromosomes prevented the preferential repression of chromatin transcription by histone H1.

DISCUSSION

Minichromosomes assembled in vitro exhibit the same properties as do isolated cellular chromatin. We have analyzed the effects of histone H1 on a defined nucleosomal template assembled in vitro. The restoration of the original structural and functional properties of the H1-depleted minichromosomes by the readdition of histone H1 confirms that histone H1 is the protein responsible for the changes observed. Appropriate controls demonstrate that transcriptional repression of the minichromosomes is not the result of artifactual aggregation induced by histone H1, that transcription is occurring on a chromatin template, and that the underlying nucleosomal structure is not disrupted after the removal of histone H1 with Biorex. Although we cannot rule out the possibility that a small number of nucleosomes might have been lost without disrupting the pattern of digestion obtained with MNase, control experiments showed that readdition of histone H1 to minichromosomes that had been depleted of H1 by Biorex restored transcriptional repression, and no significant change in transcriptional activity was observed

FIG. 7. Effect of increasing levels of histone H1 on coincubated H1-depleted 5S minichromosomes and free maxigene DNA. (a) Schematic diagram of the experimental protocol used. (b) Relaxed 5S DNA plasmid was assembled into chromatin in the presence of histone H1 (H1/n = 1.5; this preparation of histone H1 was twice as active as preparations used elsewhere in this study). Histone H1 was subsequently removed with Biorex, and the following samples containing 150 ng of minichromosomes per reaction were incubated with transcription factors in the presence of [³²P]GTP: lane 1, 5S minichromosomes assembled with histone H1 before Biorex treatment; lane 2, H1-depleted 5S minichromosomes; lane 3, control reaction with 150 ng of pUC DNA assembled into chromatin with histone H1 (H1/n = 1.5) and then treated with Biorex; lane 4, control lane 3 reaction to which 15 ng of free maxigene DNA and 135 ng of free pUC DNA had been added just before the transcription factors (the free 5S maxigene DNA produced several short aberrant transcripts, but no transcript was present in the region of the gel where the wild-type 5S transcript migrated). In lanes 5 and 6, H1-depleted 5S minichromosomes were incubated with H1 at H1/n ratios of 0 and 1.5, respectively, in the presence of 10 mM MgCl₂ for 5 min. Transcription factors were then added for 1 h, and the samples were transcribed with [³²P]GTP. Lanes 7 and 8 are as 5 and 6 except that transcription factors were first allowed to assemble for 1 h at 27°C in 10 mM MgCl₂, and then histone H1 was added for 5 min. In lanes 9 to 13, 15 ng of free maxigene and 135 ng of pUC DNA were added to each 150-ng sample of H1-depleted 5S minichromosomes. Histone H1 at H1/n ratios of 0, 0.75, 1.5, 2.25, and 3, respectively, were added, and the reaction mixtures were incubated for 5 min at 27°C in the presence of 10 mM MgCl₂. Transcription complexes were subsequently allowed to form for 1 h, and transcription was assayed in the presence of [³²P]GTP. Lanes 14 to 18 are as lanes 9 to 13 except that transcription factors were first allowed to assemble for 1 h at 27°C in 10 mM MgCl₂, and then histone H1 was added for 5 min. (c) The RNA bands from two separate sets of experiments performed as described for lanes 9 to 13 of panel b were excised and quantitated by liquid scintillation counting. Symbols: (Δ, ▲) free maxigene transcripts; (○, ●) 5S minichromosome transcripts. (d) The RNA bands from two separate sets of experiments performed as described for lanes 14 to 18 of panel b were excised and quantitated by liquid scintillation counting. Symbols are as for panel c.

after Biorex treatment of minichromosomes assembled without histone H1.

The chromatin assembled by the oocyte S-150 differs from the typical chromatin of somatic cells in that three variants of histone H2A are incorporated into the chromatin and no histone H1 is present (8, 30a, 36). We have previously shown that despite these differences, the minichromosomes assembled by the oocyte extract can incorporate exogenously added histone H1 (30a). We now confirm that the chromatin compaction (1), increased resistance to digestion by nucleases (1), and transcriptional repression (15, 34, 43, 44) seen after the addition of histone H1 to isolated cellular

chromatin are also seen when the chromatin is assembled *in vitro* on circular DNA molecules in the oocyte S-150.

Removal of histone H1 leads to transcriptional activation.

The gentle removal of histone H1 consistently leads to transcriptional activation of the 5S rRNA gene, but the degree of activation varies among experiments from about 3-fold to 100-fold. The source of this variation lies both in the levels of endogenous transcription factors present in the S-150 and in the activities of the individual transcription factors added to the transcription assays.

Variations in the levels of endogenous transcription factors in the S-150 lead to changes in the basal level of

transcription of the assembled minichromosomes. Extracts containing higher levels of endogenous factors may set up more transcription complexes on the 5S gene before chromatin assembly, although it should be noted that transcription complexes are assembled inefficiently because of the low Mg concentration (1 mM) required to form chromatin in this system. The basal levels of transcription observed under these conditions are consistent with results of many studies indicating that the prior assembly of transcription complexes can interfere with repression by chromatin (5, 13, 20, 21, 25, 45, 46).

We have also observed that in addition to the variation in the basal level of repression, the final level of transcriptional activation that can be attained after the removal of histone H1 depends on the activity of the particular preparation of transcription factors (data not shown).

Transcription complexes can prevent repression by histone H1. The prior assembly of 5S transcription complexes onto the H1-depleted minichromosomes protects the 5S gene from repression by histone H1. TFIIA and TFIIC, which are still present in the extract after Biorex treatment (Shimamura, unpublished data), are not sufficient to prevent this repression. It has been reported that TFIIA alone can prevent histone repression of 5S RNA transcription (13). However, we find that the repression that is associated with nucleosome formation is prevented by the complete transcription complex but not by TFIIA alone (D. Tremethick, K. Zucker and A. Worcel, submitted for publication).

We considered the possibility that the greater repression seen when H1 is added before the transcription complexes might merely be the result of the longer total time of exposure to histone H1 of these minichromosomes, but this possibility is unlikely because histone H1 binds very quickly to the minichromosomes. The increased resistance to MNase is established within 5 to 10 min after the addition of histone H1 to minichromosomes (30a). Transcriptional repression is also established within 5 to 10 min after the addition of H1 to the H1-depleted minichromosomes, and no further repression of transcription is observed with longer incubation times (Shimamura, unpublished data).

Whereas the prior assembly of transcription complexes offers protection from repression by histone H1, the subsequent addition of H1 consistently causes a slight decrease in the transcription level. One possible explanation for this partial inhibition of transcription is that histone H1 slows down the rate with which RNA polymerase binds and initiates transcription. The addition of histone H1 to isolated chromatin fragments has been shown to inhibit the binding of *E. coli* RNA polymerase and calf thymus RNA polymerase II (15). Another possibility is that transcription complexes are not stable over the course of transcription in the presence of histone H1.

Our findings that transcription complexes can protect the somatic-type 5S RNA gene from repression by histone H1 agree with results of previous studies that used isolated cellular chromatin (34, 44). The effect of transcription complexes on the oocyte-type 5S RNA gene is less clear. Whereas Schlissel and Brown (34) report that the oocyte-type 5S RNA gene is also protected by transcription complexes from repression by histone H1, a recent study by Wolffe (44) suggests that histone H1 may repress the oocyte-type 5S RNA gene despite the prior assembly of transcription complexes.

Histone H1 interacts with the nucleosomes to repress transcription. Levels of histone H1 that repress the 5S RNA gene assembled into chromatin fail to repress 5S RNA gene

transcription from free DNA templates. Over three times more histone H1 is required for 50% repression of free DNA transcription than is required for 50% repression of chromatin. Whereas transcription complexes can protect the H1-depleted minichromosomes from repression by histone H1, the assembly of transcription complexes onto the free DNA before the addition of histone H1 does not significantly change the shape of the H1 titration curve of the free DNA. Inhibition of transcription is not seen until high levels of histone H1 have been added, regardless of whether transcription complexes have been assembled previously on the free DNA. Such high levels of histone H1 also induce a similar abrupt inhibition in the transcription of the 5S minichromosomes. This nonspecific transcriptional inhibition, which is observed at high levels of histone H1, most likely results from DNA aggregation (A. Rodriguez-Campos, unpublished observations).

Many other studies have reported DNA and chromatin precipitation at only slightly higher than a 1:1 ratio of histone H1 to nucleosomes (1, 14, 40). Precipitation in our system fails to occur until higher levels of histone H1 are added. It is unlikely that this difference is due to a qualitative difference in our preparations of histone H1, since transcriptional repression of free DNA can be seen at the low H1 ratios reported by other workers (44) if transcription is assayed in the absence of the S-150 extract (unpublished observations). It is possible that histone H1 is rendered soluble by acidic proteins or polyanions, such as RNA, present in the S-150 extract. The formation of such complexes may account for the observation that only a fraction of the input histone H1 binds to the minichromosomes assembled in the S-150 (30a).

The solubilization of histone H1 by acidic molecules may also contribute to the preferential repression of chromatin over free DNA observed in this system. Stein and Bina (38) report that a higher preference of histone H1 for binding to nucleosomes over free DNA is observed only in the presence of polyglutamic acid, which is thought to bind and solubilize histone H1. Although specific proteins that bind and solubilize the core histones have been described (8, 18, 19, 23), no such carrier protein has yet been described for histone H1.

Mechanisms of transcriptional repression by chromatin in vitro. Previous work has shown that the transcriptional activity of a population of genes assembled into chromatin in vitro is a function of nucleosome density. As the average nucleosome density is increased, transcription is progressively repressed (21, 36). We have now shown that transcription at a given nucleosome density may be further repressed by the addition of histone H1.

Nucleosomes assembled in the oocyte S-150 at 37°C are phased on the 5S RNA gene (36), but nucleosomes assembled at 27°C are not phased (D. Tremethick, unpublished observations; the lack of nucleosome phasing is probably due to the lower nucleosome densities attained at 27°C [30a]). Thus, in some of the minichromosomes assembled at 27°C, the 5S rRNA gene will be packaged into a nucleosome, which renders the gene transcriptionally inactive (36), whereas in other minichromosomes, the gene may be centered in the internucleosomal DNA region, which could make this gene accessible for transcription.

At the low average nucleosome density of the minichromosomes assembled in the studies reported here, one nucleosome per 215 bp of DNA, transcriptionally permissive (H1-depleted) minichromosomes may carry the 40-bp internal control region of the 5S RNA gene (4, 9, 33) on the 70-bp linker DNA (215-bp nucleosome repeat – 146-bp nucleo-

some core = 69-bp linker DNA). Sliding of nucleosomes on these less densely packed minichromosomes (Fig. 1b) might also increase the accessibility of the internal control region to the transcription factors. Formation of the transcription complex on these transcriptionally permissive (H1-depleted) minichromosomes will be blocked when histone H1 binds to form a more condensed structure.

Nucleosomes bind very tightly to the DNA and are difficult to remove, although some examples of gene activation concomitant with loss of nucleosomes have been reported (2, 30). Repression by histone H1 may be modulated more readily than nucleosomal repression because histone H1 is displaced more easily than are nucleosomes from the DNA. The extremely stable repression observed in higher eucaryotes may require, in addition, the formation of higher-order structures that stabilize further the binding of both histone H1 and the nucleosomes to the DNA.

The *in vitro* system described here permits the study of the interplay between nucleosomes and histone H1 and their effects on the transcription of any template of interest. By using this system, conditions that might affect the ability of histone H1 to repress transcription of a given gene may be identified and analyzed. Parameters that might influence repression by histone H1 include the DNA sequences flanking the gene, regulatory proteins (e.g., transcription factors, high mobility group proteins) bound to specific sites within or around the gene, or the topology of the DNA. By constructing appropriate templates to be assembled into chromatin, specific questions concerning the regulation of differential gene repression by histone H1 may now be addressed.

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