The AT-rich flanks of the oocyte-type 5S RNA gene of *Xenopus laevis* act as a strong local signal for histone H1-mediated chromatin reorganization in *vitro*

Radoslaw Tomaszewski and Andrzej Jerzmanowski

**INTRODUCTION**

Transcriptional repression of the oocyte-type 5S RNA genes in early stages of *Xenopus* development is a well-documented process mediated by histone H1 (1,2). Two 5S RNA gene families are present in the oocyte and somatic cells, respectively. The AT-rich flanks of the oocyte-type 5S RNA gene are involved in the establishment of transcriptionally repressed chromatin state (3). In earlier work, we showed that the presence of physiological concentration of histone H1 in the chromatin template is necessary for transcriptional repression of the oocyte-type 5S RNA genes (4-6). Our findings suggest that histone H1 is a key factor in the regulation of 5S RNA gene transcription.

**ABSTRACT**

In *vivo*, histone H1 plays an active role in establishing the transcriptionally repressed chromatin state of the oocyte-type 5S RNA genes in the early stages of *Xenopus* development. By using fully defined *in vitro* system of chromatin assembly on plasmids with cloned oocyte- or somatic-type 5S gene repeats, we found that the oocyte repeat which comprises a 120 bp AT-rich oocyte-type 5S RNA gene placed within the few hundred bp long native AT-rich flanks, but not the somatic repeat (a similar 120 bp somatic-type 5S RNA gene placed within native GC-rich flanks) enables histone H1 to realign the nucleosomal core particles densely packed on plasmid DNA. The realignment results in creation of the repeat unit of ~240 bp and is achieved through complete removal of several core histone complexes from plasmid template with the oocyte-type repeat. This effect of H1 is independent on the plasmid sequences and seems to be solely due to the presence in the oocyte-repeat of the AT-rich flanks. The effects of H1 are completely suppressed by distamycin A, a drug that specifically recognizes and binds oligo(dA)-oligo(dT) runs in DNA. The binding of H1 results in increased protection of DNA sites within the AT-rich oocyte-type 5S repeat. In an *in vitro* transcription assay performed with reconstituted chromatin templates containing plasmids with the oocyte- or somatic-type repeats only the transcription of the oocyte-type 5S RNA gene was repressed in the presence of physiological concentration of histone H1. These results support the view that the AT-rich flanks of the oocyte-type 5S RNA gene are involved in histone H1-mediated chromatin reorganization that results in the transcriptional repression observed *in vivo*.

**INTRODUCTION**

The developmental regulation of 5S RNA gene transcription in *Xenopus laevis* has been a most thoroughly documented case of histone H1 involvement in modulation of transcription of a defined set of genes (reviewed in 1). Two 5S RNA gene families are transcribed in early stages of embryonic development of *Xenopus*:

- the major oocyte-type, occurring in 20 000 copies per haploid genome, and the somatic-type, occurring in 400 copies per haploid genome. From late gastrulation stage transcription of the oocyte-type 5S RNA genes becomes largely repressed whereas that of the somatic-type 5S RNA genes continues unaffected throughout consecutive developmental stages and during the adult life of the frog. Studies on the *in vitro* transcription of *Xenopus* somatic cell chromatin were the first to establish that histone H1 is necessary to maintain the repression of oocyte-type 5S RNA genes in somatic cells (2). This finding correlated well with the observation that normal somatic histone H1 protein was absent in *Xenopus* oocytes (3). During early embryogenesis somatic H1 is gradually accumulated, its level in chromatin correlating with the repression of the oocyte-type 5S RNA genes (4,5). A direct cause-and-effect relation between accumulation of somatic histone H1 and repression of oocyte-type 5S RNA synthesis was documented by elimination of somatic H1 using ribozyme strategies during early *Xenopus* embryogenesis. The elimination of somatic H1 *in vivo* led to continued expression of oocyte-type 5S RNA genes by embryos that would normally have these genes switched-off (6,7).

In an effort to reveal the mechanism of differential effect of H1 on transcription of 5S RNA genes, Chipev and Wolff (8) found that *in vivo* the repressed oocyte-type 5S RNA gene was protected from nuclease digestion by incorporation into nucleosome and that the entire oocyte-type 5S DNA repeats (genes with their flanks) were assembled into a loosely positioned array of nucleosomes. In contrast, the potentially active somatic-type 5S RNA gene was accessible to nuclease digestion and the majority of somatic 5S RNA genes appeared not to be incorporated into positioned nucleosomes. Chipev and Wolff showed that histone H1 had an active role in establishing these two different types of chromatin structure (8). However, the molecular basis of this gene-specific effect of H1 is not clear.

In our earlier work, based on the results of *in vitro* transcription studies, we suggested that the rationale for the selective action of H1 *in vivo* may be the difference in base composition of the flanks accompanying the two types of 5S RNA genes (9). In *Xenopus* the 5S DNA repeat consists of a 120 bp 5S RNA gene and two flanks of a total of a few hundred base pairs. The 360 bp 5'-flank and 56 bp 3'-flank of the oocyte 5S RNA gene comprise the internally repetitious sequence most of which can be represented as follows:

\[ \text{AT-rich flanks} \]

\[ \text{GC-rich flanks} \]

We have previously shown that histone H1 binds DNA with high affinity (10) and that histone H1 interacts specifically with AT-rich DNA sequences (11). Our results suggest that the different base composition of the flanks can affect the binding of histone H1 to DNA and that this may be a key factor in the selective action of H1 on transcription of 5S RNA genes.
This sequence is 76% A + T, providing a strong H1-binding region in the direct vicinity of the oocyte 5S RNA gene. The flanks of the somatic-type 5S RNA gene are G + C rich. We have shown in vitro that, due to the strong and selective binding of H1, the AT-rich oocyte-type flanks will confer the H1-dependent transcriptional repression to any of the two types of 5S RNA genes. However, the simplicity of our in vitro system did not allow to probe the relation between the different affinities for H1 of the flanks of the two types of 5S RNA genes and the differences on the level of chromatin organization revealed by the later studies of Chipev and Wolffe (8).

Here we address this problem using reconstituted minichromosomes with two types of Xenopus 5S DNA repeats. We show that the AT-rich flanks of the oocyte-type 5S RNA gene act as a strong local signal for H1-mediated reorganization of chromatin structure resulting in the increase of the spacing of nucleosomes to >200 bp, increased protection of the 5S RNA gene repeat and the inhibition of the transcription of 5S RNA gene in the in vitro transcription system.

MATERIALS AND METHODS

Preparation of histones and DNA

Core histones were isolated from chicken red blood cell nuclei as described in (10). Histone H1 was derived from calf thymus by the method described in (11). The concentration of histones was determined using the Bradford procedure with the BioRad Protein Assay (BioRad Laboratories, Inc.) and was done according to the manufacturer’s protocol. The concentration of histone H1 was calculated from absorption at 280 nm using an extinction coefficient of 2.0 for a 10 mg/ml solution of histone H1. Solutions of histones were stored in small aliquots frozen at −80°C. DNA templates used for minichromosome reconstitution were plasmids pBR327 and pUC19 with inserts consisting of Xenopus laevis somatic- or oocyte-type 5S RNA genes surrounded by their native (GC-rich or AT-rich, respectively) flanking sequences. The inserts were excised from plasmids pXlsll and pXloΔ3 sequences. The inserts were excised from plasmids pXlsll and pXloΔ3 and subsequently cloned into the HindIII site of pBR327 or pUC19 plasmid. Plasmids were prepared by the alkaline lysis method and purified twice on a CsCl/ethidium bromide gradient followed by the repetitive extraction with water-saturated n-butanol, dialysis against two changes of TE buffer and precipitation with ethanol (12). The concentration of DNA was measured spectrophotometrically at 260 nm using an extinction coefficient of 1.0 for a 50 µg/ml solution.

Reconstitution of minichromosomes

Minichromosomes were reconstituted by salt gradient dialysis (13). Defined amounts of supercoiled plasmid DNA and core histones were mixed in a final volume of 50 µl and the sodium chloride concentration was immediately adjusted to 2.0 M. The sample was incubated at room temperature for 30 min and then for 1–2 h during which it was diluted by addition of five 20 µl portions of TE buffer to obtain a final NaCl concentration of 1.0 M and a final OD260 of 1.0 in a total volume of 100 µl. The mixture was dialyzed for 5–6 h at room temperature or overnight at 4°C against 10 mM Tris–HCl pH 7.2, 1 mM EDTA with a NaCl concentration decreasing linearly from 0.8 to 0 M. After dialysis sodium polyglutamate (average molecular weight 65 000) was added to a final concentration of 2.0 mg/ml (10). The sodium chloride concentration was then increased to 0.15 M and histone H1 (from a 10 mg/ml solution in water) was added to the desired H1:core histone weight ratio. Finally, the sample was diluted with TE buffer (pH 7.2) to 200 µl and the mixture incubated at 37°C for 10–12 h with occasional gentle stirring. After incubation, the sample was dialyzed against 20 mM Tris–HCl (pH 7.2), 0.2 mM EDTA for 1.5 h at room temperature and centrifuged for 1 min in a microfuge. The supernatant designated as a soluble minichromosome fraction was stored at 4°C.

Distamycin treatment of DNA during reconstitution of minichromosomes

Before the addition of sodium polyglutamate to the minichromosome reconstitution mixture, distamycin (Distamycin A, Sigma) was added from a 1 mM stock in 96% ethanol. The final distamycin concentration was 50 µM and core histone-reconstituted DNA (~50 µg/ml). Sodium chloride was added to 15 mM. The mixture was incubated for 60 min at 37°C and subsequently subjected to H1 binding experiments with sodium polyglutamate as described before.

Digestion with micrococcal nuclease

The sample for digestion with micrococcal nuclease contained 2 µg of histone-reconstituted DNA (in a soluble minichromosome fraction) diluted with 20 mM Tris–HCl (pH 7.2), 0.2 mM EDTA to a final volume of 50 µl. Digestion was in the presence of 1 mM CaCl2. The mixture was preincubated for 1 min at 37°C prior to the addition of 10 U micrococcal nuclease (Eurogentec). The incubation with the enzyme was for 4 min at 37°C. The reaction was stopped by the addition of an equal volume of stop buffer containing 20 mM Tris–HCl (pH 7.2), 150 mM NaCl, 15 mM EDTA and 0.3% (w/v) SDS. Following nuclease treatment the samples were deproteinized with proteinase K (Gibco BRL) (200 µg/ml) and extracted with phenol–chloroform and chloroform before precipitation with ethanol. The extracted DNA was separated on 1.5% agarose in a 14×19 cm gel at a constant voltage of 70 V for at least 3 h. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV light using the GDS camera (UltraViolet Products Ltd).

Supercoiling assay

Minichromosomes were incubated for 45 min at 37°C with 2.5 U topoisomerase I (Fermentas MBI) per microgram of DNA. The incubation was stopped by the addition of SDS and EDTA to final concentration of 0.2% and 16 mM, respectively (14). The samples were subsequently incubated for at least 1 h at 37°C with proteinase K (1 mg/ml) and extracted once with phenol–chloroform before precipitation with ethanol. DNA was analyzed by two-dimensional electrophoresis in 1.2% agarose on 20×20 cm gels (15). The electrophoresis was carried out for 16–18 h in each dimension at constant voltage of 3 V/cm, in buffer recirculating at a constant temperature of 5°C. Following the first dimension, the gel was soaked for 6 h at 5°C in the dark in TBE buffer containing 1.4 µg/ml of chloroquine. Electrophoresis in the first
dimension was in TBE buffer and in TBE buffer containing 1.4 µg/ml of chloroquine in the second dimension. After electrophoresis the gel was soaked in deionized water and stained with 0.5 µg/ml ethidium bromide solution. The gel was photographed under UV light using the GDS camera. Standard topoisomerase mixtures of DNA templates were prepared according to (16).

**In vitro transcription assay**

Transcription extracts from mouse Ehrlich ascites tumour cells were prepared by the method of Dignam et al. (17). The reaction mixture (50 µl) for a standard in vitro transcription experiment contained: 12.5 mM HEPES (pH 7.7), 10% glycerol, 70 mM KCl, 0.5 mM dithiothreitol, 5 mM MgCl₂, 600 µM ATP, 600 µM CTP, 600 µM GTP, 60 µM UTP, 20 µM [α-32P]UTP (3000 Ci/mmol) (Amersham), 1 µg DNA template and 25 µl of a crude cell-free extract. The DNA template consisted of the oocyte- or somatic-type 5S RNA gene repeat from *X. laevis* cloned into the HindIII site of the pBR327 plasmid. DNA templates were used in a form of naked DNA or reconstituted minichromosomes with or without histone H1. Before the addition of the ribonucleoside triphosphates, the mixtures were pre-incubated with the transcription reaction for 30 min at 30°C. The transcription reaction was carried out for 60 min at 30°C and was terminated by addition of an equal volume of solution containing 0.5% SDS, 0.1 M sodium acetate (pH 5.5) and 50 µg carrier yeast RNA. The RNA was isolated from the reaction mixture according to (18), redissolved in deionized formamide, heated at 95°C for 3 min and loaded on a 12.5% polyacrylamide (acylamide:bis-acrylamide 16:1) gel containing 4 M urea. Electrophoresis was carried out for 18 h at 4–5 mA. In the partially denaturing conditions of this electrophoresis system the somatic and oocyte 5S RNA moves at different distances despite their identical length (19). The radioactive gels were exposed with Fuji RX films.

**RESULTS**

**In vitro assembly of minichromosomes on pBR327 plasmid**

In order to compare the effects of the oocyte- and somatic-type *Xenopus* 5S DNA repeats on chromatin assembly in vitro we choose, as a method of chromatin assembly, the reconstitution from pure DNA and histones by dialysis from high salt. The DNA was that of pBR327 plasmid into which was cloned (in the HindIII site of the polylinker) the full repeat (120 bp gene accompanied by native 5'- and 3'-flanks) of *Xenopus* 5S RNA gene of the oocyte- or somatic-type (Fig. 1A). The orientation of the inserts was confirmed by mapping the restriction enzyme cutting sites in the insert and the nearby plasmid sequences. The final plasmid constructions had 3943 and 4157 bp for plasmids containing the oocyte- and somatic-type repeats, respectively. For both constructs the final DNA length was an integer multiple of the repeat length of 210 ± 3 bp (19 x 207.5 bp and 20 x 207.85 bp for the shorter and longer plasmid, respectively). Core histones were purified from chicken erythrocytes and were homogeneous and fully distinguishable from one another as determined by SDS-polyacrylamide electrophoresis (results not shown). Histone H1 was prepared from calf thymus and consisted of a set of native variants as checked by SDS-polyacrylamide electrophoresis (results not shown). We determined the optimal core histone:DNA ratio by performing reconstitutions with varying input histone:DNA ratio. Chromatin samples with varying degrees of template saturation were digested with micrococcal nuclease (MNase) to find the highest core histone:DNA ratio at which the digestion pattern was still a 146 bp DNA ladder. The complexes formed under these conditions were analyzed by topological assay. After the reconstitution, complexes were incubated with excess of topoisomerase I to remove any supercoils not constrained by nucleosomes (20). The only negative stress remaining in plasmids after deproteinization was that constrained by histone octamers. Preliminary experiments were performed to adjust the amount of topoisomerase I required to relax superhelical tension completely from the DNA template and assure that topoisomerase activity was not the limiting step in the assay. Distribution of DNA topoisomers was assessed by two-dimensional gel electrophoresis. The supercoiling assay generates a Gaussian distribution of topoisomers about a mean and the linking number of the most intense spot corresponds to the number of core particles in the minichromosome examined (21). The reconstituted complexes had on average 27 core particles, as shown in Figure 1B for plasmid with the oocyte-type insert (28 for plasmid with the somatic-type insert), indicating that at saturation the core particles on both DNA templates were closely packed with spacing of ~150 bp. The estimation of protein and DNA content in reconstituted minichromosomes showed that the ratio (w/w) of DNA to core histones was close to 1 (results not shown).

Histone H1 was incorporated at various amounts into the core histone saturated H1-free chromatin. The complexes were then digested in exactly the same conditions with MNase to check the effect of H1 on the extent of digestion and the size of the monomer product (Fig. 1C and D). The comparison of the digestions of the minichromosomes with somatic- and oocyte-type inserts in the absence of H1 (Fig. 1C and D, lane 3) reveals some notable differences. The minichromosome with the somatic insert gives a clear digestion product of 150 bp whereas that with the oocyte insert, except the 150 bp band, also gives a broad smear of di- to hexanucleosomal size as well as a shorter diffuse band of ~120 bp. It is possible that the presence of the oocyte-type insert, presumably because of its AT-rich flanks, results in partial stabilization of the core particles in respect of the preferred MNase cutting sites in the pBR327 DNA. Increasing the amount of H1 added to the complex reconstituted on plasmid containing the somatic-type repeat resulted in increased protection of DNA reflected by the shift of DNA seen on gel into higher oligomers (dimers, trimers and tetramers). However, the incorporation of H1 had no effect on nucleosomal spacing which at the ratio of H1:core histones of 1.0 was still ~150 bp (Fig. 1C, lane 5). Higher proportion of H1 led to aggregation of the complexes and their inaccessibility to MNase (Fig. 1C, lane 6). It can be thus concluded that H1, while binding to the reconstituted complex, was unable to compete with densely packed core particles and generate a correct spacing of nucleosomes. Such a result is considered typical for chromatin reconstituted from pure components by stepwise dialysis from high salt (10). A markedly different pattern was observed upon titration with H1 of the complex reconstituted on plasmid containing the oocyte-type 5S repeat. The presence of H1 at the ratio to core histones of 0.24 resulted in no visible change of the pattern of MNase digestion. However, at the ratio of H1:core histones of 1.0, in addition to the increased DNA protection, there was a dramatic change of the spacing periodicity which now jumped to >240 bp (Fig. 1D, lanes 3–5). The above results are indicative of a different type of histone H1 binding than that occurring in complexes with plasmids containing the somatic-type repeat.
Figure 1. Histone H1-induced nucleosome alignment on pBR327 DNA containing different types of *Xenopus* 5S DNA repeats. (A) Oocyte- and somatic-type 5S DNA repeats from *X. laevis* and plasmid constructs used for the reconstitution of minichromosomes. (B) Negative linking number change after minichromosome assembly with saturating amount of core histones. After assembly minichromosomes were incubated with excess of topoisomerase I, deprotenized, extracted with phenol–chloroform and precipitated with ethanol. DNA was subjected to two-dimensional electrophoresis in 1.2% agarose. The sample was loaded onto the same well as the standard topoisomerase mixture, 1.5 h before the standard. The direction of migration is indicated by arrows marked 1 and 2, positions of nicked circular and linear plasmid are indicated by n and l, respectively. The number of nucleosomes was estimated to be ∼27. Shown is the analysis of minichromosome reconstituted on pBR327 plasmid with the 669 bp oocyte-type 5S repeat. The results for minichromosome reconstituted on pBR327 with the 883 bp somatic-type 5S repeat were similar with the average total number of nucleosomes estimated to be 28. (C and D) Micococal nuclease digestion of chromatin reconstituted on pBR327 containing somatic- (C) and oocyte-type (D) repeat. Chromatin was assembled at defined weight ratio of core histones to DNA and histone H1 was incorporated at various concentrations. Samples were digested with the same amount of M1nase at identical conditions. Purified DNA was run on 1.5% agarose gel. In (C) and (D): lane 1, MNase digest of native chicken erythrocyte chromatin; lane 2, naked supercoiled DNA used for chromatin assembly. Lanes 3–6 (in C) and lanes 3–7 (in D), MNase digests of chromatin reconstituted at H1:core histones ratio (w/w) indicated. Lane 7 (in C) and lane 8 (in D), 123 bp ladder; the DNA lengths of lower multiples are shown.

**Protection of DNA sites in minichromosomes with somatic- and oocyte-type inserts**

In order to find out whether the formation of nucleoprotein complexes on the two types of plasmids resulted in stable protection of specific sites on DNA we digested the naked DNA, minichromosomes with and without histone H1 with restriction enzymes cutting at sites located in various distance to the 5S RNA gene (Fig. 2A and C). Each sample containing an equal amount of naked or histone complexed DNA was cut with 2-fold excess of restriction nuclease required for complete digestion of the DNA as calculated for the naked template. After completion of digestion the DNA was deproteinized and analyzed by electrophoresis in agarose gel (Fig. 2B and D). Reconstitution of core particles resulted in partial protection of restriction sites in both types of plasmid templates. An exception was the *Bam*HI site which was not protected in the plasmid containing the somatic-type 5S insert. This difference could reflect the increased stabilization of core particles induced by the presence of the AT-rich flanks of the oocyte-type insert, a feature reflected also by the pattern of MNase digestion seen in Figure 1C and D (lanes 3). The addition of H1 had no effect on the protection of any of the analyzed sites in the plasmid containing the somatic-type 5S insert. In contrast, in the plasmid containing the oocyte-type 5S insert the incorporation of H1 resulted in over 2-fold increase in protection of *Hind*III and *Stul* sites compared with protection resulting from reconstitution of core particles. The increased protection of these two sites which are located on the boundaries and within the oocyte-type 5S DNA repeat, but not the *Bam*HI site located further away could result from strong binding of H1 to DNA in this region and/or from H1-induced stabilization of positioned nucleosomes over the 5S DNA repeat.

**Effect of histone H1 binding to minichromosomes on the total number of nucleosomes**

In order to find the extent of H1-mediated reorganization of nucleosome alignment observed with templates containing the oocyte-type insert we determined the linking number change related to minichromosome reconstitution and thus the number of nucleosomes deposited on the templates. Minichromosomes were reconstituted without H1 on plasmid containing somatic- or oocyte-type 5S DNA insert. Half of the sample was treated with topoisomerase I whereas the other half was used for reconstitution with histone H1 at H1:DNA ratio of 1.0 and then treated with topoisomerase I. As shown in Figure 3, before the addition of H1
Figure 2. Protection of DNA sites in the vicinity of 5S RNA gene by reconstituted nucleoprotein complexes. Products of restriction enzyme digestion of naked DNA, minichromosomes reconstituted with core histones without H1 and histone H1-containing minichromosomes for template with somatic-type (B) and oocyte-type (D) 5S DNA repeat. DNA or minichromosomes were cut with restriction nucleases and the purified DNA products were separated on 1% agarose gel. Two-fold excess of restriction nucleases was used compared with the amount required for complete digestion of 1 µg of naked DNA. In (B) and (D), core histones: minichromosomes reconstituted with core histones without histone H1; histone H1: minichromosomes reconstituted with core histones and histone H1; 1 kb: 1 kb DNA ladder. (A) and (C), sites of restriction nucleases cutting in plasmid containing somatic-type (A) and oocyte-type (C) 5S DNA repeat. Length of the fragments between cutting sites (in bp) is indicated. Thick solid line denotes 5S RNA gene. Fragments are not drawn to scale.

the average number of core particles in minichromosomes was 26–27 (28 for the plasmid with the somatic-type insert). Thus, on both plasmids the core particles were densely packed along the entire length of DNA. The reconstitution with H1 of minichromosomes containing plasmid with the somatic-type insert had no effect on the total number of nucleosomes. In contrast to that, the reconstitution with H1 of minichromosomes containing plasmid with the oocyte-type insert resulted in a decrease of the total number of nucleosomes to 16 (Fig. 3A and B). This corresponds to the average nucleosomal spacing of 245 bp, a value similar to that estimated on the basis of MNase generated DNA ladder (Fig. 1).

Distamycin A abolishes the histone H1-induced remodelling of minichromosome with oocyte-type insert

To check whether the effect of H1 observed with minichromosomes reconstituted on plasmids containing the oocyte-type insert was due to the preferential binding of H1 to AT-tracks present in DNA sequences flanking the oocyte 5S DNA and not due to other differences between the two types of inserts (like slightly larger size of the somatic insert or eight point differences between base sequences of the two types of 5S genes) we treated the minichromosomes containing the oocyte-type insert with distamycin A before the reconstitution with H1. Distamycin A is known to recognize and strongly bind the oligo(dA)-oligo(dT) tracts in DNA and to inhibit the specific binding of H1 to these sites (22). We have checked that the drug indeed prevents the selective binding of H1 to isolated oocyte-type DNA repeat (Fig. 4A). Reconstitution of H1 on distamycin A-treated minichromosomes containing the oocyte-type insert did not result in the change of the total number of nucleosomes (Fig. 4B). Thus, the observed effect of H1 in minichromosomes depends on its specific binding to the AT-rich tracts in DNA.

The effect of H1 on minichromosomes with oocyte-type insert is independent on the plasmid DNA sequences

Plasmid pBR327 is known to possess a chromatin organizing region which stimulates the nucleosome alignment reaction provided the plasmid DNA is an integer multiple of 210 ± 3 bp and its total length is between 2400 and 3600 bp (23). While the constructs with the oocyte- and somatic-type inserts used by us had both the integer multiple of 210 ± 3 bp, the total length of DNA in these pBR327 derivatives was >3900 bp. However, we could not exclude the possibility that the observed effect of H1 is due to some specific features of pBR327 sequences that manifest selectively, i.e. in the presence of the oocyte-type but not the somatic-type insert. To check this we prepared plasmid templates based on pUC19 instead of pBR327 plasmid. The pUC19 templates containing somatic- or oocyte-type inserts (the same as used in pBR327 constructs) were reconstituted with core histones and H1 identically as described for pBR327 derivatives. The
from Ehrlich ascites cells to transcribe H1-induced chromatin remodelling occurring in plasmid bearing In order to check directly the effect on transcription of the histone and from reconstituted minichromosome templates In vitro A T-rich oocyte-type insert. on the plasmid sequence but is solely due to the presence of the that the H1-induced remodelling of minichromosome is independent prevented the above effect of H1 (Fig. 5C). Thus, we conclude minichromosome of the distamycin A before the addition of H1 nuclease from 22 to 14 (Fig. 5C). The addition to the nucleosomes from 22 to >240 bp) occurred only for the template with the oocyte-type insert (Fig. 5A and B), similarly as for the minichromosomes based on pBR327 derivatives. Interestingly, in the case of pUC19 templates the differences in digestion patterns of minichromosomes with somatic- and oocyte-type inserts in the absence of H1 were much less pronounced compared with those for pBR327 templates (Fig. 5A and B, compare lanes 1 and 2). It is possible that the chromatin organizing region of pBR327 and the AT-rich oocyte type insert produce some cumulative stabilizing effect on nucleosomal core particles. The topological assay showed that the alignment of H1 on the pUC19 minichromosome with oocyte-type insert resulted in a drop of the total number of nucleosomes from 22 to 14 (Fig. 5C). The addition to the minichromosome of the distamycin A before the addition of H1 prevented the above effect of H1 (Fig. 5C). Thus, we conclude that the H1-induced remodelling of minichromosome is independent on the plasmid sequence but is solely due to the presence of the AT-rich oocyte-type insert.

**In vitro transcription of 5S RNA genes from naked DNA and from reconstituted minichromosome templates**

In order to check directly the effect on transcription of the histone H1-induced chromatin remodelling occurring in plasmid bearing the oocyte-type insert we used the cell-free transcription extract from Ehrlich ascites cells to transcribe *in vitro* the 5S RNA genes. As templates for the *in vitro* transcription were used constructs in pBR327 plasmid containing the somatic- or oocyte-type inserts, in a form of naked DNA or in the form of reconstituted minichromosomes with or without histone H1. Prior to the addition of nucleoside triphosphates, the templates were preincubated for 30 min with the transcription extract. The transcription reaction was then carried on for the next 60 min. The isolated RNA products were separated on the semi-denaturing gel which allows distinction between the somatic and oocyte 5S RNA. No noticeable differences were seen in the amount of somatic 5S RNA transcribed from naked DNA, minichromosomes lacking H1 and minichromosomes containing H1 (Fig. 6, lanes 1–3). The amount of the oocyte 5S RNA transcribed from naked DNA was only slightly smaller compared with that transcribed from minichromosomes lacking H1. However, it dropped dramatically for minichromosomes with H1 (Fig. 6, lanes 4–6). Thus, in the cell-free *in vitro* transcription system using reconstituted minichromosomes as templates, only the transcription of the oocyte-type gene surrounded by its native AT-rich flanks was sensitive to inhibition by histone H1.

**DISCUSSION**

Nucleosomes reconstituted from DNA and purified histones are irregularly spaced and tend to pack closely together independent of whether linker histones are present in the reconstitution system. The results of numerous experiments also suggest that it is not possible to convert DNA molecules that are already covered with closely packed nucleosomes into a chromatin-like arrangement.
Figure 5. Histone H1-induced alignment of nucleosomes on pUC19 DNA containing somatic- or oocyte-type 5S DNA repeat. (A and B) Micrococcal nuclease digestion of chromatin reconstituted on pUC19 containing somatic- (A) and oocyte-type (B) repeat. Chromatin was assembled at defined weight ratio of core histones to DNA and histone H1 was incorporated at various concentrations. Samples were digested with the same amount of MNase at identical conditions. Conditions of electrophoresis were as described in the legend to Figure 1. Lanes 1–6 (in A and B) MNase digests of chromatin reconstituted at indicated H1:core histones ratio (w/w). Lane M (in B) DNA size markers. (C) Negative linking number change after minichromosome assembly on pUC19 DNA containing the oocyte-type 5S DNA repeat, in the presence and absence of distamycin. Conditions of assay and electrophoresis and the order of loading samples on gel were as described in the legend to Figure 4B. Linking numbers for the spots marked by arrows are indicated.

Characterized by nucleosomes that are spaced 50 or more base-pairs apart (10,24). Complete in vitro reconstitution of chromatin-like structures characterized by highly ordered and physiologically spaced nucleosomes could only be generated on the synthetic polynucleotide poly[d(A-T)]-poly[d(A-T)], probably because of higher affinity of the poly[d(A-T)] duplex for H1 compared with average DNA (25), and on certain plasmid DNAs containing specific, chromatin organizing regions (13). In the present work we have shown that a specific fragment of DNA comprising the *Xenopus* oocyte-type 5S DNA repeat is capable of directing the histone H1-mediated major realignment of already formed DNA:core histone complexes on long stretches of DNA. The realignment results in creation of regular long gaps between packed core particles and is achieved through complete removal of several core histone complexes from DNA. The DNA templates that we initially used were the derivatives of the pBR327 plasmid which is known to possess its own specific sequence able to induce the correct alignment of H1, provided the DNA is an integer multiple of 210 ± 3 bp (23). We wanted to obtain physiologically spaced nucleosomes on plasmids with both types of inserts in order to compare the differences in stability brought about by the presence of AT-rich flanks of the oocyte-type gene. However, despite the presence in both constructs of an integer multiple of 210 ± 3 bp, the correct alignment of H1 was only seen for the plasmid with the oocyte-type insert. One reason for the lack of the effect of the pBR327 chromatin organizing sequences could be the total length of the DNA which, for both plasmids used, exceeded the limit of the reported optimal size of 2400–3600 bp (23). This notwithstanding, and since the oocyte insert was smaller than the somatic insert, we could not exclude that the observed effect of H1 was somehow linked to the difference in length between the two pBR327 DNA templates. To check this we repeated the reconstitutions with DNA derived from the pUC19, a plasmid lacking any chromatin organizing regions (23). The identical results to those obtained with the pBR327 derived templates strongly suggest that the observed correct alignment of H1 is independent of the plasmid sequence but is solely due to the presence of the oocyte-type insert. The reason for this specific effect must be the highly AT-rich flanks of the oocyte-type 5S RNA gene. No such effect is observed for somatic-type 5S repeat which consists of a very similar 5S RNA gene that is flanked by GC-rich DNA.

Figure 6. In vitro transcription of *Xenopus* 5S RNA genes. Autoradiogram of electrophoretic analysis of transcription products of in vitro transcription in Ehrlich ascites cells extract with pBR327 plasmid containing somatic or oocyte 5S DNA repeat. DNA template was in the form of naked DNA or reconstituted minichromosomes with or without histone H1 as indicated above the lanes. Transcription was for 60 min with conditions as described in Material and Methods. RNA was analyzed on 12.5% polyacrylamide gel containing 4 M urea. o, oocyte 5S RNA, s, somatic 5S RNA.
The binding of H1 results in a partial protection of DNA sites within the AT-rich oocyte-type but not within the GC-rich somatic-type repeat inserted into plasmid DNA. The above agrees well with the results of our earlier studies (see also Fig. 4A) showing a highly preferential interaction of H1 with isolated Xenopus oocyte-type 5S repeat (9). The major structural effect of H1 observed in minichromosomes containing the oocyte-type repeat is completely suppressed by distamycin A, a drug shown to block the accessibility of the AT tracts in DNA for H1.

The unusually long repeat length (~240 bp) observed in H1 containing templates with the oocyte-type repeat can be the result of the over-stoichiometric amount of H1. A similar effect of the increase in the repeat length (from 180 to 220 bp) as a result of increasing the amount of H1 was observed by Rodriguez-Campos et al. (26) and by Kamakaka et al. (27) upon reconstituting of chromatin with extracts from Xenopus and Drosophila embryonic cells.

In an in vitro transcription assay using as template the naked DNA, minichromosomes containing H1 and minichromosomes lacking H1 the inhibitory effect of H1 manifested for the transcription of the oocyte-type 5S RNA gene but not for the transcription of the somatic-type gene. Interestingly, there was not much difference in the transcription efficiency of the somatic-type gene from the naked DNA and the DNA in a form of minichromosome with or without H1. The same concerned transcription of the oocyte-type gene on the naked DNA and on of minichromosome with or without H1. The same concerned transcription of the oocyte-type gene on the naked DNA and on the DNA in a form not much difference in the transcription efficiency of the DNA, minichromosomes containing H1 and minichromosomes lacking H1. Earlier, O’Neill et al reported that on the deposition of histone H1 onto core particles reconstituted on tandem repeats of 5S rRNA gene inhibited both initiation and elongation of transcripts by the T7 RNA polymerase (28). However, the transcription system we used, based on the crude cell-free extract, is completely different from the system based on the purified T7 polymerase. It should also be noted that we have only measured the net effect of the 60 min incubation with the transcription extract and did not monitor the kinetics of the initial phase of the reaction. Nucleosome movements, most likely their sliding, occurring even in the presence of histone H1, were reported for plasmids reconstituted into chromatin and incubated in the ATP enriched cell free system (29). Similar sliding probably occurred in the H1-lacking minichromosomes containing the 5S RNA gene inserts during the 60 min incubation in the cell free, crude transcription system supplemented with ATP. The binding of H1 to the minichromosome with the somatic insert did not induce the physiological spacing and was also ineffective in preventing the sliding of nucleosomes (see Fig. 2B) and thus in restricting the accessibility of the 5S RNA gene to the transcription apparatus.

In contrast to this, histone H1 binding to the plasmid containing the oocyte-type insert strongly inhibited the transcription of the 5S rRNA gene. The AT-rich flanks of the oocyte-type gene could serve as a nucleation center for the correct binding of H1 (presumably cooperatively) to its primary nucleosomal sites. Such interpretation would account for the observed forceful removal of almost half of the bound core histone complexes from template DNA. In addition, a strong nucleation center of H1 binding located in the vicinity of a gene could form an effective barrier for the sliding of local nucleosomes and thus block the access of transcription factors to their cognate promoter sequences. Chipev and Wolfe (8) showed that H1 had an active role in inducing the loosely positioned arrays of nucleosomes over oocyte-type but not over somatic-type Xenopus 5S RNA genes. The existence near the oocyte-type 5S RNA gene of the AT-rich DNA sequences which can act as the nucleation center enabling the specific binding of H1 could be an important factor in the mechanism underlying the in vivo effects of H1. Judging from the observed displacement by the H1 binding of densely packed core histone complexes in vitro, a similar displacement could affect the transcriptional complex prebound to the 5S RNA gene in vivo. It should be also noted that in vivo the oocyte-type 5S DNA repeats occur in tandem arrays, thus the effect of H1 binding on reorganization and stabilization of nucleosome positions can be stronger than that observed by us in vitro with a template containing a single repeat.

ACKNOWLEDGEMENTS

We thank Beata Kilianczyk for excellent technical assistance. This work was supported by Polish Committee of Scientific Research grants 662759203 (A.J) and 6P04A02208 (R.T.) and by Howard Hughes Medical Institute grant 79195-543403 (A.J.).

REFERENCES