The enhancers and promoters of the *Xenopus laevis* ribosomal spacer are associated with histones upon active transcription of the ribosomal genes

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ABSTRACT

The presence of histones on the enhancer-promoter region of the *X.laevis* ribosomal spacer has been studied in embryos at stage 40, where the ribosomal genes are actively transcribed. Isolated tadpole nuclei were either fixed with formaldehyde or irradiated with UV laser to crosslink histones to DNA. The purified protein-DNA complexes were immunoprecipitated with antibodies to the histones H1, H2A and H4 and the DNA fragments carrying the respective histones were analyzed for the presence of spacer enhancer-promoter sequences by hybridization to specific DNA probe. The two independent crosslinking procedures revealed the presence of these DNA sequences in the precipitated DNA. The quantitative analysis of the UV laser-crosslinked complexes showed that histones H2A and H4 were associated with enhancer-promoter DNA in amounts similar to those found for bulk DNA, whilst the content of H1 was reduced.

INTRODUCTION

The highly transcribed genes, whose chromatin structure has been studied most extensively are the genes for ribosomal RNA (for reviews see 1–3). Yet their nucleoprotein organization is not clear. Evidence has been presented suggesting that active ribosomal genes (rDNA) are packed in altered nucleosomes or even in non-nucleosomal structures (4–7), but a nucleosome-like organization of transcribed rDNA is also well documented (8–11). If nucleosomes disappear as distinct entities it is not known whether the nucleosomal DNA is released from interactions with core histones or they remain attached to the extended DNA. While the electron microscopy of *X. laevis* oocytes failed to detect proteins other than those involved in transcription, associated with rDNA (5), these sequences were reported to be rather resistant to nuclease digestion, probably because of the protection inferred by the binding of chromosomal proteins (12).

Available data concern mainly the coding regions of ribosomal genes. Much less is known about the structure of the sequences involved in the regulation of the activity of these genes. Sequence motifs with transcriptional role were proposed (13) and then identified and precisely mapped (13–18) within the ribosomal gene spacer of *X. laevis* (for a review, see 19). The activation of ribosomal genes were found to correlate with the appearance of DNAse I hypersensitive sites and with demethylation of DNA in the spacer region consisting of enhancer elements (20,21). Very recently, *X. laevis* enhancer repeats and spacer promoters were found to be organized as non-nucleosomal structures in both inactive erythrocytes and in the active tissue culture cells (Karagyozov,L. and Moss,T., manuscript submitted). The aim of the present work was to see whether the spacer enhancers and promoters, being in an anucleosomal conformation, retain the histones attached to them. We show that histones H2A and H4 are associated with this DNA region in amounts similar to those associated with bulk DNA, while the content of H1 is twice less.

MATERIALS AND METHODS

Isolation of nuclei. Formaldehyde treatment

X. laevis eggs were collected from individual females, injected with horionic gonadotropin hormone. After fertilization in vitro, embryos were grown until stage 40 and used for isolation of nuclei according to Wolffe (22), including 5 mM sodium butyrate in all solutions to inhibit histone deacetylation. Nuclei from *X. laevis* erythrocytes were isolated by the method of Hewish and Burgoyne (23). The nuclear preparations were fixed with formaldehyde for 8 min at 30°C following the protocol of Solomon et al.(24). After fixation, the nuclei were washed in 50 mM Tris-HCl, pH 7.5 and prepared for centrifugation in CsCl to remove the non-crosslinked proteins.

UV laser irradiation of nuclei

A picosecond Nd:YAG UV laser was used to irradiate isolated nuclei as described elsewhere (25). Irradiation at 266 nm was performed in rectangular fused silica cuvettes at pulse energy 4 mJ, diameter of the beam 0.5 cm and repetition rate 0.5 Hz. The condition of irradiation were chosen in such a way as to achieve 20 absorbed photons per nucleotide at a constant laser

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intensity 0.7 GW/cm^2. The energy of irradiation was measured with pyroelectrical detectors, calibrated with Model Rj 7200 energy meter (Laser Precision Corp.). The electric signal was transmitted to and processed by microcomputer.

Separation of the crosslinked protein-DNA complexes

The crosslinked protein-DNA complexes, obtained by either formaldehyde treatment or UV laser irradiation were sonicated with Sonicator Model W-35, Heat Systems Ultrasonics Ins., using a microtip at power setting 5 for ten 30 sec bursts in an ice bath, to reduce the size of DNA to about 150 bp, made 1% in sarkosyl and centrifuged through a preformed gradient of CsCl (25).

Immunochemical procedures

Polyclonal antibodies to H2A and H4 were raised as described by Angelov et al.(25), those against H1 according to Russanova et al.(26) and immunospecifically purified from sera by affinity chromatography with the respective antigen conjugated to CNBr-Sepharose (27). The specificity of the antibodies to histones H1, H2A and H4 was demonstrated in previous papers (26,33).

Immunoprecipitation of the crosslinked protein-DNA complexes was performed by modifying the procedure of Solomon et al.(24). 0.05 ml IgGsorb (The Enzyme Center, Malden, MA) were suspended in 0.5 ml 1% solution of bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and shaked for 30 min at room temperature to block the sites of nonspecific absorption. After centrifu-gation, the pellet was suspended in a 0.5 ml mixture of the antibody and the crosslinked material (w:w ratio 1:2.5) in 50 mM HEPES, pH 7.5, 2M NaCl, 0.1% SDS, 1% TRITON X-100, 1% Na-deoxy-cholate, 5 mM EDTA, 0.1% BSA. Following 2 hours shaking at room temperature, the suspension was washed five times with 0.5 ml of the same solution, and three times with 0.5 ml 50 mM HEPES, pH 7.5, 0.15 M NaCl, 5 mM EDTA. The remaining material was eluted by washing with 0.1 ml 3.5 M KSCN, 20 mM Tris-HCl, pH 8.2. The eluates were treated with RNAse (0.015 mg per probe, 30 min, 24°C), followed by pronase digestion (1 mg/ml for at least 4 h) and finally precipitated with ethanol. The pellet was resuspended in 10 mM Tris-HCl, pH 7.5, 0.25 mM EDTA. The reversal of crosslinks in the formaldehyde-induced protein-DNA complexes was performed as described in (24). The laser-induced complexes were processed in the same way except the heat treatment.

Hybridization analysis

The 320 bp BamHI- PstI fragment, containing most of spacer promoter and the first enhancer unit of *X. laevis* ribosomal spacer (Fig. 1) was purified from clone pXl108c (28).

The DNA samples were alkali denatured and loaded on Zeta-Probe Blotting membranes (Bio-Rad). Prehybridization was in 6×SSC, 10×Denhardt's solution, 0.1 mg/ml denatured *E. coli* DNA, 1% SDS, 0.2% Na-pyrophosphate, 50% formamide. Hybridization was at 42°C for 16−20 hours with 50−100 ng of DNA probe, ^{32}P-labeled by random priming. The filters were extensively washed with 0.5×SSC, 0.5% SDS and finally with 0.1×SSC at 65°C, and autoradiographed at −70°C using Dupont Cronex intensifying screens.

The hybridization signals obtained after the autoradiography were scanned by Quick Quant II Autoscanner (Helena Laboratories). The amount of dotted DNA as well as the exposure of the film were selected in such a way as to ensure linearity of the film response.

RESULTS

The experimental approach we used to study the association of histones with the enhancer repeats of *X. laevis* ribosomal spacer consisted in (a) crosslinking histones to DNA in the nuclei, (b) separation of protein-DNA complexes, carrying a given histone by immunoprecipitation with the respective antibody, and (c) analysis of DNA, precipitated by each antibody, for the presence of enhancer and promoter sequences by hybridization to specific DNA probe.

Crosslinking histones to DNA

Histones were crosslinked to DNA in the nuclei by two alternative procedures: chemical crosslinking and photocross-linking by irradiation with UV laser. After the treatment, the nuclei were sonicated to reduce the size of DNA to about 150 bp (Fig. 2b) and centrifuged through CsCl to remove the non-crosslinked proteins. The profiles in CsCl show (Fig. 2a) that the treatment

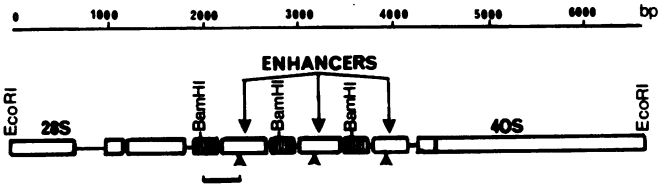

Figure 1. Structure of a typical *X. laevis* rDNA spacer. The stretched sequences show the spacer promoters. The cleavage sites for EcoRI, BamHI and PstI (▲) are indicated. The extent of the DNA probe used is shown below the diagram.

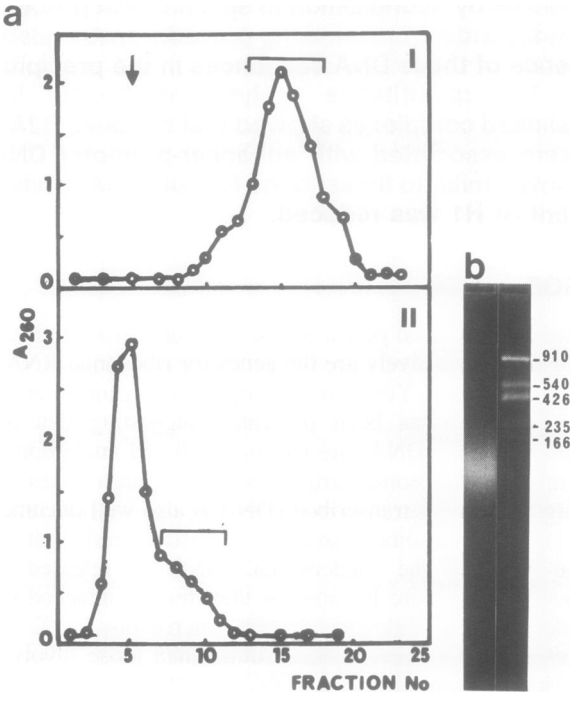

Figure 2. (a) Purification of formaldehyde (I) and UV laser (II) crosslinked protein-DNA complexes by centrifugation in CsCl. The fractions collected are indicated by the bar; the arrow marks the banding of free DNA; (b) Electrophoresis in 1.5% agarose gel of DNA isolated from crosslinked protein-DNA complexes (left side); the right side is marker DNA fragments obtained by digestion of pUC19 with DdeI.

with formaldehyde resulted in formation of protein-DNA complexes in which practically all of DNA has been involved. In the case of laser-induced crosslinking the yield of covalently linked complexes was much lower, thus forming a shoulder to the peak of the free DNA. Such a finding is reasonable, since under the conditions of irradiation not more than 10–15% of histones could be crosslinked to DNA (25). The fractions containing the crosslinked complexes (the separate peak and the shoulder, respectively) were used in the immunoprecipitation experiments.

Separation of DNA fragments, carrying a given covalently linked histone

This was carried out by immunoprecipitation of the protein-DNA complexes with antibodies to histones H1, H2A and H4. These antibodies, although raised by injecting rabbits with purified calf thymus histones, demonstrated a considerable crossreaction with the respective histones from *X. laevis*. Their ability to immunoprecipitate histone-DNA complexes is clearly shown by the presence of DNA in the precipitated material (Fig. 3).

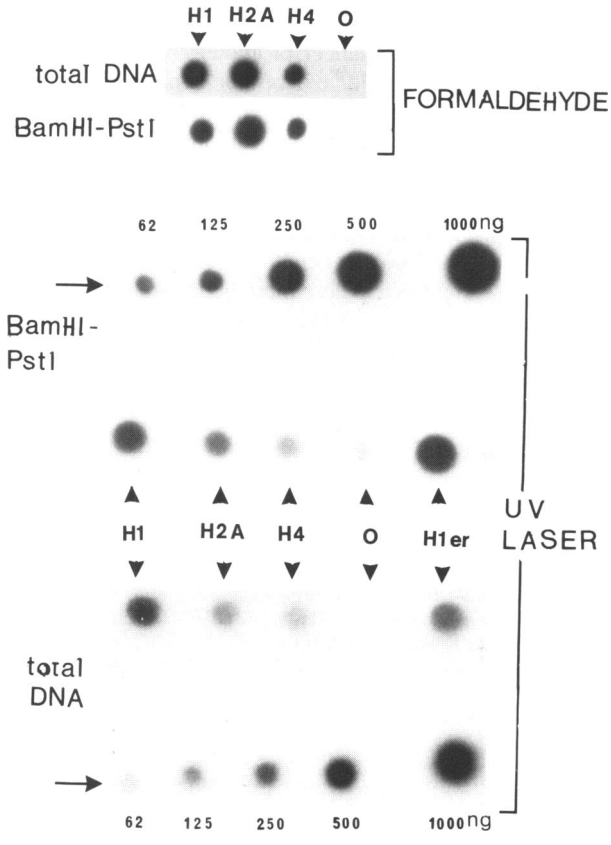

Figure 3. Dot hybridization of DNA immunoprecipitated with antibodies against histones H1, H2A and H4, and with non-immune IgG (0) to ^{32}P-labeled *X. laevis* total DNA and to BamHI- PstI DNA fragment, containing the enhancer-promoter unit of *X. laevis* ribosomal spacer. DNA to be analyzed originated from formaldehyde and from UV laser crosslinked nuclei of *X. laevis* embryos. In addition, a control experiment was performed, in which protein-DNA complexes from laser-irradiated nuclei of *X. laevis* erythrocytes were immunoprecipitated with anti-H1 antibody (H1er) and hybridized to BamH1-PstI DNA fragment. To illustrate the quantitation of the enhancer-promoter sequences, precipitated by each antibody (see Results), the hybridization dots, used to build the internal calibration curves for the respective DNA sequences, are also presented (marked by an arrow), indicating the amounts of dotted *X. laevis* DNA in micrograms.

Identification of the enhancer-promoter unit of *X. laevis* ribosomal spacer in the DNA fragments, carrying a given crosslinked histone

DNA from the immunoprecipitated protein-DNA complexes was isolated, dotted on filters and hybridized to ^{32}P-DNA, containing enhancer-promoter sequences of the *X. laevis* ribosomal spacer. Fig. 1 shows the location of the hybridization probe we used. Two different sets of protein-DNA complexes were immunoprecipitated and analyzed: those derived from formaldehyde fixed nuclei and those from the UV laser irradiated nuclei. Fig. 3 (top panel) presents the hybridization of DNA, immunoprecipitated by anti-H1, anti-H2A and anti-H4 from the formaldehyde crosslinked protein-DNA complexes to the specific DNA probe. As seen, enhancer-promoter sequences have been detected in all antibody-precipitated DNA samples. To avoid a case when short enhancer sequences might be a part of long stretches of bulk DNA, carrying crosslinked histones, the crosslinked chromatin fragments were sonicated to reduce the size of DNA to about 150 bp (Fig. 2b).

A basic motivation of the experimental approach we used is that the amount of particular DNA sequences, precipitated by the antibody depends directly on the amount of the respective histone crosslinked to these sequences. For reasons to be discussed below formaldehyde crosslinking does not allow a reliable quantitation of the amount of the antibody-precipitated DNA. Such an analysis was performed by the experiments with the laser-induced crosslinking. Aliquots from the different antibody-precipitated DNA preparations were dotted on a Zeta-Probe filter; increasing amounts of *X. laevis* genomic DNA (in the range 60–1000 ng) were applied on the same filter in order to be used as reference dots for building a calibration curve. Exactly the same set of dots was repeated on a second filter and the two filters were hybridized to *X. laevis* genomic DNA and to enhancer-promoter DNA, respectively. An autoradiography of one typical experiment is shown in Fig.3 (middle and bottom panels). The hybridization signals thus obtained were scanned and the amount of DNA, precipitated by each antibody, was estimated in nanograms by using the respective calibration curves. The introduction of reference dots allows a given quantity of DNA to be independently determined both as bulk DNA and as enhancer-promoter sequences. If the antibody-precipitated DNA is an average DNA sequence (derived from bulk chromatin), the signals obtained with the two hybridization probes, although differing in magnitude, should correspond to one and the same quantity of DNA. If, however, the amount of a given histone, associated with the enhancer-promoter DNA is reduced, less of these DNA sequences would be precipitated by the respective antibody. In such a case the quantity of dotted DNA, estimated as enhancer-promoter sequences should be lower than that estimated as bulk DNA. The quantitative analysis of Fig.3 (middle and bottom panels) is presented in Table 1. It shows that for anti-H2A- and anti-H4-precipitated DNA, the hybridization signals obtained with the two probes corresponded to similar quantities of DNA. As mentioned, this is to be expected if the enhancer-promoter DNA is associated with H2A and H4 as is bulk DNA. In the case of anti-H1-precipitated DNA, however, the hybridization signal obtained with the enhancer-promoter probe corresponded to twice less DNA than that corresponding to the signal with the bulk DNA probe. In other words, the amount of H1, associated with the enhancer-promoter sequences is twice less than that associated with bulk DNA. In a control experiment, the same experiment procedure was performed using anti-

H1-precipitated DNA from *X. laevis* erythrocytes, where the ribosomal genes are silent. Unlike the embryos, the amount of H1 bound to the spacer enhancer-promoter sequences in the erythrocytes was similar to that bound to bulk DNA.

DISCUSSION

The aim of this work was to see whether the enhancer- promoter unit of the *X. laevis* ribosomal spacer, shown to be organized as a non-nucleosomal structures in both expressed and non-expressed genes (Karagyozov,L. and Moss,T., manuscript submitted), were associated with histones. The experiments were carried out with *X. laevis* embryos at stage 40, where the highly expressed ribosomal genes (29) have also been reported to exhibit anucleosomal organization (12).

Crosslinking of histones to DNA and the subsequent analysis of DNA from the covalently linked complexes have been recently used in studying histone-DNA interactions (24,25,30–33). We applied this general approach using two independent methods for crosslinking histones to DNA in isolated nuclei: treatment with formaldehyde and irradiation with a UV laser. The crosslinked material was purified and fractionated by immunoprecipitation with antibodies to histones H1, H2A, and H4. Precipitated DNA was analyzed for the presence of enhancer-promoter sequences by hybridization to specific DNA probes. The identification of these sequences in the antibody-precipitated DNA means that they had been associated in vivo with the respective histones: otherwise these sequences would have not been crosslinked to DNA and, respectively, not precipitated. The amount of particular DNA sequences in the precipitated DNA will depend on the density of the histones along these sequences, unless the interactions of the histones with them have been altered in a way not allowing crosslinking to occur.

The two techniques for crosslinking showed that histones H1,H2A and H4 have been bound to the enhancer-promoter DNA (Fig. 3). An important point is the quantitation of these results. This analysis was carried out with the UV laser irradiated nuclei for the following reasons: (a) extremely short times of irradiation (one or several picosecond pulses) were used, so that a redistribution of histones is unlikely to occur (25); (b) crosslinking is not influenced by the extent of chromatin condensation (33); (c) no protein-protein crosslinks have been detected (25,34), and (d) the transient acetylation of histones, believed to be a property of the active chromatin structure, does not affect crosslinking (35).

The absence of protein-protein crosslinks upon UV laser irradiation turned out to be a particularly useful property for the general approach we used. Immunoprecipitation with the antihistone antibodies might be seriously affected by the 'chemical' loss of epitopes due to the formaldehyde-induced protein-protein crosslinks, as was already reported for glutaraldehyde (36). Such a possibility was suggested by the finding that practically no DNA was precipitated from formaldehyde crosslinked material with anti-H2B antibody, although the reaction of this antibody with the free histone or with non-treated chromatin was well demonstrated (not shown).

Our main conclusion is that the core histones H2A and H4, as well as the lysine-rich linker histone H1, are associated with the enhancer-promoter unit of the *X. laevis* ribosomal spacer in tissue where the ribosomal genes are actively transcribed. The quantitative analysis shows that the amounts of histones H2A and H4 bound to these DNA sequences are similar to those bound to bulk DNA, while the content of H1 is twice reduced. Such a finding is consistent with the very recent communication that the *X. laevis* enhancer/spacer promoter repeats, although non-nucleosomal, show kinetics of nuclease digestion unlike that expected for naked DNA (Karagyozov,L. and Moss,T., manuscript submitted). This result, supposed to indicate the presence of sequence specific DNA binding proteins on both the spacer promoters and enhancers might well be due to the binding of histones to those DNA sequences either alone or together with some specific protein(s). As for the reduced amount of the histone H1 associated with the enhancer-promoter region, as compared to bulk DNA, a possibility exists that a part of histone H1 molecules have their interactions with this particular DNA region affected in a way that prevents crosslinking. Such a possibility does not seem unlikely having in mind that the laser-induced crosslinking was found to proceed solely via the nonstructured tails of H1. What is obvious, however, is that such a result reflects the situation in the tadpoles, where the ribosomal genes are highly active, because in a tissue where these genes are silent (*X. laevis* erythrocytes), the amount of H1 bound to the spacer enhancer-promoter sequences did not differ from that bound to bulk DNA (Table I).

Table I. Quantitation of the hybridization analysis in Fig. 3 (middle and bottom panels). The dots of the antibody-precipitated DNA were scanned and estimated in nanograms using the respective reference dots. Two additional independent experiments, each one in duplicate, showed the same enhancer-promoter/bulk DNA ratios.

Antibody used for precipitation	precipitated DNA (in ng) estimated by hybridization to X.laevis	
	genomic DNA	enhancer-promoter DNA
anti-H1	460	240
anti-H1er	300	290
anti-H2A	130	120
anti-H4	80	60

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