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Compact structure of ribosomal chromatin in *Xenopus laevis*

Corrado Spadafora and Marco Crippa

Laboratoire d'Embryologie Moléculaire, Département de Biologie Animale, Ecole de Médecine, 20, rue Ecole de Médecine, CH-1211 Genève 4, Switzerland

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ABSTRACT

Micrococcal nuclease digestion was used as a tool to study the organization of the ribosomal chromatin in liver, blood and embryo cells of X. laevis. It was found that in liver and blood cells, ribosomal DNA is efficiently protected from nuclease attack in comparison to bulk chromatin. Although ribosomal chromatin is fragmented in a typical nucleosomal pattern, a considerable portion of ribosomal DNA retains a high molecular weight even after extensive digestion. A greater accessibility of the coding region in comparison to the non-coding spacer was found. In embryos, when ribosomal DNA is fully transcribed, these genes are even more highly protected than in adult tissues: in fact, the nucleosomal ladder can hardly be detected and rDNA is preserved in high molecular weight. Treatment of chromatin with 0.8 M NaCl abolishes the specific resistance of the ribosomal chromatin to digestion. The ribosomal chromatin, particularly in its active state, seems to be therefore tightly complexed with chromosomal proteins which protect its DNA from nuclease degradation.

INTRODUCTION

The organization of the chromatin structure of active ribosomal genes in eucaryotic cells is still not clearly defined. Extensive studies have used electron microscopic analysis as well as biochemical methods, yet a number of conflicting results were obtained. In particular, electron microscopic evidence indicates the absence of nucleosomes on actively transcribing ribosomal genes and very little compaction, if any, of the DNA (1-3). In contrast biochemical evidence suggests the existence of a nucleosomal packaging along the ribosomal DNA (4-7). (For a review see 8) One of the reason for such conflicting results is the possibility that only a subset of genes might be active in the ribosomal multigene family, while the others remain silent. Distinct chromatin structures may therefore coexist in the same organ or cell, reflecting the different levels of expression of the ribosomal genes.

Genes transcribed by Polymerase II, in contrast, have been studied in

more detail. It is now well established that substantial differences exist in the chromatin structure of these genes in their active versus inactive state (see Review 9). Active genes are highly accessible to nuclease degradation: they are degraded much faster than the same genes in an inactive state, particularly in regions of the promoter sites. The disruption of the nucleosomal structure in the transcribed genes (10-13) correlates with the enhanced sensitivity to nuclease.

Here we present a biochemical analysis of the chromatin structure of the ribosomal genes in X. laevis, comparing tissues in which these genes are expressed at different levels, i.e. blood and liver cells on one hand and embryos at stage 40 on the other (14). By the use of micrococcal nuclease digestion we find that ribosomal chromatin is highly protected as compared to bulk chromatin, particularly in embryos where these genes are fully active.

Furthermore we show that the resistance to digestion of the ribosomal chromatin is abolished after treatment with 0.8 M NaCl. The fact that salt treatment changes the accessibility of the chromatin to nuclease strongly supports the idea that we are dealing with a ribosomal DNA-protein complex.

MATERIALS AND METHODS

X. laevis embryos culture

X. laevis eggs were collected from individual females injected with estrogen (14). Embryos were grown under aeration until stage 40. Healthy embryos were manually selected for nuclei preparation.

Preparation of nuclei and of "native" chromatin fragments

Nuclei were isolated from X. laevis liver, blood and embryos according to Burgoyne et al. (15). Chromatin fragments were prepared essentially as described earlier (16). Freshly prepared nuclei were diluted to 1 mg/ml with buffer A (Tris-HCl pH 7.4 15 mM, KCl 15 mM, NaCl 15 mM, Mercaptoethanol 15 mM, Spermine 0.15 mM, Spermidine 0.5 mM) and were mildly digested with micrococcal nuclease using 0.05 units/µg of chromatin in presence of 1 mM $CaCl_2$ for 2' at 37°C. Digestions were stopped by adding EDTA to 2 mM final concentration. Nuclei were spun down and lysed by resuspension in 0.2 mM EDTA pH 8.0. Aliquots of this chromatin preparation were used for salt treatments.

Salt treatment of chromatin, micrococcal nuclease digestion and DNA extraction

Chromatin fragments were exposed to high salt concentrations by adding appropriate volumes of 5 M NaCl. After incubation at 37°C for 45', salt

was removed by step dialysis through Collodion bags (Sartorius SM 13200) in the following way:

Control chromatin (not treated with salt): 10 mM Tris-HCl pH 7.5

0.4 M " : 10 mM Tris-HCl pH 7.4

0.8 M " : 0.6 M NaCl 0.4 M NaCl 10 mM Tris-HCl

Dialysis was performed at 4°C for 2 hours against Tris-HCl 10 mM and for 30' against all other solutions.

Salt treated chromatin was digested at 37°C or at 4°C with 0.1 - 0.2 units/µg, taking into account the enzyme used for the first mild digestion, in presence of 1 mM $CaCl_2$. Aliquots were withdrawn from the incubation mixture at different times and diluted with EDTA to a final concentration of 10 mM. Samples were incubated with 50 µg/ml of DNase-free RNase A at 37°C for 1 hour and then overnight with 100 µg/ml of Proteinase K in the presence of 0.5% SDS. DNA was purified with two phenol and one chloroform extractions, adjusted to 0.3 M NaAcetate pH 5.5 and precipitated overnight at -20°C by the addition of 3 volumes of ethanol. Micrococcal nuclease digestion of nuclei and of chromatin not treated with salt, was always performed at 37°C with 0.5 units nuclease/µg chromatin. Reactions were stopped by adding EDTA to a final concentration of 10 mM. Other manipulations were done as described above for salt treated chromatin.

Cloning and purification of DNA restriction fragments

Recombinant clone HM456 was derived from clone pXL108 described by Boseley et al. (17).

For the isolation of specific restriction fragments recombinant plasmids were digested to completion with appropriate amounts of restriction enzymes. On a preparative scale 0.5 units/µg DNA were used overnight at 37°C. The digestion mixture was then loaded on an horizontal preparative gel low gelling agarose (Seakem) 1 - 1.5%, containing 0.5 µg/ml of ethidium bromide, and allowed to migrate until the bands were well separated. All electrophoresis was performed in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 2 mM EDTA). Agarose containing the stained DNA fragments was sliced with a sterile rasor blade, the volumes of the slices were roughly estimated and were placed in plastic or glass tubes with 1/10 the volume of 5 M NaCl. The slices were allowed to melt for 5' at 68°C. The mixtures were then extracted several times with saturated phenol and one or two times with chloroform. DNA was recovered by ethanol precipitation.

Analytical agarose electrophoresis, Southern blots and hybridizations

For analytical purposes DNA was routinely fractionated on 0.8 - 1.5%

horizontal agarose gels (Sigma Type II, medium EEO) containing TBE buffer and 0.5 µg/ml ethidium bromide. Electrophoresis was usually run overnight at 50 Volts, until the bromphenolblue marker was 1 or 2 cm from the end of the gel. The gel was photographed under U.V. transillumination and then soaked for 30' in a solution containing 0.2 M NaOH 0.6 M NaCl. The gel was then neutralized for 30' in a buffer containing 1 M Tris pH 7.5 - 1.5 M NaCl. The DNA was transferred to nitrocellulose filter according to Southern (18). After baking (80°C, 2 hours), filter was sealed in a plastic bag and prehybridized for about 6 hours at 42°C in prehybridization buffer: 5x SSC, 5x Denhart's, 50 mM phosphate buffer pH 6.8, 50% formamide, 100 µg/ml denatured, sonicated calf thymus DNA (SSC 1x : 0,15 M NaCl, 0,015 M Na Citrate; Denhardt's solution 100x : 2% BSA, 2% polyvinyl pyrrolidone, 2% ficoll). Hybridizations were performed in hybridization buffer (5x SSC, 1x Denhart's, 20 mM phosphate buffer, 50% formamide, 100 µg/ml calf thymus DNA) at 42°C for 12 - 16 hours, with about 10^7 cpm of the radioactive DNA probe for each filter. The filters were then washed two times with washing buffer I (2x SSC, 0.1% SDS) and two additional times in washing buffer II (0.1x SSC, 0.1% SDS). All washings were performed by shaking at 42°C for 30'. Filters were then air dried and exposed to Kodak X-Omat SO-282 films plus intensifying screens.

In some experiments the same filter was hybridized a second time with a different probe. The first radioactive probe was stripped by boiling the filter for ten minutes in 0.1x SSC, 0.1% SDS. The filter was then air dried and rehybridized with the second probe as described above, avoiding the prehybridization step. DNA size markers were obtained by digesting λ DNA (Biolabs) with Hind III and pBR 322 with HpaII restriction enzymes. P32 end labelling of these DNA fragments was performed according to Maniatis et al. (19). Differential labelling of the different restriction fragments obtained by HpaII digestion of pBR is likely caused by the different accessibility of the recessed 5 ends to the Polynucleotide kinase. Nick translation of DNA probes was done essentially as described by Rigby et al. (20).

RESULTS

a) Micrococcal nuclease digestion reveals that ribosomal genes in blood and liver cells of X.laevis are highly resistant to degradation.

Nuclei prepared from blood and liver cells were digested with Micrococcal nuclease for different lengths of time. The resulting DNA, after fractiona=

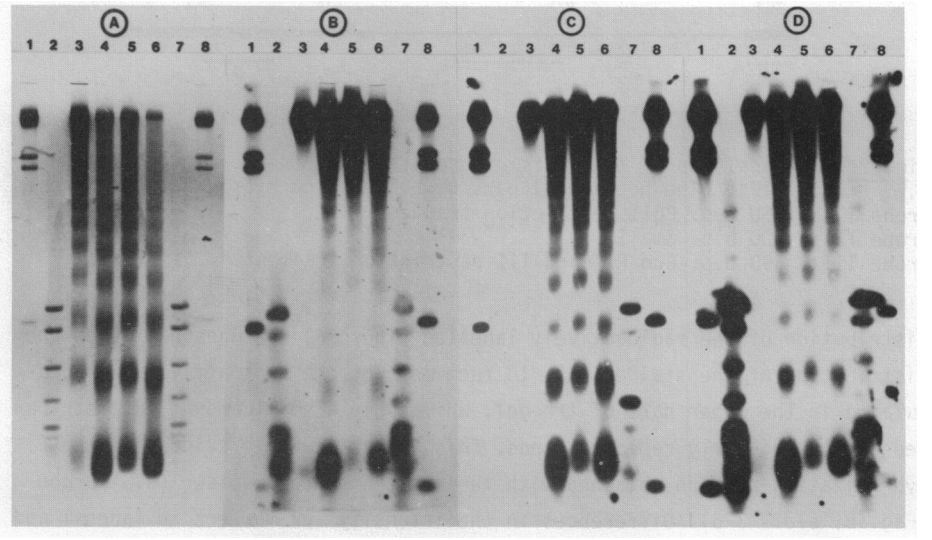

Fig. 1. Electrophoretic separation of DNA fragments obtained from X. laevis liver and blood nuclei digested with Micrococcal nuclease, and Southern blot analysis using different ribosomal DNA probes.
A: Ethidium bromide staining of the DNA fragments obtained from Micrococcal nuclease digestions of liver (slots 3, 4) and blood (slots 5, 6) nuclei. Digestions were performed with 0.5 units/µg of nuclease at 37°C for 2' (slots 3, 6), for 5' (slot 4) and for 30" (slot 5). Slots 1,8 are DNA markers obtained by restricting λ DNA with Hind III and slots 2,7 are markers of pBR 322 restricted with Hpa II. The gel is 1.5% agarose. About 5 µg of DNA are loaded on each slot. The same DNA samples as in A were hybridized with: B, probe I prepared from NTS region, C, probe II 18S region and D probe III 28S region. Slots 1,8 and 2,7 of each picture contain λ/Hind III and pBR 322/Hpa II DNA markers respectively with the exception of slot 2, panel C, that is empty.

tion on agarose gel, was hybridized with ribosomal DNA probes corresponding to different parts of the repeat unit. Fig.1A shows the ethidium bromide staining of the DNA patterns obtained after digesting nuclei from blood and liver cells with Micrococcal nuclease for two different lengths of time. Samples in slots 4 and 6 are digested to a greater extent than samples in slots 3 and 5 and most of their DNA is distributed in the repeated nucleosomal pattern. (The repeat pattern of blood chromatin is 190 b.p. about 5 b.p. longer than the repeat of liver chromatin.) Fig. 1B,C and D show the same DNA samples hybridized with non transcribed spacer probe (I), 18S probe (II) and 28S probe (III) respectively (see Fig. 2).
A comparison of the stained with the radioactive patterns shows that the

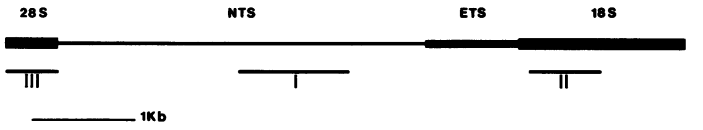

Fig. 2. Physical map of X. laevis ribosomal clone HM456 based on Boseley, P.G. et al. (17). The horizontal bars symbolize the hybridization probes.
Probe I : 1060 b.p. BglI restriction fragment
Probe II : 700 b.p. Sma I " "
Probe III: 490 b.p. Eco RI/Hind III restriction fragment.

distribution of the radioactively labelled ribosomal DNA does not match the distribution of the stained DNA. In fact most of the radioactivity is accumulated in the upper part of the gel, while only a small amount is distributed on the regularly repeated bands. This is particularly evident in the hybridization pattern obtained with the non transcribed spacer (NTS) probe (Fig.1B, slots 3-6). Differences in the intensity of markers in lanes 1 and 8, and 2 and 7, are caused by slightly different amounts of radioactive marker (Fig.1D, slots 1 and 8, and 2 and 7). A discrepancy in the intensity of the largest pBR/HpaII fragment is observed in Fig.1 B comparing slots 2 and 7. Such difference is probably caused during the blotting procedure and is limited to a small area of the filter since the adjacent Hind III fragment (slot 8) is normally transferred.

To roule out possible artefacts, such as differences in the sensitivity between ethidium bromide staining and radioactive hybridization and, more likely, loss of DNA during Southern transfer, we compared the hybridization patterns obtained by hybridizing "nick translated" total genomic DNA, NTS probe or 18S probe (Fig.3 B,C and D respectively), with DNA samples from a time course digestion of blood nuclei. In this experiment the amount of DNA loaded in each slot was twice the amount used in the former experiment. The visible pattern (Fig. 3 A) and the hybridization pattern obtained using total DNA as probe (Fig.3 B) are very similar. In both cases the DNA is totally resolved in bands which contain multiples of 185-190 base pairs and it accumulates increasingly in the fastest migrating bands at the later stages of digestion (Fig.3 A,slot 4, and Fig. 3 B, slot 4). In contrast, hybridization with ribosomal NTS and 18S probes (Fig. 3 C, slots 1-4 and 3 D, slots 1-4) shows that a considerable portion of the ribosomal DNA remains at a higher molecular weigth even after extensive digestion, when bulk chromatin is essentially reduced to monomeric, dimeric and trimeric DNA fragments. It is of interest that the ribosomal gene is not uniformly

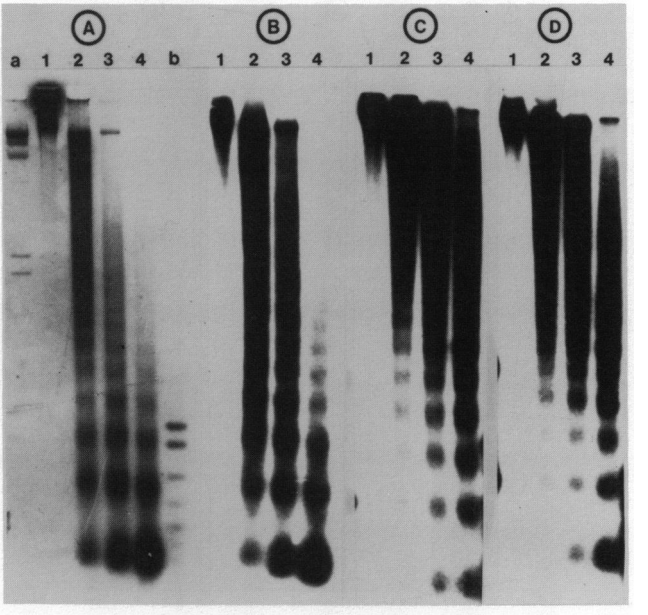

Fig. 3. Electrophoretic separation of DNA fragments obtained by digesting X. laevis blood nuclei with Micrococcal nuclease, and Southern blot analysis using genomic DNA and rDNA as probes.
A: Ethidium bromide staining of a Micrococcal nuclease digestion of blood nuclei. Digestion was performed as already described for the following lengths of time: slot 1: 0', slot 2: 30", slot 3: 2' and slot 4: 5'. Slots a and b are λ restricted with Hind III and pBR 322 with Hpa II respectively. The gel is 1.2% agarose. 10 µg of DNA was loaded in each slot. The same DNA samples as in A were hybridized with: B total genomic DNA prepared from X. laevis, C ribosomal DNA probe I and D probe II. Panels B, C and D are three different gels.

resistant to Micrococcal nuclease along its entire length. In fact,the NTS region shows a higher resistance to nuclease attack than the 18S and 28S region (Fig. 1B, slots 3-6, Fig. 1C, slots 3-6 and Fig. 1D, slots 3-6), and the 18S coding region seems to be the most nuclease sensitive part of the gene (Fig.1 C, slots 3-6). Digestion in this region produces a distinct nucleosomal pattern that, in contrast, is hardly visible in the NTS region (Fig. 1 B, slots 3-6). In addition, although both regions are generally more resistent than bulk chromatin (Fig. 3 B, slots 1-4), the 18S region is degraded slightly faster than the NTS (Fig. 3 C, slot 4 and 3 D, slot 4). Control experiments were performed to rule out the possibility that resistance to nuclease degradation was caused by a high concentration of chromatin in suspension. Digestions of nuclei and chromatin, were made at concentra-

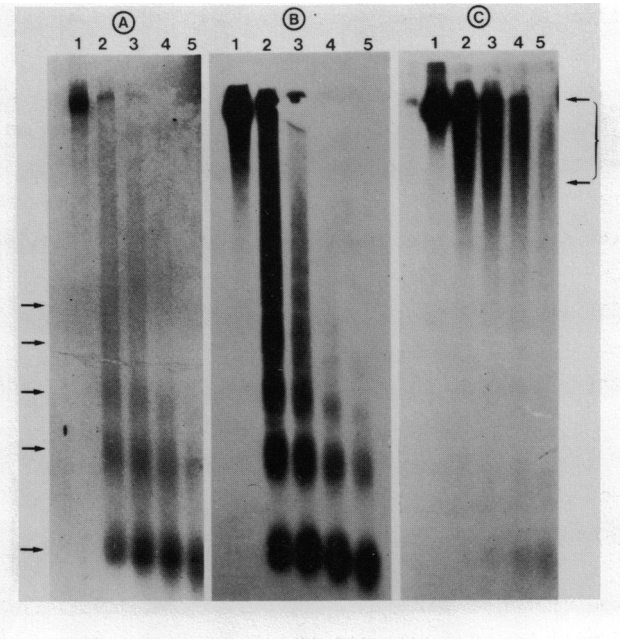

Fig. 4. Micrococcal nuclease digestion of nuclei prepared from embryos at the stage 40 and Southern blot analysis using genomic DNA and rDNA I as probes.
A: Ethidium bromide staining of a Micrococcal nuclease digestion of embryo nuclei at stage 40. Digestion was performed using 0.2 units/µg at 37°C for the following lengths of time: slot 1: 0', slot 2: 30", slot 3: 1', slot 4: 2' and slot 5: 5'. The gel was 1.5% agarose.
B: Same samples as in A were hybridized with total genomic DNA.
C: Same DNA samples were hybridized with rDNA probe I. Panels B abd C are two different gels.

tions between 100 and 200 µg/ml (5 to 10 fold less concentrated than our standard working conditions) and the results remained the same (data not shown). No correlation was found between the resistance of ribosomal chromatin to digestion and the concentration at which the chromatin was digested. Also no appreciable difference was found between liver and blood chromatin.

b) In embryonic tissues of X. laevis, ribosomal DNA is more resistant to nuclease degradation than ribosomal DNA in adult tissues.

Similar experiments were performed with nuclei prepared from embryos at stage 40. Fig. 4 A shows the Micrococcal digestion pattern obtained with nuclei prepared from embryos. Embryonic bulk chromatin is digested more rapidly than the chromatin from adult (Fig.4 A). Fig. 4 B and C show the same DNA samples hybridized with total genomic DNA and NTS probes respectively. Hybridization with total genomic DNA (Fig. 4 B) shows the normal nucleo-

somal fragmentation pattern with an accumulation of DNA at the monomer and dimer level at the longest times of digestion (slots 4 and 5). This radioactive pattern is similar to the ethidium bromide stained one (Fig. 4 A). In contrast, hybridization with the NTS probe (Fig.4 C) demonstrate (i) the absence of significant nucleosomal ladder and (ii) the absence of other detectable degradation product. Some ribosomal DNA maintains its high molecular weigth even after 5' of digestion (slot 5) without an accumulation of fragments of lower molecular weight. The amount of ribosomal DNA however decreases substantially while the digestion proceeds, suggesting that this DNA is converted rapidly from a high molecular weight species to soluble fragments. If the gel is however exposed longer, the embryonic DNA shows a very faint nucleosomal periodic ladder. The same results are obtained hybridizing with the 18S (II) and 28S probes (III) (data not shown).

c) Salt treatment of the chromatin abolishes the resistance of ribosomal DNA to nuclease degradation.

In order to learn more about the structure of the ribosomal chromatin and to understand the nature of its specific protection against nuclease degradation, we perturbed the chromatin structure by salt treatments of increasing ionic strength. If the protection is due to chromosomal components, then the salt wash might be able to loose their binding to DNA, which would then become accessible to degradation. Fig. 5 shows the results of such experiments. Aliquots of blood cell nuclei were lysed and the chromatin exposed to 0.1, 0,4 and 0.8 M NaCl (see Materials and Methods). Salt was removed by step dialysis and the chromatin was digested with Micrococcal nuclease in 10 mM Tris-HCl pH 7.5. It is clear that treatment of the chromatin with 0.1 and 0.4 M NaCl does not introduce any relevant changes in its structure (Fig. 5, slots 1-3 and 4-6 respectively). The ribosomal DNA is still highly protected and the periodic nucleosomal pattern remains unmodified. In contrast, treatment with 0.8 M NaCl reduces this protection drastically, causing a rapid degradation of the ribosomal NTS region (Fig. 5, Slot 7-9). Treatment with 0.8 M NaCl increases the accessibility of the ribosomal chromatin to nuclease degradation and makes the digestion pattern of ribosomal chromatin similar to that of bulk chromatin, under identical experimental conditions (Fig. 6). Comparison of the two hybridization patterns (Fig. 5, slots 7-9 and Fig. 6, slots 7-9) shows however that even after 0.8 M NaCl treatment the average size of the NTS DNA is still slightly larger than the bulk DNA. This may be due to the fact that the factors protecting the ribosomal DNA were not completely extracted, although salt

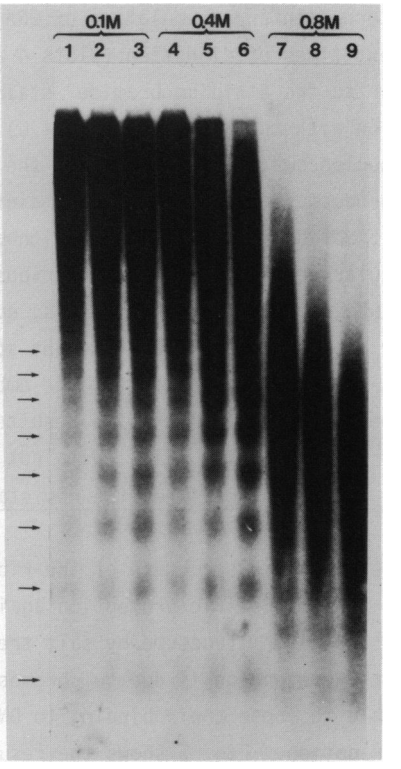

Fig. 5. Accessibility to Micrococcal nuclease digestion of ribosomal chromatin after exposure to increasing ionic strengths. Chromatin samples were exposed to different ionic strengths (see Materials and Methods) and after dialysis, digested for increasing lengths of time in 10 mM Tris pH 7.4. Digestions were performed at $4^{o}C$ using 200-250 units of Micrococcal nuclease. Hybridization was performed with porbe I NTS.
Slots 1-3 : control digestions (about 0.1 M NaCl) for 3', 9' and 15'.
Slots 4-6 : 0.4 M treatment, digestions for 3', 6' and 15'.
Slots 7-9 : 0.8 M treatment, digestions for 3', 6' and 15'.
Arrows on the left hand site of the picture indicate the nucleosomal fragments multiple of about 190 b.p.

treatments up to 1.6 M NaCl did not modify this pattern (not shown). Similar results are obtained probing for the 18S coding region; the pattern of hybridization with the 18S probe shows resistance to nuclease degradation at 0.4 M NaCl and accessibility after treatment with 0.8 M NaCl. These results strongly suggest that at least some of the chromatin components, which protect ribosomal DNA from nuclease digestion, are removed at 0.8 M, but not at 0.4 M NaCl. The effects of 0.6 M treatment on chromatin were similar to those obtained with 0.4 M.

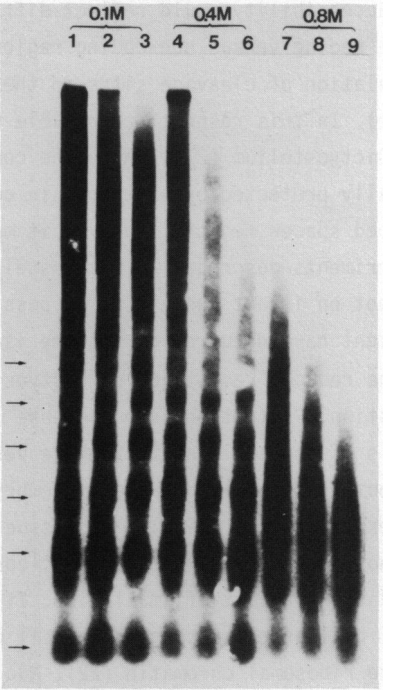

Fig. 6. The same filter of Fig. 5 is stripped of the first hybridized probe (see Materials and Methods) and is hybridized again with total genomic DNA. Arrows in the left indicate the nucleosomal ladder.

Control experiments have shown that short treatment of nuclei or chromatin with limited amounts of trypsin, followed by trypsin inhibitor, also render the ribosomal chromatin extremely sensitive to Micrococcal nuclease (data not shown).

DISCUSSION

Ribosomal chromatin shows a higher resistance to nuclease digestion than bulk chromatin. This suggests that ribosomal DNA is organized in a more compact chromatin structure than bulk DNA. The nuclease resistant state, perhaps due to a complex of ribosomal DNA with either proteins or RNA, is found in both, inactive and active state of the gene. The protection extends the entire length of the gene, although reproducible differences in the degree of accessibility were found between the coding and the non coding parts. The non transcribed (NTS) region of the gene is somewhat more protected from the nuclease digestion than the coding part (Fig. 1 B,C and D). This

variation in nuclease accessibility could reflect differences in the chromatin organization of the coding versus non-coding regions of the gene, resulting in a higher accumulation of cleavage sites in the coding part (in particular in the 18S gene). In this respect, comparable but not identical results,were found in Dictyostelium (21), where the coding regions of the gene were found "minimally protected by proteins, in contrast to the highly protected non-transcribed spacer region". However it must be pointed out that all digestion experiments described in Dictyostelium were performed on isolated nucleoli and not on intact nuclei. It is possible that a loss of nucleolar components might have occurred during the isolation of nucleoli, which would explaine the reduced resistance of Dictyostelium ribosomal chromatin to nuclease digestion. Protection from nuclease digestion is observed in blood and liver cells of the adult animal, where very little or no transcription is occurring, but is strongly enhanced in embryos at stage 40, where these genes are fully expressed. In this latter tissue, degradation of ribosomal chromatin, does not produce a significant nucleosomal ladder (Fig. 4 C), or any other degradation product of small size. It was recently reported the lack of the typical nucleosomal packaging in cell culture enriched in transcriptionally active ribosomal chromatin (22). Nuclease digestion of nuclei prepared from embryos cause a slow and progressive disappearance of the ribosomal DNA which nevertheless, maintains its relatively high molecular weight, for up to 5' of nuclease digestion. This behaviour would suggest that ribosomal chromatin, in actively transcrbing embryos, is in a structure almost impermeable to nuclease, yet once the nuclease penetrates this structure, the ribosomal DNA is totally degraded into very small fragments. In general activation of Polymerase II transcribed genes results in a greater accessibility of the genes to Micrococcal nuclease degradation (see Reviews 8,9), with the exception of immunoglobulin genes that were shown to keep, in their active state, the same degree of accessibility as the bulk chromatin (23). In parallel to the increased digestibility, these genes, once activated, lose the nucleosomal periodic structure (10-13) and their degradation patterns resemble to a smear without any specific fragmentation. Only few exceptions are known (24,25). Our results show that ribosomal genes of actively transcribing tissues behave in a strikingly different way: their protection is enhanced in comparison to bulk chromatin and, in a minor degree, to ribosomal chromatin of adult tissues. Also the nucleosomal repeat is very much reduced and becomes visible only by overexposing the films. It is not possible at the present stage of the work, to draw clear conclusions

on the organization of the ribosomal chromatin. Two explanations seems to be reasonable: the first one is that ribosomal chromatin,in its active state contains proteins which interact with the normal nucleosomal components and cause a more efficient protection against nuclease degradation. The second is that proteins, tightly interacting with ribosomal DNA, replace the nucleosomal structure, totally or in part, explaining the resistance and the loss of nucleosomal ladder in the embryos.
Fractionation and analysis of the protein component of the ribosomal chromatin would help to clarify the problem. A second intriguing question is wheather DNA sequences, other than ribosomal DNA, are also highly protected. At present we are cloning this nuclease resistent DNA in order to establish the complexity of its sequence and to determine the fragmentation pattern caused by Micrococcal nuclease digestion. A 0.8 M NaCl treatment makes the ribosomal chromatin almost accessible to nuclease as the bulk chromatin (Fig. 5 and 6). This result again strongly supports the idea that proteins are involved in the structure of the ribosomal chromatin. It is of interest to note that although differences exist in the accessibility of the coding versus non-coding regions, the loss of proteins from these two parts of the gene seems to occur at the same ionic strength. It is therefore possible that the entire repeat unit interacts with the same protein (s) leaving the differences in digestibility due to differences in the amount or in the compaction of such proteins with the DNA. Since 0.4 M NaCl only slightly modifies the digestion pattern (Fig.5), it is unlikely that is the heterogeneous class of proteins called "non histone proteins" responsible for the protection. Most of these proteins are in fact extracted by 0.3 to 0.4 M NaCl (26).

A likely candidate is Polymerase I: this molecule could strongly interact with ribosomal chromatin, causing the enhanced protection. On the other hand it is well known that 0.8 M NaCl strips H3A, H2B and H1 histones from chromatin (this latter is removed at 0.5 M NaCl) (26). The loss of these histone classes could account for the reduced resistance after salt treatment. As an alternative ribosomal DNA could be complexed with nucleolar proteins that would be tightly bound to ribosomal DNA.

The last point we would like to make is that the salt treatment causes an irreversible effect. Step dialysis after salt extraction does not restore the original protection, suggesting that once these proteins have been detached, the original structure is unable to be reconstituted.

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