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Different Chromatin Structures along the Spacers Flanking Active and Inactive *Xenopus* rRNA Genes

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The accessibility of DNA in chromatin to psoralen was assayed to compare the chromatin structure of the rRNA coding and spacer regions of the two related frog species *Xenopus laevis* and *Xenopus borealis*. Isolated nuclei from tissue culture cells were photoreacted with psoralen, and the extent of cross-linking in the different rDNA regions was analyzed by using a gel retardation assay. In both species, restriction fragments from the coding regions showed two distinct extents of cross-linking, indicating the presence of two types of chromatin, one that contains nucleosomes and represents the inactive gene copies, and the other one which is more cross-linked and corresponds to the transcribed genes. A similar cross-linking pattern was obtained with restriction fragments from the enhancer region. Analysis of fragments including these sequences and the upstream portions of the genes suggests that active genes are preceded by nonnucleosomal enhancer regions. The spacer regions flanking the 3′ end of the genes gave different results in the two frog species. In *X. borealis*, all these sequences are packaged in nucleosomes, whereas in *X. laevis* a distinct fraction, presumably those flanking the active genes, show a heterogeneous chromatin structure. This disturbed nucleosomal organization correlates with the presence of a weaker terminator at the 3′ end of the *X. laevis* genes compared with those of *X. borealis*, which allows polymerases to transcribe into the downstream spacer.

In the last few years, much progress has been achieved in identifying the different sequence elements located in the intergenic spacers that are involved in the regulation of the rRNA genes. Despite the fact that the DNA sequence of the ribosomal spacers has diverged very rapidly between different organisms, recent studies indicate a general consensus in the function as well as in the arrangement of transcriptional regulatory elements in almost all higher eukaryotes (for reviews, see references 35, 44, and 45). However, some exceptions seem to exist even among closely related species. The most striking difference resides in the function of the spacer elements close to the 3′ end of the rRNA precursor coding regions in the two frog species *Xenopus laevis* and *Xenopus borealis*. In fact, a series of transcriptional analyses failed to detect a true termination site at the 3′ end of the *X. laevis* rRNA genes (9, 18), whereas the related sites in *X. borealis* behave like efficient terminators (21) (sites T2, see Fig. 1). It seems that in *X. laevis* a single natural point mutation at this site has abolished its termination function, allowing transcription to proceed into the intergenic spacer (22). In contrast, the structural and functional organization of the spacer segments adjacent to the 5′ end of the coding regions is very similar in these two frog species, and recently, the corresponding regulatory elements have also been identified in mammalian spacers (34, 36). This region includes several duplicated spacer promoters followed by blocks of enhancer elements which have been shown to bind in vitro the same transcription factor that also binds to the ribosomal gene promoter (11, 32) (see Fig. 1).

In this report, we describe the relationship between these different transcriptional regulatory elements and their chromatin structure in *X. laevis* and *X. borealis*. One can imagine that spacer transcription or transcription factor binding to enhancer regions might be reflected in peculiar chromatin structures. Most of the structural data in the literature come from electron microscopic studies of the amplified rRNA genes of *X. laevis* oocytes. While it is widely accepted that the coding region of an active ribosomal transcription unit occurs in an extended, nonnucleosomal state, contradictory results were obtained with regard to the spacers, which have been shown to appear as packaged in nucleosomes (33) or as smooth fibers covered by a uniform layer of unknown proteins (40) or even as thin filaments similar to coprepared naked DNA (16). A confusing picture also emerges from biochemical analyses of different somatic cells. An increased DNase I sensitivity of both *X. laevis* coding and spacer regions has been correlated with transcriptional activity (23, 25), whereas from micrococcal nuclease digestion experiments, the same sequences appear to be more protected than bulk chromatin even in cells in which the ribosomal genes are thought to be highly expressed (46).

The main difficulty in interpreting biochemical studies on ribosomal chromatin is due to the fact that in a typical somatic cell only a fraction of the tandemly repeated rRNA genes may be active in transcription (5, 12, 41). In fact, using the psoralen cross-linking technique, it has recently been shown by our group that in a mouse cell line the rDNA coding regions exist in two distinct chromatin structures. One type is packaged in nucleosomes and represents the inactive copies, whereas the other type lacks a repeating structure and corresponds to the transcribed genes (5). Using the same technique, we compared the ribosomal chromatin structure of the coding and of different spacer regions in *X. laevis* and *X. borealis* tissue culture cells. We show that in both cell types, as for the mouse (5), active and inactive genes coexist in the same cell. In *Xenopus* species, the two distinct chromatin structures extend into the 5′-flanking spacer sequences containing the enhancer elements. The nature of the chromatin in the spacer segments downstream of the genes is different in the two frog species and correlates to the different functional features of the 3′ ends of the coding regions.

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MATERIALS AND METHODS

Preparation of nuclei. *X. laevis* Xl K2 cells (27) and *X. borealis* Xb 693 cells were grown at room temperature in 50% L15 medium (GIBCO) supplemented with 10% fetal calf serum (Biological Industries). The cells were harvested at the late log phase with ice-cold phosphate-buffered saline containing 1 mM EDTA, and the nuclei were isolated essentially as described by Crampton and Woodland (7). Cells were disrupted on ice in the homogenization buffer (0.3 M sucrose, 10 mM Tris HCl [pH 8], 2 mM magnesium acetate, 3 mM $CaCl_2$, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1 mM EDTA, 1 mM dithiothreitol) with a Dounce homogenizer. After cell lysis, 1.5 volumes of centrifugation buffer were added (2 M sucrose, 10 mM Tris HCl [pH 8], 5 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM EDTA), and the mixture was layered over a 6-ml cushion of centrifugation buffer in 15-ml Corex tubes. Nuclei were pelleted by centrifugation in an HB-4 rotor at 10,000 rpm for 30 min, and the nuclear pellets were washed once in storage buffer (25% glycerol, 50 mM Tris HCl [pH 8], 5 mM magnesium acetate, 5 mM dithiothreitol, 0.1 mM EDTA). Nuclei were used fresh or were stored in aliquots in storage buffer at −70°C.

X. laevis erythrocyte nuclei were prepared by a modification of the method of La Volpe et al. (23). All the following steps were performed on ice. Blood was collected from a single animal, and the cells were washed in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate). Cells were resuspended in buffer A (15 mM Tris HCl [pH 7.4], 60 mM NaCl, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine) containing 0.25 M sucrose, 0.1 mM EDTA, and 0.1 mM EGTA, and cell lysis was obtained by adding 0.25% Nonidet P-40 and mixing gently for a few minutes. The above solution but with 2 M sucrose and without Nonidet P-40 was added (0.5 volume), and the mixture was layered over a cushion of the same solution. Nuclei were pelleted as described above, and the nuclear pellets were washed and resuspended in final solution (buffer A containing 0.34 M sucrose and 25% glycerol).

Psoralen cross-linking. Nuclei at a concentration of about 2×10^6/ml in storage buffer (native conditions) were placed in open multiwell plates on ice and were irradiated for 5 h in the presence of 4,5′,8-trimethylpsoralen (Sigma) as previously described (43). Psoralen stock solution in ethanol (0.05 volume of 200 μg/ml) was added five times at intervals of 1 h. An aliquot of the nuclei was incubated on ice for 20 min in storage buffer with 500 μg of heparin (Sigma) per ml before psoralen addition. In some experiments, nuclei were irradiated with a strong UV lamp (model B-100 A; Ultra Violet Products, Inc., San Gabriel, Calif.), and the irradiation time was reduced to 15 min (the psoralen was added three times at intervals of 5 min). Both irradiation procedures gave similar extents of cross-linking.

Gel electrophoresis, transfer, and hybridization. DNA extraction, restriction enzyme digestions, agarose gel electrophoresis, reversal of psoralen cross-linking, and alkaline Southern blotting were done as previously described (5). The filters were hybridized according to the standard protocol from the Zeta-Probe membrane instruction manual (Bio-Rad). The hybridization probes were obtained by labeling the rDNA plasmid inserts with [^{32}P]dCTP by using the multiprime DNA-labeling system from Amersham. The locations of the various probes are shown in Fig. 1: pXlr14B (14B) is an *Eco*RI-*Bam*HI subclone of pXlr14 (1); in pG14F (14F), the *Sal*I-*Bam*HI enhancer fragment of pXlr14F (17) was blunt ended and cloned into the *Pvu*II site of pGEM-3 (Promega Biotec); ITSBB (BB) contains the *Bam*HI coding fragment of the *X. laevis* rDNA. pGXb800 (800) and pGXb988 (988) are pGEM-4 vectors (Promega Biotec) containing the *Bst*NI (+874)-*Sma*I (+1674) and the *Hin*fI (3033 to 4021) fragments, respectively (the nucleotide positions are relative to the *Hin*dIII site at T1 [20]).

RESULTS

Psoralen cross-linking of ribosomal chromatin in *X. laevis* tissue culture nuclei. The accessibility of DNA in the different ribosomal chromatin regions for psoralen was analyzed by using the gel retardation assay developed by our group (5, 42, 43). This method is based on the fact that the more a DNA fragment is cross-linked with psoralen, the slower it migrates in a native agarose gel (2, 42). Isolated nuclei were extensively photoreacted with psoralen, and the purified, cross-linked DNA was digested with various restriction enzymes and electrophoresed alongside non-cross-linked control DNA. The separated DNA fragments were irradiated with short-wavelength UV light to reverse the psoralen cross-linking (43) and then blotted and hybridized with different rDNA probes. The locations of the restriction fragments analyzed in this work are shown as open boxes in the restriction maps in Fig. 1. When *X. laevis* tissue culture nuclei were cross-linked under native conditions (see Materials and Methods), the 4.8-kb *Eco*RI fragment of the coding region was resolved into two distinct bands in the gel retardation assay (Fig. 2a, lane 2). The lower band (f) has a mobility close to that of the non-cross-linked control DNA (lanes 1 and 4), i.e., it has a low extent of cross-linking, whereas the upper band (s) represents fragments with a large extent of cross-linking. On the basis of the results obtained by our group with a mouse cell line (5), we interpret the f and s bands as representing DNA fragments originating from inactive and active rRNA genes, respectively (see below). It has long been recognized that nucleosomes protect DNA from being cross-linked (3, 13), and therefore, DNA fragments originating from inactive genes packaged in nucleosomes are only slightly cross-linked (i.e., preferentially only in the linker regions) and migrate almost as fast as non-cross-linked DNA. In contrast, when the rRNA genes are transcribed, the nucleosomes are lost or drastically modified and the psoralen molecules have full access to the coding sequences, which become then heavily cross-linked (5, 24, 43) (see also Fig. 2b, coding, lane 2). From the intensity of the two distinct bands, it can be determined that in this *X. laevis* cell line, the active gene copies represent only about 25% of the total population. When the nuclei are cross-linked in the presence of heparin, which removes the histones from the DNA (15, 43), the same ribosomal coding fragment is resolved in a single retarded band (Fig. 2a, lane 3) with mobility similar to that of the s band, indicating that the inactive copies have also been heavily cross-linked.

To prove that in *X. laevis* the DNA from the transcriptionally active gene copies also migrates in the s band, we labeled this DNA in situ by cross-linking radioactive nascent RNA to the rDNA template (5). Nuclei were pulse-labeled in the presence of radioactive ribonucleoside triphosphates and then photoreacted with psoralen under native conditions. The purified DNA was digested with *Eco*RI, treated with RNase (to shorten the cross-linked RNA chains), and separated on a gel. In the direct exposure of the gel, only the s band is detected (Fig. 2c, lane 1), indicating that this DNA represents *Eco*RI coding fragments arising from the transcribed gene copies. When an aliquot separated on the same

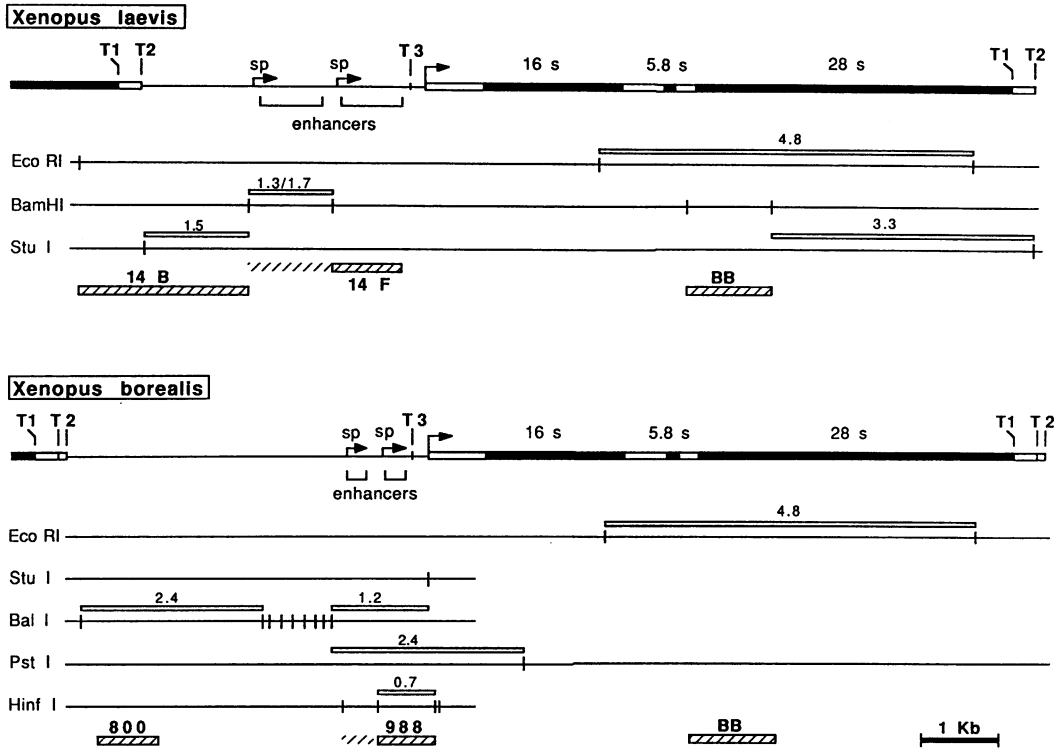

FIG. 1. Structural organization and restriction maps of the ribosomal repeat units of *X. laevis* and *X. borealis*. In the rDNA maps, the 40s precursor coding region is indicated as a box; the arrow and T2 correspond to its 5′ and 3′ ends, respectively (note that *X. borealis* has two T2 elements), and the filled boxes show the positions of the sequences coding for the 16s, 5.8s, and 28s rRNAs (T1 is the 3′ end of the 28s coding region). The intergenic spacer is represented by a solid line; the positions of the spacer promoters (sp), the enhancers, and the upstream terminator (T3) are indicated. In the restriction maps, the rDNA fragments analyzed in this study are shown as open boxes, and the numbers indicate their lengths (in kilobases). The positions of the probes used (stippled boxes) are also indicated (stippled regions mark rDNA sequences homologous to the probes).

gel was blotted and hybridized with a probe from the ribosomal coding region, both s and f bands were again visualized (Fig. 2c, lane 2). Similar results were obtained in the *X. borealis* cell line (data not shown).

In *X. laevis*, the RNA polymerases transcribing the ribosomal coding regions do not terminate at the 3′ end of the gene but appear to continue into the intergenic spacer, producing highly unstable RNA (9, 18). Furthermore, a second type of spacer transcription can also originate from the duplicated spacer promoters (see map in Fig. 1), which have been shown to be active in this particular cell line (21). To study the influence of the first type of transcription on the chromatin structure of the ribosomal spacer, we analyzed the 1.5-kb *Stu*I-*Bam*HI fragment containing spacer sequences between the 3′ end of the gene and the first spacer promoter (Fig. 1). Because of spacer length heterogeneities, the major 1.5-kb band is flanked by two faint bands representing two minor size classes (see non-cross-linked control in Fig. 2a, 3′ downstream lanes 1 and 4). The same 3′ downstream fragment obtained from nuclei photoreacted under native conditions shows a cross-linking pattern different from that of the coding region: the main 1.5-kb fragment is resolved in a slightly retarded band (f) followed by a short smear with a lower electrophoretic mobility (bracket labeled s in Fig. 2a, 3′ downstream, lane 2; note that in the same lane, the 3.3-kb coding fragment which is also detected by the same probe (Fig. 1) is resolved in the two distinct bands described above for the *Eco*RI coding fragment). The small mobility shift of the f band is similar to that observed for the *Eco*RI coding fragment and is consistent with the presence of a population of 3′ downstream spacer regions which are packaged in nucleosomes. In contrast, the smear represents fragments with different extents of cross-linking, indicating the presence of a population with an heterogeneous chromatin structure (Fig. 2b, 3′ downstream). The same peculiar chromatin structure was also obtained by in vivo cross-linking of intact *X. laevis* tissue culture cells, ruling out any possible protein rearrangements or degradations during the isolation of nuclei (data not shown). After removal of the histones with heparin, these spacer sequences become uniformly cross-linked and the 1.5-kb fragment is resolved as a single retarded band (Fig. 2a, 3′ downstream, lane 3).

The analysis of the *Bam*HI spacer fragments spanning the enhancer sequences revealed again the two f and s bands, as for the coding region, indicating the presence of two different types of chromatin with two distinct psoralen accessibilities (Fig. 2a, enhancers). Since *Bam*HI digestions of psoralen-cross-linked DNA always gave a considerable amount of partial digestion products (see bracket in Fig. 2a, enhancers, lane 2), we could not accurately quantitate the proportions of the two distinct chromatin types. To reduce these partials, we incubated the nuclei with the restriction enzyme before psoralen cross-linking. The isolated DNA was then redigested with *Bam*HI and analyzed as usual. It is apparent from Fig. 2a, enhancers, lane 5, that this additional digestion step has very much reduced the partial digestion products (compare lane 5 with lane 2). The ratio between the intensities of the f and s bands in lane 5 indicates that there are

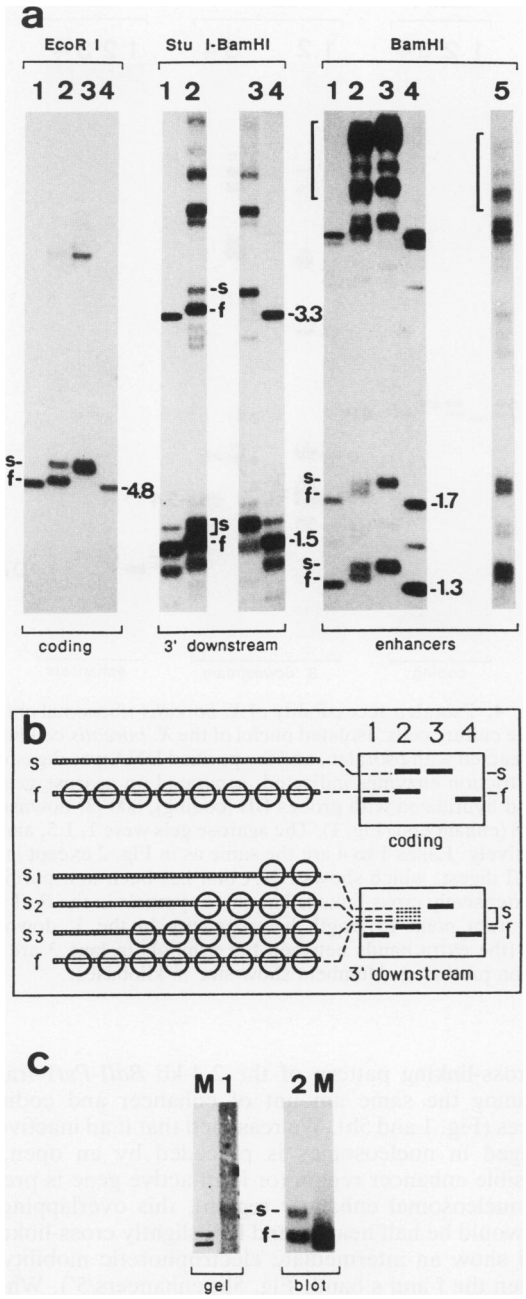

FIG. 2. (a) Psoralen accessibility of *X. laevis* ribosomal chromatin in tissue culture cells. Isolated nuclei of the *X. laevis* cell line were photoreacted with psoralen, and the purified DNA was digested with the restriction enzymes indicated on top of each panel, separated on agarose gels, blotted, and hybridized with various rDNA probes. The fragments from the coding, the 3′ downstream, and the enhancer regions were detected with probes BB, 14B, and 14F, respectively (Fig. 1), and the agarose gels were 1, 1.5, and 1.5%, respectively. The nuclei were cross-linked under native conditions (lanes 2) or in the presence of heparin (lanes 3). Lanes 1 and 4, non-cross-linked control DNA. The lengths of the fragments (in kilobases) are indicated on the right of each panel. In lane 5, the nuclei were digested with *Bam*HI before irradiation to reduce the partial digestion products (see brackets). (b) Schematic drawing representing the results in panel a from the coding and the 3′ downstream regions. Heavily cross-linked DNA fragments (s) migrate slowly in the agarose gel and are represented by a cross-hatched double line. Nucleosomes (open circles) protect the DNA from being cross-linked; DNA fragments that were organized in nucleosomes (f) are less cross-linked and migrate faster. S1, S2, and S3 represent DNA fragments with intermediate extents of cross-linking, i.e., intermediate electrophoretic mobilities. (c) Cross-linking of nascent RNA to the transcriptionally active rRNA gene copies. *X. laevis* nuclei were incubated under run-on conditions in the presence of 150 μg of α-amanitin per ml and ^{32}P-labeled CTP, UTP, and GTP (5) and then photoreacted with psoralen. The isolated DNA was digested with *Eco*RI, treated with RNase, and separated on a 1% agarose gel. The gel was either dried and exposed (lane 1) or blotted and hybridized with probe BB (lane 2). Lanes M are markers consisting of a mixture of non-cross-linked and faintly cross-linked, cloned *Eco*RI coding fragments which have been end labeled.

about as many enhancer sequences in the accessible chromatin conformation (s band) as enhancer packaged in nucleosomes (f band).

Psoralen cross-linking of ribosomal chromatin in *X. laevis* erythrocyte nuclei. To see whether the cross-linking patterns seen in the different ribosomal spacer and coding regions correlate to the transcriptional activity of the genes, we applied the same technique to study the ribosomal chromatin structure in *X. laevis* erythrocytes, in which it has been shown that these genes are not expressed (14). The appearance of only the slightly retarded f band in the gel retardation analysis of the *Eco*RI coding fragment is consistent with most of the rRNA coding sequences not being transcribed and packaged in nucleosomes (Fig. 3, coding, lane 2). As for the nuclei from cells growing in tissue culture, the same sequences become heavily cross-linked after removal of the histones with heparin, and the mobility of the *Eco*RI coding fragment is strongly decreased (Fig. 3, coding, lane 3). A similar cross-linking pattern was obtained for the 3′ downstream and the enhancer fragments (see the respective gel panels in Fig. 3). Because of spacer length variations between different frogs, these fragments show lengths different from those from the tissue culture cells; nevertheless, each size class is resolved into only the f band when nuclei are cross-linked under native conditions (Fig. 3, lanes 2) indicating a chromatin structure with a psoralen accessibility similar to that of the nontranscribed coding region.

Psoralen cross-linking of ribosomal chromatin in *X. borealis* tissue culture nuclei. As in the *X. laevis* tissue culture cells, the cross-linking pattern of the *Eco*RI coding fragment of *X. borealis* indicates the presence of an active and an inactive population of rRNA genes with two distinct psoralen accessibilities (Fig. 4, coding, lane 2). By comparing the intensity of the two bands, it is apparent that in this *X. borealis* cell line, the active genes (s band) are slightly more represented than the inactive ones (f band).

To study the chromatin structure of the 3′-flanking spacer region, we analyzed the cross-linking pattern of the *Bal*I fragment containing 2.4 kb of sequences downstream of T2 (Fig. 1). The presence of several bands in the non-cross-linked control DNA indicates length heterogeneities in this particular spacer region (see the different symbols in Fig. 4, 3′ downstream, lane 1). When tissue culture nuclei were cross-linked under native conditions (lane 2), the same bands appeared slightly retarded with respect to the control DNA (lanes 1 and 4), indicating DNA fragments with a low extent of cross-linking. Only a very minor proportion of these fragments shows a lower electrophoretic mobility and is represented by a faint smear (see bracket labeled s in Fig. 4, 3′ downstream, lane 2; the high-molecular-weight bands

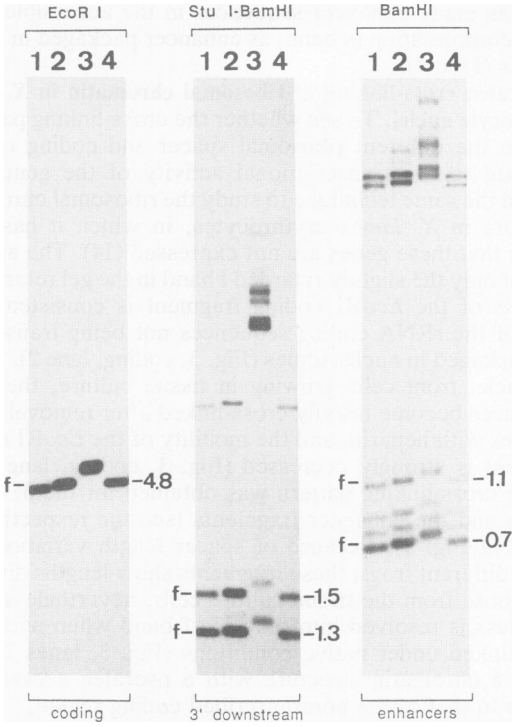

FIG. 3. Psoralen accessibility of ribosomal chromatin in *X. laevis* erythrocytes. Isolated erythrocyte nuclei were photoreacted with psoralen, and the extent of cross-linking in the different rDNA regions was analyzed exactly as described in the legend to Fig. 2. Numbers show size in kilobases.

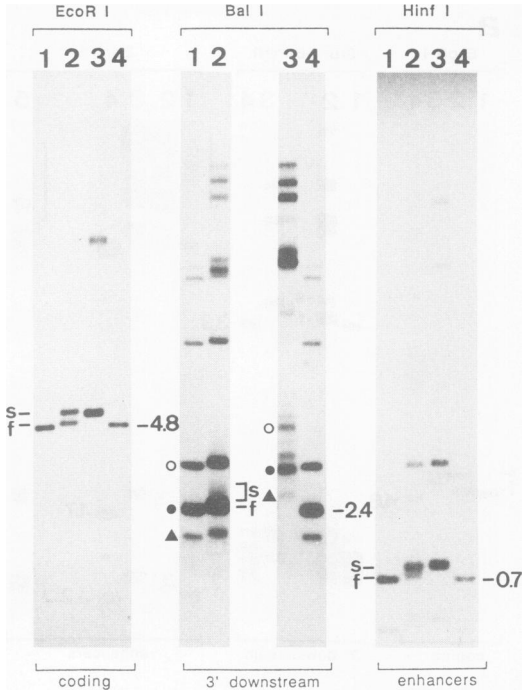

FIG. 4. Psoralen accessibility of *X. borealis* ribosomal chromatin in tissue culture cells. Isolated nuclei of the *X. borealis* cell line were photoreacted with psoralen, and the purified DNA was digested with the restriction enzymes indicated, separated on agarose gels, blotted, and hybridized with probes BB (coding), 800 (3′ downstream), and 988 (enhancers) (Fig. 1). The agarose gels were 1, 1.5, and 1.8%, respectively. Lanes 1 to 4 are the same as in Fig. 2 except lane 3 of the *Bal*I digest, which shows DNA that has been first purified and then extensively cross-linked. The three symbols in the *Bal*I restriction pattern point to length heterogeneity in the 3′ downstream region (the extra bands between the symbols in lane 3 are partial digestion products). Numbers show size in kilobases.

corresponding to partial digests were not analyzed in detail). On the other hand, the same sequences show a good cross-linkability when the DNA is first deproteinized and then photoreacted with psoralen (Fig. 4, 3′ downstream, lane 3). These results indicate that the majority of these 3′ downstream spacer sequences are packaged into a chromatin structure that protects them from being highly cross-linked.

It has been shown that in this particular *X. borealis* cell line, the spacer promoters are not active (21). Despite the fact that the enhancer sequences are not transcribed, the 0.7-kb *Hin*fI fragment containing the gene promoter and the first spacer promoter-enhancer block shows a cross-linking pattern similar to that of the *Eco*RI coding fragment, indicating the presence of the two distinct types of chromatin (compare the respective gel panels in Fig. 4). The same result is obtained by analyzing the 1.2-kb *Bal*I-*Stu*I fragment spanning both spacer promoter-enhancer blocks (Fig. 5a, enhancer). By comparing the ratio between the intensities of the f and s bands, it seems that there are slightly more enhancer regions in the open chromatin structure (i.e., highly accessible to psoralen; see s band in Fig. 4a, enhancers, lane 2) than active genes (s band in Fig. 4a, coding, lane 2). In this context, one could ask whether enhancers and coding regions with similar psoralen accessibilities are adjacent or randomly distributed along the rDNA locus. If they are random, we should expect to find some enhancer regions in an open structure which are followed by inactive genes that are packaged in nucleosomes and vice versa. To study the structural link in situ between active and inactive rRNA genes and their flanking enhancer sequences, we analyzed the cross-linking pattern of the 2.4-kb *Bal*I-*Pst*I fragment containing the same amount of enhancer and coding sequences (Fig. 1 and 5b). We reasoned that if an inactive gene packaged in nucleosomes is preceded by an open, fully accessible enhancer region (or if an active gene is preceded by a nucleosomal enhancer region), this overlapping fragment would be half heavily and half slightly cross-linked and would show an intermediate electrophoretic mobility (i.e., between the f and s band; Fig. 5b, enhancers/5′). When the same fragment isolated from nuclei photoreacted under native conditions was analyzed in the gel retardation assay, only the two distinct f and s bands were seen (Fig. 5a, enhancer/5′, lane 2). The mobility shift of the s band is identical to that of the heparin-treated sample in lane 3, indicating that it represents DNA fragments which have been extensively cross-linked. These results indicate that in the majority of the rDNA repeats, adjacent enhancer and coding regions have a similar chromatin structure which can be of two distinct types, either slightly or fully accessible to psoralen.

To confirm that such an intermediate band could be detected, we analyzed the *Eco*RI fragment containing the entire ribosomal spacer and a portion of coding sequences (Fig. 5b). Since in *X. borealis* most of the 3′ downstream spacer region is nucleosomal and the enhancer and coding sequences show a considerable proportion of s-band DNA,

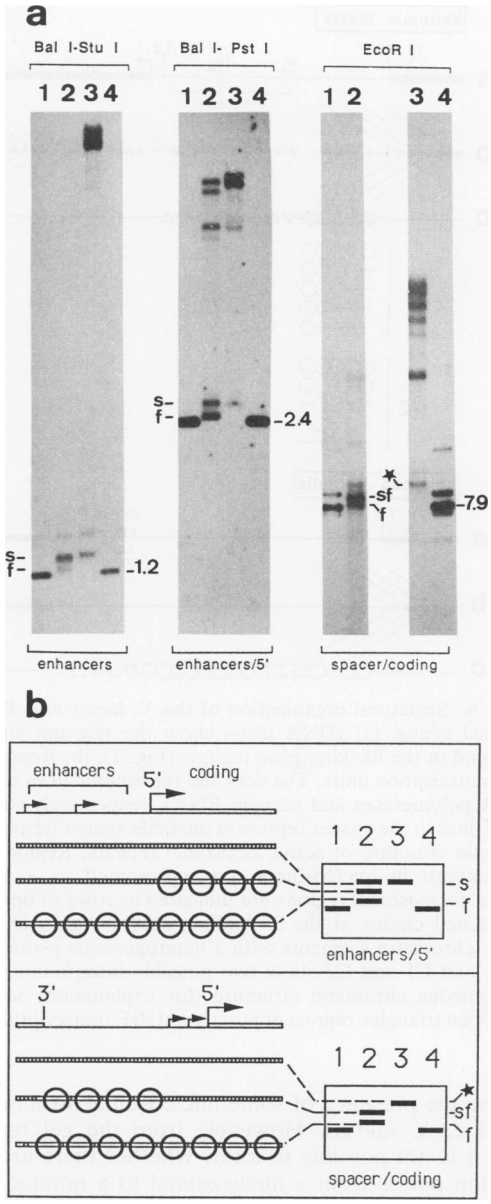

FIG. 5. (a) Psoralen accessibility of *X. borealis* ribosomal chromatin segments containing spacer sequences and portions of the coding region. The extent of cross-linking was analyzed as described in the legend to Fig. 4, using 1.5% gels (for enhancers and enhancers/5') and a 1% gel (for spacer/coding) that were blotted and hybridized with probe 988 (Fig. 1). In the *Eco*RI digest, the star points to the 7.9-kb fragment isolated from nuclei cross-linked in the presence of heparin. (b) Schematic drawing representing the possible results in panel a from the overlapping fragments enhancers/5' and spacer/coding. Symbols are as in Fig. 2b; for interpretation, see text.

there must be *Eco*RI fragments containing a nucleosomal 3'-flanking DNA followed by a coding region in an open chromatin structure (Fig. 5b). The analysis of such a hybrid molecule shows in fact the presence of an intermediate band (sf in Fig. 5a, spacer/coding, lane 2) with an electrophoretic mobility between those of the f band and the heparin-treated sample.

DISCUSSION

Chromatin structure of the rRNA coding region indicates the presence of active and inactive gene copies. Most of the biochemical probes that have been used to study the chromatin structure of specific genes and their surrounding sequences are nucleases which mark accessible regions of DNA in chromatin by introducing cuts into the DNA. Structural information is then usually inferred from the analysis of the size of the digestion products, i.e., from the distance between the accessible sites. Since active and inactive rRNA genes might coexist in the same cell as has been clearly suggested from the distribution of topoisomerase I on rDNA (30), nuclease digestion experiments would produce an indistinguishable mixture of digestion products from both types of genes, which would not allow one to make any definitive statement about their distinct chromatin structures. In this context, the psoralen cross-linking technique in combination with the gel retardation assay provides a useful method to distinguish between active and inactive ribosomal chromatin (5). One of the advantages of psoralen with respect to nucleases is that it can mark accessible sites in chromatin by binding covalently to the DNA without degrading it. The extent of psoralen cross-linking of the total population of a specific DNA restriction fragment can then be analyzed in a native agarose gel in which fragments with different extents of photoreaction will be separated because of their different electrophoretic mobilities.

Using this technique, we showed that in both *Xenopus* cell lines analyzed there are in fact two distinct types of rRNA precursor coding regions which are characterized by two different chromatin structures. The first type is more accessible to psoralen, and the resulting highly cross-linked restriction fragments migrate slower in the agarose gel (s band), whereas the other type is protected from being heavily cross-linked and the corresponding fragments migrate faster (f band). These results confirm recent findings obtained in a similar study done by our group on mouse rRNA genes in which several lines of evidence indicated that these two distinct types of coding regions derive from active and inactive rRNA gene copies, respectively (5). By treating isolated photoreacted DNA with exonuclease, which stops at the cross-link sites (47), it has been shown that f-band DNA contains psoralen cross-links about every 200 bp, similar to DNA isolated from photoreacted inactive bulk chromatin, whereas s-band DNA is randomly cross-linked and has some nascent RNAs cross-linked to it, indicating that it has originated from transcribing genes (5). In this study, by cross-linking radioactive RNA to the DNA template, we confirmed the presence of nascent RNA in the s band. We were also able to confirm the peculiar cross-linking pattern of the f and s bands by eluting the respective DNA from the gel and analyzing it in the electron microscope after having purified the rDNA in a cesium chloride gradient (data not shown). The fact that in the transcriptionally silent erythrocytes only the slightly retarded f band is seen confirms that this DNA originates from inactive genes which are packaged in nucleosomes, as has been previously suggested from micrococcal nuclease (46) and DNase I (6) digestion experiments. On the other hand, cross-linking of stage IV oocytes in which virtually all the rRNA genes are thought to be active (29) revealed only the retarded s band, suggesting that it contains in fact transcriptionally active DNA (data not shown).

The higher extent of cross-linking of the coding region of active genes indicates the absence of nucleosomes and is in

agreement with similar studies done by our group on active *Dictyostelium* (43), *Physarum* (24), mouse (5), and human and yeast (unpublished data) rRNA genes. Micrococcal nuclease digestion patterns of *X. laevis* ribosomal chromatin in different cell types have always been consistent with the presence of a considerable fraction of nucleosomes in the coding regions (38, 39, 46), which in the light of our results can be interpreted as being released from the transcriptionally inactive sequences present within the mixed population. Although s-band DNA shows an extent of cross-linking similar to that obtained after removal of the histones with heparin, we cannot rule out the presence of histones on the coding region of the active gene copies. In fact, we have shown that in H1-depleted chromatin at very low ionic strength and pH 10, the DNA is permeable to psoralen (4). An underlying nucleoprotein structure in the active amplified rRNA genes in *X. laevis* oocytes has been recently suggested from the distinct pattern of topoisomerase I binding to the transcribing DNA (8). However, if all or part of the set of histones is present, their interactions with the DNA must be different from those in normal nucleosomes in order to allow extensive access of psoralen to the histone-complexed DNA.

Different chromatin structures in *X. laevis* and *X. borealis* 3′-flanking spacer regions correlate with the functional difference of the 3′ ends of the genes. The original finding which suggested that in *X. laevis* the RNA polymerase I does not terminate at the 3′ end of the rRNA precursor coding region was the detection of highly unstable transcripts complementary to almost the entire ribosomal spacer (18, 19). However, the fact that in the electron microscope the intergenic spacer generally (but not always, e.g., see reference 28) appears free of both polymerase and nascent transcripts does not indicate high levels of spacer transcription (for a review, see reference 37). In contrast, as in almost all other eukaryotes, the related frog species *X. borealis* seems to have true termination sites at the 3′ end of the rRNA genes which prevent polymerases from continuing into the downstream spacer (21).

From psoralen cross-linking experiments in several different eukaryotes, it appears that RNA polymerase I elongation correlates with the absence of nucleosomes (5, 24, 43; this study), whereas most of the intergenic spacer sequences are nucleosomal. Therefore, it is likely that transcription across the *X. laevis* spacer is somehow reflected in a peculiar chromatin structure, whereas one might expect to find unperturbed nucleosomes in the corresponding *X. borealis* sequences. In the present study, we showed that in fact the 3′-flanking ribosomal spacer regions of *X. borealis* are packaged into a chromatin structure resistant to psoralen cross-linking, which is compatible with these sequences not being transcribed and organized in nucleosomes (Fig. 6b and c, *X. borealis*). In contrast, the corresponding fragments from the *X. laevis* tissue culture cells show a different cross-linking pattern in the gel retardation assay. Most of these sequences appear also to be packaged in nucleosomes, whereas a minor proportion are more accessible to psoralen but do not show a distinct and sharp retarded band, indicating a heterogeneous chromatin structure. On the basis of the results obtained with the *X. laevis* coding regions, we interpret these two populations as representing spacer fragments downstream of inactive and active rRNA genes, respectively. The absence of a well-defined s band representing heavily cross-linked nonnucleosomal DNA could be an indication that not all the RNA polymerases transcribing the upstream gene traverse these 3′-flanking spacer sequences,

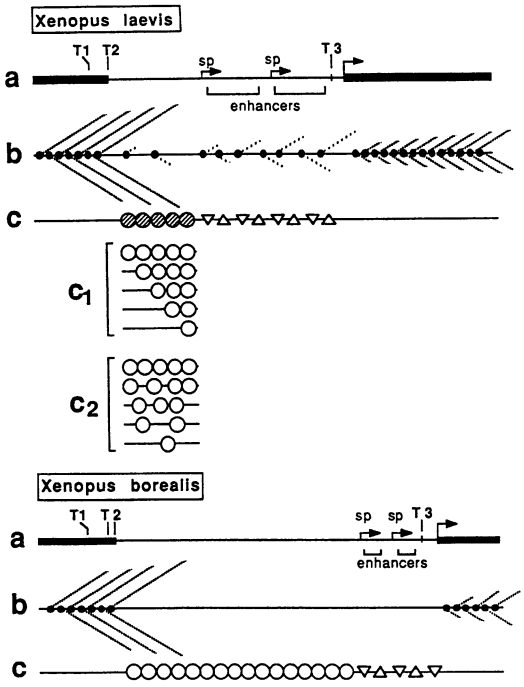

FIG. 6. Structural organization of the *X. laevis* and *X. borealis* ribosomal genes. (a) rDNA maps show the relevant sites in the spacer and in the flanking gene regions (Fig. 1). (b) Representation of the transcription units. The dots and the oblique lines correspond to RNA polymerases and nascent RNAs, respectively (the dashed oblique lines in the spacer represent unstable spacer transcripts). (c) Chromatin structure of active ribosomal repeats. Regions that are not packaged in nucleosomes are represented as a solid line, whereas nucleosomal regions are indicated as rows of open circles. The hatched circles at the 3′-flanking spacer region in *X. laevis* indicate chromatin segments with a heterogeneous psoralen accessibility, and C1 and C2 show two possible interpretations of this heterogeneous chromatin structure (for explanation, see Discussion). Open triangles represent putative xUBF transcription factors.

allowing the presence of some nucleosomal structures (Fig. 6b and c, *X. laevis*). However, from the gel retardation assay, it is not possible to know whether there are distinct transition points from a nucleosomal to a nonnucleosomal structure at different sites on different molecules or whether the heterogeneous chromatin structure is present along the entire 3′ downstream region, as represented speculatively in Fig. 6, C1 and C2, respectively. In an attempt to precisely localize the nucleosomes on single ribosomal spacers flanking active genes, we purified rDNA from psoralen cross-linked *X. laevis* tissue culture cells and spread it for electron microscopy under denaturing conditions. By this method, nucleosomal DNA can easily be recognized as single-stranded bubbles of about 150 nucleotides (3, 13, 42). Unfortunately, since some single-stranded bubbles of similar size were also present in spacers that were photoreacted as purified cloned DNA, it was not possible to identify unambiguously the nucleosomal bubbles (data not shown). The faint smear of the *X. borealis* 3′-flanking fragments showing a slightly higher psoralen accessibility could also indicate the presence of very few polymerases leaking into the downstream spacer and perturbing nucleosomal structures.

Since previous electron microscopic studies from our laboratory (16) and from others (40) have suggested that in *X. laevis* oocytes the ribosomal spacers are free of nucleo-

somes, we decided to apply the psoralen technique to the same system. In contrast to the tissue culture cells, the spacer sequences flanking the 3′ ends of active oocyte genes are fully accessible to psoralen, indicating the complete absence of nucleosomes. These results suggest that the extrachromosomal amplified rRNA genes in *X. laevis* oocytes might represent a special case (a detailed study on the oocyte genes will be published elsewhere).

Active rRNA genes are preceded by a nonnucleosomal enhancer region. As for the rRNA coding regions, we showed that in both *Xenopus* cell lines there are two different types of enhancer sequences which are characterized by two distinct chromatin structures. In the gel retardation assay, enhancer fragments are resolved in the characteristic f and s bands, indicating the presence of a nucleosomal and a nonnucleosomal fraction, respectively. A recent study using UV-laser irradiation to cross-link proteins to DNA has suggested the presence of histones in the ribosomal enhancer region in *X. laevis* embryonic cells (10). Since we showed that these sequences may be packaged in two different structures, it is not excluded that in this other study (10) mainly the enhancers of inactive gene copies were recorded.

The analysis of overlapping fragments containing both *X. borealis* enhancers and coding sequences indicated that each active rRNA gene is preceded by a nonnucleosomal enhancer region, whereas inactive genes are flanked by sequences that are packaged in nucleosomes. What are the mechanisms responsible for keeping a fraction of the enhancer sequences free of nucleosomes? Since in the *X. borealis* cell line the spacer promoters are inactive and since there is no spacer transcription coming from the upstream genes (21), we postulate factors other than the polymerase itself that can exclude nucleosomes from the enhancers. It is reasonable to propose the transcription factor xUBF as one of the possible candidates since it has been shown to bind in vitro to both promoter and enhancer sequences (32).

In the *X. laevis* cell line, transcription from the spacer promoters might also contribute to maintain the enhancer sequences in an open chromatin structure. Although spacer length heterogeneities in the enhancer region didn't permit the analysis of overlapping fragments, it is reasonable to propose, in analogy to the results from the related frog species, that active *X. laevis* genes are also preceded by a nonnucleosomal enhancer region. However, since the cross-linking pattern of the enhancer fragments seems to indicate more enhancer regions in the open chromatin structure than active genes, we cannot exclude the presence of active spacer promoter-enhancer transcription units that are not linked to active rRNA genes.

A series of transcriptional studies seem to favor a model in which the polymerase I enhancers act before the onset of transcription by facilitating the establishment of the initiation complex at the gene promoter (17, 31, 34). The enhancer repeats may in fact bind the transcription factor xUBF, thereby increasing its concentration in the vicinity of the promoter, where this factor has been recently shown to be itself a component of the stable initiation complex (26). In analogy to this model and to our results, we can view the active rRNA gene copies as the subset of genes that have managed to have their flanking enhancer sequences occupied by xUBF (and possibly other factors) in contrast to the inactive copies in which all these regulatory sequences have been packaged in nucleosomes. Recent findings from our laboratory have indicated that a cell of a defined type is characterized by a distinct ratio of active and inactive rRNA gene copies which is maintained constant throughout cell division (5, 48). In this context, it is very possible that one of the functions of the polymerase I enhancers is to maintain active the same specific subset of genes. Furthermore, we have preliminary results indicating that the active rRNA gene copies may be organized in clusters. This situation might help to keep the two populations of active and inactive rRNA genes physically separated and to concentrate the transcription factors only around active genes, thereby favoring the stable propagation of these two distinct activity states.

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