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Morphology of Nucleolar Chromatin in Electron Microscopic Spread Preparations

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I. Introduction

The structure of nucleolar chromatin at different stages of transcriptional activity, as visualized in electron microscopic spread preparations, has been reviewed in this series (Franke *et al.*, 1979; McKnight *et al.*, 1979) and elsewhere (Scheer and Franke, 1976; Franke and Scheer, 1978; Franke *et al.*, 1978a,b, 1980; Foe, 1978; McKnight *et al.*, 1978). Therefore, in this chapter, we shall recapitulate only briefly the appearance of rRNA genes and their interspersed nontranscribed spacer regions in "Miller spread preparations," i.e., after dispersal in buffers of very low ionic strength at slightly alkaline pH values. There is now general agreement that actively transcribed rRNA genes from a variety of species, when prepared for electron microscopy under such low salt conditions, exist in a nonnucleosomal configuration. In the first part of this chapter, we shall summarize the available evidence that this apparent absence of nucleosomes reflects the full or almost full extension of the intragene chromatin equivalent to the length of the B form DNA. Then, we shall take up a more controversial issue: Is the so-called nontranscribed spacer also largely extended or compacted into nucleosomes or even in higher-order (supranucleosomal) chromatin structures? As we shall show, there are two main reasons for the present disagreement on the presence or absence of nucleosomes in the spacer regions. The first reason is that spacer chromatin between tandemly arranged rRNA genes often exhibit a variable number of irregularly shaped particles of nucleosomal size which have been interpreted by various authors as evidence for an arrangement in nucleosomal particles. However, as we shall demonstrate here, the morphology of spacer chromatin can be experimentally altered from a beaded to a smooth appearance simply by raising the ionic strength of the dispersal medium to 1 m*M* alkali salt or above, so that the occurrence of "spacer particles" does not necessarily correspond to foreshortening of the DNA, as is typical of nucleosomal chromatin. Moreover, spacer particles often can still be seen after high salt extraction of nucleolar chromatin, followed by dispersal in buffer of very low ionic strength. The second reason for the present controversy is the observation that the intergene region of the extrachromosomal nucleolar chromatin of *Physarum polycephalum* displays a beaded organization and is contracted by a factor of about 2, relative to B form DNA (Grainger and Ogle, 1978). In fact, as we show below, the structural organization of spacer chromatin between rRNA genes of the same polarity on the one hand,

and the intergene chromatin of palindromically arranged rRNA genes on the other hand, is basically different under almost identical preparative conditions. One has to bear in mind that both situations are not equivalent and therefore are not directly comparable.

Then we shall address the question of whether the extended conformation of nucleolar chromatin reflects a higher sensitivity of nucleosomal structures to forces generated during the low salt spreading, or whether it represents a persistently altered conformation different from the bulk of transcriptionally inactive chromatin arranged in nucleosomes. To clarify this issue, we have mixed transcribed nucleolar chromatin of amphibian oocytes with inactive chromatin of avian erythrocytes and compared the structural appearance of both types of chromatin prepared simultaneously in buffers of various ionic strengths and pH values. We shall demonstrate that the extended conformation of nucleolar chromatin is maintained even under conditions that preserve the supranucleosomal packaging structures of erythrocyte chromatin. Finally, we consider whether electron microscopic spread preparations of transcriptionally active genes can be combined with *in situ* hybridization techniques to identify their specific genetic content. Using the rRNA genes as a model system, this approach is technically feasible and should eventually lead to a method of identifying unequivocally the transcripts and transcriptional units of specific genes.

II. Comments on the Chromatin Spreading Technique

The chromatin spreads were prepared essentially as described first by Miller and Beatty (1969a,b,c,d) and subsequently outlined in more detail (Miller and Bakken, 1972; Miller and Hamkalo, 1972). To examine the effects of the different steps of the spreading procedure on the morphology of chromatin and transcriptional units, we have made numerous spreads in parallel under various ionic strengths and fixation conditions, using various modifications of the original protocol.

A. pH and Ionic Strength

The appearance of nucleolar chromatin dispersed in buffers of various ionic strengths and pH values is discussed in detail below. In media of very low ionic strength (0.1–1.0 m*M* borate buffer), the "beads-on-a-string" aspect of transcriptionally inactive chromatin is

indistinguishable in the pH range from 7.4 to 9.0. When inactive chromatin is dispersed in the presence of 5–10 m*M* monovalent cations and about 0.1 m*M* divalent cations, the degree of compaction of the primary nucleofilament into globular higher-order structures appears to be higher at pH 7.4 than at pH 9.0 (for detailed discussions, see, e.g., Zentgraf *et al.*, 1980a,b, 1981; Labhart *et al.*, 1981).

B. Buffers

Sodium borate or Tris buffer in the dispersal medium have given identical results. Tris buffer, however, should not be included in the formaldehyde-containing sucrose solution because of the formation of Schiff's bases.

C. Fixative

Routinely, we have used 1% formaldehyde freshly prepared from paraformaldehyde. Replacement by 1% glutaraldehyde or omission of the fixative does not influence to any significant extent the appearance of nucleolar chromatin.

D. Detergent (Photoflo) Treatment of the Specimen after Centrifugation

This step can be avoided by washing the grid after centrifugation in distilled water, followed by negative staining with, e.g., 1% uranyl acetate (Woodcock *et al.*, 1976a; Scheer, 1980). The morphology of nucleolar chromatin is unaltered.

E. Metal Shadowing

The contrast of all structures is greatly enhanced by additional metal shadowing of the positively stained preparations (Angelier and Lacroix, 1975; Scheer *et al.*, 1976). We routinely rotary-shadow our preparations using Pt/Pd (80:20) at an angle of about 7°. Provided the metal layer is not too thick, the appearance of chromatin is identical to that seen in preparations only positively stained.

We want to emphasize that all chromatin preparations presented in this chapter, with the exception of that in Fig. 13, have been dispersed without the addition of detergent to the spreading medium. This is especially important in view of the possibility that structural changes of chromatin can be induced by ionic detergents such as

Sarkosyl or Joy, which are known to remove histones from a certain concentration on (Scheer, 1978). However, very low concentrations of these detergents may help in the dispersion of chromatin without affecting its basic structural organization (Glätzer, 1975; Trendelenburg *et al.*, 1976; Trendelenburg and Gurdon, 1978; Trendelenburg and McKinnell, 1979; Trendelenburg, 1981; Scheer *et al.*, 1981).

III. Transcribed Nucleolar Chromatin at Low Ionic Strength and pH 9

Visualization of transcriptionally active rRNA genes under the electron microscope requires dispersal of nucleolar chromatin. This is achieved, as originally shown by Miller and Beatty (1969a,b,c,d), by transferring nucleolar material to hypotonic buffer at a slightly alkaline pH (ca. 0.1 m*M* borate buffer, pH 9.0). Active rRNA genes, for instance, from amphibian oocytes (Fig. 1), can then be readily identified by their (1) tandem arrangement, (2) relatively regular contour length (in the case of amphibian species, ranging from 2.2 to 2.6 μm), (3) high packing density of transcriptional complexes, (4) the characteristic "terminal knobs" of the nascent RNP fibrils, and (5) the separation of adjacent genes by normally nontranscribed spacer regions. Similar morphological properties exhibit the pre-rRNA transcription units of many other species (see the review articles mentioned in Section I). The amplified palindromically arranged rRNA genes occurring in several lower eukaryotes such as *Tetrahymena*, *Dictyostelium*, and *Physarum*, however, are different because they are not tandemly arranged. Their morphology will be considered separately in Section VII.

Figure 1 presents a survey micrograph showing hundreds of maximally active rRNA genes isolated from a *Pleurodeles* oocyte. In each transcription unit (or matrix unit, i.e., the morphological equivalent of a transcribed rRNA gene), the nascent RNP fibrils are arranged in a length gradient which allows the precise localization of the initiation and termination sites of transcription. After brief swelling of the nucleolar chromatin in low salt buffer, most of the rRNA genes are closely juxtaposed, forming large aggregates (Figs. 1, 2a), probably reflecting their *in vivo* concentration in one or a few centrally located dense regions ("cores") of the amplified nucleoli (Macgregor, 1972; Miller and Beatty, 1969d; Franke *et al.*, 1981; Scheer *et al.*, 1982). At higher magnification, the maximal packing density of the RNA polymerase particles usually creates

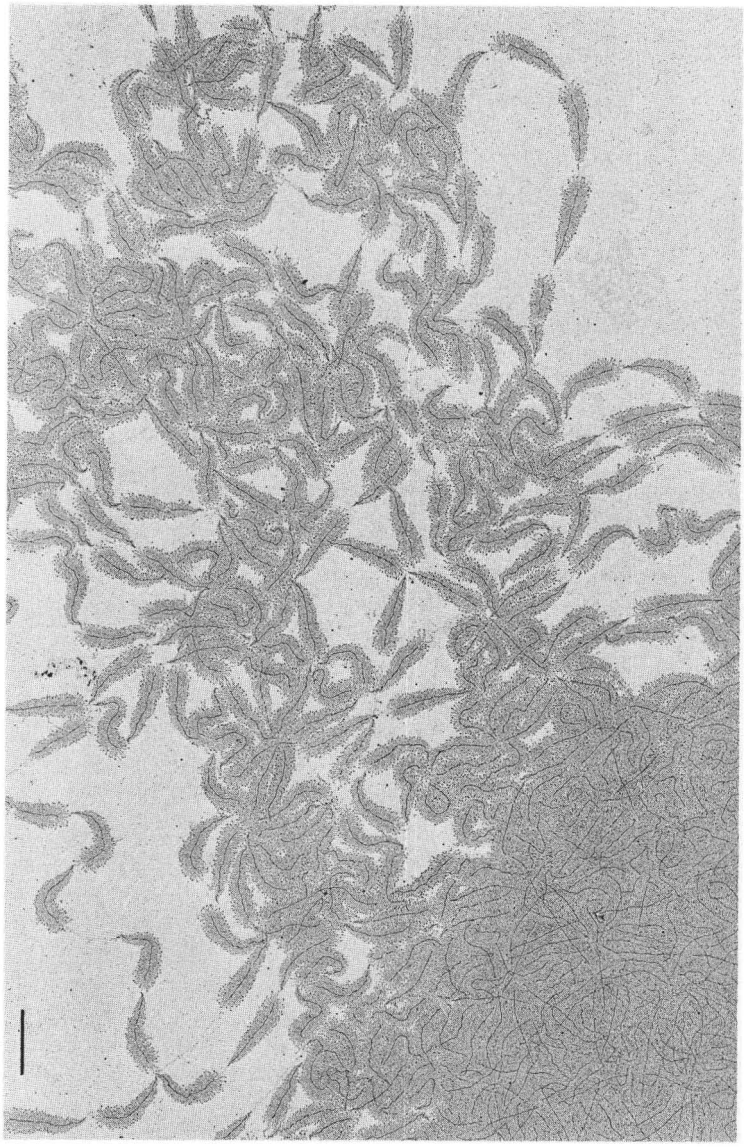


Fig. 1 Survey electron micrograph of nucleolar chromatin from a growing *Pleurodeles waltlii* oocyte after brief dispersion in pH 9 water. The tandem arrangement of transcribed (fibril-covered) "matrix units" and fibril-free spacers is recognized in regions with a more extended arrangement of the nucleolar chromatin. Bar indicates 2 μm.

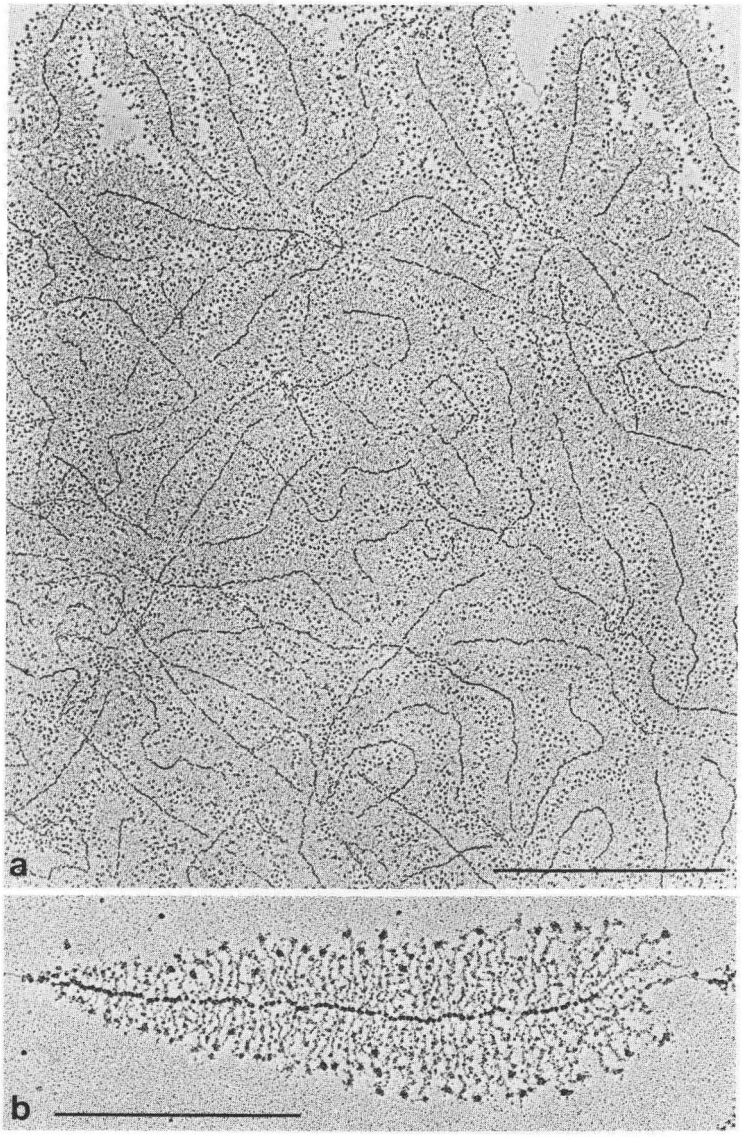


Fig. 2 Same preparations as in Fig. 1. Transcribed rRNA genes form compact aggregates in central parts of the spread nucleoli but can still be identified by the enhanced contrast of their axial intercepts (a) due to the close spacing of the RNA polymerase particles (b). Note the characteristic terminal knobs at the free ends of the lateral RNP fibrils. Bars indicate 2 μm (a) and 1 μm (b).

the impression of a continuous thick line forming the axis of the pre-rRNA transcription units (Figs. 2a,b) (Angelier and Lacroix, 1975). Therefore, the intragenic chromatin *sensu strictu* cannot be seen in such arrays (Fig. 2b). The lateral RNP fibrils reveal a somewhat knobby or beaded contour with a terminal knob of unknown nature, probably reflecting coiling up at their free 5′-ends. From comparisons of the lengths of individual RNP fibrils with the lengths of the specific chromatin stretches from which they are transcribed, it is clear that the RNA of the nascent transcripts is considerably foreshortened (by a factor of 5–12; Glätzer, 1975; Laird *et al.*, 1976; Foe *et al.*, 1976; Franke *et al.*, 1976a; Popenko *et al.*, 1981; Sommerville, 1981). By combining electron microscopic spreads with immunological localization techniques, it has recently also been possible to identify the first points of assembly of specific ribosomal proteins with the nascent RNA transcripts (Chooi and Leiby, 1981a,b). Such techniques should eventually allow a description of the topological distribution of all proteins along the nascent rRNA precursor. Of special interest is the question of which of the ribosomal proteins present in cytoplasmic ribosomes are already associated with the nascent RNA precursor and which proteins bind to the RNA regions that are discarded during the subsequent processing and maturation steps (for detailed discussion, see Kalthoff and Richter, 1982).

The spacers interspersed between the tandemly arranged rRNA genes reveal great length differences not only between different species, as illustrated in Figs. 3a and 3b, but also, though to a lesser extent, within a single cell nucleus (see, e.g., Spring *et al.*, 1976; Trendelenburg *et al.*, 1976; Scheer *et al.*, 1977; Franke *et al.*, 1979; Popenko *et al.*, 1981). These chromatin length differences appear to reflect length heterogeneity of the spacer DNA (reviewed in Long and Dawid, 1980) rather than differences in the degree of spreading (Scheer *et al.*, 1973). The term "nontranscribed" is somewhat misleading since in spread preparations of amphibian oocyte nucleoli, it is not uncommon to find individual spacers which are partly, sometimes almost entirely, covered by transcriptional units (Scheer *et al.*, 1973, 1977; Franke *et al.*, 1976a; Rungger and Crippa, 1977; Trendelenburg, 1981; Williams *et al.*, 1981). We have found similar spacer transcripts also in spread preparations of cultured *Xenopus laevis* kidney cells (Scheer, unpublished observations). Since the start regions of the spacer transcription units observed in oocytes of *Xenopus laevis* seem to correspond to the position of promoterlike sequences present in the spacer (Trendelenburg, 1981), it seems

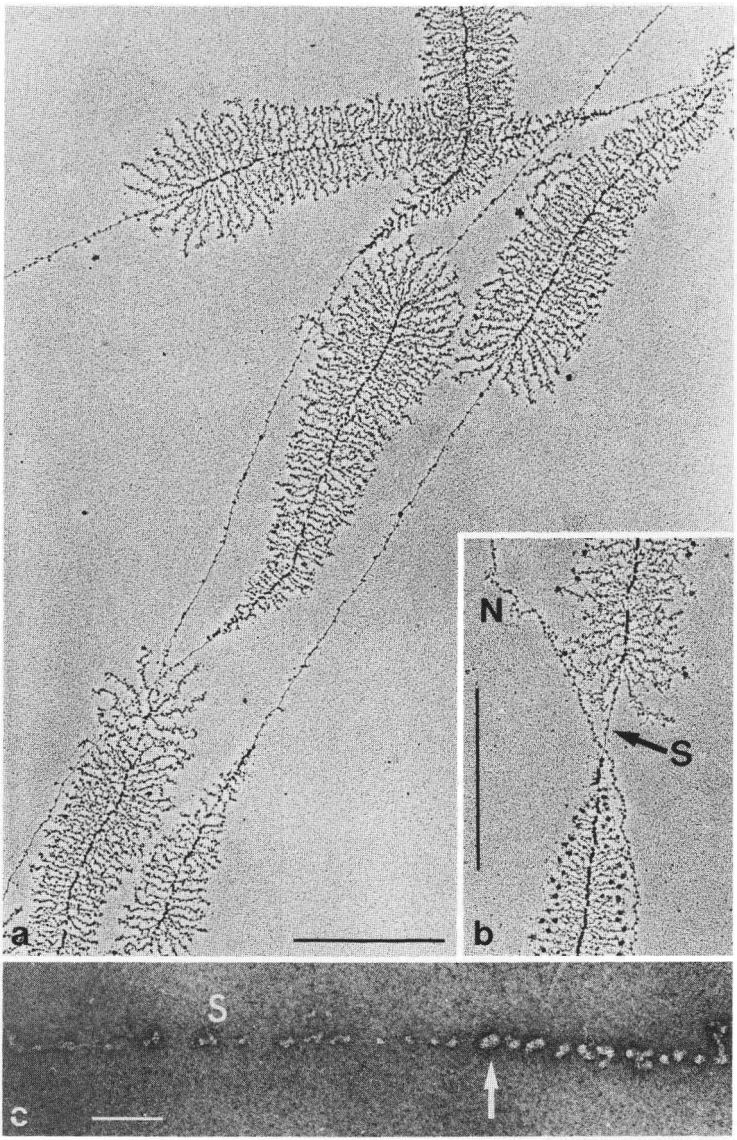


Fig. 3 Appearance of transcribed rRNA genes and interspersed spacer chromatin (S) as revealed after dispersal and spreading of nucleoli from oocytes of *Triturus helveticus* (a), *Pleurodeles waltlii* (b), and *T. cristatus* (c) in pH 9 water, followed by positive (a,b) or negative staining (c). Numerous irregularly shaped and distributed particles are associated with the spacer regions. Inactive chromatin with the characteristic beaded (nucleosomal) conformation (N) is clearly distinguished from the spacer chromatin (b). The arrow in (c) denotes the beginning, i.e., the first RNA polymerase, of a transcriptional unit. Note that the spacer of *Pleurodeles* is much shorter than that of *Triturus*. Bars indicate 1 μm (a,b) and 0.1 μm (c).

likely that such spacer transcripts in general reflect erroneous initiation events. However, at the moment, it cannot be ruled out that RNA molecules transcribed from spacer sequences are of special functional significance (see also Rungger and Crippa, 1977; Rungger *et al.*, 1979). In any event, the occurrence of transcripts in some spacers indicates that such regions, at least in certain species, are potentially transcribable.

The appearance of spacer chromatin is clearly different from that of the bulk of transcriptionally inactive chromatin. Whereas the latter has the regular beads-on-a-string appearance indicative of a nucleosomal organization (Olins and Olins, 1974; Oudet *et al.*, 1975; Woodcock *et al.*, 1976b), spacer chromatin is often, but not always, characterized by the occurrence of varying numbers of irregularly shaped and distributed particles that are generally slightly smaller than those of nucleosomes (Fig. 3a–c,6b). This difference can be shown in positively stained and metal-shadowed (Fig. 3a,b) and in negatively stained (Fig. 3c) spread preparations. A similar beaded appearance of spacer chromatin has also been reported in other species (Laird *et al.*, 1976; Woodcock *et al.*, 1976a; Foe, 1978; Reeder *et al.*, 1978; Puvion-Dutilleul and Bachellerie, 1979; Martin *et al.*, 1980; Greimers and Deltour, 1981; Popeńko *et al.*, 1981). The occurrence of such granular particles is confined to the spacer regions. This can be clearly demonstrated in situations in which the adjacent rRNA genes are not fully loaded with RNA polymerases, thus allowing identification of the gene chromatin proper (Fig. 4a–d). In stages of reduced rRNA synthesis of oocytes of urodelan amphibia (previtellogenic and full-grown oocytes), for example, the coverage of the rRNA genes by transcriptional complexes is drastically diminished, indicating that the expression of the rRNA genes is regulated at the level of transcription (Scheer *et al.*, 1976). A reduced number of lateral fibrils per gene results either in more or less extended "gaps," i.e., transcript-free regions within a matrix unit (Fig. 4a–c) or in an uniformly distributed, sparse population of lateral fibrils (Fig. 4d). Irrespective of the specific arrangement of the fibrils, the intragenic chromatin invariably appears as a thin, smooth axis and is clearly distinguishable from the spacer chromatin (Fig. 4a–d). Even when the start and the terminus of a gene are not covered by transcriptional complexes, the structural transition between smooth gene and beaded spacer chromatin is still recognized (Fig. 4c) (Woodcock *et al.*, 1976a; Foe, 1978; Scheer, 1980; see however Martin *et al.*, 1980). Taken together, these observations suggest that the architecture and probably also the biochemi-

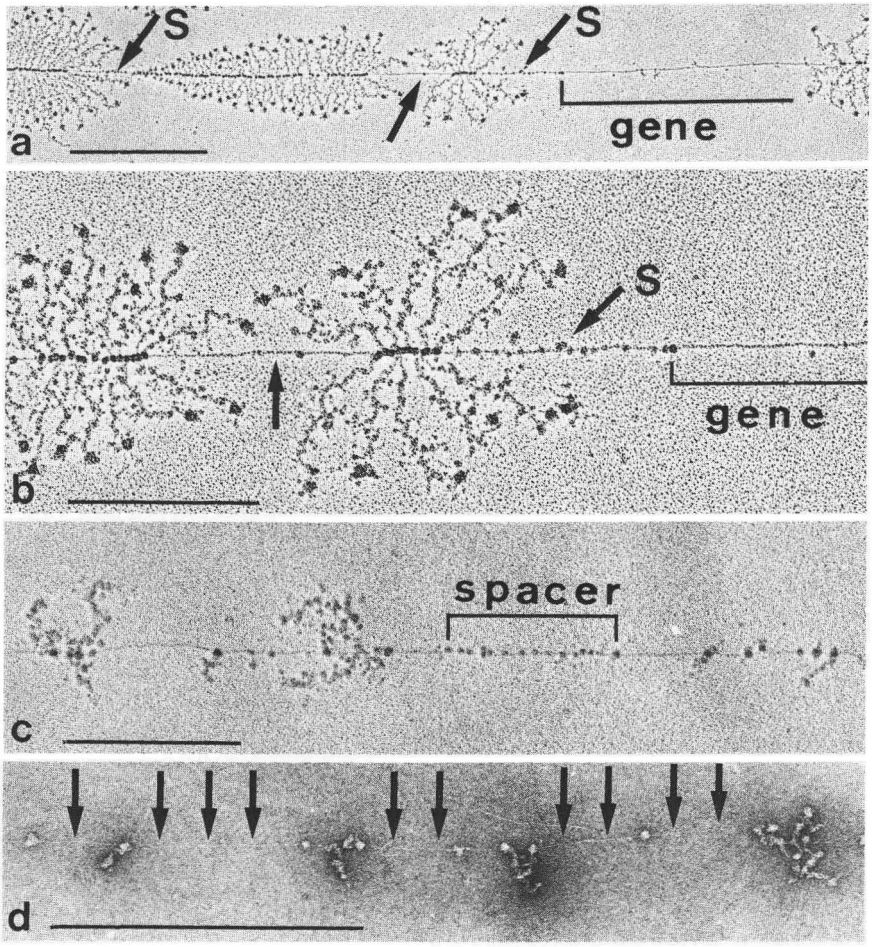


Fig. 4 Structural differences of spacer (S) and intragene chromatin as seen in spread preparations of nucleoli from oocytes of *Pleurodeles waltlii* (a–c) and *Triturus cristatus* (d). The nucleolar material was dispersed in pH 9 water and positively (a–c) or negatively (d) stained. Whereas the spacer intercepts are readily identified by their beaded appearance, the chromatin axis of the transcription units, visible in transcript-free regions, is uniformly smooth. Such gaps are located in central (arrows in a, b, and d) or proximal (brackets labeled "gene" in a and b) parts of the transcription units. (b) Higher magnification of a part of (a). Bars indicate 1 μm (a) and 0.5 μm (b–d).

cal composition of these two functionally different nucleolar chromatin regions are different. Thus, the thin, smooth contour of the intragene chromatin is maintained, in genes of reduced transcriptional activity, in those regions which at the very moment of prepa-

ration are not transcribed. This observation suggests that this specific configuration is not a direct consequence of the rapid succession of transcriptional events but rather reflects an intrinsic property of transcribed rRNA genes. The conclusion is in agreement with evidence provided by Foe (1978) for a change in the chromatin structure of rRNA genes of *Oncopeltus fasciatus* preceding transcriptional activation. How is this specific structural alteration established and maintained? This issue is related directly to the hitherto unresolved general problem of the regulation of gene expression at the chromatin level.

Several arguments clearly deny the possibility that the spacer particles described above represent nucleosomes.

1. When amplified nucleoli from *X. laevis* oocytes are extracted in a high salt buffer containing 1 *M* KCl and residual structures are pelleted and dispersed in a low ionic strength buffer, followed by spreading for electron microscopy, spacer particles are still visible (Fig. 5a). Since such extraction conditions effectively remove histones and high mobility group (HMG) proteins, these spacer particles are apparently formed by tightly bound, high salt-resistant non-histone and non-HMG proteins. It is also interesting to note, in this context, that the structure of the transcriptional complexes is also not significantly altered by the high salt treatment. Thus, not only the binding of the RNA polymerase I to the template is stable in 1 *M* KCl (see also Naito and Ishihama, 1975), but apparently also the association of at least some proteins with the nascent pre-rRNA, as judged from the preserved high electron density of the lateral fibrils (Fig. 5a).

2. When nucleolar chromatin from *Xenopus* or *Triturus* oocytes is spread in the presence of 1–20 m*M* NaCl, spacer particles are much less frequent and often appear to be totally absent (Fig. 5b,c) (Scheer, 1980). Under such conditions, spacer chromatin displays a smooth contour with a uniform thickness of 6–8 nm and resembles deproteinized DNA mounted under identical conditions, but it is clearly distinguished from adjacent transcriptionally inactive, beaded chromatin strands (Fig. 5c; Scheer, 1980). A possible explanation for this behavior of spacer chromatin at elevated ionic strength, which is in striking contrast to that of nucleosomal inactive chromatin, may be the stabilization of a uniform protein coat on the spacer DNA by the increased hydrophobic protein–protein interactions. The change observed in the morphology of the spacer regions at a monovalent cation concentration of about 1 m*M* may also explain why different authors have described spacer chromatin as

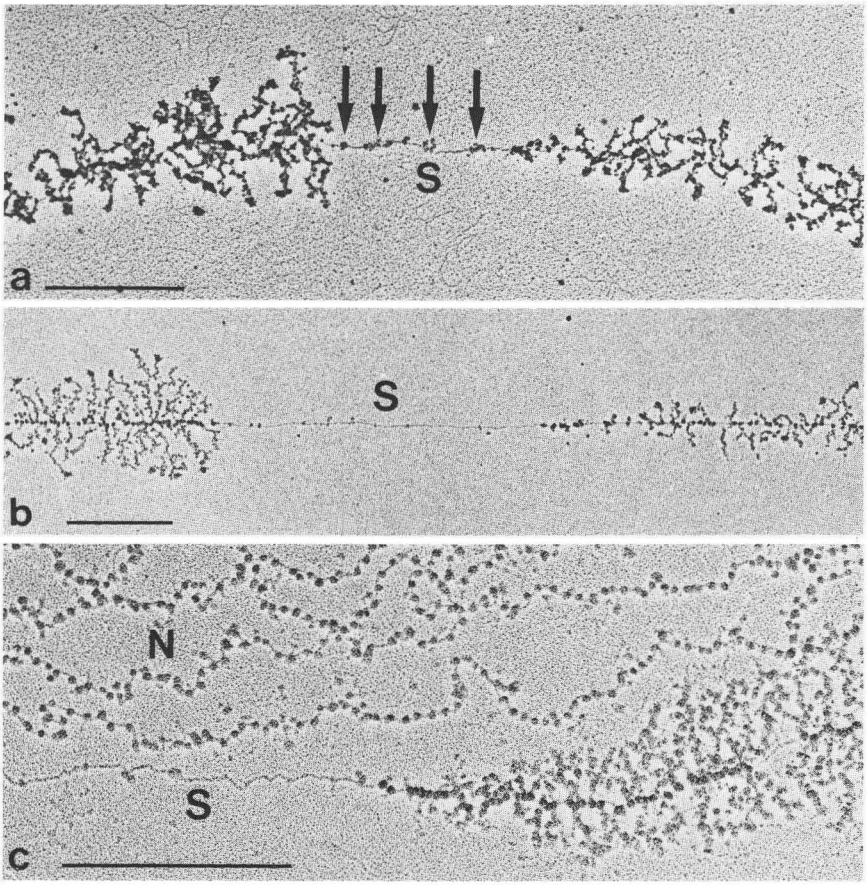


Fig. 5 Nucleolar chromatin of *Xenopus laevis* oocytes, extracted in high salt buffer and spread for electron microscopy in a low salt buffer (a). Nuclear envelopes, together with some tightly adhering peripheral nucleoli, were manually isolated from growing *Xenopus* oocytes in a buffer containing 25 m*M* NaCl, 75 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* Tris-HCl, pH 7.2 (for technical details, see Scheer, 1972), centrifuged, and resuspended in a large volume of 1 *M* KCl, 10 m*M* Tris-HCl, pH 7.4, for 30 min at 4°C. After centrifugation, the pellet was resuspended 3 times in 2 m*M* Tris-HCl, pH 7.0, and finally centrifuged through a cushion consisting of 1% formaldehyde and 0.1 *M* sucrose on a freshly glow-discharged, carbon-coated electron microscopic grid. Particles with irregular sizes and contours can still be identified on the spacer axis (arrows in a). After spreading of nontreated nucleolar chromatin in 1 m*M* borate buffer (pH 9.0) containing 5 m*M* (b) or 1 m*M* (c) NaCl, spacer chromatin appears uniformly smooth [preparations from oocytes of *Xenopus laevis* (b) and *Triturus cristatus* (c)]. Note the structural difference between inactive chromatin arranged in nucleosomes (N) and spacer chromatin (S in c). Bars indicate 0.5 μm.

either beaded or smooth, simply by working above or below this critical ionic strength (for references, see Jamrich *et al.*, 1979; Scheer, 1980).

3. Consistent with the absence of nucleosomes in transcribed nucleolar chromatin is the fair correspondence of the lengths of repeating units (gene plus spacer) in chromatin and in isolated rDNA (Franke *et al.*, 1978b; Trendelenburg *et al.*, 1976; Scheer *et al.*, 1977; Reeder *et al.*, 1978; Renkawitz *et al.*, 1979; Glätzer, 1979). This correspondence is also seen when one compares the contour length of amplified nucleolar chromatin rings of oocytes of the water beetle, *Dytiscus marginalis,* with the lengths of the corresponding isolated rDNA molecules (Fig. 6a,c) (Trendelenburg *et al.*, 1976). It is obvious that the actively transcribed circles, including the spacer regions, cannot be compacted into nucleosomes, since this would cause an apparent foreshortening of the rDNA by a factor of about 2 from the extended (B) conformation (Reeder *et al.*, 1978; Franke *et al.*, 1979; McKnight *et al.*, 1979).

IV. Transcribed Nucleolar Chromatin at Elevated Ionic Strength and pH 7.4

In discussing the structural organization of active nucleolar chromatin, a central question is whether its apparently nonnucleosomal, extended form observed in electron microscopic spread preparations is artificially induced or reflects a stable *in vivo* alteration from inactive chromatin. Although it is clear that nucleosomes are preserved in low salt buffers (in fact, they were first described using this technique; see Olins and Olins, 1974), it could be argued that nucleolar chromatin *in vivo* is compacted into nucleosomes which are altered in such a way that they are more susceptible to forces generated during the spreading process. Since higher-order structures of chromatin are unstable under very low salt conditions (for references, see Zentgraf *et al.*, 1980a,b, 1981), a possibly existing supranucleosomal packaging of nucleolar chromatin might be destroyed in such spread preparations. We have therefore dispersed nucleolar chromatin of amphibian oocytes in the presence of elevated salt concentrations at almost neutral pH, i.e., conditions that are known to stabilize nucleosomal particles (see, e.g., Gordon *et al.*, 1978; Dieterich *et al.*, 1979; Martinson *et al.*, 1979; Thoma *et al.*, 1979; Burch and Martinson, 1980) and to maintain higher-order

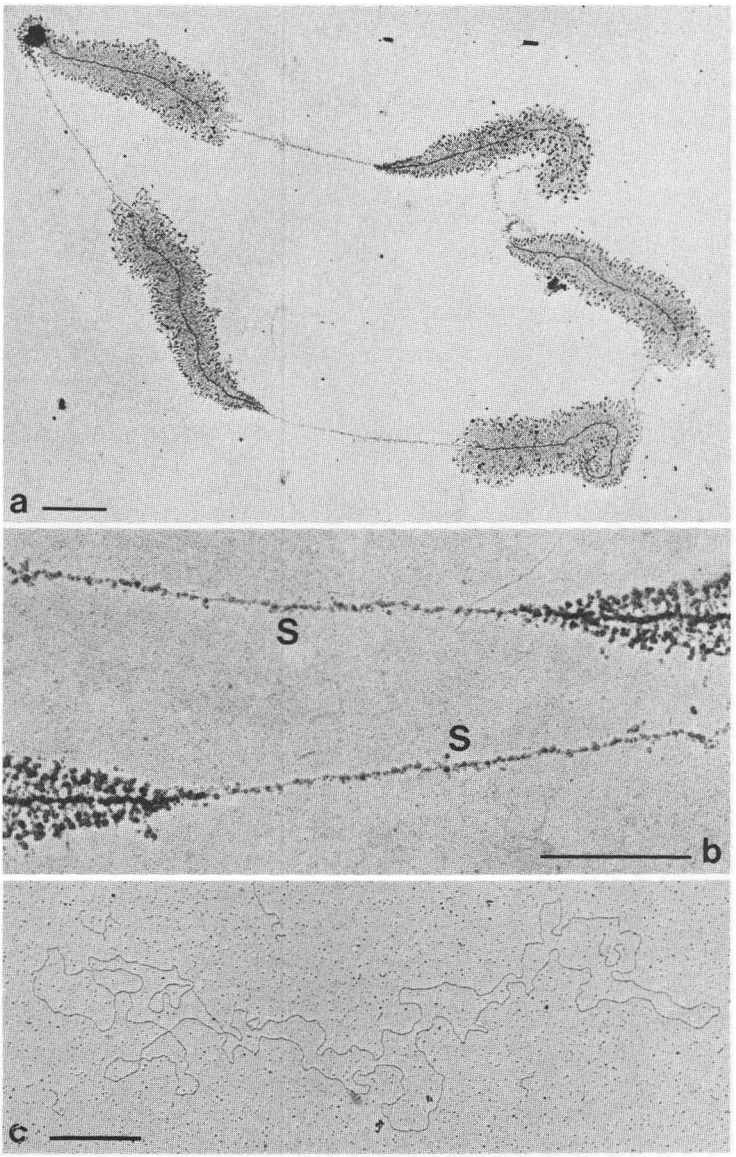


Fig. 6 Amplified nucleolar chromatin circle containing five repeating units from an oocyte of *Dytiscus marginalis* after spreading in pH 9 water (a). The spacers (S) have an irregularly beaded appearance (b). The contour length of the isolated rDNA circle shown in (c) (35 μm) corresponds to that of the chromatin ring of (a). For details, see Trendelenburg *et al.* (1976). Bars indicate 1 μm (a,c) and 0.5 μm (b).

chromatin structures (see, e.g., Zentgraf *et al.*, 1980a,b, 1981; for rDNA spacer regions, see Pruitt and Grainger, 1981). To monitor the conformation of transcriptionally inactive chromatin prepared under identical conditions, we have added chicken erythrocyte chromatin to the spreading medium containing the nucleolar genes. The two mixed chromatin samples are shown in the survey micrograph of Fig. 7 and at higher magnification in Fig. 8. The erythrocyte chromatin material is easily recognized as spheroidal masses

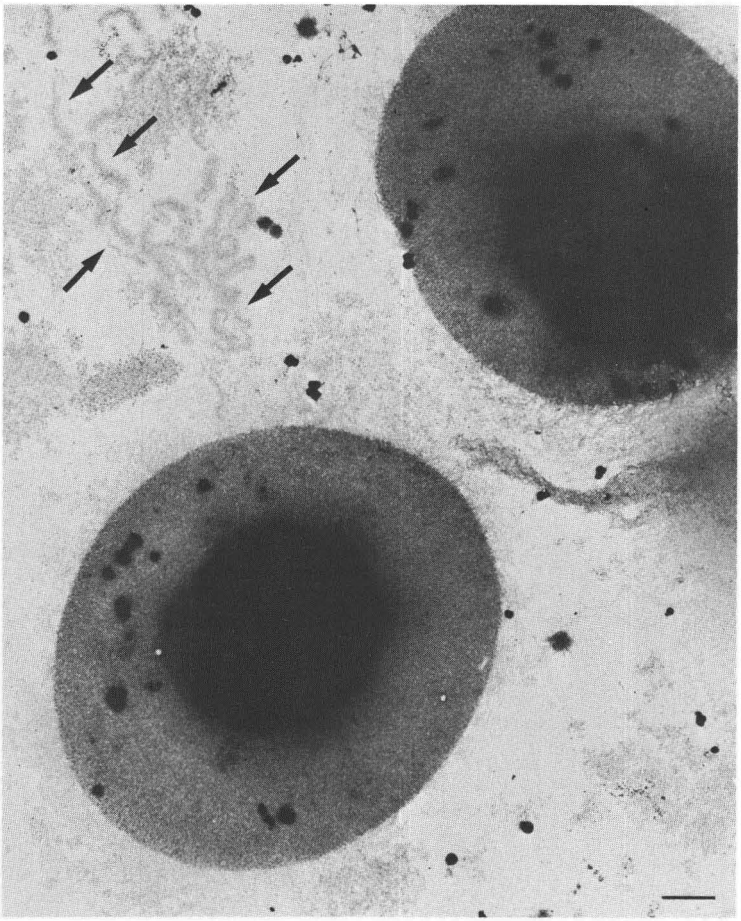


Fig. 7 Nucleolar chromatin from an oocyte of *Triturus helveticus* (denoted by arrows) mixed with chicken erythrocyte chromatin. The inactive erythrocyte chromatin is recognized as individual spheroidal aggregates surrounded by zones containing extended chromatin fibers. Bar indicates 2 μm.

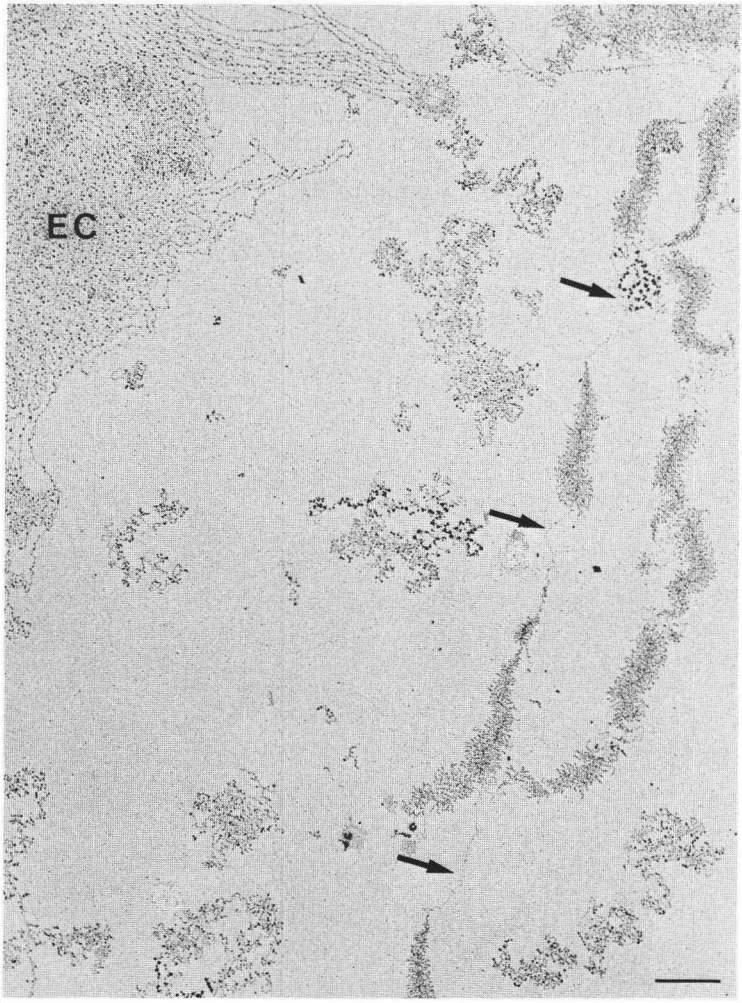


Fig. 8 Preparation similar to that shown in Fig. 7 (chromatin dispersion in 1 m*M* borate buffer, pH 9.0, containing 6 m*M* monovalent and 0.07 m*M* divalent cations). Avian erythrocyte chromatin (EC) is shown next to transcribed amphibian nucleolar chromatin (spacers are denoted by the arrows). The larger granules interspersed in the erythrocyte chromatin represent globular supranucleosomal units. Bar indicates 1 μm.

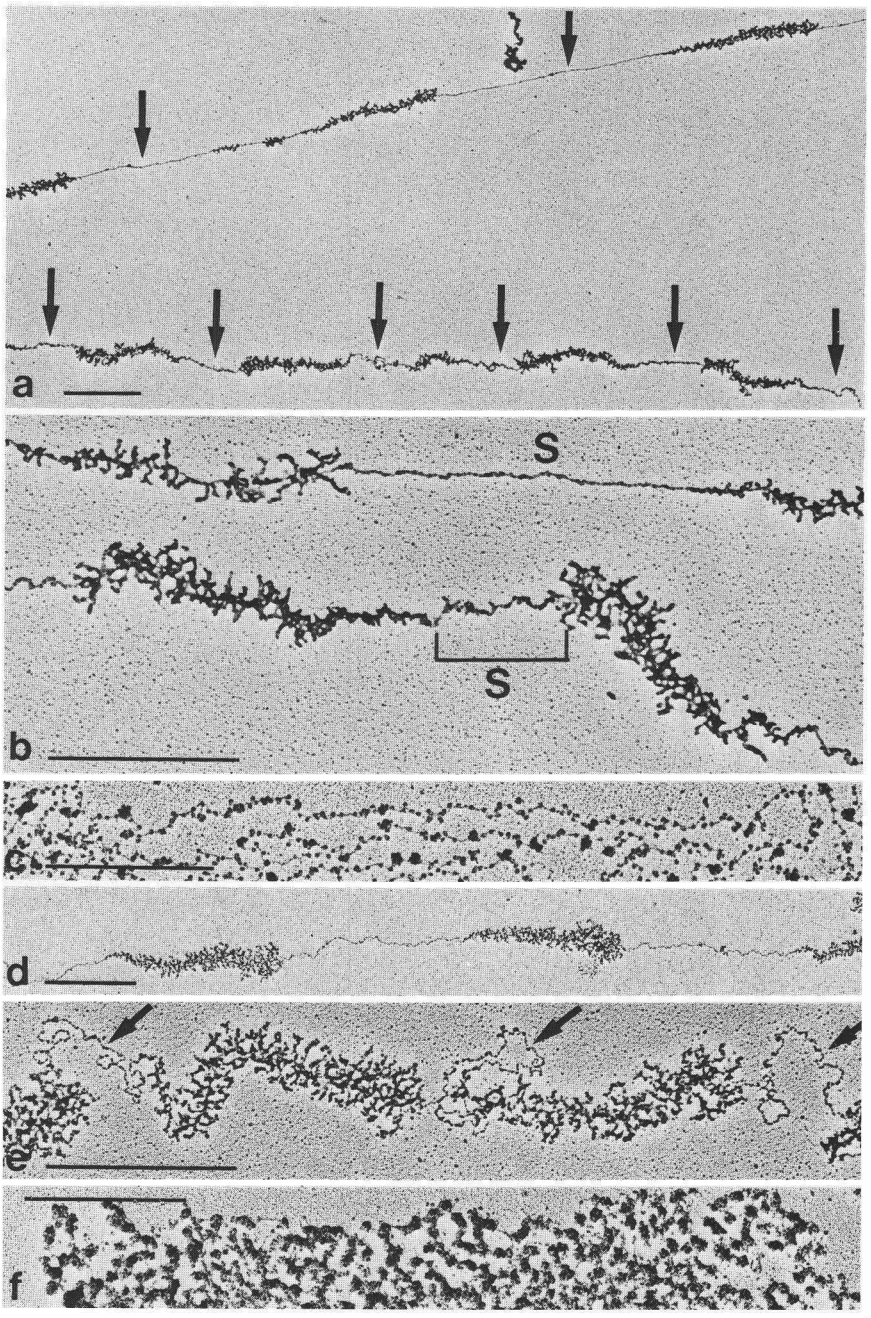
with a central dense aggregate surrounded by more loosely packed nucleofilaments (Zentgraf *et al.*, 1980a) and is clearly distinguished from the pre-rRNA transcription units of amphibian origin. In addition, we have measured the contour lengths of transcribed rRNA gene regions and spacers, obtained under various spreading condi-

tions, to examine possible chromatin packaging (see also Pruitt and Grainger, 1981). The results are illustrated in Fig. 9, and the quantitative evaluation is presented in Fig. 10.

At pH 9, the length distributions of spacer and gene regions are identical, regardless of the ionic strength of the medium, up to 20 m*M* NaCl. The only structural changes observed at elevated ionic strengths are the smoothly contoured spacers (see Section III) and the increasing tendency of the matrix units to form large aggregates.

In spread preparations made in low salt buffer (0.1–1.0 m*M* sodium borate or Tris-HCl) at almost neutral pH (7.4), the RNP fibrils of the matrix units are not fully extended (Fig. 9a,b). The gradual increase in their length, however, is still recognized. Spacer chromatin appears as a relatively thick (ca. 15 nm) axis showing irregularly spaced and sized "knobs" (Fig. 9b). Added erythrocyte chromatin is largely unfolded into chains of nucleosomal particles, with some interspersed higher-order globules (Fig. 9c). About two-thirds of the repeating units analyzed have been found to be considerably shorter than those prepared in "pH 9 water" (by a factor of about 1.7), and this foreshortening has been noted in both the gene and the spacer regions (compaction factors of 1.5 and 2.0, respectively). About one-third of the repeating units have not shown a considerable reduction in size. As a consequence, two size classes can be distinguished in the total length distributions of spacer, matrix, and repeat units (Fig. 10d,e). In spread preparations, such differently sized repeat units often lie side by side (Fig. 9a,b), suggesting that the foreshortened regions do not represent a very stable configuration. The compacted spacers reveal many projections, possibly folds or hairpinlike structures, whereas the chromatin axes of the gene regions are usually obscured by numerous tangled and aggregated

Fig. 9 Appearance of transcriptionally active nucleolar chromatin of *Triturus helveticus* oocytes (a,b,d,e) and of coprepared inactive avian erythrocyte chromatin (c,f) after spreading in 0.1 m*M* borate buffer, pH 7.4 (a–c) and 1 m*M* borate buffer, pH 7.4, containing 6 m*M* monovalent and 0.07 m*M* divalent cations (d–f). Without inclusion of salt, erythrocyte chromatin is largely unraveled into chains of nucleosomes (c). Under such spreading conditions, two different length classes of nucleolar repeating units can be distinguished: repeats with extended (upper arrows in a) and apparently foreshortened (lower arrows in a) spacers. Both types of repeating units are often seen side by side (a and b; S, spacer). When spreads are made in the presence of salt, the contour lengths of the spacers correspond to those measured in pH 9 preparations, although the spacer axis is often coiled and entangled (d,e). Added erythrocyte chromatin is arranged in arrays of closely spaced, globular supranucleosomal units ("superbeads," f). Bars indicate 1 μm (a,b,d,e) and 0.5 μm (c,f).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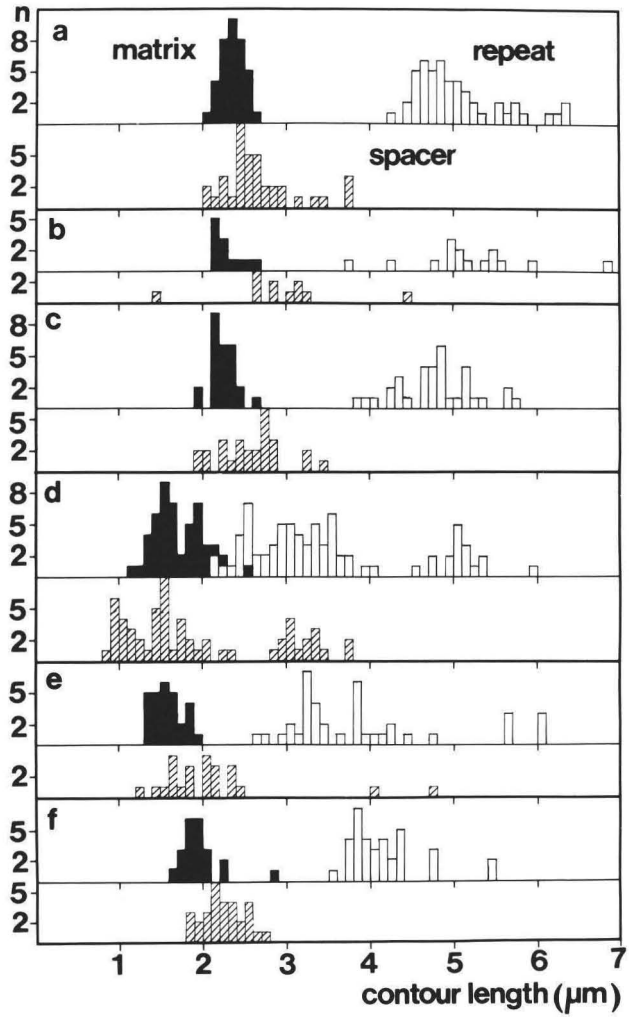

Fig. 10 Contour length distributions of matrix units, spacers, and the resulting repeat units as measured in spread preparations of nucleoli from *Triturus helveticus* oocytes under various ionic and pH conditions. Dispersion and spreading of nucleolar chromatin was done in 0.1 m*M* borate buffer, pH 9.0 (a); 1 m*M* borate buffer, pH 9.0 (b); 1 m*M* borate buffer, pH 9.0, containing 6 m*M* monovalent and 0.07 m*M* divalent cations (c); 0.1 m*M* borate buffer, pH 7.4 (d); 1 m*M* borate buffer, pH 7.4 (e); 1 m*M* borate buffer, pH 7.4, containing 6 m*M* monovalent and 0.07 m*M* divalent cations (f). The formaldehyde containing sucrose solution was adjusted to the corresponding pH and ionic strength.

RNP fibrils (Fig. 9b). The apparent foreshortening is not due to the formation of nucleosomes since it has not been observed after the ionic strength of the spreading medium has been increased, i.e., under conditions that stabilize the integrity of nucleosomes (see below).

Nucleolar chromatin prepared in a buffer containing 6 m*M* monovalent cations and 0.07 m*M* divalent cations at pH 7.4 appears almost identical to material prepared at pH 9 (Fig. 9d). Erythrocyte chromatin added to such preparations is arranged in tightly packed arrays of supranucleosomal globules (Fig. 9f), indicating that these ionic and pH conditions maintain supranucleosomal levels of chromatin folding. In spite of the recent finding of Pruitt and Grainger (1981), however, we have been unable to detect nucleosomal particles or any supranucleosomal structures in spacer or gene regions, nor have we measured any remarkable foreshortening of these regions (Fig. 10f). However, we have often observed conspicuous, close associations of adjacent matrix units (Fig. 9e) that might give the erroneous impression of spacer chromatin foreshortening. On closer inspection, however, it becomes clear that the chromatin of the spacer region is not at all foreshortened, but rather forms many loops and is occasionally highly convoluted and tangled (Fig. 9e). From this, we conclude that the extended state of active nucleolar chromatin, including both the gene and the spacer regions, reflects a fundamental difference in protein composition and/or arrangement compared to that of most transcriptionally inactive chromatin.

V. Nontranscribed Nucleolar Chromatin

Full-grown amphibian oocytes synthesize rRNA at a much slower rate than vitellogenic oocytes (Scheer *et al.*, 1976; Reeves, 1978a,b). Inactivation of nucleolar chromatin often occurs in a heterogeneous mode, resulting in pronounced local differences: Within a single oocyte nucleus, nucleoli can be found in which all genes are maximally active, whereas other nucleoli are completely inactive, and a third category contains both inactive and active regions (for a detailed study in *Rana pipiens*, see Trendelenburg and McKinnell, 1979; Williams *et al.*, 1981). Some of the active rRNA genes reveal a highly variable coverage by transcriptional complexes in different patterns of arrangement (Scheer *et al.*, 1976; Trendelenburg and McKinnell, 1979). Figure 11 illustrates, in a spread preparation of full-grown oocytes of

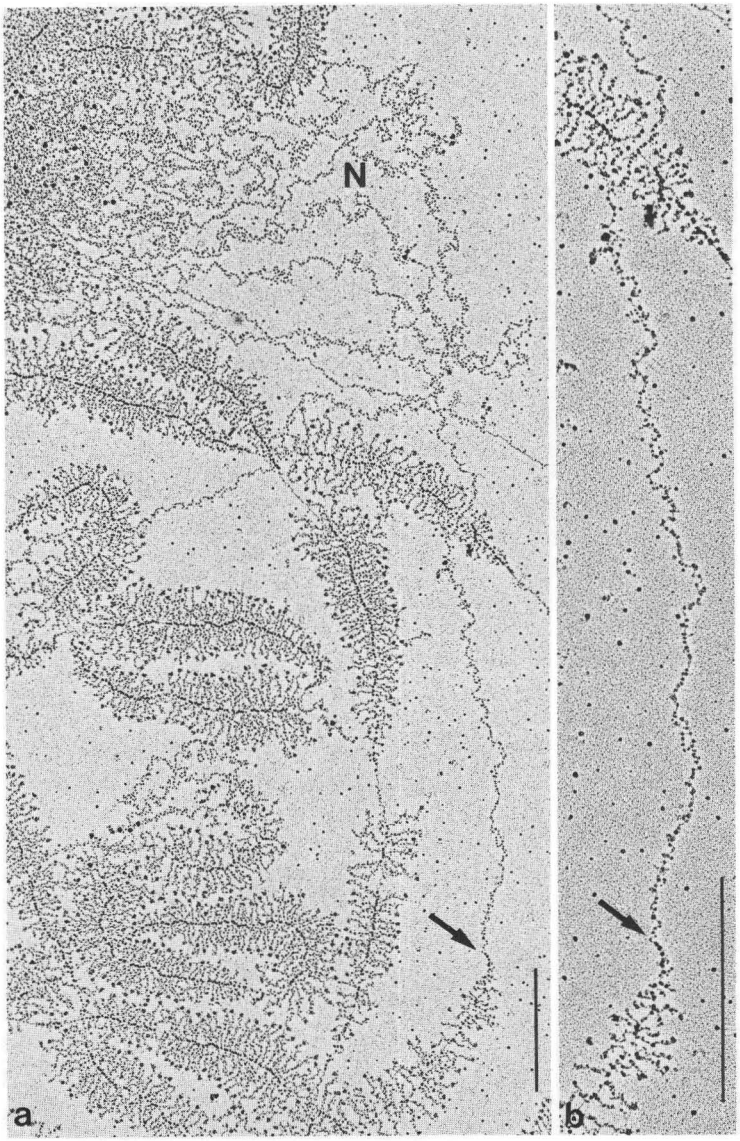

Fig. 11 Spread preparation of nucleolar chromatin from almost full-grown oocytes of *Pleurodeles waltlii*. Transcribed regions are in direct structural continuity with nontranscribed chromatin strands which exhibit the characteristic beaded morphology indicative of a nucleosomal organization (N). The arrow in (a) denotes the transition from transcribed to nontranscribed regions. The same situation is shown at higher magnification in (b). Bars indicate 1 μm.

Pleurodeles, the coexistence and direct structural continuity of chromatin strands containing fully active transcriptional units and completely inactive rRNA genes. The inactive chromatin strands have a beaded appearance indicative of an organization in nucleosomal particles, in contrast to the nonbeaded state of adjacent chromatin containing still active genes (Trendelenburg and McKinnell, 1979; Scheer, 1978; Franke *et al.*, 1979). A similar reorganization of nucleolar chromatin can also be induced after inhibition of transcription by drugs such as actinomycin D (Scheer, 1978). The appearance of beaded nucleolar chromatin is accompanied by an apparent foreshortening of the rDNA by a factor of about 2.3 from its extended (B) form, as has been shown in the amplified nucleolar chromatin rings of oocytes of the water beetle, *D. marginalis* (Scheer and Zentgraf, 1978; see also Section VI).

In general, cessation of transcriptional activity, whether natural or induced by drugs, is followed by conformational changes in nucleolar chromatin. However, this rearrangement into nucleosomes is seen only in chromatin strands completely devoid of transcriptional complexes. Matrix units still containing a few transcriptional complexes, as well as single inactive genes located between active ones, do not have a beaded, foreshortened appearance (Section III). Thus, complete inactivation of a large proportion of nucleolar chromatin seems to be a prerequisite for the conformational change from the extended to the nucleosomal state. These observations also suggest that the quantitative regulation of transcription of the rRNA genes is not directly controlled by the specific chromatin configuration but rather by factors regulating the initiation frequency at the level of individual genes (see also Foe, 1978). The nucleosomal organization seems to reflect a long-term inactivation of the rRNA genes (Franke *et al.*, 1976b).

VI. Chromatin Structure of Cloned rDNA after Microinjection into Nuclei of Amphibian Oocytes

When recombinant plasmids containing a full repeating unit of *X. laevis* rDNA are injected into amphibian oocyte nuclei, most of the DNA is rapidly assembled with the stored endogenous histones into chromatinlike structures showing a nucleosomal configuration (Trendelenburg and Gurdon, 1978; Scheer *et al.*, 1980; Bakken *et al.*, 1982). However, a small fraction of the total population injected is efficiently and correctly transcribed (Trendelenburg and Gurdon, 1978; Bakken

et al., 1982). Such experiments therefore offer the possibility of analyzing the structure of transcribed and nontranscribed chromatin circles containing identical DNA sequences prepared from a single cell nucleus.

Transcriptionally inactive chromatin circles always display a beaded configuration (Fig. 12c), with an apparent contraction ratio of the DNA by a factor of about 2 (Trendelenburg and Gurdon, 1978; Scheer *et al.*, 1980). The assembly of injected DNA into nucleosomal chromatin structures is independent of the specific DNA sequence (Wyllie *et al.*, 1978; Zentgraf *et al.*, 1979) and includes both the plasmid vector and the eukaryotic gene insert (Scheer *et al.*, 1980). The nucleosomal chromatin circles are further folded into distinct globular supranucleosomal units (Fig. 12d) (Scheer *et al.*, 1980) resembling the "superbeads" described in various kinds of transcriptionally inactive chromatin (Fig. 9c,f) (see Hozier *et al.*, 1977; Renz *et al.*, 1977; Zentgraf *et al.*, 1980a,b, 1981; Pruitt and Grainger, 1980). The transcribed circles are easily identified by the presence of transcriptional units and by the absence of typical nucleosomal particles, except for a minor region containing the plasmid vector (Fig. 12b) (Trendelenburg and Gurdon, 1978; Bakken *et al.*, 1982). It is remarkable that this "prokaryotic" region is arranged in nucleosomelike particles, as opposed to the smooth contour present in the spacer preceding the transcriptional unit and in the transcribed chromatin itself. Length measurements of the contours of (1) the DNA molecules used for injection (Fig. 12a), (2) the transcribed chromatin (Fig. 12b), and (3) the nontranscribed chromatin circles (Fig. 12c) demonstrate that, in actively transcribed circles, the DNA of the gene and the spacer regions is similar in length to that of the fully extended DNA (Trendelenburg and Gurdon, 1978). Thus, the ultrastructural organization of newly assembled chromatin containing cloned rDNA repeating units is identical to

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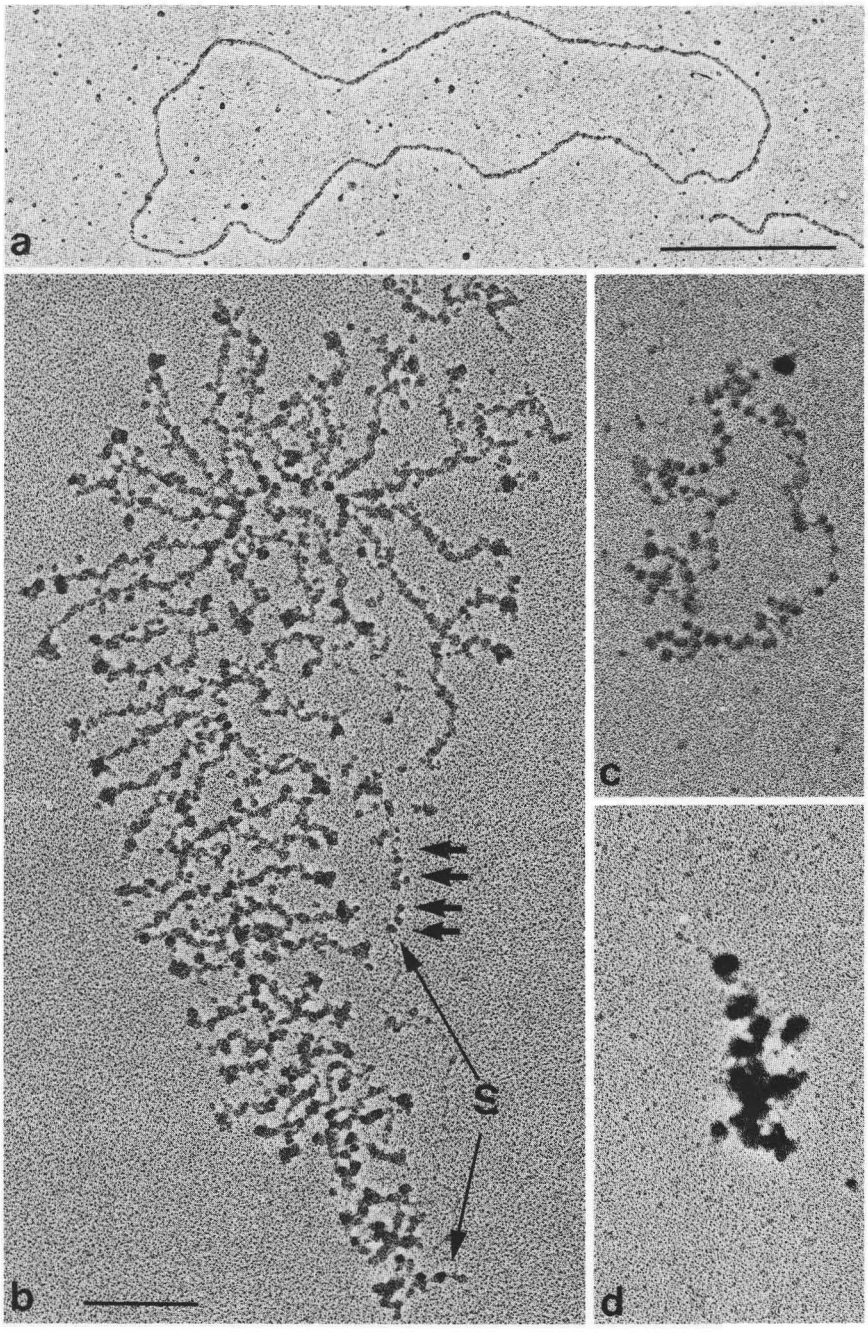
Fig. 12 Recombinant plasmid HMT containing a single full repeat unit of *Xenopus laevis* rDNA inserted into pBR 322 at the *Hin*dIII sites (a) kindly provided by Dr. M. Crippa, University of Geneva, Switzerland). After injection into nuclei of *Xenopus laevis* oocytes, the cloned rDNA is either transcribed (b) or the recombinant plasmids are assembled into regularly beaded chromatin circles (c), which are often further folded into globular, supranucleosomal units (d). Note that the nontranscribed spacer (S in b) appears thin and smooth, in contrast to the beaded ultrastructure of the region containing the prokaryotic vector (denoted by arrows in b). For details, see Trendelenburg and Gurdon (1978), Scheer *et al.* (1980), and Bakken *et al.* (1982). Parts (b–d) are magnified to the same scale to demonstrate the apparent foreshortening of the DNA by the nucleosomal and supranucleosomal packaging. Bars indicate 0.5 μm (a) and 0.2 μm (b). (b courtesy of Dr. M. F. Trendelenburg.)


a
b
S
c
d

that of the endogenous nucleolar chromatin. This finding supports the concept that transcribed rRNA genes and their adjacent spacers are organized differently from inactive chromatin.

VII. Nucleolar Chromatin Containing Palindromically Arranged rRNA Genes

In several lower animal species, amplified nucleolar chromatin occurs in the form of long linear strands with two rRNA genes at either end which are arranged in opposite polarity, i.e., in a palindromic arrangement (*Physarum:* Molgaard *et al.*, 1976; Vogt and Braun, 1976; *Dictyostelium:* Cockburn *et al.*, 1978; Grainger and Maizels, 1980; *Tetrahymena:* Engberg *et al.*, 1976; Karrer and Gall, 1976). This kind of nucleolar chromatin is of special importance since it can be isolated from the bulk of chromatin and analyzed by biochemical methods (see Section VIII and Chapters 7 and 8 of this volume).

The general appearance of a transcribed nucleolar chromatin strand from a plasmodium of *Physarum* is shown in Fig. 13a (see also Grainger and Ogle, 1978). Transcription is initiated in the central part of the palindrome and terminates near its free ends in such a way that two "nontranscribed terminal spacers" are left (Johnson *et al.*, 1979). The transcribed chromatin regions exhibit a nonbeaded structure, whereas the terminal spacers and the nontranscribed intergene region are arranged in nucleosomelike particles (Fig. 13b–e) (Grainger and Ogle, 1978; Scheer *et al.*, 1981). This morphological finding is corroborated by the apparent contraction of the intergene region by a factor of about 2, relative to the length of the corresponding rDNA inter-

Fig. 13 Transcribed nucleolar chromatin of plasmodial nuclei from *Physarum polycephalum* after spreading in 0.1 m*M* borate buffer, pH 9.0, containing 100 μg/ml tRNA and 0.1% Sarkosyl NL-30. For details, see Scheer *et al.* (1981). A palindromic unit containing two rRNA transcription units separated by a nontranscribed intergenic region (IG) is shown in (a). The opposite transcriptional polarity of the two rRNA genes is indicated by the arrows. Note the occurrence of nontranscribed terminal spacers (T). One end of a palindromic unit is shown in (b). The transcription initiation site is indicated by the vertical arrow (this region is shown at higher magnification in e). The curved arrow indicates the contour of the chromatin axis. The transcribed chromatin appears thin and smooth (b,c), in contrast to the beaded appearance of the intergene chromatin and the terminal spacers (b,c,e). Note, however, that the intergenic region directly preceding a transcription unit exhibits only a few widely scattered particles (denoted by the series of arrows in b, d, and e). N is an inactive chromatin strand with a beaded (nucleosomal) organization. Bars indicate 1 μm (a) and 0.5 μm (b–e).

a
T
IG
N
T
N
IG
b
T
c
IG
d
N
IG
e

cept (Grainger and Ogle, 1978). However, the nontranscribed chromatin stretches immediately preceding the transcriptional units display an organization different from that of the more centrally located intergenic chromatin. About 0.5 μm before the start region of a transcriptional unit, the nontranscribed chromatin contains very few particles of nucleosomal size and thus resembles the spacer chromatin located between tandemly arranged rRNA genes described above. In fact, these regions might be analogous to typical spacers of tandem rRNA genes, in terms of both their function and their nonnucleosomal arrangement.

VIII. Relationship between Morphological and Biochemical Data

The results of the morphological analysis of nucleolar chromatin of *P. polycephalum* seem to be in agreement with biochemical data (summarized in Johnson *et al.*, 1979; Allfrey, 1980). The absence of nucleosomal particles in the transcribed gene regions correlates with the extended state of chromatin subunits derived from digestion of this chromatin by micrococcal nuclease (Johnson *et al.*, 1978a). It is remarkable that this extension (DNA compaction ratio 1.2–1.3) is maintained even in isolated chromatin subunits. Another significant finding is the relative depletion of certain histones in these extended gene chromatin subunits as compared to nucleosomes (Allfrey *et al.*, 1978; Johnson *et al.*, 1978b). On the other hand, the occurrence of nucleosomal particles on nontranscribed terminal spacers and intergene regions appears to be related to the finding that rDNA restriction fragments containing the sequences derived from these regions reanneal preferentially to DNA of monomers and oligomers of nucleosomes (Johnson *et al.*, 1979).

Other investigators have reported differences in the chromatin structure of transcribed and nontranscribed nucleolar chromatin regions of *T. pyriformis*, as assayed by digestion with micrococcal nuclease (Borchsenius *et al.*, 1981) and by cross-linking experiments *in vivo* with trimethylpsoralen (Cech and Karrer, 1980). The results of both experiments fit the general concept of an extended state of the transcribed gene chromatin. Whether the much lower histone/DNA ratio of total nucleolar chromatin of *Tetrahymena*, compared to that of bulk chromatin (Jones, 1978), is directly related to the nonnucleosomal organization of the transcribed gene chromatin remains to be clarified.

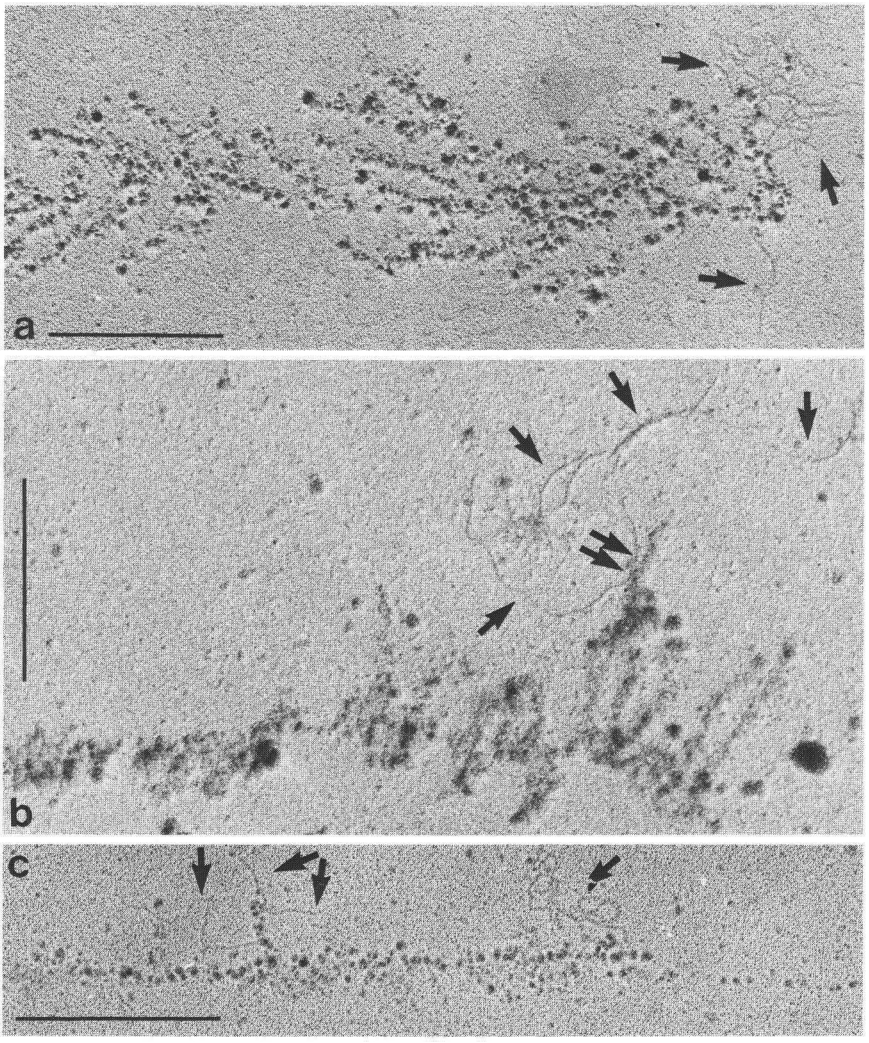


Fig. 14 *In situ* hybridization of cloned rDNA from *Xenopus laevis* to nascent pre-rRNA containing RNP fibrils in electron microscopic spread preparations. Transcribed rRNA genes from *Pleurodeles waltlii* oocytes were dispersed in pH 9 water and centrifuged through a cushion of 0.1 *M* sucrose with (a) and without (b,c) formaldehyde on carbon-coated grids. The preparation was fixed in 70% ethanol and rehydrated in distilled water. Finally, the grid was placed on a droplet of the hybridization mixture. This mixture contained nicked and denatured recombinant plasmids pXlr 101 (a single repeat unit of *Xenopus* inserted into pMB 9; see Botchan *et al.*, 1977). The specific hybridization conditions were chosen to permit both DNA–RNA and DNA–DNA hybridization, resulting in complex networks of DNA molecules attached to individual RNP fibrils (denoted by arrows). The pair of arrows in (b) depicts the site of attachment of a DNA molecule to a lateral RNP fibril. Bars indicate 0.5 μm. (From Scheer and Sommerville, unpublished observations; pXlr 101 was kindly provided by Dr. R. Reeder, Hutchinson Cancer Research Center, Seattle, Washington.)

In contrast, the morphological results obtained with amphibian nucleolar chromatin at first seem to be at variance with several biochemical data. Although the transcribed gene chromatin is invariably extended in electron microscopic spread preparations made at various ionic strengths and pH values, experiments employing micrococcal nuclease have been interpreted to indicate that these regions are digested to DNA fragments of about 200 bp, which is indicative of a nucleosome particle arrangement (Reeves, 1978a,b). However, two important points should be considered in this connection: (1) The data presented by Reeves (1978a,b) indicate that in *Xenopus* oocytes there is an inverse relationship between the transcriptional activity of rRNA genes and the amount of rDNA recovered in nucleosomes after micrococcal nuclease digestion. Thus, in oocytes of maximal rRNA synthesis, only about 37% of the rDNA is recovered in nucleosomes, a finding which might be explained by assuming that the majority of the rRNA genes occur in a nonnucleosomal state, whereas only a minority (presumably the inactive ones) are arranged in nucleosomes. (2) The demonstration of a periodic protection structure of DNA against the action of micrococcal nuclease, resulting in DNA fragments of about 200 bp, is not necessarily indicative of an organization in nucleosomal particles. This is also clear from the results of Johnson *et al.* (1978a,b, 1979), who have shown that the periodic structure of transcribed DNA of *Physarum* rRNA genes is maintained in its extended conformation. Furthermore, chromatin can be induced to unfold at high concentrations of urea, while retaining its characteristic protection pattern toward micrococcal nuclease digestion (Jackson and Chalkley, 1975; Woodcock and Frado, 1978). Therefore, the results obtained by Reeves (1978a,b) are not necessarily in conflict with the morphological observation of an absence of nucleosomes in transcribed nucleolar chromatin.

IX. Combination of Chromatin Spread Preparations with Localization Techniques

Electron microscopic chromatin spread preparations not only allow the morphological analysis of transcribed and nontranscribed regions and of the nascent RNP products but can also be combined with localization techniques that allow the identification of defined proteins or DNA or RNA sequences.

Immunological localization techniques have been successfully used to study the histone composition and accessibility of nontranscribed (Bustin *et al.*, 1976; Goldblatt *et al.*, 1978; McKnight *et al.*,

1978; Di Padua Mathieu *et al.*, 1981) and transcribed nonnucleolar chromatin (McKnight *et al.*, 1978). In the near future, similar techniques should allow us to determine whether histones are associated with transcribed nucleolar chromatin. The topological distribution of defined ribosomal proteins along the nascent rRNP fibrils has been studied by Chooi and Leiby (1981a,b), using an indirect immunolocalization technique with the second antibodies coupled to large polymethacrylate spheres.

In situ hybridization techniques should provide a means of identifying transcriptional units in electron microscopic spread preparations. Figure 14a–c shows that a denatured DNA probe containing a total repeating unit of *Xenopus* rDNA binds specifically to the transcripts of rRNA genes spread from *Pleurodeles* oocytes. The hybridized DNA molecules form extensive networks because under the specific hybridization conditions, not only are RNA–DNA hybrids formed, but complementary DNA fragments also reanneal to each other. Using further modifications and refinements of the hybridization and spreading conditions, it should be feasible not only to identify transcriptional units of defined genetic content at the electron microscopic level but also to determine the position of specific sequences of nascent RNA molecules within the lateral RNP fibrils.

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NOTE ADDED IN PROOF: After completion of the manuscript a paper appeared (Labhart, P., and Koller, T., 1982. *Cell* **28**, 279–292) that essentially confirmed observations described in this chapter. Using nucleolar chromatin of *Xenopus laevis* oocytes prepared at different ionic strengths and pH-values, these authors have shown that the chromatin of transcriptional units and spacer regions appears as smooth, thin, nonnucleosomal filaments.

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