The Characterization of Ribosomal RNA Gene Chromatin from *Physarum polycephalum*^

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We have isolated ribosomal RNA gene (rDNA) chromatin from *Physarum polycephalum* using a nucleolar isolation procedure that minimizes protein loss from chromatin and, subsequently, either agarose gel electrophoresis or metrizamide gradient centrifugation to purify this chromatin fraction (Amero, S. A., Ogle, R. C., Keating, J. L., Montoya, V. L., Murdoch, W. L., and Grainger, R. M. (1988) *J. Biol. Chem.* 263, 10725–10733). Metrizamide-purified rDNA chromatin obtained from nucleoli isolated according to the new procedure has a core histone/DNA ratio of 0.77:1. The major core histone classes comigrate electrophoretically with their counterparts on Triton-acidurea/sodium dodecyl sulfate two-dimensional gels, although they may not possess the extent of secondary modification evident with the nuclear histones. This purified rDNA chromatin also possesses RNA polymerase I activity, and many other nonhistone proteins, including two very abundant proteins (26 and 38 kDa) that may be either ribonucleoproteins or nuclear matrix proteins. Micrococcal nuclease digestion of the metrizamide-purified rDNA chromatin produces particles containing 145-base pair DNA fragments identical in length to those in total chromatin and which contain both transcribed and nontranscribed rDNA sequences. Some smaller fragments (30, 70, and 110 base pairs) are also seen, but their sequence content is not known. These particles sediment uniformly at 11 S in sucrose gradients containing 15 mM NaCl, and at 4–11 S in gradients containing 0.35 mM NaCl. Particles enriched in gene or nontranscribed spacer sequences are not resolved in these sucrose gradients or in metrizamide gradients. Our findings suggest that the rDNA chromatin fraction we have identified contains transcriptionally active genes and that an organized, particle-containing structure exists in active rDNA chromatin.

Many studies have suggested that ribosomal RNA genes may have a chromatin structure quite different from that of nonribosomal genes. This view of ribosomal RNA gene chromatin (rDNA chromatin)^

structure is derived in part from electron microscopic observations. For instance the visualization of ribosomal RNA genes from the milkweed bug *Oncopeltus fasciatus* reveals smooth and, more generally, bead chromatin fibers within active transcription units, in contrast to the beaded chromatin fibers in adjacent inactive chromatin and in nonribosomal transcription units (Foe et al., 1976; Foe, 1977). Also, we have observed active ribosomal RNA genes from amoebae of *Physarum polycephalum* which appear to contain numerous nucleosomes in nontranscribed spacer regions but few nucleosomes in gene regions (Grainger and Ogle, 1978). Labhart and Koller (1982) argue from electron microscopic visualizations that nucleolar chromatin from *Xenopus laevis* oocytes is associated with very few macromolecular constituents other than the transcription apparatus.

Many biochemical studies have supported the view that rDNA chromatin structure may be distinctive. Jones (1978) reported a histone/DNA ratio of only 40% in preparations of rDNA-enriched nucleolar chromatin isolated from *Tetrahymena thermophila*. Also, the ribosomal RNA genes of *Physarum* (Butler et al., 1978; Stalder et al., 1978), *Tetrahymena pyriformis* (Palen and Cech, 1983), and *Dictyostelium discoideum* (Ness et al., 1983) have been shown to be more sensitive to micrococcal nuclease digestion than total chromatin. Moreover, the frequency of cross-linking induced by the reagent trinethylpsoralen into the rDNA of nuclei in *Physarum* and *in vivo* in *Tetrahymena* is higher in the transcribed regions than in the nontranscribed regions, and the spacing between the cross-links is different between the two regions (Cech and Karrer, 1980; Jadelson and Vogt, 1982).

One model of rDNA chromatin structure which might explain these results was suggested from the finding in *Physarum* nucleoli of a particular fraction of 5 S chromatin particles (Johnson et al., 1978), which are enriched in transcribed rDNA sequences (Johnson et al., 1979) and reveal an extended conformation in the electron microscope (Prior et al., 1983). These investigators suggest that active transcription units in rDNA chromatin are composed of these unfolded nucleosomes, or “lexosomes,” and thereby retain a nucleosome-like periodic structure.

Although the lexosome model argues that transcriptionally active ribosomal genes have a different conformation than inactive chromatin, the model does not propose that rDNA chromatin is depleted of histones, as suggested by some of the studies mentioned above.

Other studies argue that ribosomal RNA genes possess typical nucleosome-like features and a nearly full complement of histones. For instance, identical nucleosome repeat lengths...
have been reported in the products of micrococcal nuclease digestions of both nucleoli and nuclei from Physarum (Butler et al, 1978; Stalder et al., 1978) and of rDNA chromatin and total chromatin from Tetrahymena pyriformis (Mathis and Gorovsky, 1976). Sequences that hybridize to ribosomal RNA have also been detected in the 200-base pair DNA fragments in micrococcal nuclease-derived chromatin subunits from X. laevis (Reeves and Jones, 1976; Reeves, 1976, 1977). These studies are supported by the finding of similar histone/DNA ratios, for each of the five major histone classes, in bulk macronuclear chromatin and nucleolar chromatin of Physarum, in which ribosomal DNA constitutes 75–82% of the total DNA (Colavito-Shepanski and Gorovsky, 1983).

Our approach to resolving these issues involves first the purification of rDNA chromatin from P. polycephalum, using procedures which minimize the loss of chromatin protein (Amero et al., 1988) so that we have been able to analyze directly the proteins, both histone and nonhistone proteins, associated with rDNA chromatin. These procedures have also led to assays of RNA polymerase activity and nuclease digestion studies using the purified chromatin fraction. Finally, since the Physarum rDNA sequence is palindromic, containing a central nontranscribed spacer region and distal, transcribed gene regions (Campbell et al., 1979), it has been possible to compare directly the structures of active "gene" and inactive "spacer" rDNA chromatin.

**MATERIALS AND METHODS**

**RESULTS**

Characterization of Proteins Associated with Purified rDNA Chromatin—We first characterized the proteins associated with rDNA chromatin using a two-dimensional gel electrophoresis procedure. A nucleolar lysate was initially fractionated in an agarose slab gel (Amero et al., 1988). Non-nucleolar chromatin does not enter these agarose gels, rDNA chromatin migrates as a band in the center of the gel, and free ribonuclease (RNP) particles migrate with the gel front. To visualize the proteins in different regions of the gel, strips of agarose gel were silver stained (Fig. 1). Although in Fig. 1 the top of the agarose gel was cut off, in preparations in which this region was retained, we consistently observed a complex set of proteins, including the four core histones, associated with the nuclear chromatin that does not enter the agarose gel. In this particular gel, the core histones can be seen trailing from the origin of the gel, which we believe results from broken chromatin fragments which are able to enter the gel. The rDNA chromatin band in the center of the gel also contains all four core histones and one uncharacterized protein which migrates just below the top histone band. Two conspicuous nonhistone proteins of lower mobility were seen in the rDNA chromatin region and also in the RNP region at high levels. However, the larger of the two proteins migrates throughout the agarose gel. We have not determined the molecular weights of these proteins in this gel system, since it is very difficult to align the mobilities of standard proteins with those of rDNA chromatin proteins which are electrophoresed from an agarose gel.

We also analyzed by SDS-polyacrylamide gel electrophoresis the proteins associated with rDNA chromatin that had been purified by metrizamide gradient centrifugation. These gradients resolve two chromatin peaks, a lighter density chromatin peak, which contains only rDNA, and a heavier density chromatin peak, which contains both rDNA and non-nucleolar DNA (Amero et al., 1988). As shown in Fig. 2A, we observed that the four core histone proteins, the uncharacterized histone-like protein, and the two abundant nonhistone proteins seen in agarose gels were associated with the lighter, rDNA chromatin peak, as were many other nonhistone proteins. The electrophoretic mobility of the histone-like protein, as well as its preferential association with transcriptionally competent chromatin (see below) suggests it is likely to be the HMG 14/17 protein of Physarum (Czupryn and Toczko, 1984). In comparison to the protein pattern shown in Fig. 1, it can be seen that many more high molecular weight proteins were represented in the metrizamide-purified sample than in the agarose-purified sample. This is presumably due both to stripping of chromatin protein during electrophoresis in agarose gels (Amero et al., 1988) and to inefficient recovery of large proteins from the agarose gels. We might expect to see RNA polymerase I subunits associated with rDNA chromatin, and although there are many proteins in the gel in the appropriate molecular weight range for such peptides (Hildebrand and Sauer, 1973; Burgess and Burgess, 1974), none can be unambiguously identified as RNA polymerase subunits at this time.

In this gel system the two abundant proteins have apparent molecular masses of 26 and 38 kDa. As in the agarose gel system the 26-kDa protein is associated only with rDNA chromatin, but the 38-kDa protein can be seen to trail throughout the metrizamide gradient (Fig. 2A). Since proteins have density near the bottom of these gradients (1.25 g/cm³) and nucleic acids near the top (1.12 g/cm³), the observation

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* "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
We compared nuclear histones from *Physarum* to core histone proteins associated with metrizamide-purified rDNA chromatin by electrophoresis in Triton-acetic acid-urea polyacrylamide tube gels, followed by second dimension electrophoresis in SDS-polyacrylamide slab gels. As shown in Fig. 3, we found that the major forms of the core histone proteins from purified rDNA chromatin (A) comigrated with their nuclear counterparts (B). Since histone H1 from *Physarum* displays an aberrant electrophoretic mobility (Côté et al., 1982; Mende et al., 1983), we were unable to identify histone H1 in our protein pattern. However, we found that highly modified histones (Smith, 1984), or variant histone isoforms (Newrock et al., 1977) evident in the nuclear histone samples as discrete spots adjacent to the major histone proteins, are absent from purified rDNA chromatin, and that in this gel system the histones associated with rDNA chromatin are

![Fig. 2. SDS-polyacrylamide gel analysis of proteins associated with metrizamide gradient-purified rDNA chromatin. rDNA chromatin was purified from genomic chromatin by centrifugation in metrizamide density gradients as described by Amero et al. (1988). Chromatin-containing fractions were identified by scintillation counting to detect [H]DNA. A, proteins in fractions collected across the gradient (lanes 1-7) were fractionated by electrophoresis in a 5-22% gradient polyacrylamide-SDS gel and silver stained. The bottom of the metrizamide gradient is to the left. The circles mark the positions of two abundant nonhistone proteins associated with rDNA chromatin; the asterisks mark the positions of the four core histones. The 38-kDa nonhistone protein is concentrated over the genomic chromatin peak (lane 2) and the rDNA chromatin peak (lane 5) but is also present in all of the gradient fractions; the 26-kDa nonhistone protein is only present in chromatin-containing fractions. B, metrizamide-purified rDNA chromatin was further purified by recentrifugation in metrizamide. Proteins in the rDNA chromatin-containing fractions were analyzed by electrophoresis in an 18% SDS-polyacrylamide gel and stained with Coomassie Blue. It is evident that some protein loss occurs during metrizamide gradient centrifugation.](image1)

![Fig. 3. Analysis of proteins associated with metrizamide-purified rDNA chromatin by two-dimensional polyacrylamide gel electrophoresis (PAGE). A, rDNA chromatin was purified by two rounds of metrizamide gradient centrifugation as described by Amero et al. (1988). Proteins associated with purified rDNA chromatin were fractionated first by electrophoresis in Triton-acetic acid-urea polyacrylamide tube gels and by electrophoresis in SDS-polyacrylamide slab gels in the second dimension. The proteins were stained with Coomassie Blue. Asterisks indicate the core histone proteins. B, total nuclear histone proteins were analyzed by electrophoresis in identical gels. The patterns of the major histone proteins in the two gels are indistinguishable.](image2)
homogeneous. None of the other minor proteins in the histone region of the gel corresponds in terms of electrophoretic mobility to the hv-1 and hv-2 histone variant proteins found in the macronucleus of Tetrahymena (Allis et al., 1982) or to ubiquinated H2A (Goldknopf and Busch, 1978).

Quantitation of Core Histones Associated with Purified rDNA Chromatin—We have used spectrophotometric scans of protein and DNA gels to quantitate core histone protein and rDNA content in rDNA chromatin purified by two rounds of centrifugation in metrizamide. From the results listed in Table I, it can be seen that the values from three different preparations agree quite well, with an average histone/DNA mass ratio in purified rDNA chromatin of 0.77 (±0.03):1:00. Since the total protein loss is about 20% from first to second round of centrifugation (Amero et al., 1988), this estimate may be somewhat low, although we do not know if this is due to the loss of histones or other proteins associated with rDNA chromatin. However, we do know that the four core histone proteins are present in ratios similar to those in our nuclear histone preparation. Therefore, the average histone/DNA mass ratio above does not reflect significant loss of certain histone proteins.

Quantitation of the Abundant Nonhistone Proteins Associated with Purified rDNA Chromatin—We have used the spectrophotometric scans of protein gels from the previous experiment to estimate the abundance of the 38- and 26-kDa nonhistone proteins with respect to the core histone proteins. The results from three preparations are listed in Table I. The average molar ratio of the 38-kDa protein to an “average” core histone is 1.46 (±0.61):1 and of the 26-kDa protein to an “average” core histone is 1.11 (±0.36):1. Therefore, in terms of molar ratios, these nonhistone proteins in purified rDNA chromatin are at least as abundant as any one of the core histone proteins. From Table I we can also estimate that the average molar ratio of the 38-kDa protein to the 26-kDa protein is 1.32 (±0.53):1.

The large variation in the protein ratios involving the two major nonhistone proteins in Table I and their fractionation properties in agarose gels and metrizamide gradients suggested that they may be nonintegral components, such as RNPs or nucleolar matrix proteins. We performed a mixing experiment to investigate whether these proteins can bind to calf thymus DNA (10 µg/ml) added to the initial nucleolar homogenization of [¹⁰H]thymidine-labeled plasmodium. This added DNA subsequently copurified with rDNA chromatin isolated in metrizamide gradients from the resulting lysate, as determined by measurements of specific activity of DNA-
We are unable to compare the repeat lengths in the DNA fragment patterns, since the nucleolar and rDNA chromatin samples are over-digested compared to the nuclear samples. This difference in digestion conditions is probably due to the greater accessibility of the rDNA substrate to the nuclease in these samples, since variables such as substrate concentration and enzyme diffusion rates cannot be accurately controlled. Extensive nuclease digestion may account for the absence of a typical ladder pattern in purified rDNA chromatin samples, and for the 110-, 70-, and 30-bp fragments in DNA samples derived from nucleoli and from purified rDNA chromatin, which are not evident in nuclear samples. The 110-bp fragment, which hybridizes weakly to rDNA (Fig. 5C, and 6 below), is particularly noticeable in samples derived from purified rDNA chromatin.

Characterization of rDNA Chromatin Particles by Metrizamide Gradient Centrifugation—We tried to fractionate nuclease-generated rDNA chromatin particles by metrizamide gradient centrifugation to attempt the direct analysis of active and inactive rDNA chromatin structures. As shown in Fig. 6A, two fractions of chromatin particles (ρ = 1.20 g/cm³ and ρ = 1.18 g/cm³) were resolved in these gradients. These densities are intermediate to the densities of deproteinized rDNA fragments (ρ = 1.12 g/cm³) and of histone proteins (ρ = 1.27 g/cm³) in metrizamide, indicating that these fractions contain deoxyribonucleoprotein. The appearance of two peaks of nucleosomes in metrizamide gradients has been described previously (MacGillivray and Rickwood, 1978), but the basis for this fractionation is not clear. To test whether these fractions contained specific rDNA sequences, DNA fragments purified from pooled fractions across the gradient were electrophoretically fractionated and transferred as above, and hybridized sequentially to cloned DNA fragments containing sequences from within the rDNA transcription unit and then to spacer-specific sequences. We included cloned gene and spacer rDNA fragments on each membrane as internal controls for cross-hybridization (not shown); we estimate that cross-hybridization accounts for less than 10% of the final signals.

The autoradiograms shown in Fig. 6 clearly indicate that the dense peak is enriched in both gene and spacer DNA sequences and that resolution of gene and spacer rDNA chromatin particles was not achieved by metrizamide gradient centrifugation. Since we have not probed these fractions with other cloned rDNA sequences, it is possible that the light peak is enriched in sequences from the nontranscribed terminal, or other spacer regions. Furthermore, a comparison of Fig. 6, B and C reveals that the major, nuclease-resistant gene and spacer rDNA fragment length is identical. In overexposed autoradiograms a less prominent band corresponding to 280 bp is also evident with either probe (not shown); this band is likely to comprise DNA fragments from dimer nucleosomes. We observed the four core histones in those gradient fractions containing DNA, which is again indicative of nucleosome structure, but did not observe the 38- and 26-kDa nonhistone proteins (not shown). Taken together these observations suggest that a nucleosome–type particle exists on DNA sequences within the rRNA gene, and that these particles do not differ substantially in terms of protein content from those in the nontranscribed spacer region. One difference is evident, however, in the prominent bands corresponding to 220–240 bp; these bands do not overlap in the two autoradiograms. These bands may represent the dimer forms of the 110-bp fragments seen in Fig. 5 and reflect sites of nuclease digestion within the core particles.

Characterization of rDNA Chromatin Particles by Sucrose
Gradient Centrifugation—A fraction of chromatin particles with a sedimentation constant of 5 S has been resolved in sucrose gradients from micrococcal nuclease digests of nucleoli from *P. polycephalum* (Prior et al., 1983). This fraction, called peak A, is enriched in transcribed DNA sequences. We sought to identify such small chromatin particles from rDNA chromatin purified according to our procedures, as a way of purifying active chromatin.

In our first attempt micrococcal nuclease digests of metrizamide-purified rDNA chromatin were analyzed by centrifugation in linear sucrose gradients containing 15 mM NaCl. These digests were prepared in the same way as those used in the previous experiments, wherein both gene-specific (Fig. 6B) and spacer-specific (Fig. 6C) DNA sequences were found to be resistant to digestion. As shown in Fig. 7A, we found a single peak of radioactivity with a sedimentation constant of 11 S in the chromatin-containing gradients; clearly no resolution of gene and spacer particles was achieved.

Since it has been noted that the resolution of peak A particles from nucleosome particles declines as the concentration of NaCl is reduced from 0.35 to 0.1 M (Prior et al., 1983), we analyzed duplicate portions of the micrococcal nuclease digests by centrifugation in linear sucrose gradients containing 0.35 mM NaCl. These gradient profiles often revealed a broad chromatin peak in the sucrose gradients, ranging from 9 to 4 S (not shown). Elevated ionic strength consistently reduced the sedimentation coefficient of particles from purified rDNA chromatin, which we interpret to represent protein loss or conformational changes. Again, however, no heterogeneity of subunit particles, which may reflect gene and spacer sequences, is evident in these analyses.

In the experiments wherein peak A particles have been identified (Johnson et al., 1978, 1979), nucleoli were isolated in the presence of divalent cations, whereas in our experiments, nucleoli were isolated in the presence of spermine and spermidine. To test whether the nucleolar isolation procedure affects the sedimentation properties of particles from purified rDNA chromatin, we repeated the sucrose gradient analyses with chromatin from nucleoli that had been isolated by the divalent cation procedure. In all three NaCl concentrations, we found a broad chromatin peak in the sucrose gradients, ranging from 9 to 4 S (Fig. 7C) and no 11 S chromatin peak. We believe that the spreading of the chromatin peak probably results from stripping of histone proteins from rDNA chromatin during the divalent cation isolation technique (Amero et al., 1988). Additionally, the change in sedimentation coefficient which results from elevated ionic strength is superimposed on this pattern. Nonetheless, we were unable to resolve gene and spacer chromatin particles.

Electron Microscopic Visualization of Purified rDNA Chromatin—We analyzed metrizamide-purified rDNA chromatin preparations in the electron microscope utilizing the Miller chromatin spreading technique (Miller and Beatty, 1969). We consistently observed that well-dispersed molecules were devoid of nucleosome-type particles but that some molecules (approximately 10%) possessed clusters of 200 Å particles, which we presume to be RNA polymerase molecules. Since these preparations have been treated with RNase prior to
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**Fig. 7.** Analysis of rDNA chromatin particles by sucrose gradient centrifugation. rDNA chromatin was purified by metrizamide gradient centrifugation from nucleoli that had been isolated either in the presence of divalent cations or of spermidine and spermine, as described by Amero et al. (1988). Following digestion of the purified rDNA chromatin with micrococcal nuclease, the digestion products were fractionated by centrifugation in 5-20% sucrose gradients. Chromatin-containing fractions were identified by scintillation counting, and sucrose concentrations were determined by refractometry. The positions of RNA markers are indicated at the top of each panel. Panel A, digestion products from spermine/spermidine-isolated rDNA chromatin were fractionated in sucrose gradients containing 15 mM NaCl. Panel B, digestion products from the same mixture shown in A were fractionated in sucrose gradients containing 0.35 M NaCl. Panel C, digestion products from divalent cation-isolated rDNA chromatin were fractionated in sucrose gradients containing 15 mM NaCl. The exposure of rDNA chromatin particles to 0.35 M NaCl results in a lower sedimentation coefficient; this effect is enhanced with chromatin prepared by divalent cation methods.

metrizamide gradient centrifugation, it is difficult to localize the transcription units in these micrographs. However, the finding of particle clusters of greater than 4 μm (Fig. 8, A and B), the length expected for the transcribed ribosomal RNA gene (Grainger and Ogle, 1978), suggests that the particles in these clusters do represent residual RNA polymerase molecules. These clusters (Fig. 8C) and the naked DNA stretches adjacent to them (not shown) are clearly devoid of other particles.

Chromatin molecules which are not well dispersed were uniformly associated with smaller, less dense particles which measure approximately 100 Å in diameter (Fig. 8D). Since all the molecules in these purified preparations contain only rDNA, it appears that a nucleosome-type conformation exists

**Fig. 8.** Analysis of purified rDNA chromatin by electron microscopy. rDNA chromatin was purified by metrizamide gradient centrifugation and prepared for electron microscopy by the Miller spreading technique. In each panel, the black bars represent 0.25 μm of B-form DNA. Panels A and B, clusters of dense particles which cover greater than 4 μm of DNA, the length of the rRNA gene transcription unit. Panel C, the cluster from B is displayed at a higher level of magnification to demonstrate the absence of other particle structures in the clusters. The particles in the clusters, which are 200 Å in diameter, correspond to the size of RNA polymerase molecules. Panel D, less extensively dispersed molecules possess smaller (approximately 100 Å in diameter) less dense particles (on the right hand side of the micrograph) than those seen in clusters of presumed RNA polymerase molecules (on the left).
on these molecules, as suggested by our biochemical analyses. However, the nucleosome-type conformation appears to be sensitive to the spreading technique.

DISCUSSION

We have shown that ribosomal RNA gene chromatin from Physarum possesses a slightly reduced core histone protein content, which is reflected in the density of rDNA chromatin in metrizamide gradients and in a measured core histone protein to rDNA mass ratio of 0.77:1.00. This value may represent a low estimate of the histone complement on purified rDNA chromatin, since we have demonstrated a reduction in the density of rDNA chromatin upon successive rounds of centrifugation in metrizamide gradients consistent with an overall loss of 20% total protein (Amero et al., 1988). Our results are in agreement with the histone/DNA ratios reported by Colavito-Shepanski and Gorovsky (1983) for rDNA-enriched nucleolar chromatin fractions of Tetrahymena and with the finding that nucleosome-like particles containing transcriptionally active rDNA sequences from Physarum also contain core histone proteins (Johnson et al., 1979; Prior et al., 1983). Our results in Physarum argue for a higher histone/DNA ratio than the ratio of 0.4:1 reported by Jones (1975) for nucleolar chromatin fractions of Tetrahymena. Although both Tetrahymena studies and our own analysis involved polyamine-containing nucleolar isolation methods, it is possible that subsequent techniques used to solubilize chromatin from nucleolus may account for this difference. The procedure employed by Jones (1978) involved solubilization in cyclohexane diamine tetraacetic acid, dimethyl sulfoxide, and Triton X-100, and the possible effects of these treatments on chromatin structure were not addressed. A major concern in studies of this type is the possibility that soluble histones have become bound artifactually to rDNA, and that the resulting histone/DNA ratio is misleadingly high. Since micrococcal nuclease digestions of isolated nucleolus yield DNA fragments that form a nucleosome-type ladder pattern, these rearrangements would have to take place during the nucleolar isolation procedure. Our results from the experiment in which calf thymus DNA was added to nucleolar isolation buffers argue that such rearrangement is unlikely. These results also argue that nucleosomes are not produced by an assembly factor, which probably will not be active in our buffer systems (Amero et al., 1988) and support the suggestion made by Prior et al. (1980) that a limited histone pool is present in Physarum. The presence of histones on active rDNA chromatin in vivo is also implied from the psoralen cross-linking studies of Judelson and Vogt (1982), in which gene sequences in intact nuclei were protected from cross-linking, and from our earlier electron microscopic visualizations of nucleosomes in the rDNA genes of Physarum amoebae (Grainger and Ogle, 1978). It is important to note, however, that in both the cross-linking and electron microscopic studies, the gene sequences appear to be associated with fewer nucleosomes than do spacer sequences.

We have shown by two-dimensional gel electrophoresis that in Physarum the major core histone classes in rDNA chromatin and genomic chromatin comigrate electrophoretically. It is important, however, to point out the limitations of this analysis. First, since we have not used long first-dimensional gels we are unable to resolve acetylated histone species (Chahal et al., 1980). Histone acetylation, particularly acetylation of histones H3 and H4, occurs during both S phase and G2 phase of the cell cycle in Physarum, when transcription occurs (Waterborg and Matthews, 1983).

Second, we have employed no measures to inhibit histone deacetylase activity, which is maximal when ribosomal RNA synthesis occurs during G2 phase (Waterborg and Matthews, 1982). Therefore, it is possible that acetylated histones are present in our rDNA chromatin preparation and that we have been unable to detect them. The absence of histone H1 in our protein samples from purified rDNA chromatin may be due to preferential loss of histone H1. Alternatively, it is conceivable that levels of histone H1 are reduced in Physarum rDNA chromatin, since the rDNA molecules in Physarum (and several other lower eukaryotes) are linear and discrete and may not possess the particular torsional strains induced by histone H1 in nuclear chromatin (Cole et al., 1977).

We have described two nonhistone proteins that are more abundant than the core histone proteins in purified rDNA chromatin and have shown that these may either be loosely associated with rDNA chromatin or may bind to rDNA chromatin during our isolation procedures. Therefore, caution is warranted in interpreting the nature of the association of these proteins to rDNA chromatin at this time. These proteins do not correspond in any obvious way to other nonhistone proteins which have also been reported to be specifically associated with the telomeres or center of symmetry of the Physarum rDNA molecule (Rehun et al., 1979; Cheung et al., 1981).

It is possible that the two abundant nonhistone proteins are nucleolar matrix proteins. Fields et al. (1986) have used a polyclonal serum directed against a 38-kDa nucleolar matrix protein in rat liver to stain Physarum nucleoli in an immunofluorescence assay and to probe Physarum nuclear extracts in Western blots. They report recognition in Western blots of a 55-kDa doublet by the serum, yet also in the blot one can see a signal corresponding to a protein of 38 kDa. Unfortunately, the region of the blot where a 26-kDa protein would be expected to migrate is not shown in this paper. Therefore, we may speculate that at least the 38-kDa nonhistone protein corresponds to this nucleolar matrix protein. Consistent with this hypothesis is our observation that both nonhistone proteins colocalize with large chromatin fragments and insoluble material in sucrose gradients used to fractionate nucleolar micrococcal nuclease digestion products (not shown); the proteins do not colocalize with mononucleosomes.

Alternatively, the two nonhistone proteins may be RNP molecules since they are present in the RNP region of chromatin agarose gels and the 38-kDa protein in particular trails throughout both agarose gels and metrizamide gradients. Even after RNase treatment, we still detect some RNA associated with purified chromatin preparations to which these proteins might be bound.

Our results suggest that an organized, histone-containing particle structure exists in active rDNA chromatin. First, the transcription units comprise over 45% of the total rDNA in Physarum (Grainger and Ogle, 1978; Campbell et al., 1979); our histone/DNA ratio indicates that a substantial fraction of these genes must possess histone protein. Second, we believe the histones in the rRNA genes are arranged in a nucleosome-type array, since mononucleosome-length, genespecific rDNA fragments are protected from micrococcal nuclease digestion. Our inability to resolve sequence-specific chromatin subunit particles on the basis of metrizamide or sucrose density centrifugation strongly supports this hypothesis. The considerations outlined above also suggest that the histones found associated with our purified chromatin preparations are not likely to have arisen artificially. Finally, we believe our purified rDNA chromatin fraction contains only active transcription units, since the resolution of rDNA chro-

3 P. Ferris, personal communication.
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atin from nonnucleolar chromatin in metrizamide gradients depends on prior RNase treatment (Amero et al., 1988), and this purified rDNA chromatin fraction possesses RNA polymerase I activity.

Several proposals have been put forth to describe the structure of active chromatin regions. For example, one model suggests that nucleosomes in actively transcribing chromatin unfold into pairs of "half-nucleosomes" to permit passage of RNA polymerase molecules (Weintraub et al., 1976; Oudet et al., 1977). An alternative model suggests that histone proteins are displaced completely by RNA polymerase molecules in ribosomal RNA genes (Davis et al., 1983) giving rise to protein-free stretches of DNA similar to those observed in certain electron micrographs. On the other hand, a fraction of chromatin particles from Physarum nucleoli highly enriched in rRNA genes, the peak A particles, has been identified in sucrose gradients (Johnson et al., 1978, 1979). From electron microscopic visualizations of peak A particles, Prior et al. (1983) propose the formation of "lexosomes" in active chromatin, which consist of two "semi-nucleosomes" separated by 50 bp of DNA. In addition, this 50-bp stretch of DNA is suggested as the probable binding sites for the nonhistone proteins LP30 and LP32.

Our results suggest that the 5 S particles in our high salt sucrose gradients may arise from salt-induced transitions from an 11 S particle, and therefore that intact nucleosomes are present in active rDNA chromatin. We do not know if the 5 S particles in our study are peak A particles, since our samples are extensively digested and lack histone H1, as well as the nonhistone proteins LP30 and LP32. Nonetheless, in our experiments, all subunit particles from purified rDNA chromatin are subject to the salt-induced transition, not just those from the transcription unit. In support of this suggestion, we note that Yager and van Holde (1984) have demonstrated that a shift in sedimentation characteristics of chicken chromatin are subject to the salt-induced transition, not just our experiments, all subunit particles from purified rDNA chromatin fraction possesses RNA polymerase molecules. On the other hand, a fraction of chromatin fibers which possess a compaction ratio of 20 (compared to B form DNA length) in spacer regions and a ratio of 2 in gene regions when spread in high ionic strength buffers, undergo disassembly when spread in low ionic strength buffers to produce smooth fibers. The difference in compaction ratio was clearly evident in this study in stage 2 oocytes, prior to the onset of rDNA transcription, at a time when the free histone pool is limited. Grainger and Ogle (1978) also observed scattered nucleosomes in the rDNA genes of Physarum amoeboae.

Our micrographs of purified rDNA chromatin resemble the naked units described by Labhart and Koller (1982) and by Ness et al. (1983), yet our biochemical studies indicate the presence of histone protein, and subunit particles, in rDNA transcription units. Our studies suggest that certain nucleolar lysis and fixation procedures in pH 9 buffers may lead to protein stripping and nonbeaded chromatin morphology. It is clear, therefore, that the ultrastructural approach may not be the method of choice for resolving these aspects of chromatin structure. The "three-layer" technique described by Grainger and Ogle (1978) may obviate some of these problems.

We undertook these studies to reveal differences between active and inactive regions of rDNA chromatin in Physarum and as yet have not been able to detect major differences between these two regions. It is possible that the submonomer bands in DNA fragment patterns from our micrococcal nuclease digests represent an unusual structure in some portion of the rDNA chromatin molecule. Our hybridization experiments, however, show that most gene and spacer rDNA sequences exist in typical core nucleosomes, so that the submonomer-type structure must represent a small fraction of the rDNA sequences. Additionally, certain rDNA sequences may be associated with multiacetylated histone proteins, as suggested by Boffa et al. (1986) for the active rDNA region. As we pointed out above, these modified histones probably would not have been detected in any of our analyses. Although we were unable to fractionate gene and spacer nucleosomes in either metrizamide or sucrose density gradients, it is possible that immunochemical approaches, such as immunoprecipitation of chromatin particles or fragments with antibodies specific for certain nonhistone proteins or for multiacetylated histone proteins, may result in the purification of these chromatin fractions. The availability of purified rDNA chromatin which possesses a nearly full complement of histones and retains many features of native chromatin structure should provide unprecedented opportunities for further analyses of active and inactive rDNA chromatin domains.

REFERENCES


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Specific activity of 0.24 A⁶⁶⁴/mg/mg. EDTA solutions were titrated by the addition of 
linear polyacrylamide of 96.5% and of 7.4% acetic acid. The mixtures were incubated at 38°C for 30 min, and 
filtrated through Whatman no. 1 glass fiber disks. The disks were washed with 0.1 M NaOH, 1 M NaCl, and then 
neutralized with 1 M NaOH and 1 M NaCl, and were air-dried and counted.

We found that incubation of chromatin samples at 65°C for 10 min 
produced no detectable incorporation of background levels, as determined 
treatment of these mixtures with 0.1 M NaOH at 37°C for 15 min after the addition.

Microscopic Nucllease Digestions of Physarum Nuclei or Nuclear Fractions

nuclease digestions were performed as described. Chromatin fragments were prepared from nuclei 
of Physarum polycephalum (strain ATCC 3951) by digestion with nuclease P1 to a final concentration of 10 mM. DNA fragments were isolated by precipitation with ethanol.

Intronuclease Digestions of Purified rDNA Chromatin

rDNA was purified by centrifugation in sucrose gradients to a final concentration of 10 mM. Tyrphostin A6 was added to the digestion mixture to a final concentration of 10 mM. DNA fragments were isolated by precipitation with ethanol.

Microscopic Nucllease Digestions of Purified rDNA Chromatin

rDNA was purified by centrifugation in sucrose gradients to a final concentration of 10 mM. Tyrphostin A6 was added to the digestion mixture to a final concentration of 10 mM. DNA fragments were isolated by precipitation with ethanol.

Characterization of rDNA Chromatin Particles by Sedimentation Equilibrium

Sedimentation of rDNA particles was performed and the number of nucleosomes was determined by centrifugation in sucrose gradients containing 1 M KCl and 0.1 M NaCl. The gradients were centrifuged at 120,000 rpm for 16 h at 4°C in a Beckman rotor. Fractions (approx. 100 µl each) were collected at the bottom of the tubes. The distribution of chromatin particles was determined by precipitating fractions into 20% TCA for 30 min and filtering the samples through Whatman no. 1 filters as described above.

Electron Microscopy

Electron microscopy revealed the presence of nucleosomes in the gradient. The peaks were identified by comparison of the electron microscopy images with those of standard samples of nucleosomes. The peaks were identified by comparison of the electron microscopy images with those of standard samples of nucleosomes. The peaks were identified by comparison of the electron microscopy images with those of standard samples of nucleosomes.