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Sally A. Amero

Vicky L. Montoya

Wendy L. Murdoch

Roy C. Ogle
Old Dominion University, rogle@odu.edu

John L. Keating

See next page for additional authors

Original Publication Citation

Amero, S.A., Montoya, V.L., Murdoch, W.L., Ogle, R.C., Keating, J.L., & Grainger, R.M. (1988). The characterization of ribosomal RNA gene chromatin from physarum polycephalum. *The Journal of Biological Chemistry, 263*(22), 10734-10744.

Authors
Sally A. Amero, Vicky L. Montoya, Wendy L. Murdoch, Roy C. Ogle, John L. Keating, and Robert M. Grainger

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 263, No. 22, Issue of August 5, pp. 10734-10744, 1988
Printed in U.S.A.

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

(Received for publication, February 9, 1988)

Sally A. Amero‡, Vicky L. Montoya, Wendy L. Murdoch, Roy C. Ogle§, John L. Keating, and Robert M. Grainger¶

From the Department of Biology, University of Virginia, Charlottesville, Virginia 22901

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 nuclear counterparts on Triton-acid-urea/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 nucleolar 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 M 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)[1] 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 nonbeaded 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 is total chromatin. Moreover, the frequency of cross-linking induced by the reagent trimethylpsoralen 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; Judelson 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 which 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

* This investigation was supported by National Institutes of Health Grant GM-24430. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Microbiology, University of Virginia School of Medicine, Charlottesville, VA 22908.

§ Present address: Dept. of Anatomy and Cell Biology, Medical University of South Carolina, Charleston, SC 29425.

¶ To whom correspondence should be addressed: Dept. of Biology, University of Virginia, Charlottesville, VA 22901.

[1] The abbreviations used are: rDNA, ribosomal RNA gene; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; SDS, sodium dodecylsulfate; RNP, ribonucleoprotein; TEA, triethanolamine; TCA, trichloroacetic acid; bp, base pairs.

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 *Tetrahymena*, 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[2]

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 ribonucleoprotein (RNP) particles migrate with the gel front. To visualize the proteins in different regions of the gel, strips of agarose were laid horizontally on top of a SDS-polyacrylamide slab gel; following electrophoresis, the proteins in the second dimension 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 electroeluted 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. 2*A*, 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. 2*A*). Since proteins have density near the bottom of these gradients (1.25 g/cm^3) and nucleic acids near the top (1.12 g/cm^3), the observation

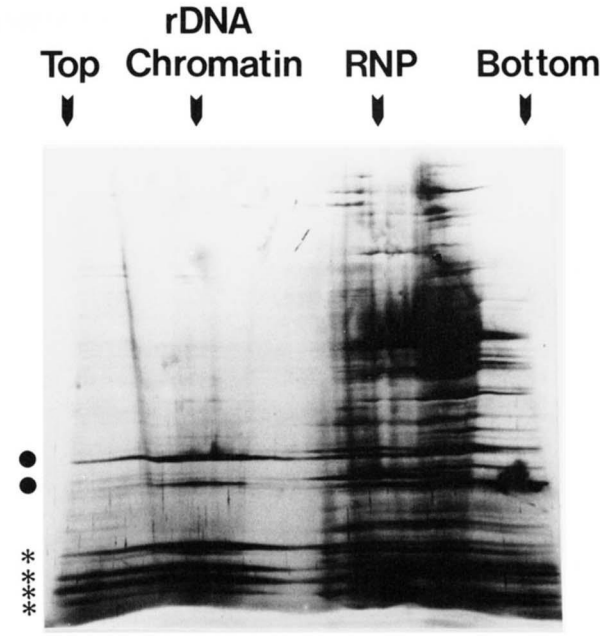

FIG. 1. **SDS-polyacrylamide gel analysis of proteins associated with agarose gel-purified rDNA chromatin.** rDNA chromatin was purified from nuclear chromatin and RNPs by electrophoresis in a 0.4% Sea/Plaque agarose gel as described by Amero *et al.* (1988). Proteins associated with these nucleolar components were analyzed by electrophoresis in a 5–22% gradient polyacrylamide-SDS gel which was subsequently silver stained. The *circles* mark the positions of two abundant nonhistone proteins associated with rDNA chromatin; the *asterisks* mark the positions of the four core histones. Both nonhistones are found in the rDNA chromatin region and RNP region of the gel; the top nonhistone protein trails throughout the gel.

[2] "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.

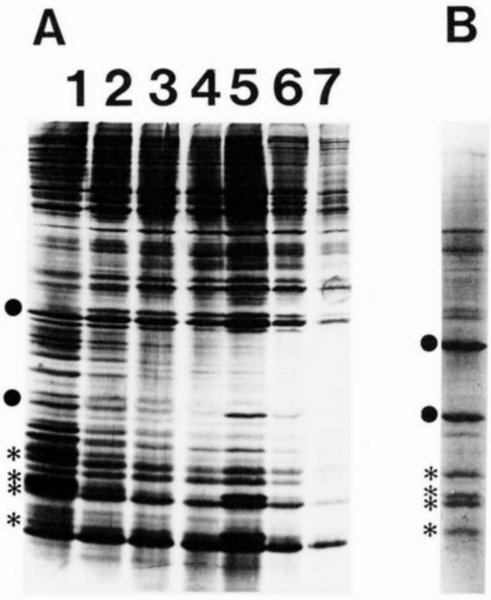

FIG. 2. **SDS-polyacrylamide gel analysis of proteins associated with metrizamide gradient-purified rDNA chromatin.** rDNA chromatin was purified from genomic chromatin by centrifugation in metrizamide density gradients as described by Amero *et al.* (1988). Chromatin-containing fractions were identified by scintillation counting to detect [^{3}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.

of trailing in regions of the metrizamide gradient devoid of radiolabeled DNA suggests that the 38-kDa nonhistone protein is associated with varying amounts of RNA. The RNase treatment which precedes centrifugation in metrizamide may account for the heterogeneity in these RNP particles.

The rDNA chromatin peak fractions from a metrizamide gradient could be further purified by a second round of centrifugation. We observed a similar protein complement associated with the twice-purified rDNA chromatin (Fig. 2*B*) and detected much less protein in other gradient fractions, although a few fractions on the dense side of the rDNA chromatin peak contained a small amount of protein that comigrates in polyacrylamide gels with the histones (not shown). This trailing effect is probably due to breakage and loss of chromatin fragments rather than protein stripping, since the densities of these gradient fractions correspond to nucleoprotein, rather than free protein. Also, histones were not observed in those dense regions of the gradient where free histones would be expected to band. Although it appears from Fig. 2 that substantial protein loss occurs, it is important to note that silver-stained gels are probably not quantitative and that many proteins which are not integral to rDNA chromatin will be lost in the second gradient. It is clear that some protein loss, and possibly histone loss, does occur during metrizamide centrifugation, however, since we found that the density of rDNA chromatin decreases slightly after a second round of centrifugation (Amero *et al.*, 1988).

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. 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.

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 [^{3}H]thymidine-labeled plasmodia. 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.

TABLE I

Stoichiometry of major rDNA chromatin components

Chromatin sample[a]	Mass ratio of total core histone proteins: μg rDNA	Molar ratio of nonhistone protein: average histone[b]	
		38-kDa protein	26-kDa protein
A.	0.791	1.895	0.989
B.[c]			
1.	0.752	1.711	1.514
2.	0.802		
C.	0.752	0.766	0.834

[a] These samples were derived from three individual preparations.

[b] Molar ratios were determined by first scanning Coomassie Blue-stained SDS-polyacrylamide gels to obtain mass ratios. The average core histone value used to calculate molar ratios was the average apparent molecular weight of the four core histones from *Physarum* as reported by Mende *et al.* (1983).

[c] This sample was divided, and independent assays were conducted on duplicated aliquots of histones.

rDNA chromatin was also purified from nucleolar lysates prepared from the same plasmodium in the absence of exogenous DNA.

The proteins associated with equal amounts of rDNA (as assessed by [^{3}H]thymidine levels) were then determined by SDS-polyacrylamide gel electrophoresis. The two aliquots contained comparable levels of the core histone proteins (not shown) indicating that histones did not become bound to the exogenous DNA, but the two nonhistone proteins were enriched in the chromatin associated with exogenous DNA (not shown). We interpret these results to suggest either that the two nonhistone proteins were solubilized during the nucleolar isolation and became associated with the exogenous DNA, or that the exogenous DNA in some way suppressed the loss of these proteins which may normally occur during chromatin isolation.

Assays of RNA Polymerase I Activity—We performed assays of RNA polymerase I activity to see if this activity was associated with metrizamide-purified rDNA chromatin. As shown in Table II in standard RNA polymerase assays, nucleolar lysates containing roughly 60 ng of DNA supported significant incorporation of roughly 5 ng of [^{3}H]UTP into RNase-sensitive, acid-precipitable material. Incorporation by nucleolar lysates, which contain some contaminating nonnucleolar chromatin, was only slightly inhibited by levels of α-amanitin known to block RNA polymerase II (Burgess and Burgess, 1974; Losick and Chamberlain, 1976). Purified rDNA chromatin containing roughly 300 ng of rDNA from metrizamide gradients supported the incorporation of roughly 1 ng of UTP in these assays; this activity was completely resistant to α-amanitin. We assume the different efficiencies in the two assays result from the purification of rDNA chromatin away from soluble factors and energy-regenerating systems or may reflect an effect of metrizamide on enzymatic activity.

Micrococcal Nuclease Digestions of Purified rDNA Chromatin—We first analyzed the kinetics of nuclease digestions as a way to learn about rDNA chromatin structure. Fig. 4 shows that digestion of metrizamide-purified rDNA chromatin with micrococcal nuclease displayed typical kinetics, reaching limit digestion when approximately 50–60% of the radioactivity in DNA had been rendered acid-soluble. Such hyperbolic kinetic curves are characteristic of micrococcal nuclease digestion on nuclear chromatin, as is our asymptotic value of 50–60% (Noll and Kornberg, 1977). These results

TABLE II

In vitro assays of RNA polymerase I activity

Template	α-Amanitin	RNase[a]	Corrected incorporation[b]
	μg/ml	μg/ml	dpm
Nucleolar chromatin[c]			5903
	5		5645
	50		5485
		500	890
Purified rDNA chromatin			1144
	5		1069
	50		1148
		500	508

[a] RNase was added to the assay after the usual 30-min incubation, and allowed to react for an additional 15 min at 37 °C.

[b] Each value represents the average of two independent trials and has been corrected for background values determined empirically to arise from unincorporated [^{3}H]UTP and from radioactivity in each chromatin sample. Each assay of nucleolar chromatin contained approximately 0.06 μg of DNA; each assay of purified rDNA chromatin contained approximately 0.3 μg of DNA.

[c] Nucleolar chromatin refers to crude nucleolar lysates prior to purification by metrizamide gradient centrifugation.

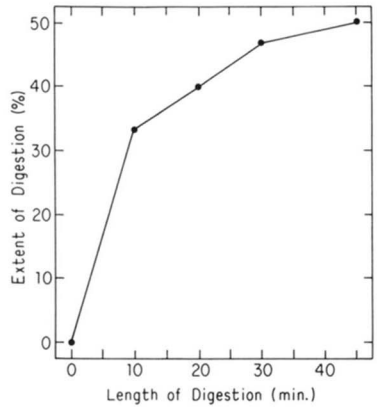

FIG. 4. **Kinetics of micrococcal nuclease digestion of metrizamide-purified rDNA chromatin.** The extent of micrococcal nuclease digestion of purified rDNA chromatin was determined at different time points as the percent reduction of trichloroacetic acid-precipitable radioactivity as described under "Materials and Methods." This curve represents typical kinetics for micrococcal nuclease digestions.

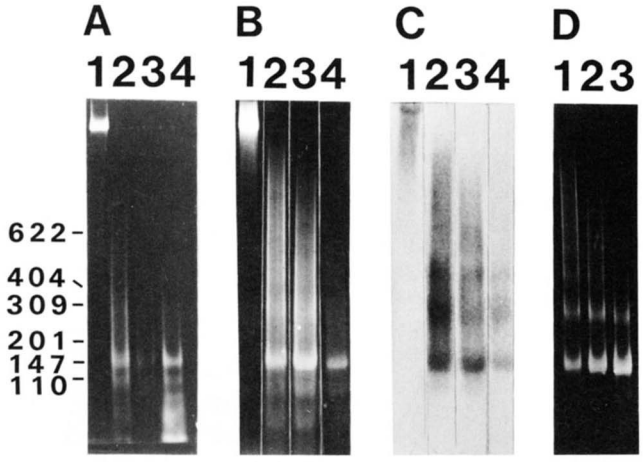

FIG. 5. **Comparison of nucleosome DNA fragment lengths in nuclear chromatin, nucleolar chromatin, and purified rDNA chromatin.** DNA fragments were purified from various chromatin samples that had been digested with micrococcal nuclease, fractionated by electrophoresis in 3.5–10% polyacrylamide gels, and visualized by staining with ethidium bromide. In each *panel, lanes 1–4* represent 0, 10, 20, and 45 min digests, respectively. *Panel A*, DNA fragments from digests of purified rDNA chromatin. *Panel B*, DNA fragments from digests of nucleolar chromatin. *Panel C*, autoradiogram from hybridization of purified, nick-translated, rDNA to the DNA fragments from *panel B* immobilized on a nylon filter. *Panel D*, DNA fragments from digests of nuclear chromatin. No major alteration of chromatin structure due to spermine/spermidine techniques is apparent by this analysis.

indicate that purified rDNA chromatin probably does not possess extensive stretches of protein-free rDNA.

We also analyzed the structure of rDNA chromatin by comparing electrophoretically the rDNA fragments from micrococcal nuclease-digested, purified rDNA chromatin (Fig. 5*A*) to those from nucleoli (*B*) and nuclei (*D*), which form a ladder-like electrophoretic pattern characteristic of a nucleosome array. Those fragments from nucleolar lysates containing rDNA sequences were identified by electrophoretic transfer to membranes and hybridization to nick-translated, purified rDNA (Fig. 5*C*). We found that the length of the major nuclease-resistant fragment, estimated to be 145 bp, is invariant between the digested samples. We conclude that nucleosome-type core particles are present on the purified rDNA chromatin.

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. 5*C*, 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. 6*A*, two fractions of chromatin particles (ρ = 1.20 g/cm^3 and ρ = 1.18 g/cm^3) were resolved in these gradients. These densities are intermediate to the densities of deproteinized rDNA fragments (ρ = 1.12 g/cm^3) and of histone proteins (ρ = 1.27 g/cm^3) 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 rDNA 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

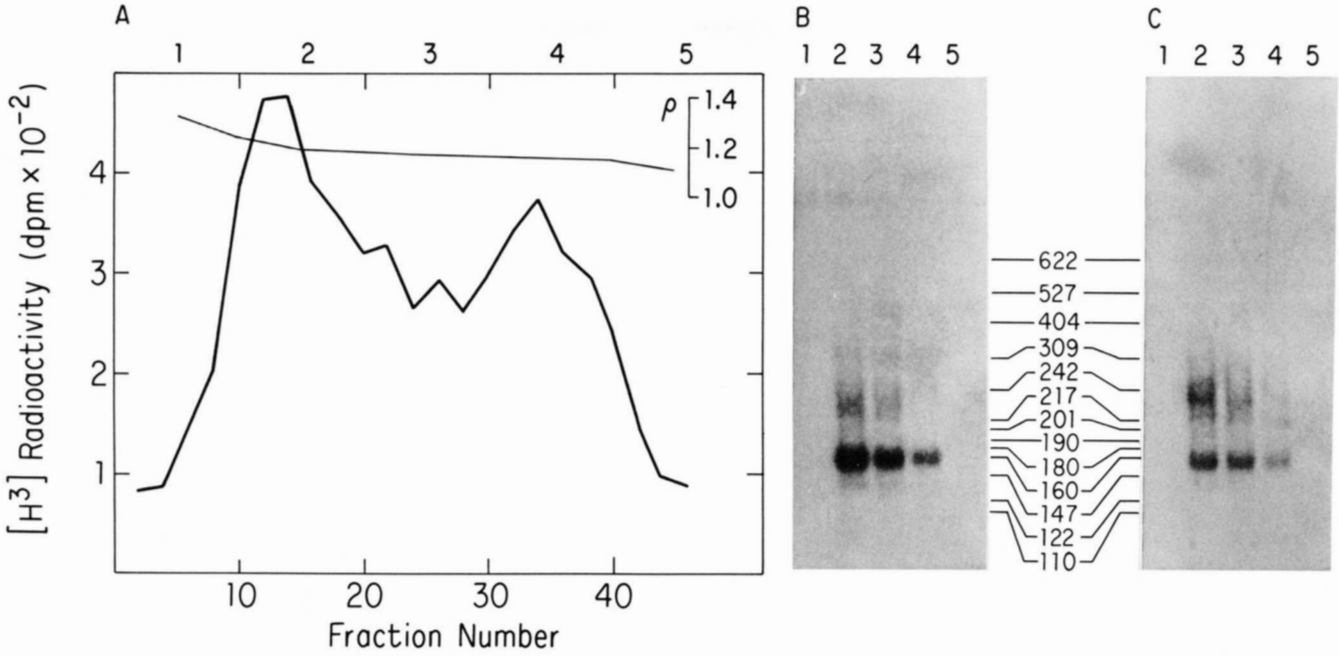

FIG. 6. **Analysis of rDNA chromatin particles by metrizamide gradient centrifugation.** Purified rDNA chromatin was digested with micrococcal nuclease, and the digestion products were analyzed by metrizamide gradient centrifugation. *A*, chromatin-containing gradient fractions were identified by liquid scintillation counting of trichloroacetic acid-precipitable radioactivity. The gradient profile was calculated from refractive index measurements of fractions collected across the gradient. *B*, DNA fragments purified from five regions of the metrizamide gradient were fractionated by electrophoresis in a 3.5–10% polyacrylamide gel and transferred to a nylon membrane for hybridization to sequence-specific, nick-translated DNA probes. *Lane 1* contains DNA from fractions 1–10; *lane 2* contains DNA from fractions 11–20, etc. The filter was hybridized to a gene-specific probe. *C*, the probe was stripped from the filter in *panel B*, and the filter was rehybridized to a spacer-specific probe. It can be seen that gene and spacer chromatin particles possess monomer DNA fragments of identical lengths and are not resolved in metrizamide gradients.

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. 6*B*) and spacer-specific (Fig. 6*C*) DNA sequences were found to be resistant to digestion. As shown in Fig. 7*A*, 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 M NaCl. These gradient profiles often revealed a single chromatin peak, for which a sedimentation constant of 4 S was calculated from the standard gradient profile (Fig. 7*B*). However, occasionally in these gradients and consistently in gradients containing 0.1 M NaCl, the chromatin peak was quite broad, ranging from 11 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. 7*C*) 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

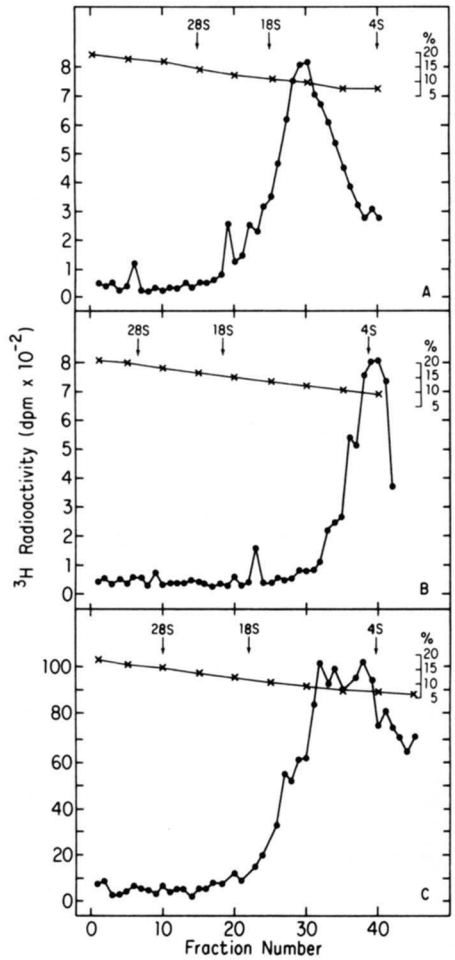

FIG. 7. **Analysis of rDNA chromatin particles by sucrose gradient centrifugation.** rDNA chromatin was purified by metrizamide gradient centrifugation from nucleoli that had been isolated either in the presence of divalent cations or of spermidine and spermine, as described by Amero *et al.* (1988). 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. 8*C*) 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. 8*D*). Since all the molecules in these purified preparations contain only rDNA, it appears that a nucleosome-type conformation exists

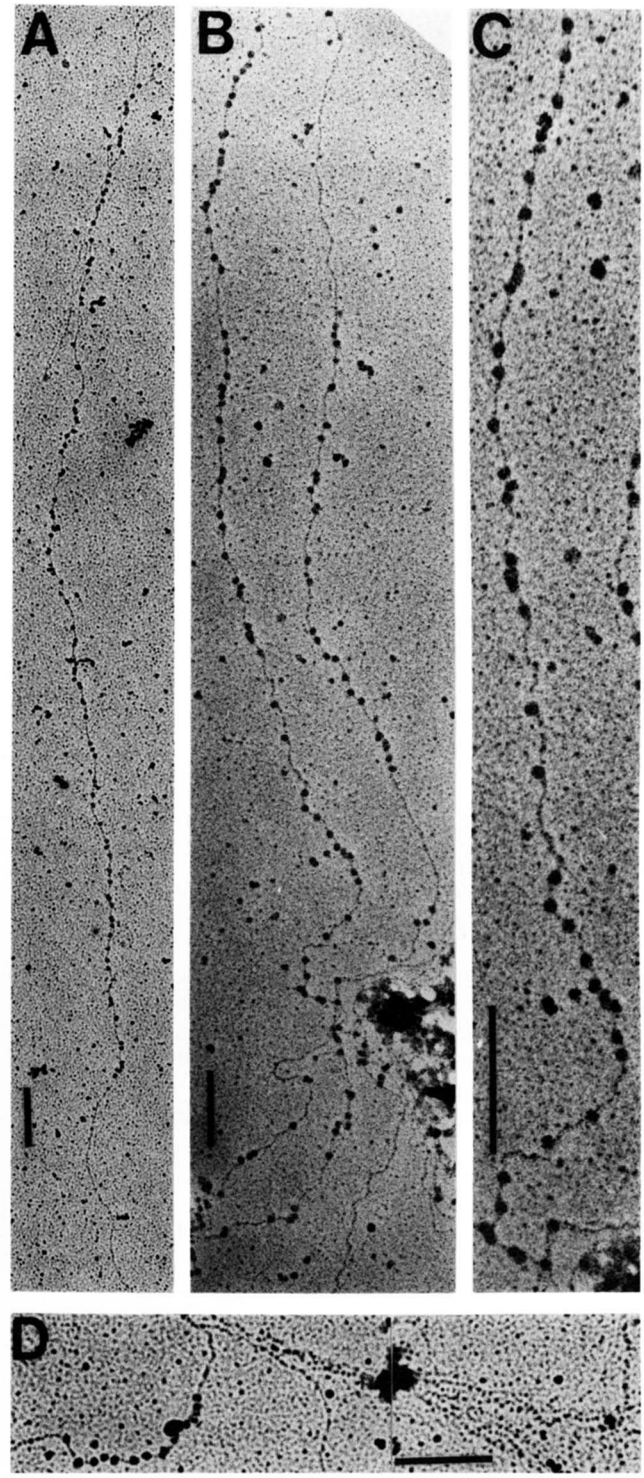

FIG. 8. **Analysis of purified rDNA chromatin by electron microscopy.** rDNA chromatin was purified by metrizamide gradient centrifugation and prepared for electron microscopy by the Miller spreading technique. In each panel, the *black bars* represent 0.25 μm of B-form DNA. *Panels A* and *B*, clusters 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 (1978) 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 nucleoli 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 nucleoli 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 (Keuhn *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 cosediment with large chromatin fragments and insoluble material in sucrose gradients used to fractionate nucleolar micrococcal nuclease digestion products (not shown); the proteins do not cosediment with mononucleosomes.

Alternatively, the two nonhistone proteins may be RNPs 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);[3] 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, gene-specific 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 artifactually. Finally, we believe our purified rDNA chromatin fraction contains only active transcription units, since the resolution of rDNA chro-

[3] P. Ferris, personal communication.

matin 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 erythrocyte nucleosomes from 10.5 to 5.5 S in increasing salt concentration results from the complete dissociation of histones from DNA, an effect that is enhanced by nucleosome dilution. This dissociation may occur even in moderate salt concentrations, 50 to 200 mM NaCl, given a prolonged incubation. These investigators also report that the nucleosomes which remain intact display a rapid, and less dramatic, 10% decrease in sedimentation value, which may reflect an unfolding of the nucleosome core particle.

The demonstration that particles from spermine/spermidine-isolated nucleoli, which we have shown does not promote extensive stripping or proteolysis (Amero *et al.*, 1988), also are subject to the changes in sedimentation behavior indicates that high ionic strength superimposes additional changes in particle structure onto those produced by the divalent cation technique.

Electron microscopic studies have yielded equally conflicting views regarding the presence of nucleosomes in active rDNA chromatin. For instance, in certain electron micrographs of *Tetrahymena* nucleolar chromatin (Ness *et al.*, 1983; Labhart and Koller, 1982), the active transcription units appear devoid of proteins other than RNA polymerase, arguing that beaded morphologies seen by others arise from nonspecific binding of nucleolar proteins during lysis and chromatin fixation steps. A nonbeaded morphology in electron micrographs has also been ascribed to gene regions of ribosomal chromatin of *Oncopeltus* (Foe *et al.*, 1976); these regions do contain protein, however, and are adjacent to beaded spacer regions. On the other hand, in electron micrographs of *X. laevis* nucleolar chromatin, Pruitt and Grainger (1981) have demonstrated that 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* amoebae.

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.

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SUPPLEMENTAL MATERIAL TO:

THE CHARACTERIZATION OF RIBOSOMAL RNA GENE CHROMATIN FROM *PHYSARUM POLYCEPHALUM*

Sally A. Amero, Vicky L. Montoya, Wendy L. Murdoch, Roy C. Ogle
John L. Keating and Robert M. Grainger

METHODS AND MATERIALS

Growth of Cultures, Nucleolar Isolations, and Chromatin Purification. *Physarum* plasmodial cultures were grown and maintained as described (Amero *et al.*, 1988). Nuclei were routinely isolated from macroplasmodial cultures by the procedures of Mohberg and Rusch (1971). Nucleoli were isolated in buffers containing spermidine and spermine (Amero *et al.*, 1988) unless specified otherwise in the text, and treated by mild RNase digestion prior to extractions of crude nucleolar chromatin in 0.1 mM EDTA (pH 7.5), 0.1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). rDNA chromatin was resolved from non-nucleolar chromatin by electrophoresis in agarose gels or by centrifugation in metrizamide equilibrium density gradients (Amero *et al.*, 1988).

Characterization of Proteins Associated with Purified rDNA Chromatin. Protein components of different regions in agarose gels used to purify rDNA chromatin were analyzed in a second dimension SDS-polyacrylamide gel (Laemmli, 1970). Following electrophoresis in the first dimension, the agarose gels were immersed in ethidium bromide (10 μg/ml) for 30 min, and the positions of nuclear chromatin, rDNA chromatin, and ribonucleoprotein (RNP) in the gel (Amero *et al.*, 1988) were determined by ultraviolet illumination. Longitudinal strips of agarose were cemented onto SDS-polyacrylamide gels with 1% agarose in 62.5 mM Tris-HCl (pH 6.8), 2.5% (w/v SDS), 5% β-mercaptoethanol for electrophoresis in the second dimension. The proteins associated with metrizamide-purified rDNA chromatin were prepared for SDS-electrophoresis by lyophilization of metrizamide gradient fractions or by precipitation of rDNA chromatin from ethanol, as described below. Proteins were visualized by silver staining (Oakley *et al.*, 1980).

Preparation and Identification of Physarum Core Histone Proteins. *Physarum* histones were isolated from nuclei as described by Mohberg and Rusch (1969), and were quantitated by the protein assay of Bradford (1976), using calf thymus histone protein standards (Worthington Biochemicals). Individual histone protein fractions were purified by the chromatographic techniques described by Johns (1977) and their identification was confirmed by amino acid analyses. Our results agree with those of other investigators (for example, Mende *et al.*, 1983). Core histone proteins from purified rDNA chromatin were identified by comparisons of their electrophoretic migrations in acetic acid-urea polyacrylamide gels (Panyim and Chalkley, 1969) and SDS-polyacrylamide gels (Laemmli, 1970) to the migrations of the nuclear core histone proteins.

Calculation of Histone to rDNA Mass Ratio. The core histone proteins from purified rDNA chromatin were quantitated by comparing spectrophotometric scans at 595 nm of samples on SDS-polyacrylamide gels stained with Coomassie Blue to scans of *Physarum* nuclear histones. Samples of rDNA chromatin that had been purified by two rounds of metrizamide gradient centrifugation were precipitated in 0.2 M NaCl from two volumes of cold 95% ethanol, stored overnight at -20°C and centrifuged at 16,500xg 10 min at 4°C. Control experiments demonstrated nearly quantitative recovery of chromatin protein and DNA by this method. The pellets were dried under vacuum and dissolved in the sample buffer of Laemmli (1970). A small aliquot was removed for liquid scintillation counting and the remaining sample was analyzed in 18% SDS-polyacrylamide gels.

rDNA in metrizamide-purified rDNA chromatin was quantitated by comparison to known amounts of phage lambda DNA in agarose gels. rDNA was recovered from ethanol-precipitated chromatin by pronase digestion, chloroform/isoamyl alcohol (24:1) extraction and ethanol precipitation. The precipitates were resuspended in 40 mM Tris-HCl (pH 7.8), 5 mM Na-acetate, 0.9 mM EDTA, an aliquot was removed for liquid scintillation counting, and samples and lambda DNA standards were electrophoresed in 1.2% (w/v) agarose gels in Tris-acetate buffer. Gels were stained with ethidium bromide, photographed, and negatives were scanned at 595 nm in a Gilford 240 Spectrophotometer with an automatic recorder.

As described above, aliquots of rDNA chromatin, protein samples, and DNA samples were removed for liquid scintillation counting immediately prior to gel electrophoresis. The values were corrected for possible variation due to quenching, providing a basis for normalization of histone mass and rDNA mass.

Characterization of Core Histone Proteins Associated with Purified rDNA Chromatin by Two-Dimensional Polyacrylamide Gel Electrophoresis. Proteins from twice-purified rDNA chromatin which were precipitated from 25% (w/v) trichloroacetic acid (TCA) on ice for 30 min, were collected by centrifugation at 17,200xg in a Sorvall SS-34 rotor for 30 min at 4°C, washed quickly with ice-cold anhydrous acetone, and dried under vacuum. The precipitates were resuspended in 8 M urea, 5% β-mercaptoethanol, 5% glacial acetic acid, 0.01% Pyronine Y for electrophoresis in Triton-acetic acid-urea 12% polyacrylamide tube gels (Franklin and Zweidler, 1977; Bonner *et al.*, 1980) which had been scavenged as described by Newrock *et al.*, 1977. The gels were then equilibrated and applied to 18% SDS-polyacrylamide gels as described by O'Farrell (1975). Proteins were visualized by staining with freshly-prepared Coomassie Blue G-250.

Assays of RNA Polymerase Activity in Purified rDNA Chromatin. RNA polymerase assays were performed essentially as described by Burgess and Burgess (1974), with the following variations: 1) reaction mixtures contained either nucleolar chromatin (0.3 μg/ml DNA) or purified rDNA chromatin (1.5 μg/ml DNA), 2) the concentration of KCl was lowered to 25 mM, the optimum KCl concentration for RNA polymerase I (Burgess and Burgess, 1974), and 3) each assay contained 0.18 μCi [^{3}H]UTP with a

specific activity of 0.24 μCi/mmole. Reactions were terminated by the addition of transfer RNA to a concentration of 1 mg/ml and of one volume of 15% (w/w) TCA. The mixtures were chilled on ice for 30 min, and filtered through Whatman GF/C glass fiber disks. The disks were washed with cold 5% (w/v) TCA, and then acetone, and were air-dried and counted.

We found that incubation of chromatin samples at 65°C for 15 min prior to the assay reduced incorporation to background levels, as did treatment of assay mixtures with 1 μg/ml RNase A at 37°C for 15 min after the assay.

Micrococcal Nuclease Digestions of Physarum Nuclei or Nucleoli. 1×10^9 nuclei or nucleoli were washed in 20 ml of micrococcal nuclease digestion buffer [10 mM triethanolamine-acetate (TEA-acetate) (pH 7.25), 15 mM NaCl, 1 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$], resuspended at a concentration of 1×10^9 per ml in micrococcal nuclease digestion buffer, and warmed to room temperature. Micrococcal nuclease (Sigma Chemical Co.) was added to 1 U/ml; after 3 min at room temperature, the digestion was terminated by the addition of 0.5 M EDTA to a final concentration of 10 mM. DNA fragments were isolated by extensive RNase and pronase digestions, followed by isoamyl alcohol/chloroform extractions and precipitation from ethanol.

Micrococcal Nuclease Digestions of Purified rDNA Chromatin. rDNA chromatin was purified by centrifugation in metrizamide gradients (Amero et al., 1988) containing 0.1 mM EDTA, rather than 1 mM EDTA, to facilitate subsequent nuclease digestions, and no ethidium bromide was added. Purified rDNA chromatin samples at room temperature were adjusted to contain 10 mM TEA-acetate (pH 7.25), 1 mM $MgCl_2$, 15 mM NaCl, 0.2 mM $CaCl_2$, 0.1 mM PMSF; micrococcal nuclease was then added to a final concentration of 2 U/μg rDNA. Samples were incubated at room temperature with occasional mixing for 10 min, unless noted otherwise, and stopped by the addition of 0.5 M EDTA to a final concentration of 2.5 mM.

To monitor micrococcal nuclease digestion kinetics, aliquots were removed from a digestion mixture at 10 min intervals, and were added to 0.5 M EDTA to a final concentration of 10 mM. One volume of 10% (w/v) TCA was added and the mixtures were incubated on ice 30 min. The samples were filtered through Whatman GF/C glass fiber disks, and washed and counted as described above.

Polyacrylamide Gel Electrophoresis of DNA Fragments and Transfer to Membranes. DNA fragments were prepared and fractionated by electrophoresis in 3%-10% gradient polyacrylamide gels as described by Jeppesen (1980). DNA fragments were transferred to Zeta-Probe nylon membranes (Bio-Rad Laboratories) according to the manufacturer's directions. The efficiency of transfer to Zeta-Probe membranes of small DNA fragments was verified utilizing Alu I or Hae III restriction fragments of plasmid pBR322 DNA (Sutcliffe, 1978) as standard markers of DNA fragment length.

Hybridizations to DNA Fragments Immobilized on Membranes. Cloned DNA fragments used as probes were the kind gifts of Dr. Patrick Ferris and Dr. Volker Vogt. Plasmid pPHR15 contains 12 to 13 kb Physarum rDNA in the proximal direction from the terminal Bam HI site (Ferris, 1984), and represents nearly the entire rDNA transcribed sequences; plasmid pPHR20 contains a 2.3 kb rDNA sequence bounded by Kpn I sites, and represents nontranscribed rDNA sequences (Ferris and Vogt, 1982). Both pPHR plasmids, as well as plasmid pBR322, were amplified and purified according to the procedures described by Maniatis et al. (1982).

Purified rDNA used as a hybridization probe was isolated from nucleoli as described (Grainger and Ogle, 1978), and purified from polysaccharide slime by centrifugation in CsCl-ethidium bromide gradients described by Maniatis et al. (1982). rDNA was then resolved from non-nucleolar DNA by centrifugation in CsCl gradients and the purity of rDNA was assessed by centrifugation in CsCl analytical gradients (Amero et al., 1988).

Purified rDNA or cloned DNA samples used as hybridization probes were labeled with [^{32}P]dCTP to specific activities of approximately 1×10^7 cpm/μg by nick translation (Maniatis et al. 1982).

Zeta-Probe membranes were prepared for hybridization reactions by incubation in prehybridization mixture (62.5 mM sodium phosphate (pH 6.8), 5 X SSC (1XSSC = 150 mM sodium chloride, 15 mM sodium citrate), 1.25% glycine, 100 μg/ml depurinated calf thymus DNA, 50% (w/v) formamide) at 42°C overnight. Hybridization reactions were conducted at 45°C for 24 h in 10 to 20 ml pre-warmed hybridization mixture (40 mM sodium phosphate (pH 6.8), 0.025% bovine serum albumin, 0.025% polyvinylpyrollidone, 0.025% Ficoll, 3.75 X SSC, 100 μg/ml depurinated calf thymus DNA, 10% (w/v) dextran sulfate, 50% (w/v) formamide) and membranes were then washed twice in 2 X SSC, 0.1% SDS at 42°C for 15 min each and twice in 0.1 X SSC, 0.1% SDS at room temperature. Membranes were air-dried and then exposed to Kodak X-Omat film with an intensifying screen for 24 to 48 h. We calculate that these washing conditions permit approximately 15% mismatch (Wetmur and Davidson, 1968; McConaughy et al., 1969).

In experiments involving rehybridization of a filter to different probes sequentially, probes were stripped from the membranes by incubation in hybridization mixture at 65°C for 30 min. Re-exposure of stripped membranes to film demonstrated the nearly quantitative removal of the probe.

Characterization of rDNA Chromatin Particles by Metrizamide Gradient Centrifugation. Purified rDNA chromatin was digested with micrococcal nuclease as described above, and re-introduced into 3 ml metrizamide gradients (ρ = 1.1811 gm/cm^3) containing 1 mM TEA-acetate (pH 7.25), 1 mM EDTA, 0.1 mM DTT, and 0.1 mM PMSF. The gradients were centrifuged at 55,000 rpm for 42 h at 4°C in a Beckman SW50.1 rotor. Fractions (approximately 150 μl each) were collected from the bottom of the tubes. The distribution of chromatin particles was determined by precipitating fraction aliquots in 20% (w/v) TCA on ice for 30 min and filtering the samples through Whatman GF/C filters as described above.

Characterization of rDNA Chromatin Particles by Sucrose Gradient Centrifugation. Purified rDNA chromatin was digested with micrococcal nuclease as described above, and layered on 16 ml 5%-20% linear (w/v) sucrose gradients containing 10 mM TEA-acetate (pH 7.25), 2 mM EDTA, 1 mM $MgCl_2$ and either 15 mM, 0.1 M, or 0.35 M NaCl. The gradients were centrifuged in the Beckman SW27.1 rotor at 26,000 rpm for 22 1/2 h at 4°C. In each experiment, total [^{14}C]-labeled Drosophila RNA was layered in the same volume of metrizamide solution onto parallel sucrose gradients to provide size standards. Fractions were collected from the bottom of the tubes to determine the distribution of radioactivity in the gradients; the concentration of sucrose in the gradients was determined by refractometer readings of fraction aliquots.

Electron Microscopy. Metrizamide gradient fractions containing rDNA chromatin were diluted tenfold and dispersed in cold pH 9.0 distilled water for 20-30 min, then 1/4 volume of 0.1 M sucrose, 10% formalin, (pH 8.5) was added. As described by Miller and Beatty (1969), the chromatin was centrifuged through a sucrose-formalin cushion onto a carbon-coated electron microscope grid, stained with phosphotungstic acid and uranyl acetate, and rotary shadowed with platinum. The grids were observed using a JEOL 100C transmission electron microscope.