The Purification of Ribosomal RNA Gene Chromatin from Physarum polycephalum*

(Received for publication, February 9, 1985)

Sally A. Ameroč, Roy C. Ogleš, John L. Keating, Vicky L. Mostoya, Wendy L. Murdoch, and Robert M. Grainger†

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

We have undertaken the purification of ribosomal RNA gene (rDNA) chromatin from the slime mold Physarum polycephalum, in order to study its chromatin structure. In this organism rDNA exists in nucleoli as highly repeated minichromosomes, and one can obtain crude chromatin fractions highly enriched in rDNA from isolated nucleoli. We first developed a nucleolar isolation method utilizing polyamines as stabilization agents that results in a chromatin fraction containing far more protein than is obtained by the more commonly used divalent cation isolation methods. The latter method appears to result in extensive histone loss during chromatin isolations. Two methods were then used for purifying rDNA chromatin from nucleoli isolated by the polyamine procedure. We found that rDNA chromatin migrates as a single band in agarose gels, well separated from other components in the chromatin preparation. Although the utility of this technique is somewhat limited by low yields and by progressive stripping of protein from rDNA chromatin, it can provide useful information about rDNA chromatin protein composition. The application of this technique to the fractionation of gene and spacer chromatin fragments produced by restriction enzyme digestion is discussed. We also found that rDNA chromatin, if RNAse-treated, bands discretely in metrizamide equilibrium density gradients with a density lighter than that of non-nucleolar chromatin. These characteristics suggest that we have identified a transcriptionally active rDNA chromatin fraction which possesses a lower protein to DNA ratio than does non-nucleolar chromatin. This technique yields sufficient purified rDNA chromatin for further biochemical studies and does not cause extensive protein stripping. The procedures developed here should be applicable to the analysis of a variety of chromatin fractions in other systems.

The ribosomal RNA genes of the slime mold Physarum polycephalum, as well as several other primitive eukaryotes, exist as discrete, linear, palindromic molecules (Gall, 1974: Molgaard et al., 1976; Karrer and Gall, 1976; Auffrey et al., 1977; Cockburn et al., 1978), that are localized in nucleoli and are highly repeated (Braun and Evans, 1969; Ryser and Braun, 1974; Yao et al., 1974; Bohnert et al., 1975; Cockburn et al., 1976, Grainger and Ogle, 1978; Borhardt and Nielsen, 1981). Because of these characteristics these genes may be studied as a chromatin fraction (rDNA chromatin) obtained from isolated nucleoli (for example, Bradbury et al., 1973; Leer et al., 1976; Colavito-Shepenski and Gorovsky, 1983; and references below).

Typical nucleolar chromatin preparations from Physarum (or other organisms with extrachromosomal rDNA) also contain many components which render the analysis of rDNA chromatin impossible without additional purification techniques. A small percentage of intact nuclei are invariably present in preparations of isolated nucleoli (ratios of nuclei/nucleoli range from 1:300 to 1:1000). Since rDNA chromatin comprises 2% of the Physarum cellular genome (Braun and Evans, 1969; Ryser and Braun, 1974; Bohnert et al., 1975; Hall and Braun, 1977), standard nucleolar chromatin fractions from Physarum may contain up to 50% non-nucleolar chromatin. Other contaminants, including nucleolar matrix and nascent ribosomal components, are found in these fractions; additionally, Physarum plasmodial cultures secrete a mixture of slime polysaccharides (McCormick et al., 1976) which copurify with chromatin and DNA. As a consequence only a small fraction of the components in crude nucleolar preparations are directly associated with rDNA chromatin.

To enable further study of Physarum rDNA chromatin structure, it was therefore important to develop methods to eliminate these components from chromatin preparations. In our experiments three criteria were used in assessing rDNA chromatin purity and integrity: 1) enrichment with respect to rDNA sequences, 2) maintenance of the native protein complement and integrity of chromatin structure, and 3) the elimination of other nucleolar components.

Several different approaches have been reported for the purification of rDNA chromatin with respect to the criterion of rDNA sequence enrichment. Leer et al. (1976) obtained a fraction of rDNA chromatin from Tetrahymena, which was at least 95% pure with respect to DNA sequence, by treating a crude nucleolar lysate briefly with trypsin and pelleting the insoluble bulk chromatin. Jones (1978a, 1978b) obtained a fraction of rDNA chromatin from Tetrahymena by fractionating a crude nucleolar lysate in glycerol gradients; the DNA in this fraction is nearly all rDNA. Minichromosomes containing the Physarum ribosomal RNA genes have been isolated by sucrose gradient centrifugation of crude nucleolar lysates alone (Seebeck et al., 1979) or following gel filtration chromatography (Cunningham et al., 1984). In addition

* This investigation was supported by National Institutes of Health Grant GM-34430. 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, Gilner Hall, University of Virginia, Charlottesville, VA 22901.

1 The abbreviations used are: rDNA, ribosomal RNA gene; PMSF, phenylmethylsulfonyl fluoride; EGTA, [ethylenediamine(oxethylenenitro)-]tetrasacetic acid; TEA, triethanolamine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.
metrizamide gradient centrifugation has been utilized to isolate a nucleolar fraction containing rDNA from *Tetrahymena* (Higashinakagawa et al., 1979) and from *Xenopus laevis* (Higashinakagawa et al., 1977). However, these studies have not assessed the purity of rDNA chromatin in terms of an appropriate protein complement, structural integrity, or contamination from components that are not normally associated with rDNA chromatin.

Our early results led us to question whether the standard procedure for isolating nucleoli from *Physarum* may disrupt the chromatin structure of the ribosomal RNA genes. This procedure for isolating nuclei and nucleoli from *Physarum* utilizes divalent cations as stabilizing agents (Mohberg and Rusch, 1971) to produce clean, intact structures. However, Clark and Felsenfeld (1971) have demonstrated that the incubation of chromatin in buffers containing comparable levels of CaCl₂ permits the random rearrangement of chromosomal proteins. Many procedures utilizing divalent cations and/or polyamines have been developed for the isolation of nuclei and nucleoli from *Tetrahymena* and *Dictyostelium*. In comparing these methods, several authors have noted differences between nuclei or nucleoli isolated by the various methods (Gorovosky, 1970; Charlesworth and Parish, 1977; Jones, 1978a, 1978b; Hamana and Iwai, 1979; see also Jockusch and Walker, 1974 and Schicker et al., 1979), but none has reported a thorough investigation of these differences. As a way to assess the possible effects of the divalent cation procedure on the integrity of *Physarum* rDNA chromatin, we devised an alternative procedure to isolate nucleoli from *Physarum* in the presence of spermine and spermidine, and have compared the properties of chromatin samples prepared by each method. We have also compared the properties of chromatin samples extracted from nucleoli by a variety of methods.

Finally, in conjunction with the new nucleolar isolation procedure we have developed two methods, one involving agarose gel electrophoresis and the other metrizamide density gradient centrifugation, to purify rDNA chromatin from many of the other components found in nucleolar chromatin preparations. In this way we have been able to achieve purity not only with respect to DNA sequence but also with respect to protein complement.

### MATERIALS AND METHODS

#### RESULTS

Purification of rDNA Chromatin by Gel Filtration Chromatography—We first tried gel filtration chromatography as an initial step in the purification of rDNA chromatin from *Physarum* nucleolar fractions. Lysates from nucleolar preparations isolated by the divalent cation procedure were applied to a Bio-Gel A-150m column, and as shown in Fig. 1A, two peaks of ultraviolet-absorbing material were resolved by the column. Analysis of nucleic acids from pooled fractions within each peak by sedimentation in CsCl gradients revealed that the first peak to elute from the column contained predominantly rDNA and nuclear DNA sequences, and that the second peak contained predominantly RNA (data not shown). As shown in Fig. 1A, the first peak contained most of the [³H] thymidine radiolabel and, therefore, most of the eluting DNA. Using this procedure rDNA chromatin is clearly not substantially resolved from any residual bulk chromatin. On the other hand, the column fractionation does remove an extensive amount of ribonucleoprotein from the chromatin preparation.

Subsequently, proteins from pooled column fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1B); the core histone proteins were identified on the basis of electrophoretic mobilities, which correspond to those of histone protein standards (Fig. 1B, lane 6). This analysis revealed that the four core histone proteins which coeluted from the column with the first UV-absorbing peak were not present in equimolar ratios; in addition, a substantial amount of protein that comigrates with core histone protein, in particular histone H2A, eluted with the second peak. These observations presented the possibility that chromatin proteins may have been removed from chromatin molecules and that the chromatin fraction may thus not have possessed a full complement of chromosomal proteins. Although samples used in these experiments were solubilized by sonication treatments, we

---

3 Portions of this paper (including "Materials and Methods," part of "Results," and Table 1) 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.
obtained the same protein profiles in fractions eluted from the column with nucleolar lysates that had not been sonicated (not shown).

Nucleolar Isolations in Buffers Containing Polyamines—Because of concerns that the divalent cation nucleolar isolations might lead to extensive protein loss in rDNA chromatin preparations, we developed a procedure using polyamines as stabilizing agents, which is described in detail under "Materials and Methods." Several tests were then used to assess the protein complement associated with chromatin preparations isolated by the two procedures. First, nucleoli were isolated according to both procedures, digested with micrococcal nuclease, and the resulting deoxyribonucleoprotein particles were solubilized and fractionated electrophoretically in 1% agarose gels. DNA extracted from these particles was also examined on these gels. As can be seen in Fig. 2, DNA from both digestes (from Mg²⁺ or Ca²⁺-isolated nucleoli, lanes 1 and 3, respectively, or spermine/spermidine-isolated nucleoli, lane 5) demonstrates the basic ladder pattern indicative of repeating chromatin subunit structure. In this system nucleosomal particles normally migrate much more slowly than DNA extracted from such particles (Bakayev et al., 1977). However, chromatin fragments from Mg²⁺ or Ca²⁺-isolated nucleoli (lanes 2 and 4, respectively) migrate at exactly the same mobility as purified DNA from these samples. Only nucleosomal particles derived from nucleoli isolated by the spermine/spermidine procedure show the typically retarded migration expected (lane 6). Therefore, nucleosome-like organization exists in nucleoli from all three kinds of nucleolar preparations, yet there is substantially less protein in chromatin from nucleoli prepared by divalent cation procedures.

We also analyzed the extent of protein loss inherent to the two nuclear isolation procedures by comparing the densities of chromatin samples in CsCl-sarkosyl density gradients (Vinograd, 1963). Nucleolar chromatin samples prepared from nucleoli isolated according to each protocol were cross-linked by formaldehyde to prevent dissociation of protein and DNA during centrifugation. In this type of analysis, most proteins band at a density close to 1.2 g/cm³ and DNA at 1.70 g/cm³ so that chromatin fractions with higher density have a smaller proportion of protein. We found chromatin prepared from Mg²⁺-isolated nucleoli formed a broad band in these gradients with an average density near 1.6 g/cm³ (Fig. 3A); chromatin prepared from spermine/spermidine-isolated nucleoli formed a sharper band with a density of 1.4 g/cm³ (Fig. 3B). Therefore, a significant proportion of the chromatin sample from Mg²⁺-isolated nucleoli possessed a lower protein/DNA ratio than chromatin originating from spermine/spermidine-isolated nucleoli.

Because chromatin from spermine/spermidine preparations appeared to have a more native chromatin structure, we attempted to fractionate this material on Bio-Gel A-150m columns as described earlier to separate the chromatin from smaller contaminants. This was not possible, however, since chromatin prepared by this method could not be successfully eluted from Bio-Gel A-150m columns. In general we have found that chromatin from nucleoli stabilized with spermine and spermidine is far less soluble than chromatin from nucleoli stabilized with divalent cations, again consistent with a higher protein to DNA ratio.

![Fig. 2. Changes in nucleosome structure occur during the divalent cation nucleolar isolation method: agarose gel electrophoresis of Physarum chromatin particles. Nucleoli were isolated from microplasmodia in the presence of 10 mM MgCl₂, 10 mM CaCl₂, or spermidine and spermine. Chromatin particles were isolated by treatment of the nucleoli with micrococcal nuclease and solubilization in low ionic strength buffers. Subsequently, DNA was purified from a portion of the soluble chromatin fractions, and both chromatin and DNA samples were fractionated by electrophoresis in 1% agarose gels. Lanes 1, 3, and 5 contain purified DNA samples; lanes 2, 4, and 6 contain chromatin samples. The samples in lanes 1 and 2 came from nucleoli isolated in MgCl₂, in lanes 3 and 4 from CaCl₂-isolated nucleoli, and those in lanes 5 and 6 from spermidine-isolated nucleoli. The similar migration of DNA and nucleosomes from nucleoli isolated in divalent cations, as well as the retarded migration of nucleosomes from nucleoli isolated in spermine and spermidine, are indicative of changes in nucleosome structure inherent to the divalent cation nucleolar isolation method.](image)

![Fig. 3. Comparison of chromatin prepared from divalent cation-isolated nucleoli and spermine/spermidine-isolated nucleoli: analysis by CsCl density gradient centrifugation. Nucleoli were isolated from microplasmodia grown in the presence of [³H]thymidine by either the divalent cation method or by the spermidine/spermine method. Chromatin fractions were solubilized from each, treated with formaldehyde to cross-link proteins and DNA, and fractionated by centrifugation in CsCl density gradients containing 0.5% sarkosyl. The presence of chromatin in individual fractions was detected by liquid scintillation counting and the densities of certain fractions were determined by refractometry. Panel A contains chromatin from nucleoli isolated by the divalent cation method; Panel B contains chromatin from nucleoli isolated by the spermidine/spermine method. The difference in densities between the two samples demonstrates that chromatin obtained from nucleoli isolated by the divalent cation method possesses significantly less protein than does chromatin isolated by the spermidine/spermine method.](image)
Purification of rDNA Chromatin by Agarose Gel Electrophoresis—Since gel filtration chromatography had failed to resolve rDNA chromatin from non-nucleolar chromatin and was incompatible with chromatin samples from nucleoli isolated with spermine and spermidine, we sought a new method for purifying rDNA chromatin.

We found that the technique of agarose gel electrophoresis, utilizing low percentage, low charge agarose gels, was successful in resolving the components of nucleolar chromatin samples. In Fig. 4A is shown a gel stained with ethidium bromide, comparing electrophoretic properties of chromatin from spermine/spermidine and from Mg²⁺ nucleolar preparations. In this spermine/spermidine preparation (lane 1), the band marked by an asterisk contains only rDNA, as shown by CsCl gradient analysis of DNA purified from this band (data not shown). At the top of this lane of the gel one can see a large amount of stained material (marked by an arrow); this is predominantly non-nucleolar DNA, as assessed by its buoyant density in CsCl gradients (data not shown). Below the chromatin band is a broad band that comigrates with, and is partially obscured by, the bromphenol blue marker dye used here. This material is completely removed by RNase treatment and is therefore thought to be ribonucleoprotein. In Fig. 4A lane 2 is shown rDNA chromatin from a Mg²⁺ nucleolar preparation. The prominent ethidium bromide-staining band, rDNA chromatin, migrates considerably ahead of rDNA chromatin from spermine/spermidine nucleolar preparations, consistent with a depleted protein complement. This material can be shown to comigrate with purified rDNA in this gel system (data not shown).

In Fig. 4B the gel from A is shown after subsequent staining with Coomassie Blue to identify protein-containing regions. While the rDNA chromatin band from spermine/spermidine-isolated nucleoli is clearly stained, that from the Mg²⁺-isolated nucleoli is not, again arguing that this chromatin preparation is depleted in protein. Subsequently, the proteins associated with the rDNA chromatin band from each type of nucleolar preparation were electroeluted from agarose gel strips and compared by SDS-polyacrylamide gel electrophoresis. The rDNA chromatin band from Mg²⁺-isolated nucleoli possessed far less histone protein/microgram of rDNA than did the rDNA chromatin band from spermine/spermidine-isolated nucleoli (not shown).

The agarose gel method successfully purifies rDNA chromatin from many contaminants, and its uses in identifying rDNA chromatin proteins will be described (Amero et al., 1988), but upon longer electrophoresis, we noticed that the rDNA chromatin band began to diffuse, as though proteins were being stripped during the electrophoresis. To test this hypothesis we determined the density in CsCl/sarkosyl density gradients of rDNA chromatin which had been purified in an agarose gel and then cross-linked (panel A) was compared to the density of rDNA chromatin which had been cross-linked and then purified by agarose gel electrophoresis (panel B). Fractions were assayed by liquid scintillation counting to detect the presence of chromatin and by refractometry to determine densities. The difference in density between the two samples indicates that significant protein stripping occurs during agarose gel electrophoresis.

![Fig. 4](image-url)  

**Fig. 4. Purification of rDNA chromatin by agarose gel electrophoresis.** Nucleolar chromatin samples were fractionated by electrophoresis in 0.4% agarose gels in low ionic strength buffers, and the nucleic acids in the gels were stained with ethidium bromide (panel A). The position of the well is marked by an arrow; and the position of rDNA chromatin is marked by an asterisk. Proteins in the gels were then stained with Coomassie Blue (panel B). This particular gel contained chromatin solubilized from nucleoli isolated by the spermidine/spermine method (lane 1 in each panel) and by the divalent cation method (lanes marked 2 in each panel). The differences in electrophoretic mobility and Coomassie Blue staining between the two samples demonstrates that rDNA chromatin obtained from nucleoli isolated by the divalent cation method contains much less protein than does rDNA chromatin from spermine/spermidine-isolated nucleoli.

![Fig. 5](image-url)  

**Fig. 5. Protein stripping occurs during agarose gel electrophoresis of rDNA chromatin: analysis by CsCl density gradient centrifugation.** The density in CsCl/sarkosyl density gradients of rDNA chromatin which had been purified in an agarose gel and then cross-linked (panel A) was compared to the density of rDNA chromatin which had been cross-linked and then purified by agarose gel electrophoresis (panel B). Fractions were assayed by liquid scintillation counting to detect the presence of chromatin and by refractometry to determine densities. The difference in density between the two samples indicates that significant protein stripping occurs during agarose gel electrophoresis.

While the electrophoretic purification scheme does provide a method for essentially complete purification of rDNA chromatin, the method has limitations. The protein-stripping problem can be minimized by using noncharged agarose, and it has been possible to analyze the protein components associated with rDNA chromatin.
purified Physarum rDNA chromatin that would match the protein-depleted chromatin from divalent cation isolated nucleoli. However, it is difficult to isolate quantities of purified rDNA chromatin required for many preparative analyses (10 µg or more).

**Purification of rDNA Chromatin by Metrizamide Gradient Centrifugation**—We sought to develop another technique for purifying Physarum rDNA chromatin that would match the purification possible by gel electrophoresis, but eliminate protein loss encountered in gel electrophoresis, and also produce larger amounts of rDNA chromatin for subsequent analyses. We thought it might be possible to separate rDNA chromatin and nonnucleolar chromatin in gradients of metrizamide, which, since it is a nonionic medium, would be expected to interact very little with chromatin samples. However, we found that crude nucleolar chromatin fractions in metrizamide equilibrium gradients band as a single chromatin peak (ρ = 1.19 g/cm³) (Fig. 6A) which contains both rDNA and nuclear DNA sequences (as revealed by CsCl gradient analysis; data not shown). However, when isolated nucleoli were treated by a mild RNase digestion to release nascent ribonucleoprotein components prior to chromatin extraction, and the resulting nucleolar extracts were centrifuged in metrizamide equilibrium density gradients, two DNA-containing peaks were resolved in these gradients (ρ = 1.19 g/cm³ and 1.163 g/cm³; Fig. 6B).

To characterize these fractions, DNA was extracted from each peak and analyzed by CsCl density gradient centrifugation. As shown in Fig. 7A, DNA from the dense metrizamide peak contained both nuclear DNA and rDNA; DNA from the light metrizamide peak contained only rDNA (Fig. 7B). We also determined the densities of purified rDNA in metrizamide (1.12 g/cm³), and of most proteins in metrizamide (1.27 g/cm³) (see also MacGillivray and Rickwood, 1978), and found that our chromatin peaks band with densities intermediate to these two values, indicating that both metrizamide peaks contained deoxyribonucleoprotein. The density of a significant proportion of rDNA chromatin was affected by RNase treatment, presumably from removal of ribonucleoprotein fibrils from active genes or to reduction of nonspecific aggregation. Since the density of this fraction is lighter in metrizamide it would appear that this rDNA chromatin fraction may possess a lower protein/DNA ratio than does bulk chromatin, a point which is investigated in the accompanying report (Amero et al., 1988).

We addressed the possibility of protein stripping during metrizamide gradient centrifugation in the following ways. First, we purified rDNA by metrizamide gradient centrifugation and reintroduced the chromatin to a second round of metrizamide gradient centrifugation. We compared the densities of rDNA chromatin after each purification step and found that the density of twice-purified rDNA chromatin (1.14 g/cm³) is lighter than the density of first-round rDNA chromatin (1.163 g/cm³). In order to estimate the amount of protein stripping this density shift represents, we assumed a direct relationship between the density of certain nucleoprotein complexes (high molecular weight chromatin, ribonucleoprotein particles) and their reported protein and nucleic acid contents (MacGillivray and Rickwood, 1978). We estimated

![Fig. 6. Purification of rDNA chromatin by metrizamide gradient centrifugation.](image-url)
that a loss of approximately 20% total protein occurs during metrizamide gradient centrifugation. We also examined the migration of metrizamide-purified rDNA chromatin in our agarose gel system. When electrophoresed with nucleolar chromatin not previously exposed to metrizamide the two samples comigrated in the gel (data not shown). If metrizamide centrifugation resulted in substantial depletion of rDNA chromatin protein, we would expect an acceleration in mobility compared to rDNA chromatin which possesses more protein.

**DISCUSSION**

Our goal in this study was to develop an effective method for purifying rDNA chromatin from *Physarum*. Our first objective was to prepare crude nucleolar chromatin fractions which retained as much of the rDNA chromatin protein complement as possible. Our second objective was to obtain rDNA chromatin from fractions purified with respect to DNA sequence as well as associated protein content, including the removal of ribonucleoprotein and nucleolar matrix components. This later point is addressed more completely in our accompanying report (Amero et al., 1988).

We compared the properties of rDNA chromatin fractions prepared from nucleoli isolated according to two different protocols. Our results suggest that the standard divalent cation isolation procedures strip chromosomes proteins from rDNA chromatin. In considering this matter it is important to discuss both histone proteins and nonhistone proteins. Experiments which are described in the accompanying paper (Amero et al., 1988) suggest that two abundant nonhistone proteins on rDNA chromatin prepared from spermine/spermidine-isolated nucleoli may bind nonspecifically to chromatin. These abundant proteins must contribute to the different densities of these chromatin samples in CsCl density gradients and may account for some of the difficulties we encountered in gel filtration chromatography with these samples.

Our results suggest, however, that the divalent cation nucleolar isolation technique leads to significant loss of histone proteins, in comparison to the spermine/spermidine technique, exemplified by differences in electrophoretic behavior of chromatin from the two procedures. Direct comparison of the proteins associated with agarose gel-purified rDNA chromatin from each type of nucleolar preparation indicates that rDNA chromatin from Mg²⁺-isolated nucleoli possesses much less histone protein than rDNA chromatin from spermine/spermidine-isolated nucleoli (not shown). In addition particles from polyamine-isolated nucleoli migrate as expected for intact chromatin structures, whereas particles from Mg²⁺-isolated nucleoli comigrate with deproteinized DNA isolated from these chromatin particles. Several studies suggest that these atypical particles do not reflect the chromatin structure that exists in vivo. First, DNA fragments from these digests display typical repeat patterns (see also Grainger and Ogle, 1978), so that some nucleosome-like features must be present in the intact nucleolus at the time of digestion. Second, the transcribed portion of the *Physarum* rDNA molecule is protected in an intact nucleus from psoralen cross-linking (Judelson and Vogt, 1982), although the frequency and spacing of the cross-links differ from those in the nontranscribed portion. Third, we have visualized by electron microscopy scattered nucleosomes in the rRNA gene of *Physarum anamoe-bae* (Grainger and Ogle, 1978); we discuss in our accompanying report (Amero et al., 1988) that these nucleosomes visualized in the electron microscope may represent only a small fraction of the nucleosomes present on these genes in vivo. Even though a simple particle composed of the histones H3 and H4 may account for the results in each of these studies (Solner-Webb et al., 1976), such a particle would be expected to migrate more slowly than DNA in agarose gels (Wu and Crothers, 1984). Clearly the particles produced in our Mg²⁺-isolated nucleoli contain even less protein than H3/H4 tetramers. Since there appears to be at least some chromatin structure in intact nucleoli it is likely that protein loss occurs during lysis. Another factor which might account for some histone loss from chromatin is proteolysis which has been shown to occur during lysis of Mg²⁺-isolated nuclei from *Physarum* (Annesley et al., 1981).

Histone loss does not appear to be extensive in chromatin from polyamine-isolated nucleoli. The histone/DNA ratio in this chromatin fraction is 0.77:1, only slightly reduced from the overall levels in total nuclear chromatin (Amero et al., 1988). It is more of a concern whether some histones are being added to rDNA chromatin during the polyamine nucleolar isolation, perhaps by a nucleosome assembly factor. The histone pool available for such assembly is likely to be quite small, based on experiments involving incorporation of acetoxypropene derivatized H3 proteins into *Physarum* chromatin (Prior et al., 1980). The well-characterized nucleosome assembly systems are dependent on Mg²⁺ and ATP (Ruberti and Worcel, 1986) and would be inefficient in our polyamine buffer systems. In addition experiments in which exogenous DNA was added during nucleolar isolations (Amero et al., 1988) argue that histone does not become bound to this DNA and suggest that this kind of histone addition does not occur.

The difference in the two isolation procedures may be the result of effects involving polysaccharide slimes which appears to be more prevalent in Mg²⁺-isolated nucleoli than in polyamine-isolated nucleoli. It is possible that polyamines also prevent interaction of negatively charged slime polysaccharides with nucleolar chromatin and thus inhibit protein loss. We conclude it is unlikely that extraneous histone proteins are becoming associated with rDNA chromatin during the spermine/spermidine nucleolar isolation procedure.

It has been suggested that chromatin particles within active and inactive rRNA gene chromatin differ, since a fraction of chromatin particles with a sedimentation value of 5 S enriched in active rDNA gene sequences (“Peak A” particles) can be resolved from typical 11 S nucleosomes by sucrose gradient centrifugation (Johnson et al., 1976a, 1976b; 1976; Johnson, 1980). These Peak A particles are associated with two nonhistone proteins and have a less compact conformation than do 11 S nucleosomes (Prior et al., 1983). Since these particles were purified from divalent cation-isolated nucleoli there may be similar concerns about protein loss as discussed above. It has been argued that Peak A particles do contain all four core histones and the sedimentation value of these particles results from conformational differences (Prior et al., 1983). Additional effects, however, may be involved; this issue is discussed further in our accompanying paper (Amero et al., 1988).

We explored several methods for the purification of *Physarum* rDNA chromatin. First we attempted the purification of rDNA chromatin by gel filtration chromatography and found that this technique allows fractionation of total nucleolar chromatin from ribonucleoprotein components but does not remove residual nuclear chromatin from rDNA chromatin. Cunningham et al. (1984) have reported the purification of *Physarum* rDNA chromatin through an initial gel filtration chromatographic step similar to the method we described followed by sucrose gradient centrifugation. However, our experience indicates that gel filtration chromatography is only useful with chromatin likely to be depleted in chromatin protein.
To circumvent the limitations of the gel filtration columns, we developed methods involving agarose gel electrophoresis in conditions of low ionic strength for the purification of rDNA chromatin from bulk chromatin and from ribonucleoprotein contaminants. To our knowledge this technique has not been used to characterize rDNA chromatin fractions and should be useful in a variety of systems. The technique can be readily used in a two-dimensional gel analysis to characterize the proteins associated with the chromatin fraction (Amero et al., 1988).

Using the agarose gel electrophoretic method, we can distinguish rDNA chromatin from divalent cation and spermast/ spermidine nucleolar preparations because of their different mobilities, and therefore it also provides a powerful test for the loss of chromatin protein. However, the agarose gel purification method also results in an intrinsic, additional loss of chromatin protein, which can be minimized by reducing the intrinsic charge of the agarose medium or circumvented altogether by chemical cross-linking prior to the electrophoretic procedure. Although this latter treatment precludes the possibility of certain subsequent protein analyses, it should be possible ultimately to reversibly cross-link chromatin samples prior to agarose gel electrophoresis to circumvent protein stripping and to facilitate recovery of chromatin protein.

Agarose gel electrophoresis may also be useful in the purification of chromatin fragments produced by restriction enzyme cleavage of DNA in chromatin samples. We have achieved the resolution of rDNA chromatin fragments produced by EcoRI, HindIII, or HaeIII digestion of chromatin samples from divalent cation-isolated nucleoli; although rDNA is cleaved in spermast/spermidine-isolated nucleoli, we failed to resolve chromatin fragments extracted from them. We attribute this difference to the cohesive effects of the additional protein content in the latter samples. It may be possible to overcome this problem by treating chromatin with reagents such as β-galactosidase or griseofulvin, as suggested by Prior et al. (1983), to overcome these effects. In addition reversible cross-linking techniques may also permit the use of detergents in the agarose gels to overcome some of these interactions and to enable one to purify chromatin fragments (e.g. gene and nontranscribed spacer)in this manner. In this way this method may allow resolution of chromatin fragments that are superior to that obtainable with sucrose gradient centrifugation (Cunningham et al., 1984; Laughlin et al., 1984) or affinity chromatography (Weisbrod and Weintraub, 1981).

In spite of the utility of the gel electrophoresis methods, we sought another way to purify chromatin that would further minimize the protein stripping problem and increase our yield of chromatin; metrizamide gradient centrifugation met these criteria. We were able to resolve Physarum rDNA chromatin from non-nuclear chromatin and from other nuclear components (Amero et al., 1988) in metrizamide gradients when the initial nucleolar preparation was first treated with low levels of RNase. Otherwise nucleolar and non-nuclear chromatin bands as a single peak in these gradients.

The densities of Physarum rDNA chromatin and non-nuclear chromatin in metrizamide gradients demonstrate that RNase treatment produces a derivative of rDNA chromatin which probably has a lower protein/DNA ratio than is found in non-nuclear chromatin. However, a significant proportion of rDNA chromatin is unaffected by RNase treatment and comigrates with most chromatin in these gradients. We have shown previously that the active transcription unit within the ribosomal RNA genes of Physarum amoebae in electron micrographs are tightly packed with RNA polymerase molecules and possess dense fibrils of ribonucleoprotein (Grainer and Ogle, 1978). We believe that this ribonucleoprotein network may contribute significantly to the overall density of the rDNA chromatin complex. Therefore, we suggest that these two types of rDNA chromatin in metrizamide gradients may represent transcriptionally active (and thus sensitive to RNase treatment) and transcriptionally inactive (and insensitive to RNase treatment) rDNA chromatin fractions. If so, the purified rDNA chromatin fraction may represent the first purified fraction of a single, active gene.

Both the agarose gel systems and metrizamide gradients have allowed us to characterize the Physarum rDNA chromatin (Amero et al., 1988) much more carefully than would have been possible without these techniques. In addition these methods are likely to be useful in analyzing a variety of other extrachromosomal genes and viral chromosomes.

REFERENCES


Borkhardt, B., and Niesler, O. F. (1981) Chromosoma 84, 131-143


Purification of rRNA Gene Chromatin


Seebach, T., Stalder, J., and Braun, R. (1979) Biochemistry 18, 484-491
Purification of rRNA Gene Chromatin

We also compared the effects of warming nucleoli recovered from a suspension containing buffer containing 10 mM MgCl₂ as in buffer A, which contains spermine and spermidine. We found that pretreatment chromatin from each preparation had a density in 20-25% sucrose density gradients of 1.07 g/mL (data not shown), indicating little difference between the two buffers. These results suggest that conditions leading to stripping of chromatin proteins in the absence of spermine and spermidine are responsible for the observed differences in non-denaturation steps, not in the later washing steps. In addition, similar analyses indicated that the inclusion of both spermine and spermidine in the buffer A wash was necessary to avoid protein loss.

Analysis of Protein Loss during Agarose Gel Electrophoresis

To investigate whether contamination of nucleoprotein aggregates and charged components of agarose contribute to the loss of chromatin protein during agarose gel electrophoresis, we repeated the cell-pulsed student analysis shown in figure 5 with two agarose gels. One agarose gel, which has a higher net charge than the HS(D) agarose gels, was used with the conclusion that HS(D) agarose, which has essentially no net charge. This analysis showed that agarose gels produce the greatest (±7.700 g/mL), and bigger against the least (±1.5 g/mL) protein losses, and revealed a direct correlation between the effect of protein loss during electrophoresis and the inherent net charge of the agarose gel.

The observation was confirmed by measurements of the mobility of DNA chromatin with respect to purified RNA in agarose media. As illustrated in Table 1, the mobility ratio indicated that a direct correlation exists between the electrophoresis mobility of the DNA chromatin and the net charge of the agarose gel.

We observed a direct correlation between the electrophoresis mobility of the DNA chromatin and the net charge of the agarose gel.

Table 1. Ratios of Relative Mobilities of DNA Chromatin and RNA in Agarose Gel

<table>
<thead>
<tr>
<th>Length (in min) of electrophoresis</th>
<th>DNase I agarose</th>
<th>RNA agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.56</td>
<td>0.59</td>
</tr>
<tr>
<td>20-25</td>
<td>0.63</td>
<td>0.67</td>
</tr>
<tr>
<td>30-40</td>
<td>0.67</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Purified DNA and nuclearic protein were electrophoresed in either DNA agarose gel or in RNA agarose gel. As progressive intervals, indicated in the first column, the electrophoresis was stopped and the gels, which contained她在图中描述，were photographed, the mobilities (k) of DNA chromatin and of RNA at each time point were measured, as the distance from the origin on the photographs, and their ratio was calculated by DNA chromatin/RNA DNA.