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

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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; Allfrey *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; Borkhardt and Nielsen, 1981). Because of these characteristics these genes may be studied as a chromatin fraction (rDNA chromatin)[1] obtained from isolated nucleoli (for example, Bradbury *et al.*, 1973; Leer *et al.*, 1976; Colavito-Shepanski 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.*, 1970) 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 nuclear 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

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[1] The abbreviations used are: rDNA, ribosomal RNA gene; PMSF, phenylmethylsulfonyl fluoride; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TEA, triethanolamine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

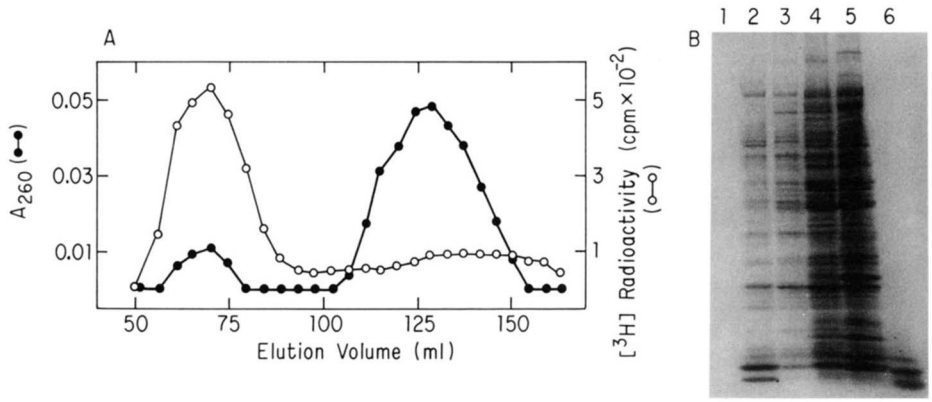

FIG. 1. **Fractionation by gel filtration chromatography of crude chromatin extracted from nucleoli isolated by the divalent cation method.** Nucleoli were isolated in 3 mM $MgCl_2$ from macroplasmodia grown in the presence of [^{3}H]thymidine, and lysates from these nucleoli were fractionated by chromatography on a Bio-Gel A-150m column. *Panel A*, eluted material was collected in 5-ml fractions, which were analyzed for UV absorbance and for radioactivity. *Panel B*, column fractions were pooled, dialyzed, and lyophilized for electrophoresis in a 5–22% gradient SDS-polyacrylamide gel. *Lanes 1* and *2* are fractions from the first peak (containing DNA), *lane 3* is from fractions between the two peaks, and *lanes 4* and *5* are fractions from the second peak (containing RNA). *Lane 6* is a *Physarum* nuclear histone sample. The histone protein pattern in lane 2, corresponding to chromatin-containing fractions, is depleted in comparison to the histone protein pattern in lane 4, corresponding to fractions containing ribonucleoprotein.

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 $CaCl_2$ 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[2]

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. 1*A*, 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. 1*A*, the first peak contained most of the [^{3}H] 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. 1*B*); the core histone proteins were identified on the basis of electrophoretic mobilities, which correspond to those of histone protein standards (Fig. 1*B*, *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

[2] Portions of this paper (including "Materials and Methods," part of "Results," and Table I) 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 extracted from both digests (from Mg^{2+} or Ca^{2+}-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 Mg^{2+} or Ca^{2+}-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/cm^3 and DNA at 1.70 g/cm^3 so that chromatin fractions with higher density have a smaller proportion of protein. We found chromatin prepared from Mg^{2+}-isolated nucleoli formed a broad band in these gradients with an average density near 1.6 g/cm^3 (Fig. 3*A*); chromatin prepared from spermine/spermidine-isolated nucleoli formed a sharper band with a density of 1.4 g/cm^3 (Fig. 3*B*). Therefore, a significant proportion of the chromatin sample from Mg^{2+}-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.

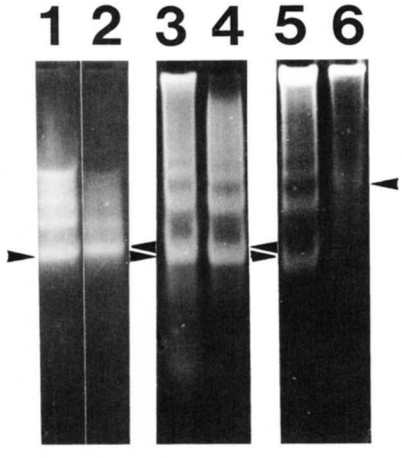

FIG. 2. **Changes in nucleosome structure occur during the divalent cation nucleolar isolation method: agarose gel electrophoresis of *Physarum* chromatin particles.** Nucleoli were isolated from microplasmodia in the presence of 10 mM $MgCl_2$, 10 mM $CaCl_2$, or spermidine and spermine. Chromatin particles 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 $MgCl_2$, in lanes 3 and 4 from $CaCl_2$-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.

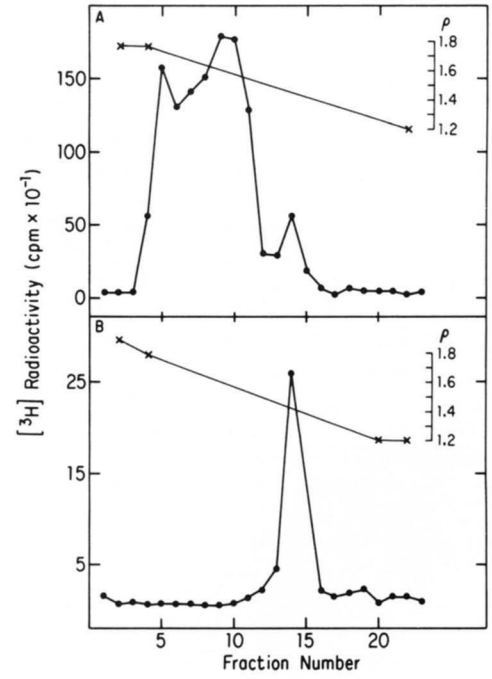

FIG. 3. **Comparison of chromatin prepared from divalent cation-isolated nucleoli and spermine/spermidine-isolated nucleoli: analysis by CsCl density gradient centrifugation.** Nucleoli were isolated from microplasmodia grown in the presence of [^{3}H]thymidine by either the divalent cation method or 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 spermine/spermidine method.

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. 4*A* is shown a gel stained with ethidium bromide, comparing electrophoretic properties of chromatin from spermine/spermidine and from Mg^{2+} 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. 4*A lane 2* is shown rDNA chromatin from a Mg^{2+} 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. 4*B* 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 Mg^{2+}-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 Mg^{2+}-isolated nucleoli possessed far less histone protein/microgram of rDNA than did the rDNA chromatin band from spermine/spermidine-isolated nucleoli (not shown).

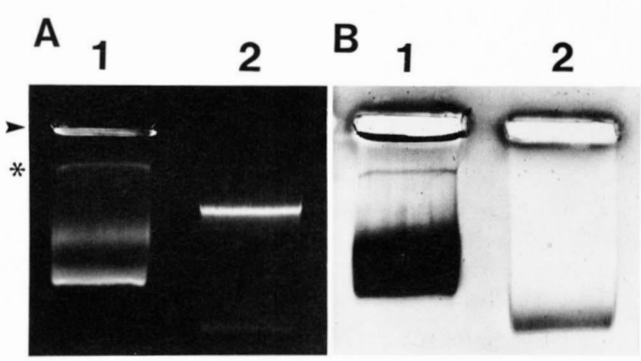

FIG. 4. **Purification of rDNA chromatin by agarose gel electrophoresis.** Nucleolar chromatin samples were fractionated by electrophoresis in 0.4% agarose gels in low ionic strength buffers, and the nucleic acids in the gels were stained with ethidium bromide (*panel A*). The position of the well is 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.

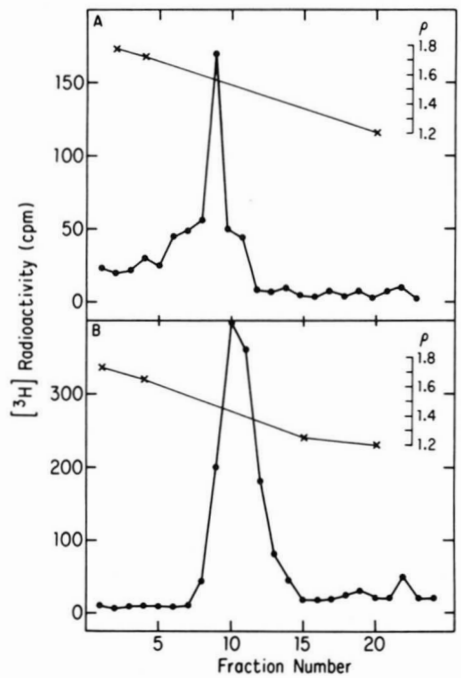

FIG. 5. **Protein stripping occurs during agarose gel electrophoresis of rDNA chromatin: analysis by CsCl density gradient centrifugation.** The density in CsCl/sarkosyl density gradients of rDNA chromatin which had been purified in an agarose gel and then cross-linked (*panel A*) was compared to the 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.

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),[3] 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 gradients of chromatin that had been purified electrophoretically and then cross-linked with formaldehyde (1.55 g/cm^3; Fig. 5*A*) with the density of chromatin that had been cross-linked and then purified electrophoretically (1.45 g/cm^3; Fig. 5*B*). Although these absolute values changed with each experiment, the sample that was purified electrophoretically without cross-linking always displayed a higher density than that of the sample that was cross-linked first. Since both chromatin samples represent purified rDNA chromatin, this experiment clearly demonstrates a loss of rDNA chromatin protein which is attributable to agarose gel electrophoresis. As described in detail in the Miniprint section, the extent of protein stripping is proportional to the inherent charge of the agarose medium, suggesting that the gel matrix interacts with chromatin proteins.

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 asso-

[3] This citation refers to the accompanying paper.

ciated with rDNA chromatin (Amero *et al.*, 1988). Additionally, we have achieved the initial purification of rDNA chromatin fragments produced by restriction nuclease digestion (not shown), although this purification was successful only with 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 in 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/cm^3) (Fig. 6*A*) 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 extractions, 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/cm^3 and 1.163 g/cm^3; Fig. 6*B*).

To characterize these fractions, DNA was extracted from each peak and analyzed by CsCl density gradient centrifugation. As shown in Fig. 7*A*, DNA from the dense metrizamide peak contained both nuclear DNA and rDNA; DNA from the light metrizamide peak contained only rDNA (Fig. 7*B*). We also determined the densities of purified rDNA in metrizamide (1.12 g/cm^3), and of most proteins in metrizamide (1.27 g/cm^3) (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).

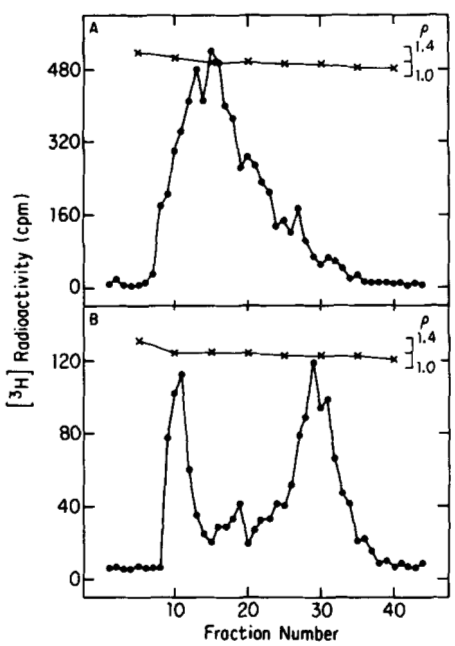

FIG. 6. **Purification of rDNA chromatin by metrizamide gradient centrifugation.** Nucleoli were isolated from microplasmodia grown in the presence of [^{3}H]thymidine; chromatin solubilized from these nucleoli was fractionated by centrifugation in metrizamide equilibrium density gradients (*panel A*). The presence of chromatin in gradient fractions was detected by liquid scintillation counting, and the densities of certain fractions were calculated from refractive indices. *Panel B* shows the distribution in metrizamide gradients of chromatin fractions from nucleoli treated by RNase digestion. This analysis demonstrates that mild treatment of nucleoli with RNase produces a derivative of rDNA chromatin that possesses a light density in metrizamide gradients.

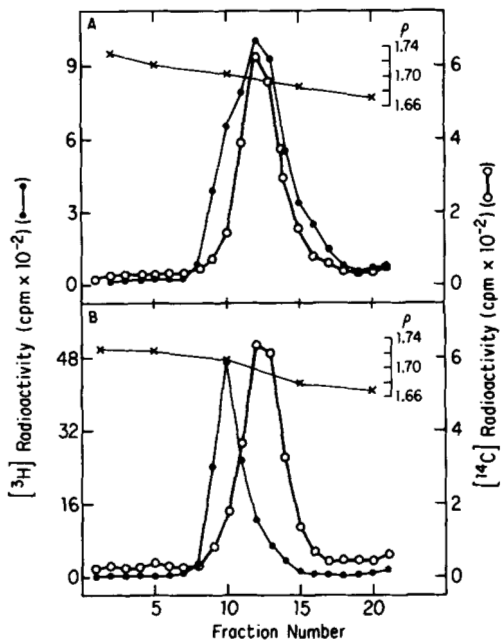

FIG. 7. **CsCl density gradient analysis of metrizamide gradient chromatin fractions.** Fractions from the dense chromatin peak and from the light chromatin peak resolved in metrizamide equilibrium density gradients were pooled. ^{3}H-labeled DNA was purified from each pooled sample and analyzed in CsCl density gradients, which contained ^{14}C-labeled *Drosophila* DNA as an internal density marker. The presence of DNA was detected by liquid scintillation counting of gradient fractions, and densities of gradient fractions were determined by refractometry. *Panel A* contains DNA from the dense chromatin peak; the densities of these DNA fractions (1.700 g/cm^3 and 1.712 g/cm^3) correspond to nuclear DNA and rDNA, respectively. *Panel B* contains DNA from the light metrizamide peak; the density of this DNA sample (1.712 g/cm^3) corresponds to that of rDNA only. This analysis demonstrates that the light metrizamide peak contains only rDNA chromatin.

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/cm^3) is lighter than the density of first-round rDNA chromatin (1.163 g/cm^3). 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

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 comigrate 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 chromosomal 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 Mg^{2+}-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 Mg^{2+}-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 scatterred nucleosomes in the rRNA gene of *Physarum* amoebae (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 (Sollner-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 Mg^{2+}-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 Mg^{2+}-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 acetoxypyrene-derivatized H3 proteins into *Physarum* chromatin (Prior *et al.*, 1980). The well-characterized nucleosome assembly systems are dependent on Mg^{2+} 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 slime which appears to be more prevalent in Mg^{2+}-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.*, 1978a, 1978b; 1979; 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 other gene 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 spermine/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 *Eco*RI, *Hin*dIII, or *Hae*III digestion of chromatin samples from divalent cation-isolated nucleoli; although rDNA is cleaved in spermine/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 is far 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-nucleolar chromatin and from other nucleolar 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-nucleolar chromatin band as a single peak in these gradients.

The densities of *Physarum* rDNA chromatin and non-nucleolar 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-nucleolar 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 (Grainger 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.

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SUPPLEMENTAL MATERIAL TO:

THE PURIFICATION OF RIBOSOMAL RNA GENE CHROMATIN FROM PHYSARUM POLYCEPHALUM

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METHODS AND MATERIALS

Growth and maintenance of Physarum cultures. Plasmodia and microplasmodia of Physarum polycephalum, strains M3CVIII and 5x2, (from Dr. J. Mohberg) were grown in the hematin-citrate medium described by Daniel and Baldwin (1964), with the substitution of yeast extract for chicken embryo extract. Surface rocker cultures were inoculated directly from microplasmodia and were grown for two days as parallel strips on Whatman #1 chromatography paper, which was supported on wire mesh screens in Pyrex baking dishes covered with aluminum foil (Mohberg and Rusch, 1969). In most experiments either 50 μCi or 500 μCi of sterile [^{3}H-methyl] thymidine (60 Ci/mmole, ICN) was added directly under sterile conditions to growth media in each dish.

Isolation of nuclei and nucleoli. In some experiments nucleoli (and nuclei) were isolated from microplasmodia or plasmodia as described by Mohberg and Rusch (1971). Either $CaCl_2$ or $MgCl_2$ was used as a stabilizing agent.

Nucleoli were also isolated in the presence of polyamines rather than divalent cations. In these experiments, nucleoli were prepared from plasmodia or microplasmodia of the 5x2 strain by homogenization in isolation buffer containing 0.34M sucrose, 10mM Tris-HCl (pH 7.25), 5mM ethylene diamine tetra acetic acid (EDTA), and approximately 0.4mM spermidine (Sigma Chemical Co.); after mixing these components the buffer was adjusted to pH 6.8 with 1M HCl. The homogenizations were done in 200 ml of buffer in a Waring blender connected to a rheostat. Each homogenization was conducted for 2 min, with the blender set on "high" and the rheostat set between 70 and 100 v. A step in which microplasmodia or plasmodia strips still on filter paper were rinsed in 50mM Tris-HCl (pH 7.25) to remove traces of culture medium was routinely included before homogenization. This step was important for obtaining a high nucleolar to nuclear ratio. It was also important that this rinse was at room temperature, since the plasmodia became refractory to breakage upon exposure to cold conditions at this point. Immediately following homogenization, spermine (Sigma Chemical Co.) was added to 0.15mM to stabilize nucleoli; the protease inhibitor PMSF (Sigma Chemical Co.) was also added to a 0.1mM final concentration. The addition of these factors prior to homogenization led to a high nuclear contamination of nucleolar preparations under any homogenization conditions. The yield and integrity of each homogenate was analyzed by light microscopy. In some experiments rDNA enrichment was assessed by CsCl gradient centrifugation, as described below.

Several parameters had to be adjusted to produce homogenates containing little slime, few nuclei, and clean, intact nucleoli. It was important to introduce a small amount of plasmodia (0.2–0.4 gm) to the homogenization (200 ml), particularly with older cultures which tend to produce greater amounts of slime. The precise concentration of spermidine in the isolation medium was also variable and critical; if the concentration was too high, nuclei were not disrupted; if too low, nucleoli were destroyed. In addition, the proper blender speed had to be determined for each preparation, so that nuclei were disrupted but nucleoli remained intact. It was also critical that the pH of the homogenization buffer be maintained at pH 6.8. An increase of even 0.5 units led to the disruption of any intact structures; a decrease of 0.5 pH units resulted in the stabilization of nuclei.

Homogenates (six or seven) were pooled and centrifuged at 1000xg for 30 min at 4°C. The pellets were resuspended in appropriate buffers for subsequent analysis and the yield, as well as the nuclear to nucleolar ratio, was determined by microscopic examination. A typical nucleolar preparation averaged 1x10^9 nucleoli from six strips of macroplasmodia.

Preparation of nucleolar chromatin fractions. We have utilized several methods for solubilizing nucleolar chromatin fractions. Early in our investigations nucleoli were isolated exclusively by the divalent cation technique of Mohberg and Rusch (1971), which renders nucleoli exceedingly refractory to breakage or solubilization. In some experiments, nucleoli were sonicated briefly as described by Kawashima et al. (1979) to solubilize slime polysaccharides. To extract nucleolar chromatin, nucleoli were lysed in 10mM Tris-HCl (pH 7.25), 5mM EDTA.

In our later experiments using the spermine/spermidine nucleolar isolation procedure we were able to eliminate mechanical breakage steps, such as sonication, since nucleoli prepared by this procedure are not as contaminated by slime polysaccharides as are those prepared by the divalent cation procedure. We also introduced several washing steps prior to chromatin extractions. In the first wash, crude nucleolar pellets obtained by centrifuging plasmodial homogenates were resuspended in buffer A modified from Hewish and Burgoyne (1973): 0.35M sucrose, 15mM Tris-HCl (pH 7.4), 15mM B-mercaptoethanol, 15mM NaCl, 1mM EDTA, 0.2mM EGTA, 0.15mM spermine, 0.5mM spermidine, and recentrifuged at 5,000 rpm for 10 min at 4°C in a Sorvall HB-4 rotor. In the second wash, nucleoli were resuspended at a concentration of 1x10^9 nucleoli/ml in a washing buffer containing 0.2M NaCl, 2mM EDTA, 10mM TEA-acetate (pH 7.25); more recently, the NaCl concentration of the wash buffer was lowered from 0.2M to 75mM, as described in the text. In certain experiments, enzymatic digestions, such as treatment with RNase A, or nuclease digestions, were performed at this point. The suspensions were chilled on ice, recentrifuged at 5,000 rpm for 5 min at 4°C as above, and the NaCl wash was repeated.

Nucleoli (at a concentration of 0.5x10^9 nucleoli/ml) were then extracted three times in 0.1mM EDTA, 0.5mM DTT (Calbiochem), 0.1mM PMSF (pH 7) at 4°C. Supernatants from the extractions were enriched in rDNA chromatin.

Assessment of rDNA Enrichment in Isolated Nucleoli or Nucleolar Chromatin. The percentage of DNA from isolated nucleoli or nucleolar chromatin that was rDNA was determined from the profile of DNA from these samples on CsCl equilibrium gradients, since rDNA bands at a density (1.712gm/cm^3) distinct from non-nucleolar DNA (1.700g/cm^3) (Molgaard et al., 1976). The protocol used for the isolation of DNA from nuclei or nucleoli has been described (Grainger and Ogle, 1978).

CsCl gradients were centrifuged in a Beckman Model E analytical ultracentrifuge (Grainger and Ogle, 1978) or in the preparative ultracentrifuge. In the latter case CsCl gradients were centrifuged in the Beckman Type 50 rotor at 35,000 rpm for 72 h at 18°C, or alternatively, in the Beckman Type 65 rotor at 42,000 rpm for 18 h at 18°C. [^{14}C]labelled DNA, prepared from the nuclei of Drosophila embryos according to the procedures of Hewish and Burgoyne (1973), was included as an internal marker of the density of Physarum nuclear DNA, since the densities of Drosophila nuclear DNA and Physarum nuclear DNA are coincident.

Nuclease digestions of nucleoli and subsequent analysis of nucleosome structure by agarose gel electrophoresis. One criterion used to assess the structural integrity of chromatin in isolated nucleoli (or nuclei) was the electrophoretic characteristics of nucleosomes derived from nuclease-treated nucleoli (or nuclei). In order to perform micrococcal nuclease digestions nucleoli (or nuclei) were washed once in a micrococcal nuclease digestion buffer (15mM NaCl, 10mM TEA-acetate (pH 7.25), 1mM $MgCl_2$, 0.1mM $CaCl_2$). The resulting pellet was resuspended at a concentration of 1x10^9 nucleoli (or nuclei)/ml in the digestion buffer and incubated at room temperature with 1-10 μg/ml micrococcal nuclease (Sigma Chemical Co.) for 3 min. Nucleoli (or nuclei) were quickly centrifuged at 5,000 rpm at 0°C for 3 min, and the resulting supernatant was removed and adjusted to a final concentration of 5mM EDTA. Other digestion products were solubilized from the remaining pellet by nucleolar (or nuclear) lysis and successive extractions in the following buffers: 1) 10mM TEA-acetate (pH 7.25), 2) 10mM TEA-acetate (pH 7.25), 5mM EDTA, 3) 5mM TEA-acetate (pH 7.25), 2.5mM EDTA, and 4) 1mM TEA-acetate (pH 7.25), 0.5mM EDTA. To monitor the extent of nuclease digestion, as well as the efficiency of solubilization, DNA was purified from the extracts and sized by agarose gel electrophoresis (Sugden et al., 1975) using 2% (w/v) agarose gels. Fragments obtained by digestion of φX174 DNA with the restriction endonuclease Hae III (Bethesda Research Laboratories) were used as size standards on agarose gels (Jeppeson et al., 1976).

Chromatin particles solubilized from nucleoli (or nuclei) treated with micrococcal nuclease were fractionated by electrophoresis in 1% (w/v) agarose gels (Varshavsky et al., 1976) containing 0.5 μg/ml ethidium bromide in Buffer E (1X buffer E is 40mM Tris-HCl (pH 7.8), 5mM Na-acetate, 9mM EDTA) or by electrophoresis in 5% polyacrylamide gels containing 1X Buffer E. Lanes in some gels included chromatin particle standards prepared from chick red blood cell nuclei (as described by Pruitt and Grainger, 1980) and from Drosophila embryo nuclei (Hewish and Burgoyne, 1973), treated with micrococcal nuclease as described above. DNA was visualized by ultraviolet illumination of gels stained after electrophoresis by incubation of the gel in 5-10 μg/ml ethidium bromide for 30 min. To determine if protein was associated with the nuclease digestion products, gels were then stained in 50% (v/v) methanol/10% (v/v) acetic acid containing 0.2% (w/v) Coomassie Blue G-250, and destained by continuous washing in 7% (v/v) methanol/5% (v/v) acetic acid.

Analysis of rDNA chromatin by gel filtration chromatography on BioGel A-150m columns. BioGel A-150m was equilibrated in 1mM Tris-HCl (pH 7.25), 0.5mM EDTA, in a 20 cm column. Solubilized chromatin samples from nucleoli prepared by either the divalent cation or the spermine/spermidine technique were prepared for chromatography by adding NaCl and B-mercaptoethanol to 50mM and 1mM final concentrations, respectively. Nucleolar chromatin components were eluted from the column in 1mM Tris-HCl (pH 7.25), 0.5mM EDTA, 50mM NaCl, 1mM B-mercaptoethanol, and fractions were collected.

Column fractions were analyzed in several ways. First, fractions were monitored individually for absorbance at 260nm. Second, with chromatin samples labelled with [^{3}H]thymidine, 1ml aliquots of individual fractions were added to 5ml of Beckman Aquasol for scintillation counting, to detect the presence of DNA. To examine protein in these fractions, samples were fractionated on 5%-22% (w/v) gradient SDS-polyacrylamide gels (Laemmli, 1970) and visualized by silver staining (Oakley et al., 1980). Total Physarum histones were prepared as described by Mohberg and Rusch (1969), and included as standards in these gels.

Analysis of rDNA chromatin by low ionic strength agarose gel electrophoresis. A number of parameters were tested in our efforts to design an electrophoretic system that would result in the purification of rDNA chromatin. The resolution of rDNA chromatin in agarose gels containing either Isogel, HGT(P), or SeaPlaque agarose (all from FMC Corporation, Marine Colloids Division) was optimal in 0.2%-0.4% (w/v) agarose gels, containing 1-5mM TEA-acetate and 1-5mM EDTA. Variations in buffer concentrations within these ranges and in pH values from 7.25 to 8.5 did not affect the resolution. Agarose gels also contained 0.5 μg/ml ethidium bromide, to observe the migration of rDNA chromatin by ultraviolet illumination. Electrophoresis at 4°C was conducted at 50-100 V. Protein in the gels was detected subsequently by staining with Coomassie blue as described earlier. Chromatin was recovered from gels by electroelution, small gel pieces were placed in dialysis tubing

containing 1mM TEA-acetate (pH 7.25), 1mM EDTA and the tubing was placed in a chamber containing the same buffer in a 300V electric field for 1 h at 4°C.

Formaldehyde treatment of chromatin and analysis by CsCl gradient centrifugation. In order to estimate the approximate protein to DNA ratio in various chromatin samples, formaldehyde crosslinked samples were analyzed by CsCl gradient centrifugation. Chromatin samples from plasmodia labeled with [^{3}H]thymidine were treated with 0.1 volume of 11% (v/v) formaldehyde (J.T. Baker Chemical Co.) for 18 h at 4°C to crosslink DNA and chromosomal proteins (Ilyin and Georgiev, 1969). Formaldehyde was then removed by dialysis against 1mM TEA-acetate (pH 7.25), 1mM EDTA for 3 h at 4°C. Formaldehyde-treated chromatin samples were analyzed in CsCl gradients (ρ=1.6 gm/cm^3) containing 5mM TEA-acetate (pH 7.25), 1mM EDTA, 0.5% sarkosyl (Sigma Chemical Co.). Gradients were centrifuged in a Beckman SW 56 rotor at 25°C at 50,000 rpm for 60 h. Fractions were collected through a 23-gauge needle from the bottom of the tubes, and the DNA peak was determined by liquid scintillation counting of fractions.

Analysis of rDNA chromatin by metrizamide gradient centrifugation. In this procedure, chromatin samples were prepared from nucleoli which in most cases were incubated at 37°C for 30 min in the presence of 0.01 µg/ml RNase 4 prior to chromatin solubilizations. Samples were then centrifuged in a Sorvall HB-4 rotor at 4100 xg for 10 min at 4°C to remove insoluble debris. 1 ml of solubilized nucleolar chromatin was added to a solution of metrizamide (Nyegaard and Co., Oslo, Norway) so that the final mixture (2.5 ml; 1.1795 gm/cm^3) contained 1mM TEA-acetate (pH 7.25), 1mM EDTA, 0.1mM DTT, and 0.1mM PMSF. Gradients were formed by centrifugation in a Beckman SW 50.1 or SW 56 rotor at 30,000 rpm for 30 to 36 h at 4°C. Fractions were collected from the bottom of the tubes. The distribution of chromatin was determined by liquid scintillation counting of fraction aliquots or by illumination of gradient fractions with a hand-held ultraviolet light source.

RESULTS

Comparisons of Nuclear Wash Buffers.

We tested how much nucleolar chromatin protein content was affected by washing nucleoli in buffers containing different salt concentrations to remove cytoplasmic protein, by performing another cross-linking experiment in conjunction with gel electrophoresis. For this analysis, equal portions of a nucleolar preparation isolated in spermine/spermidine buffer were washed in Buffer A and then washed separately in the appropriate salt-containing buffer. Chromatin was extracted from each sample and crosslinked by treatment with formaldehyde. rDNA chromatin was purified from non-nucleolar chromatin and ribonucleoprotein by agarose gel electrophoresis and recovered from the agarose gels by electroelution. The densities of the rDNA chromatin samples were analyzed by CsCl-sarkosyl gradient centrifugation. Chromatin extracted from nucleoli washed in 0.2M NaCl had a density of 1.66 gm/cm^3 while chromatin extracted from nucleoli washed in 75 mM NaCl had a density of 1.46 gm/cm^3 (data not shown). From this experiment we conclude that resuspension of nucleoli in buffers containing 0.2M NaCl results in a substantial loss of rDNA chromatin protein and that this effect is minimized if the NaCl concentration is lowered to 75 mM.

We also compared the effects of washing nucleoli recovered from a spermine/spermidine homogenate in buffers containing 10 mM $MgCl_2$ or in Buffer A, which contains spermine and spermidine. We found that crosslinked chromatin from each preparation had a density in CsCl/sarkosyl density gradients of 1.47 gm/cm^3 (data not shown), indicating little difference between the two buffers. These results suggest that conditions leading to stripping of chromatin proteins in divalent cation-containing buffers must be established in the homogenization 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.

Analyses of Protein Loss During Agarose Gel Electrophoresis.

To investigate whether interaction of chromosomal proteins and charged components of agarose contribute to the loss of chromatin protein during agarose gel electrophoresis, we repeated the CsCl-sarkosyl gradient analysis shown in Figure 5 with two other agarose preparations: SeaPlaque agarose, which has a higher net charge than the HGT(P) agarose preparation used in the previous experiment and Isogel agarose, which has essentially no net charge. This analysis showed that SeaPlaque agarose produced the greatest (ρ=1.700 gm/cm^3), and Isogel agarose the least (ρ =1.51 gm/cm^3) protein loss, and revealed a direct correlation between the extent of protein loss arising during electrophoresis and the inherent net charge of the agarose medium.

This observation was confirmed by measurements of the mobility of rDNA chromatin with respect to purified rDNA in agarose media. As illustrated in Table I, the mobility ratio data indicated an acceleration of the chromatin electrophoretic rate in SeaPlaque agarose, an observation consistent with a progressive stripping of protein occurring as the chromatin band moves through the gel. These results also revealed that while the acceleration of the chromatin electrophoretic rate was pronounced with SeaPlaque agarose, which has the highest intrinsic net charge of the three preparations used in our experiments, Isogel agarose, with the least intrinsic net charge, produced no acceleration of the chromatin electrophoretic rate. Therefore, a direct correlation exists between the electrophoretic mobility of the rDNA chromatin band and the net charge of the agarose medium.

Table I. Ratios of Relative Mobilities of rDNA Chromatin and rDNA in Agarose Gels

Length (in min) of electrophoresis	SeaPlaque agarose	Isogel agarose
10	.54	.50
20-25	.64	.55
30-40	.67	.52

Purified rDNA and nucleolar chromatin were electrophoresed in either SeaPlaque agarose gels or in Isogel agarose gels. At progressive intervals, indicated in the first column, the electrophoresis was stopped and the gels, which contained ethidium bromide, were photographed. The mobilities (R_f) of rDNA chromatin and of rDNA at each time point were measured, as the distance from the origin on the photographs, and their ratio was calculated (R_f rDNA chromatin/R_f rDNA).