Isolation of a Minichromosome Containing the Ribosomal Genes from *Physarum polycephalum*†

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ABSTRACT: The ribosomal genes of *Physarum polycephalum* are located on linear DNA molecules (rDNA) of 38 × 10^6 daltons present in each nucleolus in 100–200 copies. The rDNA is not part of the large chromosomal DNA. These molecules have now been solubilized in the form of transcriptionally active deoxyribonucleoprotein complexes from nucleoli purified through Percoll gradients. This ribosomal chromatin (rDNP) sediments in sucrose gradients as a homogeneous fraction with an approximate sedimentation coefficient of 100 S. It contains α-amanitin-resistant RNA polymerase I activity and rDNA as its major (or sole) DNA component. Buoyant density analysis indicates that ribosomal chromatin contains, besides RNA polymerase I, significant amounts of additional protein. RNA synthesized in vitro by the endogenous RNA polymerase is complementary to rDNA and is transcribed mainly from the sequences coding for 19S and 26S ribosomal RNA.

In recent years a tremendous effort has been made to gain a better understanding of the role of chromosomal proteins in the regulation of transcription. Due to the structural and functional complexity of chromatin, analysis of total chromatin from many organisms has proven rather difficult. Different approaches have therefore been employed to circumvent the problems posed by the size and complexity of chromatin.

A variety of procedures have been developed to fractionate chromatin into active and repressed components (Chiu et al., 1977; Gottesfeld et al., 1974; Strätling et al., 1976; Turner & Hancock, 1974).

Efforts have also been directed to the analysis of the nucleolar chromatin which might be more amenable to biochemical analysis due to its small DNA content and its highly active ribosomal RNA synthesis (Ballal et al., 1977; Bachellerie et al., 1977). Nucleolar chromatin has recently been isolated from *Xenopus laevis* in sufficient purity so that a study of its protein composition has been initiated (Reeder et al., 1978; Higashinakagawa et al., 1977).

In addition, small DNA viruses like SV40 and polyoma have been successfully employed as model systems for investigating chromosomal functions (Green & Brooks, 1976; Seebeck & Weil, 1974). These viruses depend largely on cellular mechanisms for their replication and transcription; viral DNA is also complexed with cellular histones into nucleosomal structures as is cellular chromatin (Griffith, 1975).

Recently, evidence has been accumulating for the occurrence of extrachromosomal DNA molecules in various eukaryotic organisms or cell lines. In several cases these extrachromosomal elements have been shown to carry ribosomal genes (Findly & Gall, 1978; Zuchowski & Harford, 1976; Karrer & Gall, 1976; Vogt & Braun, 1976). Such eukaryotic extrachromosomal elements carrying ribosomal genes offer a novel and exciting approach to the study of regulatory functions of chromosomal proteins.

So, the ribosomal genes of the ciliate *Tetrahymena pyriformis* have been investigated for their nucleosomal organization by nuclease digestion (Mathis & Gorovsky, 1976). Westergaard has reported the isolation of the ribosomal genes of *Tetrahymena* as nucleoprotein complexes (Leer et al., 1976) and they have begun an investigation of the proteins composing this nucleolar chromatin (Leer, 1979; S. Shall, personal communication). A different approach has so far been used with *Physarum polycephalum* where, after nuclease digestion and sucrose gradient centrifugation of total chromatin, a particular peak of chromatin (peak A) can be isolated, which is enriched in ribosomal genes. This material differs in its protein composition quite strongly from the remainder of the chromatin (Allfrey et al., 1978).

The ribosomal genes of *Physarum* are located extrachromosomally on linear, independently replicating DNA molecules with a discrete molecular weight of 38 × 10^6 daltons (Vogt & Braun, 1977) and a buoyant density higher than that of bulk DNA (Zellweger et al., 1972). These rDNA[1] molecules are localized in the nucleolus (200 copies per nucleolus) and are not covalently linked with either chromosomal DNA or with each other.

Digestion of intact nuclei with different nucleases has demonstrated that the ribosomal genes in *Physarum* are similarly protected from micrococcal nuclease digestion as is bulk chromatin (Stalder et al., 1979), but that they are highly susceptible to degradation by DNase I (Stalder et al., 1978).

A recent investigation of *Physarum* chromatin by electron microscopy has indicated that the ribosomal genes are organized in chromatin-like structures formed by individual rDNA molecules (Grainger & Ogle, 1978).

We have attempted to isolate the ribosomal genes in the form of nucleoprotein complexes, avoiding harsh conditions such as excessive shear, sonication, or high ionic strength, which might alter the native structure of chromatin. The present communication describes the isolation and characterization of the ribosomal genes from *Physarum polycephalum* in the form of transcription complexes or "minichromosomes" released from the nucleolus.

Materials and Methods

Cell Culture Methods. Plasmodia of *Physarum polycephalum*, strain M_3CVIII, were maintained in semidefined medium (Daniel & Baldwin, 1969) in continuously shaking cultures at 26 °C. Synchronously growing surface plasmodia were formed by plating 0.3-mL aliquots of a 1:1 mixture of

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; rDNA, ribosomal DNA; rRNA, ribosomal RNA; rDNP, ribosomal deoxyribonucleoprotein complex.

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packed microplasmodia and water onto Schleicher and Schull No. 576 filter paper disks (8-cm diameter) over supporting metal screens in plastic petri dishes. Fusion was allowed to proceed for 45 min at 26 °C, whereafter nutrient medium was added (Braun & Behrens, 1969). The first highly synchronous mitosis (mitosis II) occurred 14–15 h after plating. The time between mitosis II and mitosis III varied from 8.5–9.5 h at 26 °C. Plasmodia were routinely harvested for the isolation of nucleoli during the late G2 phase between mitosis II and mitosis III.

When labeling of DNA or stable RNA was desired, microplasmodia were grown for several generations in a complex medium containing mycological peptone (Plaut & Turnock, 1975) and the desired isotopes. This medium allows a much better uptake of labeled precursors than does the standard growth medium. Surface plasmodia were then formed from the prelabeled cultures and fed with unlabeled regular growth medium.

Isolation of Nucleoli. Nucleoli were isolated by a modification of the procedure developed by Mohberg (Mohberg & Rusch, 1969). The growing edge of 10–20 surface plasmodia [(5–10) × 10^8 nuclei] was scraped into 200 mL of ice-cold buffer N (250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 0.1% Triton X-100), and all following steps were carried out in the cold. The plasmodia were homogenized in the 1-L cup of a Waring blender at high speed for 45 s at 120 V. $CaCl_2$ (1 M, 2 mL) was added immediately, and the homogenate was stirred again in the blender at low speed for 15 s at 50 V.

After being kept on ice for 10 min to allow the foam to settle, the homogenate was filtered through two layers of milk filter (Flawa CS). The filtrate was then centrifuged in the Sorvall HS-4 rotor at 6000 rpm (7000*g*) for 15 min. The pellets from the 400-mL filtrate were resuspended in 50 mL of buffer P (250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) and were recentrifuged in an MSE swing-out rotor at 2500 rpm (1200*g*) for 15 min. The resulting pellet contained the crude nucleolar fraction.

Percoll Density Gradient Centrifugation. The crude nucleolar preparation still contains large amounts of contaminating polysaccharides. Therefore, in order to remove the bulk of the polysaccharides, nucleoli were further purified by equilibrium centrifugation in a Percoll density gradient.

Percoll stock solution was prepared by dialyzing the commercial Percoll solution (Pharmacia) against two changes of buffer P. This dialysis proved to be necessary to remove small molecular weight contaminants which otherwise lead to aggregation and precipitation of the isolated nucleolar chromatin. The dialyzed Percoll stock solution has a refractive index of 1.3710.

The crude nucleolar pellets prepared from 30–50 surface plasmodia were suspended with buffer P to a final volume of 20 mL in a Ti 60 nitrocellulose tube. Dialyzed Percoll (16 mL) solution was added and the solution was thoroughly mixed and then centrifuged in a Beckman Ti 60 rotor at 40 000 rpm for 60 min at 2 °C. Two clearly visible bands are formed. The upper thick one contains the polysaccharides, the lower, the nucleoli. This lower band was removed with a pasteur pipet, diluted with buffer P to 50 mL, and after thorough mixing centrifuged at 2500 rpm (1200*g*) for 15 min. The resulting tightly packed pellet was resuspended in 40 mL of buffer P and recentrifuged. The pellet from this centrifugation was resuspended in 40 mL of buffer NII (250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 0.01% Triton X-100) and recentrifuged again. The final pellet contains, as judged by phase-contrast microscopy, clean and morphologically intact nucleoli which are essentially free of slime. The proportion of contaminating nuclei is always small, varying from preparation to preparation by less than 1–5%.

EDTA Solubilization of Nucleolar Chromatin. The nucleolar pellet derived from 30–50 surface plasmodia was suspended in 0.5–2 mL of buffer NII and was then gently mixed with one-tenth volume of 100 mM EDTA, pH 7.2. The suspension was dialyzed for 2–3 h at 4 °C against 250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA. By that time the initially turbid suspension had cleared and was centrifuged for 10 min at 20 000 rpm (48000*g*) in a Sorvall SS 34 rotor. The resulting clear supernatant contains the solubilized nucleolar chromatin, while the tight, whitish pellet contains the nuclei (which are resistant to lysis by EDTA under these conditions) and the remaining polysaccharide. This procedure usually solubilizes between 50 and 80% of the nucleolar chromatin. However, the variation from preparation to preparation can be quite high and, in occasional batches of nucleoli, solubilization of chromatin is very poor. We haven't so far been able to determine the reason for this variability.

RNA Polymerase Assay. RNA polymerase was assayed as described previously (Smith & Braun, 1978). To one volume of sample (usually 10 μL) an equal volume of the following reaction mixture was added: 100 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 20% glycerol, 1.2 mM of each ATP, CTP, and GTP, and 2.5 μM of [^{3}H]UTP (41 Ci/mmol). Samples were incubated at 30 °C for 20 min, precipitated with 1 mL of cold 5% Cl_3AcOH and 10 mM sodium pyrophosphate after adding 50 μL of yeast tRNA (1 mg/mL) as carrier, and filtered onto Whatman GF-C glass fiber filters. When necessary, filters were individually labeled with drawing ink which is resistant to the Cl_3AcOH and the ethanol used for washing the filters.

In Vitro RNA Synthesis. For making larger amounts of in vitro transcripts, 0.5–0.8-mL aliquots of nucleolar chromatin isolated from sucrose gradients were mixed with equal volumes of the reaction mixture described above, except that its [^{3}H]UTP concentration was 6.25 μM and it contained 2 μg/mL of α-amanitin. After incubation at 30 °C for 20 min, one-tenth volume of 20% $NaDodSO_4$, one-hundredth volume of proteinase K (10 mg/mL), and 50 μL of yeast tRNA (1 mg/mL) were added, and incubation was continued for another 30 min. One volume of hot phenol (65 °C), saturated with 10 mM NaOAc, pH 5.1, was added and the mixture was vigorously shaken at 65 °C for 5 min. It was then mixed at room temperature with 0.5 mL of chloroform–isoamyl alcohol (24:1), and phases were separated by a brief centrifugation. Usually no interphase was visible. The aqueous phase was removed, mixed with one-fiftieth volume of 5 M NaCl, and precipitated with ethanol.

Metrizamide Gradients. Linear metrizamide density gradients were preformed in polyallomer tubes from 2.4 mL each of 0.2 and 0.5 M metrizamide stock solutions in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Samples (0.3 mL) were layered on top of the gradients and were centrifuged to equilibrium in a Beckman SW 50.1 rotor at 40 000 rpm for 16–20 h at 2 °C. Fractions were collected by pumping out the gradients from the bottom.

Sucrose Gradients. Sedimentation experiments were carried out in linear 15–40% (w/v) sucrose gradients in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Samples (0.3 mL) were layered on top of 4.8-mL gradients and were centrifuged in a SW 50.1 rotor at 40 000 rpm and 2 °C for the times in-

dicated in the text. Fractions were collected as described above.

RNA–DNA Hybridization. DNA from sucrose gradient fractions was alkali denatured and loaded onto nitrocellulose filters (Millipore; 13-mm diameter) as described elsewhere (Hall & Braun, 1977). Individual filters were numbered with water-insoluble ink before drying and baking. For hybridization all filters from one gradient were placed into one sterile scintillation vial containing 3 μg of ^{3}H-labeled rRNA (specific activity 130 000 cpm/μg) in a total volume of 3 mL of 2 × SSC and 0.2% NaDodSO$_4$. Hybridization was carried out for 15–18 h at 65 °C, and filters were then washed and digested with RNase as described. For competition hybridization experiments, triplicate filters for each experimental point (10 μg/filter of total nuclear DNA) were incubated in 2 mL of 2 × SSC and 0.2% NaDodSO$_4$ containing the ^{3}H-labeled RNA probe plus unlabeled RNA as specified in the text.

DNA and RNA Extraction. Total nuclear DNA and both ^{3}H-labeled and unlabeled rRNA were prepared as described elsewhere (Hall & Braun, 1977), while rDNA was extracted from isolated nucleoli and further purified according to Vogt & Braun (1976).

Restriction Enzyme Digestion and Agarose Electrophoresis. Purified rDNA or DNA isolated from nucleolar chromatin was digested with Eco-RI and Hind III restriction endonucleases according to Vogt & Braun (1976), and fragments were electrophoresed on 0.8% agarose slab gels using 36 mM Tris-phosphate, 0.5 mM EDTA, as the buffer system at pH 8.3.

Results

Isolation of Nucleolar Chromatin. Nucleoli were routinely prepared from batches of 30–50 surface plasmodia in late G2 phase of the cell cycle between mitosis II and III. The nucleolar pellet was checked for purity with a phase-contrast microscope and nucleoli were then lysed with EDTA as described. This procedure solubilizes the nucleolar chromatin, while the nuclei, which are extremely resistant to EDTA after having been exposed to calcium-containing buffers (Walker & Jokusch, 1974; and our own observations), can be removed by centrifugation, thus minimizing the contamination of nucleolar chromatin with chromatin of nucleoplasmic origin.

In order to characterize the DNA present in nucleolar chromatin, such chromatin was prepared from cultures continuously labeled with [^{3}H]thymidine. DNA was extracted from both the solubilized nucleolar chromatin and the pellet of unlysed nuclei and was analyzed in CsCl gradients. Figure 1A shows that the DNA extracted from nucleolar chromatin bands predominantly at the density position typical for rDNA (Zellweger et al., 1972), suggesting that the nucleolar chromatin contains rDNA at least as a major component. The proportion of nonribosomal DNA, as detectable in CsCl equilibrium density gradients, varies from preparation to preparation from undetectable amounts to 25%. These low and variable amounts suggest that main-band DNA represents a contamination rather than an intrinsic component of the nucleolar chromatin. Figure 1B shows the density profile of the DNA extracted from the pellet containing the unlysed nuclei and nucleoli. Here the major part of DNA bands at the density position of bulk DNA, i.e., nonribosomal DNA. The comparatively high proportion of DNA banding at the density position of rDNA is probably due to the presence of incompletely lysed nucleoli in this pellet.

DNA extracted from nucleolar chromatin was further characterized by digestion with the restriction endonucleases Eco-RI and Hind III and analysis of the resulting fragments

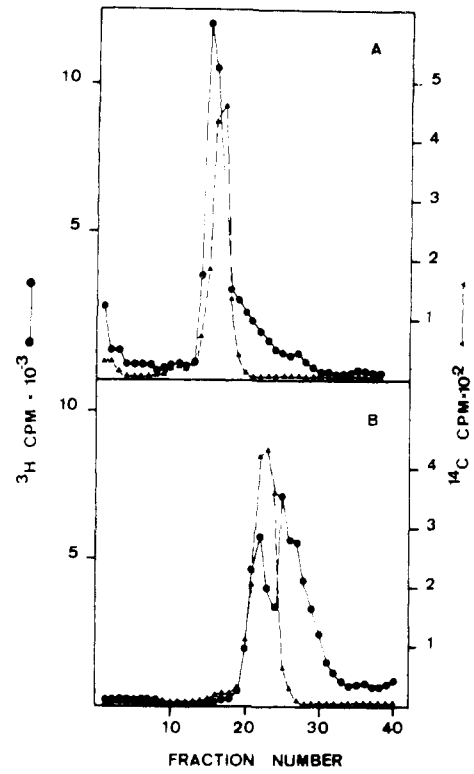

FIGURE 1: Cesium chloride density gradient of DNA extracted from rDNP. DNA extracted from nucleolar chromatin (A) and from the remaining, unlysed nuclei and nucleoli (B) was centrifuged to equilibrium in a Sorvall TV 865 vertical rotor at 35 000 rpm and 20 °C for 22 h. For radioactivity determination, 10-μL aliquots per fraction (A) or 50-μL aliquots per fraction (B) were spotted onto filter paper disks. All counts were corrected for spillover. Density of rDNA, 1.712 g/mL; density of main-band DNA, 1.702 g/mL; density of *E. coli* marker DNA, 1.710 g/mL. ^{3}H-labeled nucleolar DNA (●–●), ^{14}C-labeled *E. coli* marker DNA (▲--▲).

on 0.8% agarose gels. Restriction fragments obtained from DNA extracted from nucleolar chromatin comigrate with restriction fragments obtained from purified rDNA (Vogt & Braun, 1976; Molgaard et al., 1976). Southern transfer of such fragments to nitrocellulose filters and hybridization with ^{3}H-labeled rRNA further confirmed that the DNA from nucleolar chromatin is rDNA (results not shown).

Sedimentation Analysis of Nucleolar Chromatin. Aliquots of nucleolar chromatin were sedimented through sucrose gradients as described under Materials and Methods. Fractions (0.25 mL) were collected, and 10-μL aliquots of each fraction were assayed for endogenous RNA polymerase activity in the presence and absence of 1 μg/mL of α-amanitin. Figure 2 shows the sedimentation profile of the RNA polymerase activity detected in the presence and absence of α-amanitin with the arrow indicating the position of a λ DNA marker. In experiments where the rDNA was to be localized in such gradients, 10-μL aliquots/fraction were assayed for RNA polymerase activity while the remainder of the fractions was loaded onto nitrocellulose filters and hybridized with ^{3}H-labeled rRNA as described under Materials and Methods. Figure 3 demonstrates that the peak of hybridizable rDNA comigrates with the peak of RNA polymerase activity. The arrow indicates the position of a ^{32}P-labeled Herpes simplex virus DNA marker.

The above experiments illustrate that the nucleolar chromatin sediments as a rather homogeneous peak with an approximate sedimentation coefficient of 100 S, thus considerably faster than purified rDNA, which cosediments with λ DNA under our conditions.

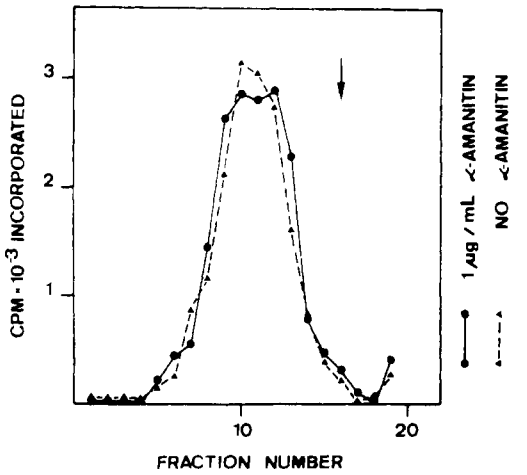

FIGURE 2: Velocity sedimentation of rDNP in a sucrose gradient. rDNP was sedimented in a SW 50.1 rotor at 40 000 rpm and 2 °C for 100 min. 10-µL aliquots of each fraction were assayed for RNA polymerase activity in the presence and absence of 1 µg/mL of α-amanitin. The arrow indicates the position of λ phage DNA cosedimented as a marker.

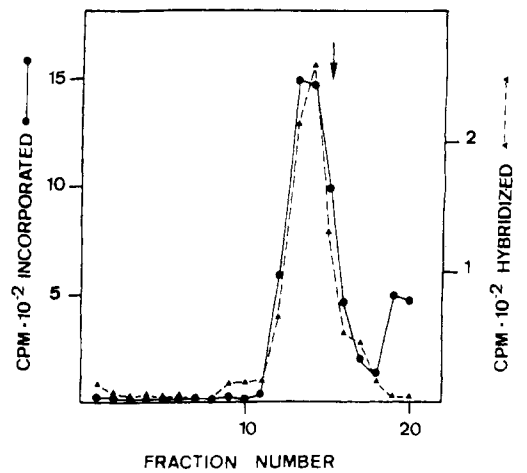

FIGURE 3: Cosedimentation of rDNA and RNA polymerase activity in rDNP. rDNP was sedimented in a SW 50.1 rotor at 40 000 rpm and 2 °C for 70 min. 10-µL aliquots of each fraction were assayed for RNA polymerase activity in the presence of 1 µg/mL of α-amanitin. The remainder of each fraction was then loaded onto a nitrocellulose filter and was hybridized with ^{3}H-labeled rRNA. The arrow indicates the position of ^{32}P-labeled Herpes simplex viral DNA cosedimented as a marker.

When [^{14}C]TdR prelabeled nucleolar chromatin was isolated and sedimented as described above, and the DNA was extracted from the peak and was analyzed on CsCl density gradients, no DNA with a density other than rDNA could be detected (results not shown). This observation indicates that nucleolar chromatin can be effectively purified, by sedimentation in sucrose gradients, from remaining fragments of nuclear chromatin which still may contaminate a crude preparation of nucleolar chromatin (see Figure 1A).

Based on the above assumption that nucleolar chromatin contains only rDNA, we expected it to be rich in RNA polymerase I activity, since in *Physarum* ribosomal genes are transcribed by an α-amanitin-resistant RNA polymerase I (Gornicki et al., 1974), as they are in all other eukaryotes. Aliquots of a peak of nucleolar chromatin (corresponding, e.g., to fraction 10 in Figure 2) were therefore assayed for RNA polymerase activity in the presence of increasing concentrations of α-amanitin. The results presented in Figure 4 show that the RNA polymerase activity in nucleolar chromatin is entirely resistant to α-amanitin concentrations up to 200 µg/mL. This

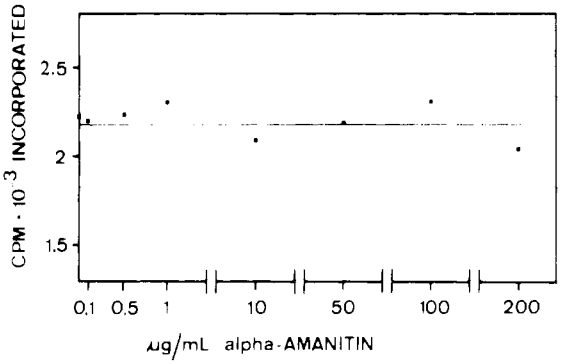

FIGURE 4: α-Amanitin resistance of RNA polymerase activity from rDNP. Aliquots from an rDNP preparation sedimented as described in Figure 2 were assayed for RNA polymerase activity as described under Materials and Methods. α-Amanitin was added to the concentrations indicated.

finding indicates the absence of RNA polymerases II and III from nucleolar chromatin. Since RNA polymerases I and II from *Physarum* have rather different salt optima, aliquots of nucleolar chromatin also were assayed for RNA polymerase activity in the absence of α-amanitin, but with increasing concentrations of ammonium sulfate. Figure 5 shows that the endogenous RNA polymerase activity has a single optimum at very low salt concentrations. This finding is in good agreement with results obtained with purified polymerase I and is in clear contrast to the biphasic salt optimum curve obtained with RNA polymerase II (Smith & Braun, 1978). Both those results suggest that nucleolar chromatin contains RNA polymerase I as its only actively transcribing enzyme, and they confirm our contention that nucleolar chromatin, purified by sucrose gradient centrifugation, is not contaminated with nuclear chromatin.

Buoyant Density of Nucleolar Chromatin. Aliquots of nucleolar chromatin were centrifuged to equilibrium in metrizamide density gradients preformed as described under Material and Methods. ^{14}C-Labeled *Escherichia coli* DNA was always run on parallel gradients as a density marker. From the gradients containing the nucleolar chromatin, 0.2-mL fractions were collected and 10-µL aliquots of each fraction were assayed for RNA polymerase activity in the presence of 1 µg/mL of α-amanitin. Figure 6 demonstrates that the nucleolar chromatin forms a rather homogeneous peak at a density position clearly higher than that of purified DNA (1.172 vs. 1.125 g/mL). Since, in metrizamide, proteins have a higher density than DNA or RNA, this finding suggests the presence of protein bound to rDNA, thus supporting the results obtained from sedimentation analysis. Moreover, when various fractions from the peak formed by nucleolar chromatin during sedimentation in sucrose gradients (front edge, center or peak, and trailing edge) are separately recentrifuged in metrizamide gradients, similar density profiles are obtained (results not shown). This would further strengthen the view that nucleolar chromatin consists of a rather homogeneous population of deoxyribonucleoprotein particles. Their density profile indicates a DNA to protein ratio of 1:1 (Birnie et al., 1973). However, this estimation bears some uncertainty since it has not yet been determined how much RNA and RNA-bound protein the ribosomal chromatin does contain. The slight skewing of the peak of RNA polymerase activity towards less dense positions, which is reproducibly observed, suggests an intrinsic density heterogeneity of the nucleolar chromatin or else it might reflect a partial decomposition of the particles during isolation.

Characterization of RNA Synthesized in Vitro by Nucleolar

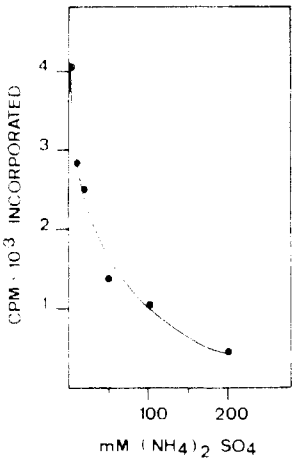

FIGURE 5: The effect of salt on the activity of RNA polymerase from rDNP. RNA polymerase activity was assayed as described under Materials and Methods in the absence of α-amanitin, at the indicated concentrations of $(NH_4)_2SO_4$. The data shown are initial velocities.

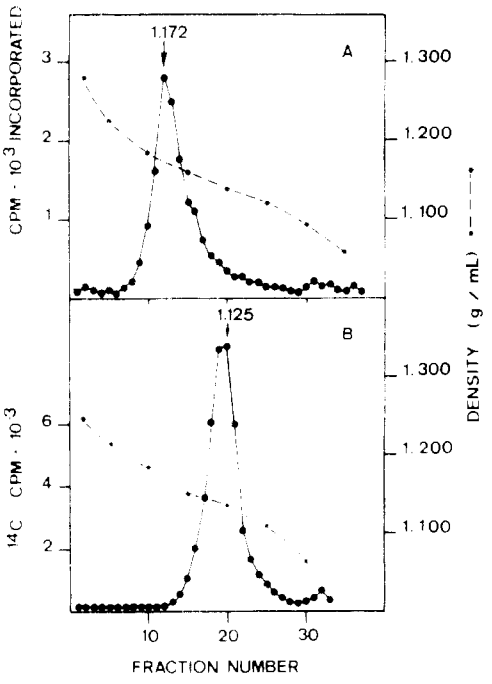

FIGURE 6: Metrizamide density gradient of rDNP. 0.3 mL of rDNP was centrifuged to equilibrium through a preformed metrizamide density gradient in a SW 50.1 rotor at 50000 rpm and 2 °C for 17 h. From the gradient containing rDNP 10-μL aliquots of each fraction were assayed for RNA polymerase activity in the presence of 1 μg/mL of α-amanitin. (A) rDNP, (B) ^{14}C-labeled *E. coli* DNA. Densities were calculated from the refractive indices (Birnie et al., 1973).

Chromatin. The results presented so far show that nucleolar chromatin can be isolated as a discrete nucleoprotein particle containing ribosomal genes and RNA polymerase I. We now wish to present results on the analysis of RNA synthesized in vitro by this particle. Nucleolar chromatin was sedimented in sucrose gradients as described above, and 10-μL aliquots of each fraction were assayed for RNA polymerase activity to localize the peak of the nucleolar chromatin. Peak fractions (similar to fractions 9-11 in Figure 2) were pooled and used to synthesize ^{3}H-labeled RNA in vitro. Aliquots of this RNA were hybridized to individual fractions of CsCl gradients containing total nuclear DNA. For such gradients, 100 μg of total nuclear DNA was fractionated to resolve rDNA from bulk DNA according to their different densities. To improve resolution, the gradients also contained 1 μg of the dye bis-

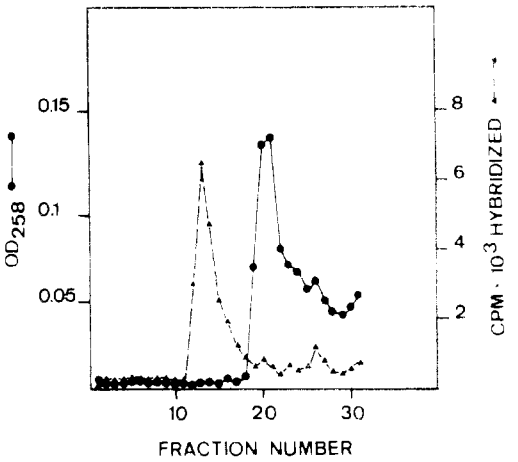

FIGURE 7: Hybridization of in vitro RNA to a density profile of total nuclear DNA. 100 μg of total nuclear DNA was centrifuged to equilibrium in a cesium chloride density gradient containing 100 μg of bisbenzimid H 33258 (Hoechst) in a Ti 50 rotor at 33000 rpm and 20 °C for 65 h. Fractions were loaded onto nitrocellulose filters and were hybridized with in vitro synthesized RNA as described under Materials and Methods.

benzimid H 33258 (Hoechst) per microgram of DNA, and the initial density was adjusted with CsCl to a refractive index of 1.3960 (U. Affolter, personal communication).

Gradients were then centrifuged as described in the legend to Figure 7. DNA from each fraction of the gradient was loaded onto nitrocellulose filters and was hybridized with the RNA synthesized in vitro. Figure 7 shows a peak of hybridization in the density region of the gradient containing the ribosomal sequences while only background hybridization takes place in the region of main band DNA, demonstrating that RNA synthesized in vitro by nucleolar chromatin is indeed complementary only to DNA with the density of rDNA. When, in control experiments, ^{3}H-labeled in vitro RNA was hybridized to the individual fractions of analogous gradients, but in the presence of 300 μg/mL of unlabeled 26S and 19S rRNA, hybridization was diminished by more than 70% (results not shown). These findings suggested that the majority of the in vitro RNA which hybridizes to DNA of the density of rDNA originates from the coding sequences, though the latter amount to not more than about 10% of the total rDNA molecule (Vogt & Braun, 1976; Molgaard et al., 1976). To corroborate these findings, in vitro RNA was analyzed by competition hybridization. Nitrocellulose filters, each carrying 10 μg of total nuclear DNA, were hybridized with 0.053 μg of ^{3}H-labeled in vitro RNA (specific activity 350000 cpm/μg) in the presence of increasing amounts of unlabeled 19S and 26S rRNA. Unlabeled yeast tRNA was added where necessary to keep total RNA concentration constant. The result of such a hybridization is shown in Figure 8 and demonstrates that ribosomal RNA is able to compete out more than 70% of the labeled in vitro RNA. This result supports the previous observation that at least 70% of the hybridizable in vitro RNA is complementary to those sequences of rDNA which code for 19S and 26S rRNA.

Discussion

We have isolated nucleoli from *Physarum polycephalum*, introducing as a novel step a density gradient centrifugation in colloidal silicon (Percoll) which allows the elimination of polysaccharides from the nucleolar preparation. From such nucleoli, we have been able to isolate the ribosomal genes as discrete transcription complexes or minichromosomes, containing rDNA and active RNA polymerase I and presumably

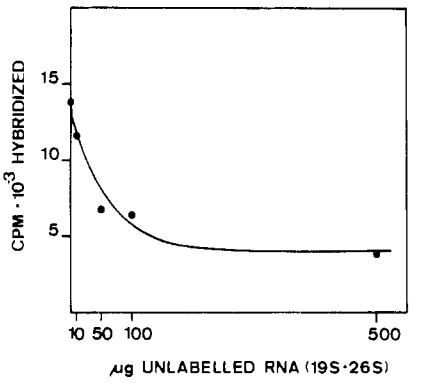

FIGURE 8: Competition of ^{3}H-labeled in vitro RNA with unlabeled 26S and 19S rRNA. Filters containing 10 µg of total nuclear DNA were hybridized in triplicate with 0.053 µg of ^{3}H-labeled in vitro RNA and the indicated concentrations of unlabeled rRNA (26 S and 19 S in equimolar amounts).

additional proteins. These particles have operationally been termed rDNP (for ribosomal deoxyribonucleoprotein), in analogy to rDNA.

Sedimentation analysis on sucrose gradients demonstrated that rDNP is isolated as a homogeneously sedimenting class of particles which sediment much faster than purified rDNA. This sedimentation behavior suggests that rDNP contains, besides RNA polymerase I, additional proteins, among them presumably histone as a major class. This conclusion is also supported by the result of the density analysis in metrizamide gradients, where rDNP migrates to a position of higher density than does purified DNA. The presence of protein complexed to rDNA is also suggested by earlier observations that rDNA in intact nuclei is protected from digestion by micrococcal nuclease to a similar degree as is bulk chromosomal DNA (Stalder et al., 1979).

Density analysis of the DNA present in nucleolar chromatin indicates that rDNA is the only DNA component of the nucleolus. This leaves us with a tentative view of a nucleolus composed entirely of rDNP particles as the sole genetic element. In addition to these, it also contains, among other components, RNP particles and ribosomal precursors of various stages. Such a structure of the nucleolus is similar to the situation observed in the nucleoli of *Xenopus* oocytes where ribosomal genes represent the total DNA content of the nucleolus (Reeder et al., 1977).

The rDNP contains RNA polymerase I as its sole transcriptional activity. This is in good agreement with the observation that so far no coding sequences, besides those for rRNA, could be detected on rDNA. However, we would like to caution that a comparison of relative activities of RNA polymerases I and II is, at least in *Physarum*, loaded with pitfalls unless purified enzymes are used. Numerous inhibitors for one or the other enzyme have been observed.

Hybridization analysis of RNA transcribed in vitro by the endogenous RNA polymerase I of rDNP shows that under our conditions this RNA hybridizes to rDNA and that 70% of the hybrizable RNA is complementary to the DNA sequences coding for 19S and 26S rRNA. Because the coding sequences represent only 10% of the entire rDNA molecule, this finding suggests that transcription in vitro is restricted to those sequences also transcribed in vivo. The remaining 30% may be complementary to the transcribed spacer sequences. However, we cannot exclude that the excess RNA might represent transcripts of regions or strands not transcribed in vivo. In any case, in vitro transcription in this system appears to reflect the in vivo situation at least to a great extent, and this system will hopefully be useful in elucidating the mechanisms of control of rRNA transcription. Furthermore, this small minichromosome containing only a single, and well defined, set of genes (those coding for rRNA) is expected to be a valuable model system for the elucidation of structure and function of chromatin at large.

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Mutual Homology of Mouse Immunoglobulin γ-Chain Gene Sequences†

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ABSTRACT: We have assessed the relative homology of mouse immunoglobulin heavy-chain gene sequences using complementary DNAs (cDNAs) synthesized against γ-chain mRNAs (γ1, γ2a, γ2b, and γ3) purified from mouse myelomas. cDNAs complementary to the γ-chain mRNAs did not cross-hybridize with the μ- and α-chain mRNAs, whereas they cross-hybridized to significant extents (22–66%) with the γ-chain mRNAs of other subclasses. The heterologous hybrids formed, however, melt at 5–13 °C lower temperatures as compared to the homologous hybrids, indicating that significant portions of the heterologous hybrids are mismatched. The rates of the cross-hybridization reactions are 2- to 17-fold slower than those of the homologous hybridization reactions. Therefore, the γ-chain gene sequences of four subclasses share a part of homology with each other, but they are different enough to be measured separately. Cross-hybridization analyses indicate that the γ2a and γ2b genes are the most closely related, while the γ1 and γ3 genes are the least related among the γ subclass genes.

Immunoglobulin heavy chains are encoded by a family of V[1] region genes and a set of C region genes (reviewed by Eichmann, 1975). In order to study the organization of immunoglobulin heavy-chain genes, it is essential to isolate the specific probe for each heavy chain gene. Recently we have succeeded in purifying mRNAs encoding mouse immunoglobulin heavy chains (Ono et al., 1977). Using purified mRNAs we have synthesized and purified complementary DNAs (cDNAs) corresponding to the heavy chains of all four γ subclasses, namely, γ1, γ2a, γ2b, and γ3 chains.

In this paper we will report the mutual homology among four γ-chain gene sequences. The extent and rate of hybridization and the thermal stability of hybrids formed showed that although these gene sequences share partial homology with each other, each cDNA is specific to its own subclass.

Materials and Methods

Materials. Mouse myeloma tumors were kindly supplied by Dr. M. Potter of National Institutes of Health, except that MC 101 was provided from Dr. Migita of Kanazawa University. Tumors were maintained as described (Swan et al., 1972). ^{3}H-Labeled dCTP (24 Ci/mmol) was obtained from the Radiochemical Center, Amersham, England, and ^{3}H-labeled dATP and dGTP (12 Ci/mmol) were purchased from New England Nuclear. Hydroxylapatite of DNA grade was obtained from Bio-Rad Lab.

Preparation and Purification of mRNAs and cDNAs. Preparation and purification of heavy-chain mRNAs derived from mouse myelomas MOPC 31C (γ1), HOPC 1 (γ2a), MPC 11 (γ2b), and J 606 (γ3) were done as described in the previous report (Ono et al., 1977). Purities of γ1, γ2a, γ2b, and γ3 mRNAs were 97, 63, 80, and 100%, respectively, as assayed by hybridization kinetic analysis. Detailed characterization of mRNAs will be described elsewhere. [^{3}H]cDNAs complementary to the mRNAs were synthesized using [^{3}H]dCTP, [^{3}H]dATP, and [^{3}H]dGTP by avian myeloblastosis virus reverse transcriptase and purified as described (Honjo et al., 1974). The specific radioactivity of [^{3}H]cDNA was 1.6 × 10^7 cpm/μg. Although each cDNA showed a single transition in hybridization kinetic analysis to corresponding mRNAs (Ono et al., 1977), γ2a and γ2b cDNAs showed, in our preliminary experiments, the presence of some quantity of contaminants in their preparations. As the contaminants seem to come from minor mRNA species present in common in tumor cells, they were removed by hybridizing γ2a and γ2b

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[1] Abbreviations used: V and C regions, variable and constant regions; cDNA, synthetic DNA complementary to mRNA; Cot (Crt) values, product of concentration of nucleotide sequences of DNA (RNA) and time of incubation (mol of nucleotides × s/L).

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