Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1116–1120, March 1978
Biochemistry

Altered nucleosome structure containing DNA sequences complementary to 19S and 26S ribosomal RNA in *Physarum polycephalum*

(ribosomal cistrons/nucleosomes/peak A/saturation hybridization)

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Communicated by Igor Tamm, December 1, 1977

ABSTRACT **The localization of DNA sequences coding for ribosomal RNA was studied by hybridization of purified ribosomal RNA to DNA from chromatin fragments prepared by limited digestion of *Physarum* nuclei with staphylococcal nuclease. The ^{32}P-labeled 19S and 26S RNA hybridized to DNA from nucleosome monomers, dimers, trimers, and higher oligomers, separated by sucrose gradient centrifugation, although the level of hybridization to DNA from nucleosome fractions was less than the level of hybridization to undigested nuclear DNA. The distribution of 19S and 26S rDNA sequences in the nucleosome fractions differed from the distribution of bulk DNA in that the rDNA sequences were recovered primarily in two fractions containing monomer-sized DNA lengths (140–160 base pairs). The percentage of DNA hybridizing to 19S plus 26S RNA was greater in peak A, the more slowly sedimenting monomer peak, than in any other chromatin fraction at all stages of digestion. Peak A and monomer particles differed in protein content and distribution. The presence of ribosomal cistrons in an altered nucleosome configuration may be related to changes in functional states of rDNA chromatin.**

Electron microscopic visualization of actively transcribing ribosomal genes indicates that the DNA comprising the transcribed segment is in a highly extended conformation (1–5). This evidence may be compared with biochemical observations indicating chromatin containing DNA coding for ribosomal RNA (rDNA) behaves biochemically as though it were organized into nucleosomes (6–9). Nucleosome fractions produced by treatment of *Xenopus* nuclei with staphylococcal nuclease contain DNA complementary to 18S and 28S RNA (6–8), and experiments performed using nuclei from cultured *Xenopus* embryonic cells indicate that rDNA from transcribing ribosomal genes may similarly be organized into subunits shielded from nuclease attack (8). Although it has been suggested that this protection of rDNA sequences from nuclease digestion indicates that transcribing regions of rDNA are organized in the beaded configuration typical of most DNA sequences (6–9), this conclusion is not readily reconciled with the numerous electron microscopic observations stressing the absence of nucleosomes from transcribing genes. For example, the nascent ribosomal RNA fibrils of *Oncopeltus* appear to be attached to an unbeaded chromatin strand with a packing ratio significantly lower than for DNA in regions of beaded chromatin (1).

There are several alternative explanations for this apparent discrepancy. The recovery of rDNA sequences in nucleosome-sized particles may represent the selective shielding of inactive rDNA sequences. This view is supported by data indicating that the ribosomal cistrons in *Xenopus* are underrepresented in nucleosomes from cells in which rRNA synthesis is high (ref. 7; cf. ref. 10). The active rDNA may be rapidly degraded because it is not as effectively shielded by associated histones. However, it has been pointed out that the transcribing strand of ribosomal chromatin appears thicker than a double-strand of free DNA, suggesting the presence of associated proteins (1, 2), and the presence of histone H2B in transcribing chromatin has been shown by immunoelectron microscopy (11). The shielding of many rDNA sequences from random nuclease attack, observed in nuclease digestion experiments, would not be inconsistent with the absence of a beaded chromatin structure, observed by electron microscopy, if extension of the DNA template were achieved by postsynthetic modifications of the associated proteins (12, 13) or by other changes in composition leading to unfolding of the nucleosomes about an axis of symmetry (14).

In order to characterize nucleoprotein structures comprising ribosomal gene chromatin, we have separated and analyzed the chromatin fragments prepared by limited staphylococcal nuclease treatment of nuclei from *Physarum polycephalum*. Studies on rDNA chromatin in this lower eukaryote are facilitated by the localization of ribosomal cistrons on a large, extrachromosomal (15), palindrome-like (16, 17) DNA molecule that constitutes 1%–2% of the total nuclear DNA. The subunit structure of *Physarum* chromatin has been described (18). Here we describe distinguishing characteristics of the localization of 19S and 26S rDNA sequences in chromatin substructures of actively growing *Physarum* microplasmodia.

MATERIALS AND METHODS

Treatment of Nuclei with Staphylococcal Nuclease and Isolation of Resultant Chromatin Fractions. Nuclei were isolated from *Physarum* microplasmodia, strain a × i, by a modification (18) of the procedure of Mohberg and Rusch (19) and were incubated with staphylococcal nuclease [nuclear DNA, 400 μg/ml; nuclease (Worthington), 50 units/ml] for various times at 37° in 1 mM $CaCl_2$/60 mM KCl/15 mM NaCl/1.0 mM phenylmethylsulfonyl fluoride (PMSF)/20 mM Tris·HCl, pH 7.8 (20). Nuclease digestion was terminated by addition of EDTA to a final concentration of 10 mM, and chromatin subfractions were released by shearing with five strokes of a tight-fitting pestle in a Dounce homogenizer. After centrifugation of 3000 × *g* for 10 min at 4°, aliquots of the supernatant fraction corresponding to 3×10^8 nuclei were layered

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Abbreviations: rDNA, DNA coding for ribosomal RNA, including transcribed and nontranscribed spacer sequences; 19S and 26S rDNA, DNA coding for 19S and 26S ribosomal RNA; PMSF, phenylmethylsulfonyl fluoride.

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over 5%–20% sucrose gradients (36 ml) containing 0.35 M NaCl/0.1 mM PMSF/2.0 mM EDTA, pH 7.4, and centrifuged for 22.5 hr at 27,000 rpm in a Beckman SW 27 rotor at 4°, essentially as described by Woodcock and coworkers (21).

Extraction of DNA and Hybridization to Labeled 19S + 26S RNA. Pooled fractions from sucrose gradients were dialyzed overnight against 50 mM ammonium acetate/0.1 mM PMSF/20 mM EDTA, pH 7.0, and lyophilized. After initial extraction of protein with phenol and chloroform/isoamyl alcohol, DNA samples were treated with RNases A and T1 and with proteinase K, reextracted with phenol and chloroform/isoamyl alcohol, precipitated with ethanol, and redissolved in 15 mM NaCl/10 mM EDTA/10 mM Tris·HCl, pH 8.0.

DNA determinations were by the diphenylamine reaction described by Burton (22). Hybridization was carried out with 1–5 g of DNA and purified excess 19S + 26S RNA labeled *in vivo* with [^{32}P]phosphate (2–5 × 10^5 cpm/μg) in 49% (vol/vol) formamide/0.9 M NaCl/0.1% sodium dodecyl sulfate/1 mM EDTA/ and 25 mM 2-{[tris(hydroxymethyl)methyl]amino}-ethanesulfonate (23), pH 7.0, in a final volume of 0.2 ml. After incubation for 16 hr at 60°, samples were treated with ribonucleases by a modification (to be described elsewhere) of the procedure of Weiss *et al.* (24), precipitated with 10% (wt/vol) trichloroacetic acid and 150 μg of yeast carrier RNA per sample, collected on nitrocellulose filters, and assayed for radioactivity by scintillation spectrometry. In all studies, background radioactivity averaged less than 0.5% of input radioactivity.

RESULTS

After treatment of nuclei with staphylococcal nuclease, chromatin subunits released from nuclei were separated on sucrose gradients containing 0.35 M NaCl along with EDTA and PMSF to inhibit DNase and proteolytic activities, respectively. Separation of peaks for monomer (1°), dimer (2°), trimer (3°), and tetramer (4°) fractions (Fig. 1) was generally similar to reported separations of nucleosome fractions from various higher eukaryotes. Polyacrylamide gel electrophoresis of DNA extracted from these nucleosome peaks confirmed that in each case the DNA length obtained was predominantly that expected for the designated multimer, although minor bands corresponding to adjacent multimers usually could be observed. In addition to these nucleosome fractions, two peaks were observed sedimenting more slowly than the monomer peak. The peak nearest the top of the sucrose gradient contained DNA fragments that migrated electrophoretically as multiple bands with lengths ranging from approximately 10–100 base pairs.

Another peak, peak A (Fig. 1), was partially resolved from the fragment peak and contained DNA of predominantly monomer length (140–160 base pairs). Peak A has been detected under all digestion conditions thus far used. When sedimented for longer times than those described in Fig. 1, peak A was clearly resolved from slower particles containing DNA fragments and from the faster monomer peak. Under conditions in which about 10% of the total nuclear DNA is digested by nuclease, corresponding to 2-min digestion in Fig. 1, peak A comprised only a small proportion of the total DNA released from nuclei, the bulk of released DNA being found in monomer and multimer fractions. Upon more extensive digestion, peak A comprised a larger proportion of the released DNA. After 10-min digestion, when about 56% of the total DNA is digested by nuclease treatment, peak A and monomer peaks each contained about 30% of the released DNA and formed a doublet resolved from the slower fragment peak.

DNA extracted from the sucrose gradient fractions shown in Fig. 1 was hybridized to purified ^{32}P-labeled 19S + 26S RNA in order to measure the percentage of the DNA complementary to those sequences. Several different hybridization procedures were used. Procedures based upon adsorption of DNA to nitrocellulose filters were found to be unsuitable for measuring hybridization of fragments of monomer length or smaller, because of variable retention of DNA on the filters during the annealing reaction. Procedures requiring *in vivo* labeled DNA were not used because it is known that rDNA in *Physarum* is labeled differently from bulk chromosomal DNA (15). The most reliable procedure was found to be hybridization in solution followed by RNase treatment and precipitation of undigested hybrids. A possible disadvantage of this method is that backgrounds due to undigested free RNA may be high relative to labeled RNA in hybrids. However, it was found that, under the conditions used in the present study, 19S + 26S sequences in total DNA are completely saturated at less than 25-fold excess RNA (approximately 0.25 μg of RNA per 5 μg of total DNA), and that backgrounds are several-fold less than hybridization values. In several experiments, samples containing DNA from nucleosome fractions or from digested nuclei were sonicated to randomize size differences among hybridizing fragments. Results of hybridization experiments performed with 80% formamide and 2 × standard saline–citrate as described by Casey and Davidson (25) were similar to those presented here, indicating that DNA·DNA reannealing is not a significant problem under the present conditions.

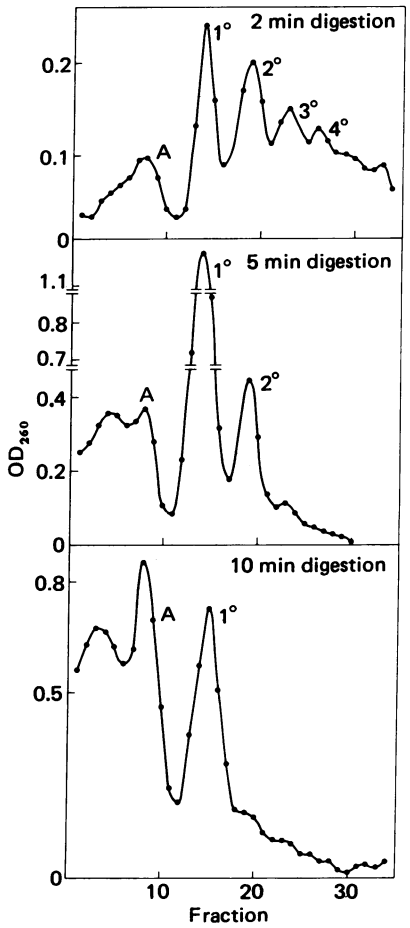

FIG. 1. Isolation of staphylococcal nuclease digestion products from *Physarum* chromatin. Nuclei were treated with staphylococcal nuclease for different times, sheared to release chromatin digestion products, and centrifuged. Fractions of 1.1 ml were collected. 1°, monomer; 2°, dimer; 3°, trimer; 4°, tetramer.

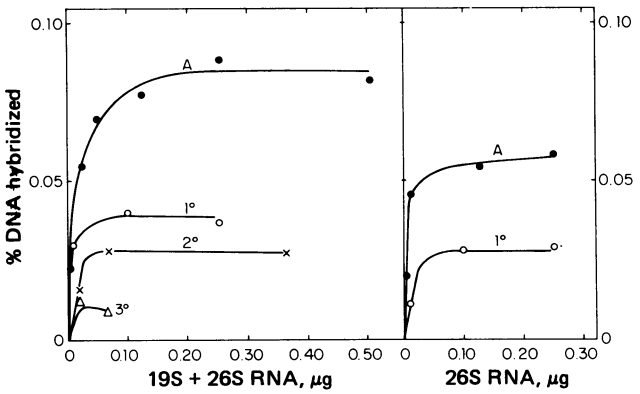

FIG. 2. Hybridization of purified 19S + 26S RNA to DNA isolated from *Physarum* chromatin digestion products. Nuclei were treated for 2 min with staphylococcal nuclease, and nucleosome peaks were isolated as in Fig. 1. Fractions containing peaks were pooled, DNA was extracted and its concentration was determined. (*Left*) Hybridization with 5 μg of DNA per point and varying concentrations of purified 19S + 26S RNA (used at a ratio of 19S:26S = 1:2; ^{32}P specific activity, 3.61×10^5 cpm/μg). (*Right*) Hybridization with 26S RNA only (specific activity, 3.61×10^5 cpm/μg).

Saturation of total undigested *Physarum* DNA with 19S + 26S RNA indicated that 0.180 ± 0.002% of the DNA hybridized to those sequences, a result in agreement with previously reported values (26). There were considerable differences in the capacity of DNA from the different chromatin fractions to hybridize to 19S + 26S RNA (Fig. 2 *left*). From each fraction, aliquots (5 μg) of DNA were hybridized to saturation with increasing concentrations of labeled ribosomal RNA. In general, DNA from the more slowly sedimenting nucleosome fractions hybridized to a greater extent than did DNA from the higher oligomers. It is notable that no significant hybridization to DNA from fragments at the top of the sucrose gradient was observed. Irrespective of the time of digestion used to prepare nucleosomes, DNA from peak A hybridized to a greater extent than did DNA from any of the other fractions. DNA from both peak A and monomer fractions hybridized with purified 26S RNA (Fig. 2 *right*). In each case, the extent of hybridization at saturation was approximately two-thirds of the value obtained with 19S + 26S RNA. This indicates that both 19S and 26S rDNA sequences are distributed equally, on a weight weight basis, in monomer fractions, because the 26S region is about twice as long as the 19S region.

Under the digestion conditions described in Table 1, approximately 9% of the total nuclear DNA was digested to non-ethanol-precipitable fragments that were not recovered after centrifugation and isolation of nucleosomes. DNA from each of the nucleosome peaks hybridized to 19S + 26S RNA, indicating that at least some of the ribosomal cistrons are packaged into structures with sedimentation characteristics of nucleosomes. However, the content of 19S + 26S rDNA sequences differed among the subunit fractions recovered by sucrose gradient centrifugation. Peak A DNA hybridized to the ribosomal RNA to an extent of approximately 0.2%. In contrast, monomer DNA hybridized only to about 0.1%, and DNA from higher oligomers hybridized to an even lesser extent. The average recovery of released DNA in all the nucleosome fractions hybridizing to 19S + 26S RNA was 0.093%.

After 2-min digestion, as for Table 1, DNA not released by digestion but pelleted with the nuclei was also extracted and hybridized to 19S + 26S RNA. DNA not released from nuclei hybridized to an extent of 0.089%, similar to the value obtained for hybridization to DNA from the released particles and considerably lower than the value for total undigested DNA.

Table 1. Saturation hybridization of 19S + 26S RNA to DNA in nucleosome fractions

Sucrose gradient peak*	% recovered DNA†	% DNA hybridizing to 19S + 26S RNA‡		% recovered 19S + 26S rDNA per fraction
Fragments	1.1	<0.001		<0.4
A	1.4	0.209	(0.196 ± 0.022)	6.4
1°	29.3	0.105	(0.087 ± 0.023)	67.3
2°	29.8	0.038	(0.030 ± 0.007)	24.7
3°	18.5	0.003	(0.012 ± 0.011)	1.2
Higher oligomers	19.9	0.001		0.4

* Fractions were pooled from the sucrose gradient peaks of the 2-min digestion depicted in Fig. 1 *top*.

† DNA was determined by the diphenylamine procedure (22). After 2-min digestion, 56.7% of recovered DNA was in nucleosomes, and 43.3% was retained in the nuclear pellet; 8.7% of the initial DNA was not recovered after 2-min digestion.

‡ Values were determined by saturation of DNA (5 μg per point) with excess RNA. Single values are from a typical experiment, shown in Fig. 1, and are averages of duplicate points taken from hybridization curves at saturation. Values in parentheses are means ±SD from three separate 2-min digestion experiments performed with different preparations of nucleosomes and different preparations of 19S and 26S RNA.

It can be seen in Table 1 that, whereas about 30% of the total DNA was recovered as monomer-length fragments in the peak A and monomer fractions, more than 70% of the hybridizable rDNA sequences was recovered in these fractions. The results indicate that after 2 min of nuclease treatment a high percentage of rDNA sequences recoverable from nuclei are digested to monomer-length DNA segments. In addition, a high percentage of the rDNA sequences were digested away more rapidly than was the bulk of chromosomal DNA. Even after 2 min of digestion, only about 62% of the initial content of sequences hybridizing to 19S + 26S RNA could be recovered. Upon digestion for longer than 2 min, the percentage of DNA in each peak complementary to 19S + 26S RNA was diminished, although at all times the highest content of the rDNA sequences was in the peak A fraction (data not shown).

The lengths of DNA segments extracted from peak A and

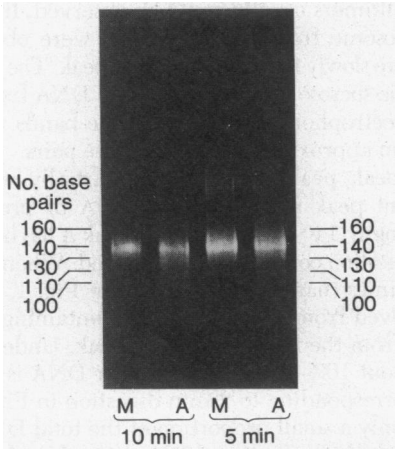

FIG. 3. Comparison of DNA lengths in peak A (A) and monomer (M) fractions isolated from *Physarum* chromatin after 5- and 10-min digestions with staphylococcal nuclease. Chromatin subunits were isolated as described in the legend to Fig. 1, and fractions containing the peak A and monomer peaks were pooled. DNA was extracted and subjected to electrophoresis on 5% polyacrylamide gels containing 0.09 M Tris·borate and 2.5 mM EDTA, pH 8.3. DNA sizes were determined by using polyoma A2 and λdv-1 restriction fragments as described (18).

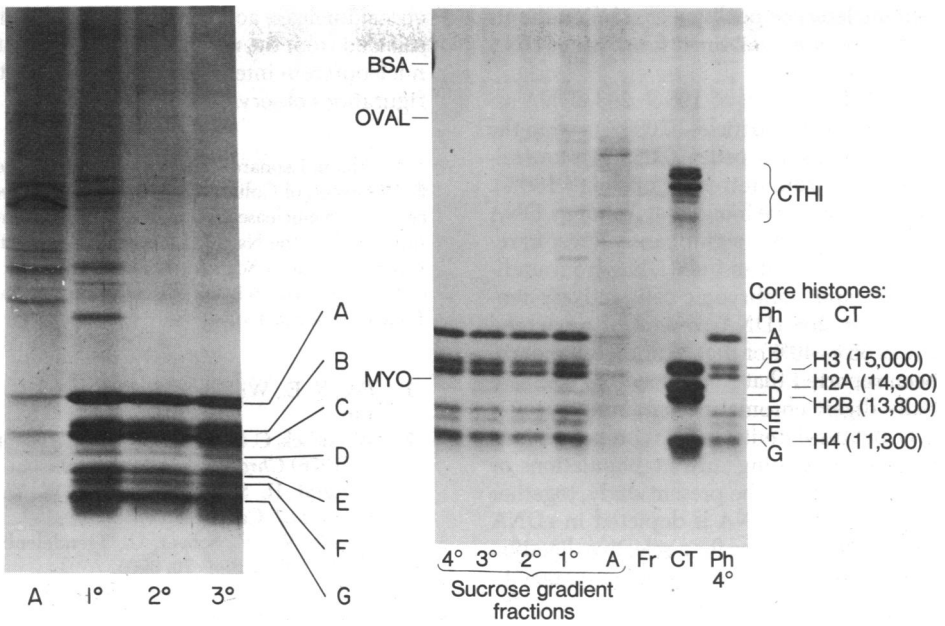

FIG. 4. Electrophoretic patterns of proteins extracted from *Physarum* nucleosome fractions after limited digestion with staphylococcal nuclease. After 5-min digestion, the chromatin fragments were isolated as described in the legend to Fig. 1. Fractions were dialyzed against 0.05 M ammonium acetate/0.1 mM PMSF, pH 7.0, lyophilized, redissolved in sample buffer containing 2% sodium dodecyl sulfate, and subjected to electrophoresis on slab gels as described (27). (*Left*) In 14%–19% polyacrylamide gradient gel run at 13 mA for 16 hr. (*Right*) In 15% polyacrylamide gel run at 9 mA for 16 hr. Positions of *Physarum* histone bands (Ph) are compared with calf thymus histone bands (CT) at right. Fr, A, 1°, 2°, 3°, 4° represent *Physarum* slowly sedimenting chromatin fragments, peak A, monomer, dimer, trimer, and tetramer, respectively.

monomer fractions are compared in Fig. 3. After 5- and 10-min digestions, both fractions contained predominantly DNA lengths averaging approximately 140 base pairs. In the case of the monomer, DNA of dimer length was present as a minor component; in the case of peak A, DNA of subnucleosome lengths ranging down to approximately 80 base pairs could be detected. This analysis of DNA lengths indicates that observed differences in hybridization between peak A and monomer DNAs are unlikely to be due to size differences between hybridizing segments. The sedimentation values of peak A and monomer particles were considerably different. Analytical ultracentrifugation studies of sedimentation velocities of peaks isolated from sucrose gradients indicated that monomers sediment at 11 S and peak A particles sediment at 5 S. Since fully extended DNA of monomer length sediments near 5 S, this result indicates that peak A DNA is in a more extended conformation than monomer DNA.

Proteins were extracted from isolated nucleosome fractions and subjected to slab gel electrophoresis as described (27). The resultant electrophoretic staining patterns are shown in Fig. 4. All of the major *Physarum* core histones (A–G) could be detected in each nucleosome fraction. However, peak A and monomer nucleosomes differed with respect to relative amounts of the different histones. Only faint bands could be detected in peak A for histones B and F, and histone G also appeared to be relatively diminished. Bands B and G correspond to calf histones H3 and H4, respectively; band F is a histone not found in mammalian nuclei. The histones detected in peak A are evidently associated with DNA in this fraction because histones extracted from peak A and monomers resedimented on shallower sucrose gradients gave similar electrophoretic patterns (data not shown). In resedimented peak A and monomer fractions, the protein/DNA ratio for peak A was lower than that for the monomers. Direct measurements indicated that this ratio for peak A was about 1.0 and that for monomers was about 1.2. It can be seen in Fig. 4 that peak A had a higher relative content of nonhistone proteins than did the monomer fraction. On most gels, at least one band depleted in peak A, band B, was detected at the top of the sucrose gradient.

DISCUSSION

Labeled 19S + 26S rRNA hybridized to DNA of nucleosome monomers, dimers, trimers, and higher oligomers (Fig. 2; Table 1), suggesting that a portion of the rDNA is packaged into repeating chromatin structures with sedimentation characteristics of nucleosomes. These results are consistent with those of Reeves and coworkers (7, 8) and of Reeder (6), who reported that DNA of ribosomal cistrons from *Xenopus* can be recovered in nucleosome fractions. The results are also consistent with a preliminary report by Grainger (28) that nucleolar chromatin of *Physarum* is digested by staphylococcal nuclease to produce repeating subunit structures (28).

The present results indicate that the distribution of 19S + 26S rDNA sequences in the nucleosome fractions differs from that of bulk DNA in that the hybridizable rDNA sequences are recovered primarily in the monomer and peak A fractions (Table 1). At all digestion times examined, the peak A fraction possessed the highest content of sequences hybridizing to 19S + 26S RNA. After 2-min digestion, the 19S + 26S rDNA content of the peak A fraction is approximately double that of the monomer fraction. At this level of digestion, the 19S + 26S rDNA content of peak A is not significantly different from that of total undigested chromatin, although at later times the hybridizable rDNA content of all chromatin subfractions is diminished. We find in the present study that the rDNA content of *Physarum* higher oligomers is relatively low compared to that of the monomers. It should be noted that, in our experiments, the hybridization conditions used do not discriminate against measurement of hybridization to smaller DNA fragments. It is likely that, in the present study, large fragments containing undigested or partially digested rDNA sequences are retained in the nuclei after nuclease digestion and centrifugation. In this study, hybridization of ribosomal RNA to DNA

from regions between nucleosome peaks on sucrose gradients was not attempted; these regions contained insufficient DNA for quantitative saturation studies.

After 2-min digestion, the content of 19S + 26S rDNA sequences in the released chromatin particles (0.093%) and in the chromatin remaining in the nuclear pellet (0.089%) is low relative to the content in DNA from untreated nuclei (0.180%). Similarly, Reeves and Jones (8) have found that *Xenopus* DNA complementary to ribosomal RNA is significantly lower in recovered nucleosome fractions than in bulk DNA from undigested chromatin. In *Xenopus* embryonic cells actively synthesizing rRNA, the 19S + 26S rDNA content of recovered nucleosomes is as low as 35%–40% of that in undigested chromatin (29). It has been reported that staphylococcal nuclease does not preferentially digest chromatin containing specific classes of DNA (30, 31), but these studies used, as probes, cDNAs either from total poly(A)-containing mRNA populations or from a specific mRNA. In contrast, the present study, together with observations that monomer DNA is depleted in rDNA sequences (8, 29, 32), provides evidence for a selective digestion of chromatin containing DNA sequences complementary to ribosomal RNA.

Despite possessing similar DNA lengths, peak A sediments at about 5 S whereas the monomers sediment at about 11 S, as indicated by sedimentation velocity analysis. This difference in sedimentation values may be due to differences in DNA conformation occurring as a result of differences in protein content or structure. It is likely that peak A DNA is in a more extended conformation than is monomer DNA. At least a portion of these extended monomers may be derived from 11S monomers by removal of histones, because the histone/DNA ratio of peak A is lower than that of monomers. Electron micrographs of peak A reveal few free DNA strands, however. Many peak A particles, although more extended than monomers, possess substructures indicative of nucleoprotein complexes (data not shown). Gel electrophoresis of proteins from isolated nucleosome fractions (Fig. 4) shows differences between peak A and monomer proteins. These differences persist even when the two peaks are further purified by resedimentation on sucrose gradients for longer times. It is clear from Fig. 1 and Table 1 that peak A is not exclusively derived from rDNA chromatin. Further work is necessary to establish whether peak A is derived from an extended nucleosome structure that exists *in vivo*, or whether rDNA-containing monomer beads are altered so that they convert to peak A after digestion. It is possible that peak A represents an extended subunit form comprising much of the ribosomal gene chromatin.

Plasmodia are the most active stage in the *Physarum* life cycle (nuclear doubling time is about 8.6 hr in the cultures used), and transcription of ribosomal RNA occurs through most of the nuclear division cycle (33). It can be argued that transcription of most if not all of the 200–400 ribosomal genes present in each nucleus is required in microplasmodia. Thus, the possibility exists that differences between rDNA chromatin and bulk chromatin in regard to nuclease susceptibility are a result of the active state of ribosomal gene transcription. Staron and colleagues (34) have reported that a peak of [^{14}C]uridine incorporation coincides on sucrose gradients with peak A after *in vivo* labeling of plasmodia and digestion of nuclei with staphylococcal nuclease (34). It remains to be determined what relationship the RNA extracted from peak A has to the DNA in this fraction. Our data provide evidence that a high percentage of 19S and 26S rDNA sequences are packaged in a structural configuration different from that of the bulk of chromosomal DNA with regard to susceptibility to staphylococcal nuclease action and to the types of nucleoprotein units shielded from the nuclease. It will be of interest to elucidate the nucleoprotein interactions that give rise to the structural configurations observed.

We thank Leonard Lothstein for excellent technical assistance. Dr. R. W. Sweet, of Columbia University, provided valuable advice concerning ribonuclease treatment after hybridization. This research was supported by the National Science Foundation (PCM 76-19926), the American Cancer Society (VC 114 F), the U.S. Public Health Service (GM 17383), the Science Research Council, and the North Atlantic Treaty Organization.

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