Isolation of transcriptionally active chromatin from mammalian nucleoli

(HeLa cells/RNA polymerase I/urea sucrose gradients/gene isolation and ribosomal RNA/gene expression)

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Communicated by Hilary Koprowski, October 12, 1976

ABSTRACT Nucleoli isolated from HeLa cells are functionally active but contain large amounts of RNA and proteins (RNA/DNA ratio 1:1; protein/DNA ratio 7:1). We have isolated from the nucleolus a DNA-protein complex that has the characteristics of nucleolar chromatin (RNA/DNA ratio <0.05:1; protein/DNA ratio 1.7:1). This nucleolar chromatin has most of the transcriptional activity of the intact nucleolus and, as assayed by circular dichroism and dye binding, has largely preserved its structure. The isolation of a transcriptionally active fragment of chromatin, which constitutes only a small part of the total genome and codes for only one recognizable product, offers several advantages for the study of chromatin structure and function.

The isolated nucleolus offers several advantages in a study of chromatin structure and function. It comprises less than 5% of the total genome of mammalian cells (1-4), and it can be isolated easily in a highly reproducible manner while maintaining its functional activity (3, 5, 6). The only gene product of the nucleolus that has been identified thus far is the precursor molecule of rRNA (6). Precursor synthesis constitutes 30-40% of total nuclear RNA synthesis (7), although the genes for rRNA represent only 0.05% of the total genome (2). The rest of the nucleolar DNA is apparently satellite DNA (8, 9), which is not transcribed in vivo; and in vitro, as chromatin it is not even transcribed by an exogenous bacterial RNA polymerase (10). Thus, the nucleolus contains very active genes next to totally inactive ones, a unique combination for the study of chromatin structure and function.

Unfortunately, the mammalian nucleolus, as isolated by standard procedures (3, 5) has a protein/DNA ratio 7-10:1 and an RNA/DNA ratio of 1:1 (3, 4, 8), both clearly in excess of the values obtained for whole nuclear chromatin that range from 1.15 to 2.7 to 1 for protein/DNA ratio and less than 0.17:1 for RNA/DNA ratio (11). To study nucleolar chromatin, it would be desirable to remove the excess RNA and proteins (presumably ribonucleoprotein particles, ref. 12). In this paper, we report the isolation of a nucleolar chromatin with a protein/DNA ratio of 1.7:1, which seems to have preserved most of the functional characteristics of the intact nucleolus.

MATERIALS AND METHODS

Chemicals. [3H]Leucine (60 Ci/mmole), [3H]uridine (25.8 Ci/mmole), [3H]UTP (27.6 Ci/mmole), and [3H]thymidine (6.7 Ci/mmole) were purchased from New England Nuclear Corp., Boston. Mass. ATP, GTP, CTP, UTP, calf thymus DNA, Escherichia coli tRNA, ethidium bromide, and heparin were obtained from Sigma Chemicals, St. Louis, Mo. a-Amanitin was obtained from Calbiochem (La Jolla, Calif.), poly d(A-T) from Miles Laboratory (Elkhart, Ill.), and ribonuclease A from Worthington (Freehold, N.J.). All other chemicals were supplied by Mallinckrodt Chemical Works (St. Louis, Mo.), and the Fisher Scientific Co. (Pittsburgh, Pa.). Analytical grades were used where available.

Preparation of HeLa Cell Nucleoli. HeLa cells growing in logarithmic phase, in suspension, were harvested in a Sorvall 2RC centrifuge at 800 × g, washed twice with phosphate-buffered saline at pH 7.2, resuspended in a buffer consisting of 10 mM Tris-HCl at pH 8.0, 3 mM CaCl₂, 2 mM Mg acetate, and 0.5 mM dithiothreitol, and lysed with a Teflon homogenizer. Triton X-100 in the same buffer was added to a final concentration of 0.25% (wt/vol) for 3 min. The nuclei were washed at least twice in a 10 mM Tris-HCl at pH 7.4, 0.25 M sucrose, and 3.3 mM MgCl₂ buffer, again resuspended in 15 ml of this buffer, layered onto 35.0 ml of 0.88 M sucrose in 10 mM Tris-HCl at pH 7.4, and 0.5 mM Mg acetate, and pelleted at 1200 × g for 10 min. Nuclei thus prepared were free of cytoplasmic contamination. These nuclei were resuspended in 0.35 M sucrose solution, kept at 2°C, and sonicated with a Ultrasonic Cell Sonifier W185 four times at 100 W for 10 sec and two to three times at 50 W for 20 sec with intermittent cooling. The sonicated material was layered onto a 0.88 M sucrose cushion and centrifuged at 1500 × g for 25 min in an International Centrifuge. The pellet contained the nucleoli. The purity of these nucleoli has been discussed in a previous paper from this laboratory (13).

Preparation of Nucleolar Chromatin. (i) The nucleolar pellet was resuspended in 50 mM Tris-HCl at pH 7.4 (0.5-1.0 A₂₅₀ units of DNA and RNA/ml), and incubated with 1 mg/ml [ribonuclease A, ribonuclease 3'-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22 (RNase A)] at 37°C for 90 min with constant shaking (13). Prior to use, the RNase A was incubated for 15 min at 90°C to inactivate contaminating DNase. After the RNase digestion, the nucleolar suspension was cooled to 2°C and pelleted at 1500 × g for 15 min in an International Centrifuge.

(ii) The nucleolar pellet obtained after RNase A digestion was resuspended in 10% (wt/vol) sucrose in 10 mM Tris-HCl at pH 7.4, 0.5 mM Mg acetate, and kept for 20 min with intermittent stirring at 2°C. This suspension was then layered gently onto a 10-40% (wt/vol) sucrose gradient, made in 10 mM Tris-HCl at pH 7.4, 0.5 mM Mg acetate, and 5 M urea. The gradient was set up on a 1.0 ml cushion of saturated CsCl or sucrose solution in cellulose nitrate tubes with 14.0 ml total capacity. The gradients were centrifuged in a SW 40 rotor for 30 min at 1400 × g in a Beckman L3-50 centrifuge and collected from the bottom, with 0.5 ml per fraction. The gradient was found not to be necessary and could be replaced by a 25% (wt/vol) sucrose solution. The location of DNA was identified either by incor-
FIG. 1. Urea-sucrose gradients of nucleoli labeled with [3H]uridine before, and after, RNase digestion. HeLa cells were grown and labeled for 90 min with [3H]uridine in minimal Eagle's medium (0.3 μCi/ml). Intact nucleoli (A) or RNase-digested nucleoli (B) were prepared and layered onto urea-sucrose gradients as described. The position of DNA was determined as described in Materials and Methods.

porated radioactive label ([3H]thymidine) or by addition of ethidium bromide (0.5 μg/ml). The urea/sucrose gradient centrifugation was repeated twice. The fractions containing the nucleolar chromatin were collected and dialyzed overnight against 10 mM Tris-HCl at pH 7.4 and 0.5 mM Mg acetate.

RNA Synthesis Assay. This was carried out as described previously (13). Where desired, 20 μg of poly d(A-T), α-amanitin (1 μg/ml), heparin (2, 5, and 10 U.S. Pharmacopeia units/0.125 ml) or t-RNA (250 μg/ml) were added.

Circular Dichroic Measurements. These were carried out as described by Huang and Baserga (13). Corrections for light scattering and absorption flattening are also discussed.

Ethidium Bromide Binding. This was also determined by circular dichroism, as reported (14).

Histone Analysis. Histone proteins were extracted twice with 0.2 M H2SO4, precipitated with 4 volumes of absolute alcohol at −20°C, and then recovered by centrifugation at 27,000 × g for 1 hr. Gels were prepared and analyzed according to the procedure of Panyim and Chalkley (15).

Determination of RNase Activity. Assay of RNase activity was performed by measuring the rate of disappearance of the acid-precipitable HeLa [3H]rRNA, which was added to nucleoli or nucleolar chromatin. Samples were incubated at 37°C for 30 min. For simplicity, the rate of digestion of endogenous RNA in nucleoli was assumed to be the same as that of the added [3H]rRNA.

Analytical Procedures. Protein was determined by the method of Lowry et al. (16) with bovine serum albumin as a standard; DNA by the Burton procedure (17).

FIG. 2. Sedimentation pattern in urea-sucrose of nucleoli labeled with [3H]leucine before (A) and after RNase digestion (B). At the time of plating, HeLa cells were continuously labeled with [3H]leucine (0.5 μCi/ml) and nucleoli prepared as described. The position of DNA was determined as described in Fig. 1.

RESULTS

The procedure we finally adopted for the isolation of nucleolar chromatin rests on the following findings: (i) RNase treatment of the intact nucleolus removes most of the RNA without disturbing the structure of the DNA/protein complex, as can be determined by electron microscopy (3), circular dichroism (13), and ethidium bromide binding (14); and (ii) uracil, while breaking protein-protein hydrophobic bonds has an effect on chromatin which is largely reversible (13, 18). We, therefore, isolated nucleolar chromatin by digestion with RNase, followed by centrifugation in an urea-sucrose gradient.

Isolation of nucleolar chromatin

Fig. 1 shows the sedimentation pattern in urea/sucrose of HeLa cells nucleoli labeled with [3H]uridine before, and after, digestion with RNase. These data confirm previous results that most of the RNA is digested by RNase and only a small amount of label (less than 5%) cosediments with DNA after digestion. Digestion with RNase has no effect on DNA. When nucleoli labeled with [3H]thymidine were centrifuged on urea-sucrose gradients, more than 98% of the radioactivity sedimented with DNA, either before or after RNase digestion (not shown). Fig. 2 shows the sedimentation pattern in urea-sucrose of nucleoli labeled with [3H]leucine after one centrifugation. A small amount of protein sediments at the top even before RNase digestion. After RNase digestion, about 40% of the radioactivity sediments at the top. Because labeling of proteins may be uneven, we decided to determine the protein/DNA ratio by chemical methods and in addition to repeat the urea-sucrose centrifugation. Table 1 shows that, after two urea-sucrose centrifugations, the protein/DNA ratio is 1:8:1, a reasonable
Table 1. Protein/DNA ratio in HeLa cell nucleoli (A) and nucleolar chromatin (B)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
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<tbody>
<tr>
<td>A</td>
<td>7.2 ± 1.3</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td>B₁</td>
<td>3.2 ± 0.3</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>B₂</td>
<td>1.9 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>B₃</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.1</td>
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HeLa cell nucleoli were isolated as described in Materials and Methods. Nucleoli were then treated with RNase, and the digested product was centrifuged over a 10-40% (wt/vol) sucrose/5 M urea gradient (B), as described. The nucleolar chromatin thus obtained was subsequently centrifuged (B₁ and B₂) over the same type of gradient. Untreated nucleoli (A) and nucleolar chromatin (B) were dialyzed overnight against 10 mM Tris-Cl at pH 7.4. Protein and DNA were determined as described under Materials and Methods. Results represent the mean value ±SD.

The samples were prepared and RNA synthesis assayed as described in Materials and Methods. The values are expressed as cpm [³H]UTP incorporated into RNA per μg of DNA, and represent the mean average of two independent experiments. One heparin unit is defined as in the U.S. Pharmacopeia.

of the intact nucleolus, confirming the results of Ferencz and Seifart (21), but has no appreciable effect on nucleolar chromatin.

Structure of nucleolar chromatin

Fig. 4 shows the circular dichroism (CD) spectra (between 250 and 300 nm) of the nucleolus before any treatment, after RNase digestion, and after one centrifugation in urea-sucrose. It has previously been shown that RNA contributes considerably to the molar ellipticity of the nucleolus, and, therefore, that RNase digestion markedly reduces the CD signal (13). Centrifugation of RNase digested nucleoli through urea-sucrose, followed by dialysis, results in a DNA-protein complex whose CD spectra are very close to those of RNase digested nucleoli, and confirms that the effects of urea on chromatin are largely reversible (13, 18).

The same conclusions are suggested by Fig. 5, where the molar ellipticity of DNA-ethidium bromide complexes (14) at various dye/DNA ratios is shown to be essentially the same for RNase digested nucleoli and nucleolar chromatin.

The histone complement of nucleolar chromatin is shown in

![Fig. 3](image-url)  
**Fig. 3.** Endogenous activity of HeLa cell nucleoli and nucleolar chromatin. HeLa cells were grown, and nucleoli and nucleolar chromatin prepared as described. After overnight dialysis, both samples were pelleted and resuspended in assay buffer. The RNA synthesis assay was carried out as detailed in Materials and Methods. The values are expressed as cpm [³H]UTP incorporated into RNA per μg of DNA. (○-○), untreated nucleoli; (●-●), nucleolar chromatin.

![Fig. 4](image-url)  
**Fig. 4.** CD spectra of isolated HeLa nucleoli (---), RNase digested nucleoli (---), nucleolar chromatin (---), and nucleolar chromatin + 1% sodium dodecyl sulfate (●-●). Spectra of nucleoli and RNase treated nucleoli are taken from ref. 13. Spectrum of nucleoli is expressed in concentration of DNA + RNA, whereas the others are in concentration of DNA only.
Fig. 6, where it is compared to the histone complement of intact nucleoli. The same amount of DNA was used for histone extraction, so that the relative amounts can be directly compared. The smaller histones (H2a, H2b, H3, and H4) are present in the same amounts in intact nucleoli and in nucleolar chromatin. However, the amount of H1 is reduced in nucleolar chromatin by about 50%, again confirming that H1 is the histone most easily removable from chromatin (22).

RNase activity

The RNase activity of intact nucleoli and of nucleolar chromatin was determined as described in Materials and Methods. The results showed that only 0.001% of the added RNase A was left in nucleolar chromatin after two centrifugations through urea-sucrose. However, even this small amount was considerably higher than the endogenous RNase activity of the intact nucleolus, that, in our experiments, was calculated at 0.15 μg of RNA digested per mg of DNA/min at 37°C.

DISCUSSION

Functional and structural studies of eukaryotic chromatin are made difficult by its complexity. In an attempt to simplify the problem, Polisky and McCarthy (23) have used simian virus-40 chromatin. Recently, a transcriptionally active chromosome has been isolated by Hallick et al. (24) from the chloroplasts of Euglena gracilis. However, from their paper, it is not possible to determine how much of the chromatin structure has been preserved.

The advantages of the nucleolus as a discrete unit of the mammalian genome have been mentioned earlier. In this manuscript, we report the isolation of a nucleolar chromatin from intact nucleoli that largely preserves the function and structure of the chromatin in the intact nucleolus, after removal of most of the RNA-protein complex that is related to the expression of nucleolar activity but is not an integral part of chromatin as commonly defined (see review in ref. 11). The nucleolar chromatin we have isolated has a protein/DNA ratio of 1.7:1, very little RNA, and 70% of the endogenous template activity of the intact nucleolus. The incubation system we have used for the RNA synthesis assay is the same as the one characterized in detail by Villalobos et al. (25), and we have therefore omitted from this paper the cofactor requirements. While there is no question that the product will have to be identified by more rigorous methods, the fact that the activity is totally resistant to α-amanitin strongly indicates that it is a product of RNA polymerase I (19, 26). CD spectra and ethidium bromide binding capacity are very close to those displayed by the DNA-protein complex of the isolated nucleolus (13, 14), but about 50% of H1 histone is removed by the isolation procedures. It is not clear yet at what stage in the procedure this loss occurs. According to previous reports (18, 27), urea treatment of chromatin removes less than 2% of the histone fraction. Another unresolved problem is the persistence of some RNase, probably due to the fact that RNase binds to DNA (28). It should be noted though that the nucleolus itself contains RNase activity (29), and that neither the endogenous nor the exogenous RNase activities seem to interfere greatly with the transcriptional activity. Even with these unresolved problems, the nucleolar chromatin described in this manuscript seems to be a good preparation for further studies on the structure of the rRNA genes and of the transcriptionally inactive satellite DNA. For instance, because the nucleolus is known to be activated and rRNA synthesis increased in quiescent cells stimulated to proliferate (3, 4, 20, 30, 32), nucleolar chromatin could be used to investigate changes, especially in nucleolar proteins (33), occurring when resting cells begin to grow.

Sonication of nuclei is necessary for the isolation of nucleoli, and sonication is known to cause shearing of chromatin (11). Although we have not yet determined whether or not the intact nucleolus still preserves its subunit structure, it is likely that very little shearing has occurred, because the size of nucleolar DNA is roughly the same as that of unsonicated nuclei (unpublished data). In addition, it has been shown that rDNA genes have the general subunit structure of whole chromatin (34).

In conclusion, we have developed a procedure for the isolation, from intact nucleoli, of a nucleolar DNA-protein complex that has preserved much of the structure and chemical composition of chromatin, and most of the endogenous template activity. This nucleolar chromatin should be useful in the study of chromatin structure and function under different physiological conditions.

This work was supported by USPHS Research Grants CA-12923 and Wistar Contract HD-06323 from the National Institutes of Health.