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# Circular Dichroic Studies of the DNA and RNA of Nucleoli†

Cheng-Hsiung Huang and Renato Baserga*

ABSTRACT: Circular dichroism (CD) in the 240–300-nm region was used to study the conformation of DNA and RNA complexed with proteins in isolated nucleoli from HeLa cells. Deoxyribonuclease or ribonuclease digestion was employed to obtain (1) the individual CD spectra of nucleolar DNA or RNA in complex form with proteins, or in free form; and (2) the experimental CD baseline correction to exclude contributions from nonnucleic acid sources such as light scattering artifacts and proteins. The CD spectrum of nucleolar DNA in DNA–protein complexes was highly reduced in ellipticity in comparison with protein-free DNA. It showed a positive peak at 283 nm with a molar ellipticity $[\theta]_{283} = 1200$ deg cm$^2$ dmol$^{-1}$ and a crossover at 262 nm. Addition of sodium dodecyl sulfate shifted the peak to 276 nm with $[\theta]_{276} = 8000$ deg cm$^2$ dmol$^{-1}$ and a crossover at 254 nm. The CD spectrum of nucleolar RNA in RNA–protein complexes was also reduced in comparison with protein-free RNA, showing a peak at 269 nm ($[\theta]_{269} = 6900$ deg cm$^2$ dmol$^{-1}$), and a crossover at 250 nm. Addition of sodium dodecyl sulfate shifted the peak to 265 nm with $[\theta]_{265} = 18\,000$ deg cm$^2$ dmol$^{-1}$ and a crossover at 246 nm. The low ellipticity of both nucleolar DNA and RNA when complexed with proteins was increased by treatment with sodium chloride, urea, or heparin. This suggests that some ionic, hydrophobic, and hydrogen bondings are involved in the nucleic acid–protein interaction in nucleolar chromatin similar to that observed in nuclear chromatin.

A number of investigators have pointed out that the functional and structural study of eukaryotic chromatin is made difficult by its complexity. In an attempt to simplify the problem, Polisky and McCarthy (1975) have used SV-40 chromatin. We have directed our attention to the nucleolus, which has the following advantages: (1) it is a component of the genome of normal cells; (2) it is isolated easily and in a highly reproducible form, preserving at the same time some of its functional activities (Busch and Smetana, 1970; Muramatsu et al., 1974); (3) it constitutes 3–5% of the total genome (McConkey and Hopkins, 1964; Steele, 1968; Busch and Smetana, 1970; Wilhelm et al., 1972; Schmid and Sekeris, 1975); (4) it has a functional specialization (Amalric and Zalta, 1975), that is, the synthesis of ribosomal RNA, which constitutes about 40% of total nuclear RNA synthesis (Reeder and Roeder, 1972); (5) the main product of the nucleolus,

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rRNA, is easy to identify; and (6) the nucleolus is known to be involved in the growth of cells, especially in the transition of resting ($G_0$) cells to the cycling state (Tsukada and Lieberman, 1964; Zardi and Baserga, 1974; Epifanova et al., 1975; Schmid and Sekeris, 1975; Bailey et al., 1975).

Conformational studies of nucleoli may provide us with information on the structure of nucleolar chromatin and its changes with growth. An attempt at a conformational study of nucleolar chromatin by thermal denaturation was reported by Wilhelm et al. (1972) who found only small differences between nuclear and nucleolar chromatin. In this paper we report the results of our studies using circular dichroism (CD)[1] to investigate the conformation of nucleolar DNA and RNA as they interact with proteins within the nucleoli.

## Materials and Methods

*Isolation of HeLa Cell Nucleoli.* Logarithmically growing HeLa cells in suspension culture were collected by centrifugation at 2500 rpm (1000*g*) for 3 min in a swinging bucket rotor in an RC-3 Sorval centrifuge. All subsequent centrifugations were performed in an International centrifuge at 0–4 °C. Nucleoli were prepared from cells essentially according to the procedure of Muramatsu et al. (1974), with slight modifications. The cell pellet was washed twice with 20 volumes of complete Hanks' solution and collected by centrifugation at 2000 rpm (715*g*) for 3 min. The cell pellet was then suspended in 20 volumes of reticulocyte standard buffer (RSB) which contained 10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4. The suspension was kept at 0 °C for 10 min and then centrifuged at 2000 rpm (715*g*) for 5 min. To the pellet were added 10 volumes of RSB and 0.25% (final concentration) of the detergent Nonidet P40. After mixing, the suspension was homogenized in a tight Dounce homogenizer to break the cells. The complete breakage of cells was monitored with a light microscope after staining with 0.1% toluidine blue. Usually 10–15 up-and-down strokes were sufficient. The suspension was then centrifuged at 2500 rpm (1118*g*) for 10 min to obtain a pellet of crude nuclei. The nuclei were suspended in 10–20 volumes of 0.25 M sucrose solution containing 3.3 mM $CaCl_2$ for 10 min. The suspension was then layered over an equal volume of 0.88 M sucrose solution and centrifuged at 3000 rpm (1610*g*) for 10 min to obtain a pellet of clean nuclei. This last procedure had to be repeated occasionally to obtain satisfactorily clean nuclei.

The clean nuclei were resuspended in 10 volumes of 0.34 M sucrose solution, mixed, and sonicated with a Branson sonifier in a beaker cooled by ice-water. The sonication was performed at a setting of 2 and an output of 65 W at 15–30-s intervals. Each sonication interval was followed by a cooling time of 30–60 s. The completion of nuclear breakage was checked with a light microscope after toluidine blue staining. Usually sonication for a total of 2–3 min was sufficient. The sonicated suspension was layered over an equal volume of 0.88 M sucrose solution and centrifuged at 3000 rpm (1610*g*) for 20 min to collect the nucleoli.

*RNA Synthesis in Isolated Nucleoli.* RNA polymerase activity was measured according to Roeder and Rutter (1970). Nucleoli equivalent to 14.4 μg of DNA were used. The final volume of the reaction mixture was 0.2 ml and contained 2 μCi of [$^3$H]UTP. The reaction was carried out at 37 °C for 10 min. Scintillation counting was performed in a Packard Tri-Carb liquid scintillation spectrometer.

*Circular Dichroic Measurements.* The circular dichroic (CD) spectra were recorded in a JASCO Model J40 recording spectropolarimeter. The instrument was routinely standardized according to Simpson and Sober (1970). The sample was placed in a fused 1-cm cylindrical quartz cell and measured at room temperature. The sample compartment has been modified so that the cell can be placed close (5.5 cm) to the photomultiplier. Usually nucleolar suspensions in 10 mM Tris-HCl, pH 8.0, with an absorbance of 1.0 to 1.8 OD at 260 nm (in 1% sodium dodecyl sulfate) were used. Scanning regions were from 240 to 340 nm. Samples were usually repeatedly scanned 2–4 times to cancel out or minimize random noise. Results are expressed as molar ellipticity, $[\Theta]_{nm}$, in degrees cm$^2$ dmol$^{-1}$ of DNA or RNA nucleotide residues, assuming a mean molecular weight of 330 for both DNA and RNA nucleotide residues.

Since nucleolar preparations formed a particulate suspension similar to those of intact nuclei and chromatin, light scattering artifacts seemed to be unavoidable. As detailed in the Results section, we have used an experimentally obtained baseline (250–320 nm) to minimize the artifact due to differential light scattering between left- and right-handed polarized lights.

The CD spectra in the presence of NaCl, urea, heparin, and sodium dodecyl sulfate were obtained by additions of solid NaCl, solid urea, and high concentrations of heparin and sodium dodecyl sulfate to the appropriate final concentrations. Corrections were made for the changes in volume due to the additions. In experiments on the reversibility of the urea effect, urea was removed by exhaustive dialysis against 10 mM Tris-HCl buffer, pH 8.0.

*RNase Digestion.* Nucleoli in 50 mM Tris-HCl, pH 7.0 (0.5–1.5 OD units of DNA + RNA/ml), were incubated with 1 mg/ml ribonuclease A at 37 °C for 2 h with constant shaking. Before use ribonuclease A was incubated at 85 °C for 15 min to denature contaminating DNase. After digestion, the nucleolar suspension was cooled to 0 °C and centrifuged in an International centrifuge at 3000 rpm (1610*g*) for 30 min. The pellet was washed once or twice with 10 mM Tris-HCl, pH 8.0, until little absorbance at 260 and 280 nm was found in the supernatant. Labeling experiments with [$^3$H]thymidine and [$^3$H]uridine indicated that RNase digestion removed about 93% of RNA and undetectable amounts of DNA from nucleoli.

*DNase Digestion.* Nucleoli suspended in 50 mM Tris-HCl, pH 7.0, and 7.5 mM $MgCl_2$ (0.5–1.0 OD unit of total nucleic acids per ml) were incubated with 0.25 mg/ml DNase I at 0 °C for at least 3 h (Unuma et al., 1968). After incubation, the pellet was collected by centrifugation at 3000 rpm (1610*g*) for 30 min and washed once or twice with 10 mM Tris-HCl, pH 8.0. Labeling experiment with [$^3$H]uridine and [$^3$H]thymidine indicated that DNase digestion removed about 90% of DNA and 14% of RNA from nucleoli.

*RNase plus DNase Digestion.* The nucleolar suspension was first digested with RNase. After washing, the RNase digested nucleoli were subsequently digested with DNase. The digestion conditions for both RNase and DNase were the same as described above.

*Electron Microscopic Studies.* The isolated nucleoli were fixed in 3% glutaraldehyde in phosphate buffer, post-fixed in 1% osmium tetraoxide in *s*-collidine buffer. The fixed nucleoli were then stained in uranyl acetate (Locke et al., 1971), dehydrated with alcohol, and embedded in Epon-Araldite. The sections were stained with lead (Venable and Coggeshall, 1965).

[1] Abbreviations used: CD, circular dichroism; RSB, reticulocyte standard buffer; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; ORD, optical rotatory dispersion.

TABLE I: Incorporation of [$^3$H]UTP into RNA by Isolated Nucleoli of HeLa Cells.[a]

| Additions | [$^3$H]UTP Incorporated (cpm per $\mu$g of DNA per 10 min) | |
|---|---|---|
| | 0.15 M KCl | 0.80 M KCl |
| Control | 92.5 | 24.4 |
| $\alpha$-Amanitin (0.5 $\mu$g/ml) | 91.3 | 24.1 |
| Heparin (0.8 USP unit/ml) | 137.2 | |

[a] Assays were performed as described in Methods and Materials. Nucleoli equivalent to 14.4 $\mu$g of DNA were incubated at 37 °C for 10 min with 2 $\mu$Ci of [$^3$H]UTP in a final volume of 0.2 ml.

*Determination of DNA, RNA, and Protein.* DNA, RNA, and protein were measured according to the procedure of Scott et al. (1956), as modified by Fleck and Munro (1962).

*Materials.* DNase I (DPFF, RNase free) and RNase A were from Worthington, Freehold, N.J. Sucrose and urea (ultra-pure) were from Schwarz/Mann, Orangeburg, N.Y. Nonidet P40 was from Shell Chemical Co. Heparin (Grade A) and nucleotide triphosphates were from Calbiochem. Radioactive [$^3$H]UTP, [$^3$H]uridine, and [$^3$H]thymidine were from New England Nuclear. All other chemicals were of the highest grade available.

## Results

Treatment of isolated nuclei with $CaCl_2$, as in the present procedure, hardens the perinucleolar chromatin which is isolated as an integral part of the nucleolus (Busch and Smetana, 1970). Therefore the isolated nucleoli in our experiments include the perinucleolar chromatin and have a ratio DNA-RNA-protein equal to 1:0.6–0.9:7–12.

*Nucleolar Function.* Table I shows that isolated nucleoli were capable of actively synthesizing RNA, confirming previous results by several investigators (Higashinakagawa et al. 1972; Ferencz and Seifart, 1975; Grummt, 1975). The presence of a polymerase-II-specific inhibitor, $\alpha$-amanitin, at 0.5 $\mu$g/ml, had little effect on RNA synthesis, even when measured in the presence of 0.8 M KCl, a condition shown to suppress polymerase I in favor of polymerase II activity (Reeder and Roeder, 1972). This is indicative of a nucleolar preparation of high purity, largely free from extranucleolar chromatin (Roeder et al., 1970; Chambon, 1975). This conclusion was further confirmed by electron microscopic studies (not shown).

Table I also shows that the naturally occurring polyanion, heparin, even at a low concentration (0.8 USP unit/ml), stimulated the polymerase activity to 148%, in agreement with the observation of Ferencz and Seifart (1975) on rat liver nucleoli.

*CD Spectra of Isolated Nucleoli.* Figure 1 shows the CD spectra of nucleolar preparation in the 240–320-nm range. Curve I is the spectrum of untreated nucleoli, with a positive peak at 270 nm and a crossover at 253–254 nm. A positive tail was found extending into the longer wavelength range above 300 nm where both free DNA and RNA are not supposed to display optical activity (Brahms and Mommaerts, 1964). This tail most likely arose from the differential light scattering between the left- and right-handed polarized lights (Dorman and Maestre, 1973), although the protein component of the nucleoli may also have contributed to it.

Sodium dodecyl sulfate is known to detach almost all proteins from nucleic acids (Steele and Busch, 1967; Kumar and Warner, 1972). Addition of 1% sodium dodecyl sulfate to intact nucleoli (curve II) greatly increased the magnitude of the positive ellipticity without changing the peak position (270 nm). However, the crossover was shifted to 249 nm. Addition of sodium dodecyl sulfate removed much of the opalescent appearance of the nucleolar suspension, but did not significantly alter the magnitude and shape of the positive tail above 300 nm.

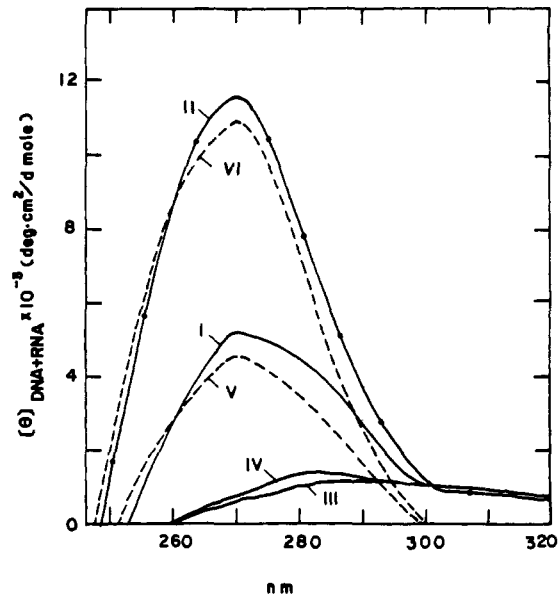


FIGURE 1: CD spectra of isolated nucleoli from HeLa cells in 10 mM Tris-HCl, pH 8.0, before and after digestion with RNase + DNase. For the preparation of digested nucleoli, the nucleoli (0.5–1.5 $A_{260}$ units of DNA + RNA/ml) were first digested with RNase A (1 mg/ml) in a buffer solution containing 50 mM Tris-HCl, pH 7.0 at 37 °C for 2 h. After washing, the nucleoli were subsequently digested with DNase I (0.25 mg/ml) in a buffer solution containing 50 mM Tris-HCl (pH 7.0) and 7.5 mM $MgCl_2$ at 0 °C for 3 h. (I) Untreated nucleoli; (II) untreated nucleoli + 1% sodium dodecyl sulfate; (III) RNase + DNase digested nucleoli; (IV) digested nucleoli + 1% sodium dodecyl sulfate; (V) untreated nucleoli after correction by using curve III as a baseline; (VI) untreated nucleoli + 1% sodium dodecyl sulfate after correction. Details of the correction are discussed in the text.

The CD spectrum of nucleoli, digested with RNase + DNase and expressed in terms of input concentration of DNA + RNA before digestion, is shown in curve III. The tail above 300 nm remained unaltered when compared with curves I and II, indicating an origin other than DNA or RNA in nucleoli. Curve III also showed a plateau between 300 and 280 nm followed by a gradual decrease to give a crossover at about 260 nm. Since little loss of proteins was found after these digestions (not shown), the diminution of the positive CD band between 240 and 300 nm (compare curves I and III) must be attributed to the loss of RNA and DNA. Addition of 1% sodium dodecyl sulfate to the digested nucleoli resulted in the spectrum shown in curve IV, which exhibited a similar type of positive tail and only slightly higher CD in the 250–300-nm range when compared with curve III. This slight difference could be due to the residual amount of DNA left after digestion (about 10% of total DNA).

*Correction for Light Scattering.* For a particulate suspension such as a nucleolar preparation ($A_{310}/A_{260}$ = 0.2–0.3), it is clear that differential CD light scattering artifacts, as evidenced by the appearance of a positive tail above 300 nm, would also contribute a CD spectrum in the region below 300 nm and thus complicate the measurement of the true spectra of DNA and RNA. However, the nucleoli were a morpho-

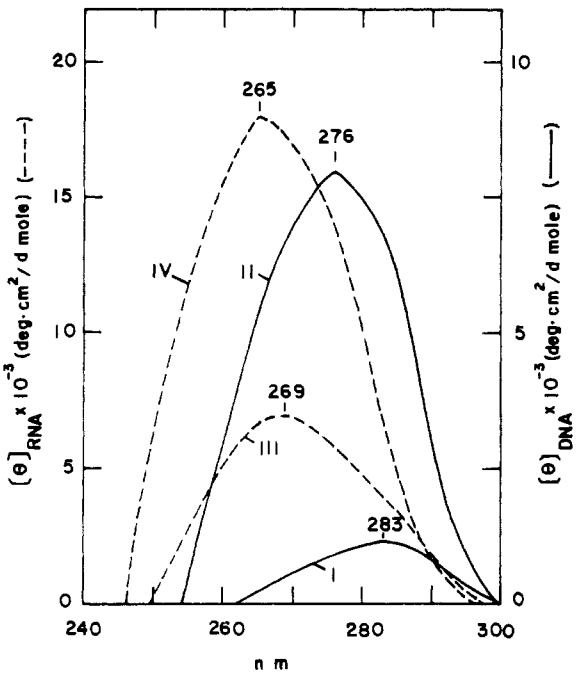


FIGURE 2: CD spectra of DNA and RNA in RNase-digested or DNase-digested nucleoli. Conditions of digestion were as described in Figure 1, except that incubation time for DNase was 3.5 h. (I) DNA in DNA-protein complex in RNase-digested nucleoli; (II) RNase-digested nucleoli + 1% sodium dodecyl sulfate; (III) RNA in RNA-protein complex in DNase-digested nucleoli; (IV) DNase-digested nucleoli + 1% sodium dodecyl sulfate. Notice the different scales for RNA and DNA on the ordinates.

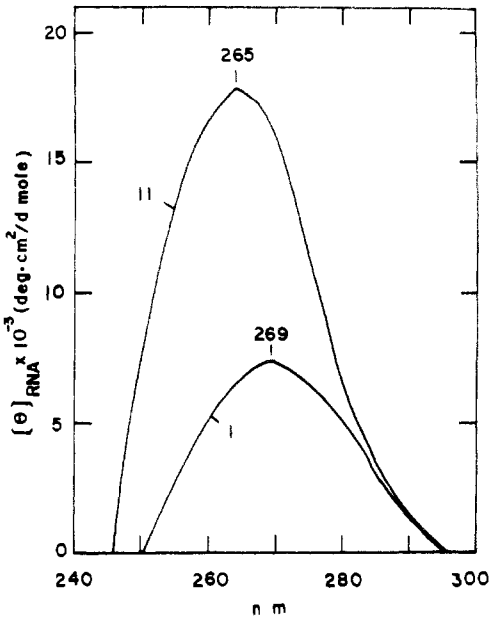


FIGURE 3: CD spectra of RNA in RNA-protein complex in nucleoli obtained from the difference in spectra between untreated and RNase-digested nucleoli. The CD spectra of RNase-digested nucleoli were subtracted from those of untreated nucleoli. (I) RNA in RNA-protein complex; (II) the same as I after treatment with 1% sodium dodecyl sulfates.

logically well-defined entity, even after RNase + DNase digestion, and the various preparations gave highly reproducible CD measurements. The positive tail above 300 nm was persistently observed and was not significantly altered in shape and magnitude by RNase + DNase digestion or by the addition of sodium dodecyl sulfate. Therefore, it seems reasonable to consider curve III as a basis for the baseline correction of the CD spectra of DNA and RNA in the 240–300-nm range to exclude nonnucleic acid contributions, such as scattering artifacts or proteins. All spectra in the 240–300-nm range reported hereafter were therefore corrected using curve III as a baseline. The CD spectra of DNA + RNA in nucleoli resulting from such correction are also shown in Figure 1. The spectrum of untreated nucleoli (curve V) has a positive peak at 269–270 nm and a crossover at 252 nm. The spectrum of sodium dodecyl sulfate treated nucleoli (curve VI) shows a 2.5-fold larger ellipticity with a peak at 270 nm and a crossover at 248 nm. Obviously the decreased ellipticity of nucleic acids in nucleoli arises from the interaction between nucleic acids and proteins.

Both homogenization with a Teflon pestle homogenizer and sonication with a Branson sonifier of the EDTA-washed nucleoli increased the positive CD to some extent (not shown). A 50–100% increase in ellipticity at 270 nm for about 100 up-and-down strokes and a 100–150% increase for 3-min sonication were observed (not shown).

*CD Spectra of DNA and RNA in Nucleoli.* The individual contribution to the CD spectrum from DNA and RNA in isolated nucleoli was resolved by the separate use of RNase and DNase digestions. The approach to the use of these digestions is similar to that employed in electron microscopic studies (see Busch and Smetana, 1970). The spectra in the 240–300-nm range of RNase-treated nucleoli are shown in curve I (no sodium dodecyl sulfate) and curve II (+1% sodium dodecyl sulfate) of Figure 2, both of which are expressed in terms of DNA nucleotide concentration. Curve I has a positive peak at 283 nm with a $[\theta]_{283} = 1150$ deg cm$^2$ dmol$^{-1}$ and a crossover at 262 nm. Addition of sodium dodecyl sulfate (curve II) caused a dramatic increase in ellipticity with a peak at 276 nm ($[\theta]_{276} = 8000$ deg cm$^2$ dmol$^{-1}$) and a crossover at 254 nm. The characteristics of this CD spectrum were similar to those of a typical DNA without RNA (Brahms and Mommaerts, 1964).

If nucleoli were treated with DNase, the resulting CD spectra in 240–300-nm range were different, as shown in curve III (no sodium dodecyl sulfate) and curve IV (plus sodium dodecyl sulfate) of Figure 2. Both curves are expressed in terms of RNA nucleotide concentration. Curve III has a positive peak at 268–269 nm with $[\theta]_{269} = 6900$ deg cm$^2$ dmole$^{-1}$, and a crossover at 250 nm. Upon addition of sodium dodecyl sulfate (curve IV), a 2.6-fold increase in ellipticity was obtained with a peak at 265 nm and a crossover at 246 nm. The features of both spectra are quite different from those of the RNase-treated nucleoli. Curve IV is similar to that of most RNAs (Brahms and Mommaerts, 1964).

The CD spectrum of RNA in nucleoli can also be deduced by subtracting the spectrum of RNase-treated nucleoli from that of untreated nucleoli. Spectra of RNA in nucleoli deduced from RNase digestion are shown in curve I (no sodium dodecyl sulfate) and curve II (plus sodium dodecyl sulfate) of Figure 3. Both curves are virtually indistinguishable from those obtained by DNase digestion (Figure 2). Similarly, the CD spectrum of DNA may be deduced by subtracting the spectrum of DNase-treated nucleoli from that of untreated nucleoli (not shown).

*Effect of NaCl, Heparin, and Urea.* The nature of the decrease in positive CD spectra of DNA and RNA in complex form with proteins in nucleoli, as compared with their respective relaxed form (in the presence of sodium dodecyl sulfate) was further studied by treatment with various reagents

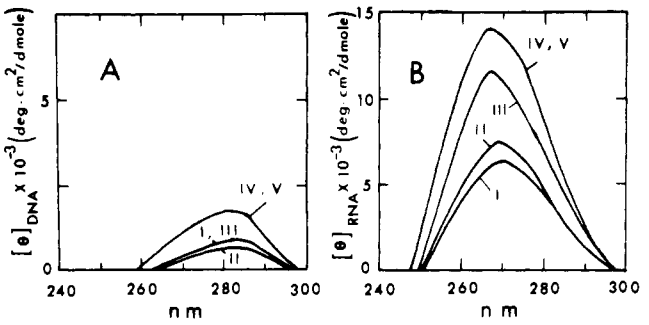


FIGURE 4: Effect of NaCl on CD spectra of nucleoli. CD spectra of nucleolar DNA (A) were obtained using RNase-digested nucleoli. CD spectra of nucleolar RNA (B) were obtained using DNase digested nucleoli. Concentrations of NaCl (M) for both A and B were: (I) 0; (II) 0.25; (III) 0.60; (IV) 1.20; and (V) 3.00.

such as NaCl, heparin, and urea. The effect of NaCl on nucleolar DNA and RNA in complex form with proteins is shown in Figure 4. Figure 4A shows the increase in positive CD spectrum of the DNA in RNase-digested nucleoli upon addition of various amounts of NaCl. At concentrations below 0.6 M, NaCl had little effect on ellipticity but caused a twofold increase at 1.2 or 3.0 M, with little change in peak position. Considering the low ellipticity ($[\theta]_{283}$ = 1750 deg cm$^2$ dmol$^{-1}$) obtained with 1.2 or 3.0 M NaCl, the effect is moderate. Figure 4B shows the effect of NaCl on RNA in DNase-digested nucleoli. Low concentrations of NaCl were effective in increasing the CD; a small increase in $[\theta]_{270}$ from 6300 to 7400 with 0.25 M NaCl and a large increase to $[\theta]_{270}$ = 11 000 with 0.6 M NaCl were obtained. A further increase to $[\theta]_{270}$ = 13 700 could be achieved with 1.2 or 3.0 M NaCl, both of which also caused a peak shift of 3 nm (from 270 to 267 nm).

The polyanion heparin has been shown to increase nucleolar polymerase activity (Table I). Figure 5A shows the increase in CD of DNA in RNase-digested nucleoli upon addition of increasing amounts of heparin. As the concentration of heparin increased, the CD increased accordingly. Heparin at 46.7 USP units/ml produced a $[\theta]_{275}$ = 3860 deg cm$^2$ dmol$^{-1}$ and a shift of the peak from 283 to 278 nm. The spectrum obtained in the presence of 46.7 USP units/ml heparin plus 1% sodium dodecyl sulfate is also shown for comparison. Figure 5B shows the increase by heparin in ellipticity of RNA in nucleoli, as deduced from the spectral difference between the untreated and the RNase-digested nucleoli after additions of heparin. The increase reached a limiting value (39% increase at 265 nm) at 27.6 USP units/ml heparin, with $[\theta]_{265}$ = 10 000 deg cm$^2$ dmole$^{-1}$.

Urea is known to perturb the interactions between proteins and nucleic acids probably by destroying hydrophobic and hydrogen bondings (Bartley and Chalkley, 1973; Simpson, 1973; Carlson et al., 1975). Figure 6A shows the CD increase of DNA in RNase-treated nucleoli caused by urea. The starting ellipticity of the RNase-treated nucleoli in this experiment is in the lower part of the ellipticity range ($[\theta]_{283}$ = 700–1200) normally obtained. Urea at concentrations below 2.7 M (curves 1, 2, and 3) had little effect on CD ($[\theta]_{283}$ = 750), but produced a definite increase at 5.4 M (curve 4, $[\theta]_{283}$ = 1450) and 7.2 M (curve 5, $[\theta]_{283}$ = 2250). The spectrum in the presence of 1% sodium dodecyl sulfate plus 7.2 M urea is also shown in curve 6 ($[\theta]_{277}$ = 6200). It is clear that urea at 7.2 M caused only about a quarter of the increase in ellipticity produced by sodium dodecyl sulfate + 7.2 M urea. The reversibility of the effect of urea was also tested (Bartley and Chalkley, 1973; Chang and Li, 1974). The RNase digested

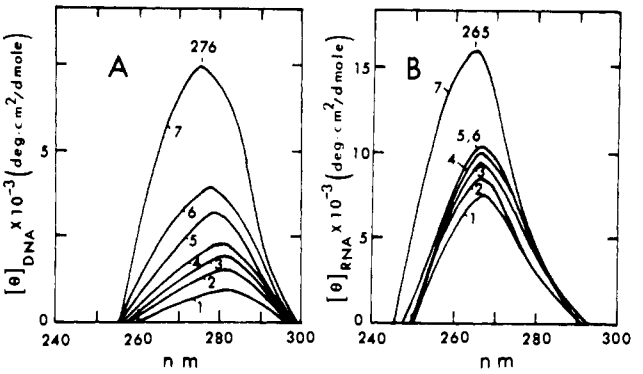


FIGURE 5: Effect of heparin on CD spectra of nucleoli. CD spectra of the nucleolar DNA (A) were obtained using RNase-digested nucleoli. CD spectra of the nucleolar RNA (B) were obtained from the spectral difference of the untreated and RNase-digested nucleoli. Concentrations of heparin (USP units/ml) were as follows: (curve 1) 0; (curve 2) 3.3; (curve 3) 6.7; (curve 4) 13.3; (curve 5) 26.7; (curve 6) 46.7; and (curve 7) 46.7 + 1% sodium dodecyl sulfate.

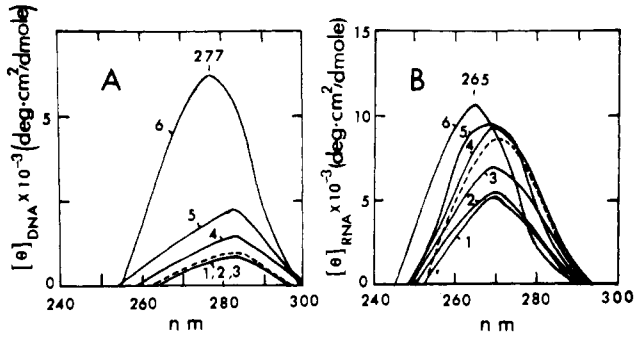


FIGURE 6: Effect of urea on the CD spectra of nucleoli. CD spectra of nucleolar DNA (A) were obtained using RNase-digested nucleoli. CD spectra of nucleolar RNA (B) were obtained from the spectral difference of the untreated and RNase-digested nucleoli. Concentrations of urea (M) in both A and B were as follows: (curve 1) 0; (curve 2) 1.0; (curve 3) 2.7; (curve 4) 5.4; (curve 5) 7.2; and (curve 6) 7.2 + 1% sodium dodecyl sulfate. The broken lines in both A and B were the CD spectra obtained after dialysis to remove 7.2 M urea.

nucleoli were first treated with 7.2 M urea and subsequently subjected to exhaustive dialysis to remove urea. The CD spectrum obtained after dialysis is shown as a broken curve in Figure 6A. This spectrum is very close to that of the untreated sample (curve 1), indicating reversibility of the urea effect.

Figure 6B shows the increase in CD of RNA in nucleoli treated with urea. These spectra were obtained from the spectral difference between the untreated and the RNase-treated nucleoli. In this case, urea at concentrations higher than 1 M produced a considerable effect on CD spectrum. At 265 nm, increases in ellipticity of 38% for 2.7 M ($[\theta]_{265}$ = 6000), 81% for 5.4 M ($[\theta]_{265}$ = 8000) and 114% for 7.2 M ($[\theta]_{265}$ = 9200) urea were obtained. The magnitude of the ellipticity in the presence of 7.2 M urea alone is close to that in the presence of sodium dodecyl sulfate + 7.2 M urea (curve 6, $[\theta]_{265}$ = 10 900). The broken curve shown in Figure 6B is the CD spectrum after exhaustive dialysis to remove 7.2 M urea. Since this spectrum is only slightly lower than the spectrum of the urea-treated sample (curve 5), the effect of urea on the RNA–protein complex seems largely irreversible.

## Discussion

Circular dichroism has been successfully used to study the molecular conformation of nucleic acids in macromolecular

TABLE II: Comparison of the Molar Ellipticity[a] of DNA in Various Macromolecular Assemblies.

| Sources | Preparation | $[\Theta]_{275nm}$[a] DNA |
|---|---|---|
| HeLa | Nucleoli[b] | 500–850 |
| HeLa | Unsonicated chromatin[c] | 2600–3300 |
| | Sonicated chromatin | 4800–5200 |
| WI-38 | Unsonicated chromatin[c] | 2200–2700 |
| | Sonicated chromatin | 4000–5000 |
| | Nuclei[d] | 2200–3400 |
| 2RA | Unsonicated chromatin[c] | 2500–3300 |
| | Sonicated chromatin | 4100–5300 |

[a] The molar ellipticity of DNA is expressed as deg cm$^2$ dmol$^{-1}$ nucleotide. [b] This is the usual range of $[\Theta]_{275}$ obtained in the course of this study. This range corresponds to $[\Theta]$ = 700–1200 deg cm$^2$ dmol$^{-1}$ at the peak position, 283 nm. [c] The unsonicated chromatin is prepared according to the procedure of Marushige and Bonner (1966) as modified by Paul and Gilmour (1968). No sonication was performed. [d] Nuclei were isolated according to the procedure of Goto and Ringertz (1974).

assemblies of biological origin, such as intact nuclei (Wagner and Spelsberg, 1971; Olins and Olins, 1972; Chiu and Baserga, 1975), chromatin (Simpson and Sober, 1970; Shih and Fasman, 1970; Bartley and Chalkley, 1973; Lin et al., 1974; Nicolini and Baserga, 1975; Hjelm and Huang, 1975), ribonucleoprotein (Favre, 1975; Ray et al., 1975), and viruses (Kay et al., 1970; Maestre et al., 1971; Dorman and Maestre, 1973; Homer and Goodman, 1975). The problems involved in using CD to study nucleoli are twofold. Firstly, due to their particulate nature, nucleoli exhibit light scattering artifacts, which include absorption flattening (Duysens, 1956) and differential light scattering between polarized lights (Gordon, 1972; Dorman et al., 1973). Secondly, nucleoli contain, besides DNA, a large amount of RNA which usually has a higher positive molar ellipticity than DNA. Methods have to be devised to resolve their individual contributions.

In the present study we employed an experimentally obtained baseline spectrum (curve III, Figure 1) to exclude contributions from non-nucleic acid sources such as light scattering artifacts and proteins. This baseline spectrum is similar to the difference spectrum attributed to the light scattering of intact $T_2$ bacteriophage obtained by Dorman et al. (1973). Their difference spectrum had a plateau extending from 320 to 270 nm (with a small peak around 290 nm) followed by a sharp leveling off to form a crossover at 265 nm.

To resolve the individual contribution of DNA and RNA to CD spectra we made use of DNase and RNase digestions based on the assumption that these digestions would not significantly alter the conformation of the remaining nucleic acid. Since the spectra of nucleolar RNA obtained by DNase digestion of nucleoli (curves III and VI of Figure 2) are virtually indistinguishable from those (curves I and II of Figure 2) deduced from the spectral difference between the untreated and the RNase digested nucleoli, it seems reasonable to believe that little conformational change in CD spectra of nucleolar DNA or RNA was observed after respective RNase or DNase digestion. From thermal denaturation studies of rat liver and kidney nucleoli, Wilhelm et al. (1972) suggested that the existence of RNA in nucleoli was responsible for the lowering (5–10 °C) of the most stable peak in nucleolar chromatin when compared with extranucleolar chromatin, although the shapes of both profiles differed only slightly. It is not known whether RNase digestion would have any effect on the thermal denaturation profile of nucleolar chromatin.

DNase and RNase digestions have been successfully employed in electron microscopic studies of nucleoli to reveal the in vivo structures of DNA and RNA (see Busch and Smetana, 1970). Under conditions similar to ours but with a lower amount of DNase (50 μg/ml) and a shorter digestion time (2 h), Unuma et al. (1968) found that in thioacetamide-treated rat nucleoli only 12% of the DNA remained after DNase digestion. Under our conditions we found that about 10% of DNA remained after DNase digestion. This is also reflected in the small CD difference between curves III and IV in Figure 1 when sodium dodecyl sulfate was added to the DNase-digested nucleoli. This difference spectrum showed a very small ellipticity with a peak between 275 and 280 nm and a crossover close to 255 nm. Since the molar ellipticity of nucleolar RNA either in the presence or absence of sodium dodecyl sulfate is much higher than that of nucleolar DNA, any significant amount of residual RNA, after RNase + DNase digestion, would dominate the spectrum in curves III or IV, especially in the 260–270-nm range. Labeling experiments with [$^3$H]uridine indicated that about 7% of nucleolar RNA remained after RNase digestion alone.

Nucleolar DNA in DNA–protein complex has a greatly reduced molar ellipticity in comparison to protein-free DNA. In Table II, we have listed the values of $[\Theta]_{275}$ for nucleoli, chromatin, and nuclei from HeLa cells, human fibroblast WI-38 cells, and 2RA (SV-40 virus transformed WI-38) (C. H. Huang and R. Baserga, unpublished data; Lin et al., 1974; Chiu and Baserga, 1975; Nicolini and Baserga, 1975). It is obvious from Table II that nucleolar DNA has a very low molar ellipticity compared with other DNAs of biological origin. This seems to be consistent with the heterochromatic (condensed) nature (Ohno et al., 1957; Mattoccia and Comings, 1971) of nucleoli where less than 1% of the genome is active (McConkey and Hopkins, 1964; Steele, 1968; Quagliarotti et al., 1970), as compared with 5–20% in nuclear chromatin (Paul and Gilmour, 1968).

The reduced positive ellipticity of DNA in nuclear chromatin has been attributed to the DNA–protein interactions (see review by Simpson, 1973). These interactions may also occur in nucleoli to a greater extent. As in the case of nuclear chromatin, the interactions in nucleoli may include at least some ionic interaction, hydrophobic force, and hydrogen bonding, as is evident from the increased ellipticity caused by NaCl and urea. The reason for the higher conformational constraint (lower ellipticity) in nucleolar DNA is not clear. In rat liver and kidney, little qualitative difference in protein composition of nuclear and nucleolar chromatin was found (Wilhelm et al., 1972). In liver, the melting behavior of the isolated DNA from nuclei and nucleoli is similar (Steele, 1968). Nevertheless, the possibility that the large quantity of proteins in nucleoli may play a role in maintaining this constraint cannot be ruled out (Sponar et al., 1970; Smart and Bonner, 1971).

The suppression of the positive CD spectrum of nucleolar RNA, similar to that observed for DNA, is also worth mentioning. Optical rotatory dispersion (ORD) studies on conformation of RNA in ribosomes (Blake and Peacocke, 1965; McPhie and Gratzer, 1966; Sarkar et al., 1967) indicated that, after correction for the protein contribution, the ORD spectra of RNA in ribosomes and in aqueous solutions were the same. Studies on RNA-containing viruses, such as Mongo virus variants (Kay et al., 1970) and icosahedral RNA bacteriophage μ2 (Isenberg et al., 1971), indicated no CD difference

of RNA in virus and in free form after correction of protein contribution. However, differences in conformation of RNA in free form and in protein-complexed form were observed by ORD in tobacco mosaic virus (Bush and Scheraga, 1967), in potato virus X (Homer and Goodman, 1975), and in duck mRNA-protein particle (Favre, 1975). In the present study, the CD spectrum of RNA in nucleoli is clearly different from that measured in the presence of sodium dodecyl sulfate; the latter resembles a typical spectrum of free RNA in solution. The difference cannot be attributed to the protein component since CD of the RNase + DNase treated nucleolar residue (curves III and IV, Figure 1), which retained most of the nucleolar proteins, showed only a small change upon addition of sodium dodecyl sulfate. In fact, this small change is probably due to the residual DNA left after digestions.

The mode of increase in positive ellipticity of DNA in nucleolar DNA-protein complex caused by NaCl is similar to that observed in nuclear chromatin. Sodium chloride at concentrations below 0.6 M caused little increase in ellipticity of nucleolar DNA (this study) and of nuclear chromatin (Simpson and Sober, 1970; Hjelm and Huang, 1974). The largest increase was seen when the concentration of NaCl was increased from 0.6 to 1.2 M, after which a plateau in ellipticity was obtained. The effect of urea in increasing ellipticity of nucleolar DNA and nuclear chromatin is also similar; a large increase was obtained at 4–7 M urea (see Simpson, 1973; Chang and Li, 1974).

The reason for the low ellipticity of nucleolar DNA in the presence of 1.2 or 3.0 M NaCl ($[\Theta]_{283} = 1750$), as compared with $[\Theta]_{283} = 7000$ in the presence of sodium dodecyl sulfate, is not clear. The CD spectrum of nuclear chromatin treated with 2 or 3 M NaCl is close to that of free DNA (Simpson and Sober, 1970; Hjelm and Huang, 1974). The study by Wilhelm et al. (1972) on rat liver nucleolar proteins indicated that a significant amount of nonhistones (43–75%), especially those with high molecular weight, was not extractable with 2 M NaCl–5 M urea, but was extracted with 1% sodium dodecyl sulfate + 1% $\beta$-mercaptoethanol. If this is also the case in HeLa nucleoli, it could be possible that these nonhistones may play a role in the suppression of ellipticity of nucleolar DNA after treatment with high concentrations of NaCl. A similar explanation may possibly be used to explain the low ellipticity of nucleolar DNA after treatment with 7.2 M urea. Compared with the effect on nucleolar DNA, NaCl and urea are more effective in releasing the suppression of the ellipticity of nucleolar RNA. After treatment with 3 M NaCl or 7.2 M urea, the CD spectra were close to those obtained in the presence of sodium dodecyl sulfate.

Several conclusions have emerged from the present study. It is obvious that both nucleolar DNA and RNA, when complexed with proteins, are in highly restrained conformational states, as detected by CD. The high restraint is consistent with the highly heterochromatic (condensed) nature of the nucleoli. The observation of the suppression in CD of nucleolar RNA provides one of the interesting cases in an RNA-containing structure where the packing of RNA into a macromolecular assembly causes a conformational change in the RNA molecule. The conformational restraint of both nucleolar DNA and RNA can be at least partially released by increasing the ionic strength with NaCl, breaking up the hydrophobic and hydrogen bonds with urea or by the addition of a polyanion such as heparin.

The preparation of isolated nucleoli, like that of chromatin, nuclei, or membrane fragments, is a particulate suspension. As has been discussed by previous research workers (Duysens, 1956; Gordon, 1972; Schneider, 1973), these suspensions, with a particle size comparable or larger than the wavelength of the incident light, may exhibit an absorption flattening effect which may distort the CD spectra. The extent of such effect in isolated nucleoli has not been fully evaluated in the present study.

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