Circular dichroism studies of ethidium bromide binding to the isolated nucleolus

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Received 31 March 1976

ABSTRACT

Circular dichroism in the 300-360 nm region and fluorescence induced by intercalating binding of ethidium bromide to both DNA and RNA components were studied in isolated HeLa nucleoli. Both DNA and RNA components contribute to the induced dichroic ellipticity. Digestion of nucleoli by RNase or DNase shows that most of the induced ellipticity comes from the DNA component. In nucleoli with an RNA/DNA = 0.8/1.0 the RNA component gives only 20% of the total ellipticity when measured at an ethidium bromide/DNA = 0.25. Spectrofluorometric titration shows that ethidium bromide intercalates mostly into DNA in nucleoli. Both circular dichroism and fluorescence studies indicate that both DNA and RNA components in isolated nucleoli are less accessible to intercalating binding by ethidium bromide when compared to purified nucleolar DNA, DNA in chromatin or purified ribosomal RNA. Circular dichroic measurements of intercalating binding of ethidium bromide to nucleoli may be used to study changes in nucleoli under different physiological or pathological conditions.

INTRODUCTION

Ethidiumbromide (EB) binds to nucleic acids or chromatin mainly in two ways: (a) a strong binding in which the dye molecule is intercalated between two base pairs of double-helical nucleic acids; and (b) a weak electrostatic binding of EB to the outside phosphate groups of nucleic acids (for review see reference 1). Intercalating binding of EB to either DNA or chromatin can be measured by circular dichroism (CD), since the intercalation induces a positive CD band between 300 and 360 nm, with a peak at 308 nm (2,3). Weakly bound and free dye molecules do not acquire optical activity. We have chosen this technique to study the intercalation binding of EB to the nucleolus isolated from HeLa cells. Selection of the nucleolus was based on the following considerations: (a) it represents a small and well-defined portion of the eukaryotic genome, the amount of DNA in the nucleolus being 3-5% of the total DNA; (b) it can be easily isolated in a highly reproducible form, preserving at the same time some of its functional activities (4,5); (c) it has a functional specialization (6), that is, the synthesis of ribosomal RNA which constitutes 40-50% of the total nuclear RNA synthesis (7,8); (d) the main product of the

nucleolus, ribosomal RNA, is easy to identify; and finally (e) the nucleolus is known to be involved in cell growth, especially in the transition of resting (G_0) cells to the cycling state (9-13).

MATERIALS AND METHODS

Materials

Ethidium bromide and calf thymus DNA (type I) were purchased from Sigma. DNase I (DPFF, RNase free) and RNase A were from Worthington. Nonidet P40 was from Shell Chem. Co. Pronase (B grade, free of nucleases) was from Calbiochem. [^3H]-uridine and [^3H]-thymidine were purchased from New England Nuclear Corp.

Isolation of HeLa Cell Nucleoli

HeLa cells logarithmically grown in suspension culture for 2 days were collected by centrifugation at $1,000 \times g$ for 3 min in a swinging bucket rotor in an RC-3 Sorvall centrifuge. All subsequent centrifugations were performed in an International centrifuge at $0-4^\circ$. The isolation of nucleoli was essentially according to the procedure of Muramatsu *et al.* (5), with slight modifications. The cell pellet was washed twice with 20 vol of complete Hanks' solution and collected by centrifugation at 2,000 rpm ($715 \times g$) for 3 min. The cell pellet was then resuspended in 20 vol of reticulocyte standard buffer (10 mM NaCl, 1.5 mM MgCl_2 and 10 mM Tris-HCl, pH 7.4). The suspension was kept at 0° for 10 min and then centrifuged at 2,000 rpm ($715 \times g$) for 5 min. The cell pellet was resuspended in 10 vol of reticulocyte standard buffer and the detergent Nonidet P40 was added to 0.25% (v/v). 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 and usually 10-15 up and down strokes were sufficient. The suspension was centrifuged at 2,500 rpm ($1,120 \times g$) for 10 min to obtain a pellet of crude nuclei which were then resuspended in 10-20 vol of 0.25 M sucrose solution containing 3.3 mM CaCl_2 for 10-15 min. The suspension was then layered over an equal volume of 0.88 M sucrose solution and centrifuged at 3,000 rpm ($1,600 \times g$) for 10 min to obtain a pellet of clean nuclei. If nuclei are not satisfactorily clean, the last step can be repeated.

The clean nuclei were suspended in 10 vol of 0.34 M sucrose solution, mixed and sonicated with a Branson Sonifier in a beaker or conical glass test tube cooled by ice water. The sonication was performed at a setting of 2 to 3 with an output of 65 to 70 watts, at 15-30 second intervals, each of which was followed by a cooling time of 30-60 seconds. 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 3,000 rpm (1,600 x g) for 20 min to collect the nucleoli.

The nucleoli collected under these conditions have a ratio DNA : RNA : protein = 1 : 0.69-0.9 : 7-12. In this procedure, treatment of isolated nuclei with CaCl_2 hardens the perinucleolar chromatin which is then isolated as an integral part of the nucleoli (4). Less than 1% of the total nucleolar DNA is rDNA (14).

Preparation of Chromatin

The procedures for the culture of WI-38 human diploid fibroblast cells and AFB cells (temperature-sensitive mutant of BHK 21/13 cells), and for the isolation of unsheared chromatin have been previously described in detail (15,16). The same procedures were followed for HeLa cell chromatin except that HeLa cells were disrupted in 20 vol of reticulocyte standard buffer instead of 20 mM EDTA-80 mM NaCl solution. Sonicated chromatin was obtained by sonication of unsheared chromatin (in 10 mM Tris-HCl, pH 8.0) in a Branson Sonifier for 60-90 sec at an output of 65 watts, with an ice-water bath for cooling.

Circular Dichroic Measurements

Circular dichroic spectra were recorded in a JASCO model J40 recording spectropolarimeter. The instrument was routinely calibrated according to Simpson and Sober (17). The sample was placed in a fused 1-cm cylindrical quartz cell and measured at room temperature. Usually nucleolar suspensions in 10 mM Tris-HCl, pH 8.0, with an absorbance (in 1% sodium dodecyl sulfate) of 0.6 to 1.8 O.D. at 260 nm were used. The CD band of nucleic acids was scanned in the 250-300 nm region. The induced CD band of the EB-nucleic acid complex was scanned in the 300-360 nm region (2,3) after increasing amounts of EB were added to a fixed amount of nucleic acids (0.1-0.2 mM total nucleic acid). The scanning was usually repeated more than twice to minimize the random noise. The results are expressed as molar ellipticity, $[\theta]_{nm}$, in degree $\cdot \text{cm}^2/\text{dmole}$ of nucleic acid-phosphate or EB, as described in Figure Legends. The ratio, r , is the concentration ratio between EB and nucleic acid-phosphate. A mean molecular weight of 330 for both DNA and RNA nucleotide-phosphate is used.

Binding Studies by Spectrofluorometric Titration

Fluorescence was measured with a Perkin-Elmer MPF-3L recording spectrofluorometer. Binding studies were performed in 10mM Tris-HCl, pH 8.0 solution at room temperature, using Scatchard plots (18). The excitation wavelength was 540 nm and the emission wavelength was 590 nm. A solution containing 5 μM EB and either purified extra-nucleolar DNA (50 $\mu\text{g/ml}$), sonicated extra-nucleolar

chromatin (85 μ g DNA/ml), nucleoli (90 μ g DNA/ml) or RNase digested nucleoli (90 μ g DNA/ml), all from HeLa cells, was systematically diluted with 5 μ M EB solution.

RNase and DNase Digestions

Procedures for both RNase and DNase digestions have been described (19). Nucleoli in 50 mM Tris-HCl, pH 7.0 or 7.5 ($A_{260} = 1 - 3$), were incubated with 1 mg/ml ribonuclease A at 37° for 2 hours with constant shaking. Ribonuclease A was incubated at 85° for 15 min before use. After digestion, the nucleolar suspension was cooled to 0° and collected by centrifugation at 3,000 rpm (1,600 x g) for 30 min in an International Centrifuge. The nucleoli were washed once with 10 mM Tris-HCl, pH 8.0. Labeling experiments with [3 H]-thymidine and [3 H]-uridine indicated that RNase digestion removed about 95% of RNA and undetectable amounts of DNA from nucleoli.

To remove DNA, isolated nucleoli in 50 mM Tris-HCl, pH 7.0 and 7.5 mM $MgCl_2$ ($A_{260} = 1-2$) were digested with 0.25 mg/ml DNase I at 0° for 3.5 hours. After digestion, nucleoli were recovered and washed once in 10 mM Tris-HCl, pH 8.0, by centrifugation at 3,000 rpm (1,600 x g) for 30 min. Labeling experiments with [3 H]-uridine and [3 H]-thymidine indicated that DNase digestion removed about 90% of DNA and 14% of RNA from nucleoli.

Isolation of DNA from Nucleoli and Extra-nucleolar Chromatin

Pure DNA from nucleoli and from extra-nucleolar chromatin of HeLa cells was isolated according to Marmur (20). The extra-nucleolar chromatin was obtained by centrifugation (SW 50 rotor, 45,000 rpm for 30 min) of the sonicated nuclear suspension left on the top of the 0.88 M sucrose solution after collection of nucleoli. The digestion by RNase and pronase were repeated three times for nucleolar DNA and twice for extra-nucleolar DNA, each time being followed by ethanol precipitation. Before use, RNase was preincubated at 85° for 15 min and pronase at 37° for 1 hr.

Isolation of RNA from Ribosomes

HeLa cell ribosomes were isolated by a previously described procedure (21) but omitting the use of bentonite. Pure ribosomal RNA was obtained by repeated extraction with phenol and chloroform-isoamyl alcohol (24 : 1 by V : V), digestion by DNase I and pronase, and precipitation in ethanol.

Determination of DNA, RNA and Protein

DNA, RNA and proteins in nucleoli were measured by the procedure of Scott *et al.* (22), as modified by Fleck and Munro (23). Pure DNA, RNA and DNA in chromatin were measured spectrophotometrically.

RESULTS**Induced Spectra**

Ethidium bromide (EB), DNA or RNA alone does not show detectable circular dichroic (CD) activity between 300 and 360 nm. The binding of EB to DNA or RNA induced a strong positive CD band in this wavelength range, with a peak at 308 nm and a shoulder around 335 nm (2,3,24). This induced CD band originates mostly from the intercalating binding of EB to the adjacent base pairs of a double-helical nucleic acid (2,3,25). We have observed that the qualitative features of the induced CD band, such as the band shape and the position of the peak and the shoulder, were very similar for EB bound to; (a) various types of purified DNA preparation from calf thymus, HeLa nucleoli and HeLa cell extra-nucleolar chromatin; (b) sonicated or unsonicated chromatin isolated from various cell types such as HeLa, WI-38, AF8 and 2RA; and (c) isolated HeLa and AF8 cell nucleoli and RNase or DNase digested nucleoli from HeLa cells. Nevertheless, on the basis of the same value for r (added EB to nucleotide-phosphate ratio), the magnitude of the CD bands from these preparations is different. Some typical EB-induced CD spectra are shown in Figure 1.

Fig.1: EB-induced CD spectra of HeLa cell nucleoli (.....), RNase digested nucleoli (—), sonicated whole chromatin (----) and DNA from nucleoli (— · — ·) at r (added EB/DNA-phosphate) = 0.27, 0.28, 0.26 and 0.23 respectively. All are expressed in DNA-phosphate concentration.

Effect of DNase or RNase Digestion

Figure 2 shows the molar ellipticity as a function of r (added EB to nucleotide-phosphate ratio), of the CD band at 310 nm ($[\theta]_{310}$) of EB bound to isolated HeLa cell nucleoli, before and after digestion with either DNase or RNase. Both $[\theta]_{310}$ and r are expressed in DNA-phosphate concentration for the untreated and RNase-digested nucleoli, but in RNA-phosphate concentration in DNase-digested

Fig.2. Variation, with r (added EB to nucleotide-phosphate concentration ratio), of the molar ellipticity at 310 nm ($[\theta]_{310}$) of CD spectra of EB complexed with HeLa cell nucleoli before and after RNase or DNase digestion. In this experiment the nucleoli have a DNA/RNA of 1/0.81. Both r and $[\theta]_{310}$ are expressed in DNA-phosphate concentration of intact nucleoli (— Δ —) and RNase digested nucleoli (— $\bullet$ —), but are expressed in RNA-phosphate concentration for DNase digested nucleoli (— $\square$ —) and for RNA complexed with proteins in nucleoli (— $\times$ —). The latter is deduced from the spectral differences between undigested and RNase digested nucleoli.

nucleoli. Both DNA and RNA components should contribute to the ellipticity of EB bound to isolated nucleoli since nucleoli are rich in RNA content (in this preparation, RNA/DNA = 0.81/1.0). However, the molar ellipticity induced by EB binding is only slightly decreased (21% at $r = 0.25$) by RNase digestion.

Digestion with DNase causes a marked decrease in $[\theta]_{310}$ (to 16% of untreated nucleoli at $r = 0.25$) when expressed in DNA-phosphate concentration before digestion. If the remaining ellipticity in the DNase digested nucleoli is expressed in RNA-phosphate concentration, as also shown in Figure 2, the resulting $[\theta]_{310}$ is only 25% when compared to that of RNase digested nucleoli at $r = 0.25$. These results seem to indicate that the RNA-protein complex in the nucleoli contributes only modestly to the induced ellipticity from the intercalating binding of EB.

Digestions with DNase and RNase of HeLa cell nucleoli under these conditions have been shown (see MATERIALS AND METHODS) to remove DNA or RNA almost selectively. The effectiveness of these digestions is further substantiated by the observation (19) that when measured in 1% sodium dodecyl sulfate (to detach proteins), the CD band in 250-300 nm (from nucleotide only) of RNase-digested nucleoli has characteristic features similar to those of purified DNA from HeLa cell nucleoli. Similarly, CD band of DNase digested nucleoli (in the 250-300 nm range) shows features characteristic of that of purified HeLa cell r-RNA. The negative ellipticity in the 200-250 nm region of isolated nucleoli, which mainly reflects the secondary structure of proteins, is not significantly altered by digestion (not shown).

If one assumes that the loss in ellipticity after RNase digestion is due to the removal of the RNA component from nucleoli, as illustrated in Figure 2, one can calculate and plot the $[\theta]_{310}$ as a function of r for this RNA component (broken curve in Figure 2) from the difference in ellipticity between the undigested and the RNase-digested nucleoli. This curve is very close to that of the DNase-digested nucleoli, implying that DNase or RNase digestion of HeLa cell nucleoli does not significantly alter the binding properties of EB to the remaining polynucleotide. Similar agreement has been observed in CD bands in the 250-300 nm region (no dye added) of the undigested and digested HeLa cell nucleoli (19).

Comparison with Chromatin and DNA

Figure 3 shows the variation of $[\theta]_{310}$ with r for calf thymus DNA, purified DNA from HeLa cell nucleoli and from extra-nucleolar chromatin, and purified HeLa cell r-RNA. Both $[\theta]_{310}$ and r are expressed in DNA-phosphate concentration for all DNA preparations, but in RNA-phosphate concentration for r-RNA. It is clear that the induced ellipticity due to EB binding for the two HeLa cell DNA preparations is somewhat lower than that for calf thymus DNA; it is even lower for r-RNA. However, the ellipticity of both purified nucleolar DNA and r-RNA is much higher than that of their respective counterparts in RNase or DNase

digested nucleoli. At $r = 0.25$, purified nucleolar DNA has a $[\theta]_{310}$ of 8,800 $\text{deg} \cdot \text{cm}^2/\text{dmole}$ of DNA-phosphate compared to 2,250 (26%) for RNase digested nucleoli, and purified r-RNA has a $[\theta]_{310}$ of 3,000 $\text{deg} \cdot \text{cm}^2/\text{dmole}$ of RNA-phosphate compared to only about 450 (14%) for DNase digested nucleoli (cf. Figure 2). Clearly, both DNA and RNA in isolated nucleoli exhibit lower induced ellipticity by EB intercalation than in free form in solution; the decrease in induced ellipticity is even more pronounced for RNA than for DNA.

Fig.3: Variation, with r , of $[\theta]_{310}$ of EB complexed with purified DNA and RNA. Both $[\theta]_{310}$ and r are expressed in DNA-phosphate concentration for pure DNA from calf thymus (—○—), pure DNA from HeLa cell extra-nucleolar chromatin (—X—) and pure DNA from HeLa cell nucleoli (—●—), whereas RNA-phosphate concentration is used for purified HeLa Cell ribosomal RNA (—△—).

One possible explanation for the different $[\theta]_{310}$ values for calf thymus DNA and HeLa DNA is the different degrees of contamination by protein. Table I shows the absorbance ratios, $A_{260\text{nm}}/A_{230\text{nm}}$ and $A_{260\text{nm}}/A_{280\text{nm}}$, for the three DNA

Table I

Absorbance ratios between 230 nm, 260 nm and 280 nm for DNA preparations purified from calf thymus, HeLa cell nucleoli and HeLa cell extra-nucleolar chromatin

Purified DNA from:	$\frac{A_{260}}{A_{230}}$	$\frac{A_{260}}{A_{280}}$
Calf Thymus	2.23	1.83
HeLa Cell Nucleoli	2.20	1.81
HeLa Cell Extra-nucleolar Chromatin	2.31	1.86

preparations. Usually, for the same DNA preparation, higher values of these ratios, especially A_{260}/A_{230} , would indicate a higher purity (20). The ratios for purified nucleolar DNA are not significantly different from those of calf thymus DNA, whereas purified DNA from extra-nucleolar chromatin shows even higher ratios. Thus it seems difficult to explain the low $[\theta]_{310}$ for HeLa cell DNA preparations when compared to calf thymus DNA.

Figure 4 compares $[\theta]_{310}$ as a function of r , of HeLa nucleoli with whole chromatin (sonicated and unsonicated) from HeLa, WI-38 and AF8 cells. Both $[\theta]_{310}$ and r are expressed in DNA-phosphate concentration for all preparations, including nucleoli. In chromatin preparations, the RNA contribution to ellipticity at 310 nm is negligible due to the low RNA content (26). The sonicated chromatin preparations show the highest ellipticity, whereas the unsonicated chromatin preparations are close to the isolated nucleoli, especially at r lower than 0.6. The $[\theta]_{310}$ of the unsonicated chromatin preparations levels off rapidly at r higher than 0.3, when compared to that of the sonicated chromatin and nucleoli preparations.

The ratios of $[\theta]_{310}$ at $r = 0.25$, in the order of DNA from extranucleolar chromatin : sonicated chromatin : unsonicated chromatin : RNase digested nucleoli, are 1 : 0.40 : 0.28 : 0.20. For comparison the ratios of $[\theta]_{275}$ (from DNA component alone in the 250-300 nm region) in the same order are 1 : 0.5 - 0.6 : 0.32 - 0.42 : 0.06 - 0.10.

Fig.4: Variation, with r , of $[\theta]_{310}$ of EB complexed with nucleoli, and sonicated and unsonicated chromatin preparations from various types of cells. For all preparations (nucleoli included), both $[\theta]_{310}$ and r are expressed in DNA-phosphate concentration. $[\theta]_{310}$ is the average value taken from several experiments. The number of experiments taken for average are shown in the parentheses following each sample preparation indicated as follows : (---○---) : HeLa cell nucleoli (2); (—●—) : unsonicated WI-38 whole chromatin (5); (---●---) : sonicated WI-38 whole chromatin (3); (—□—) : unsonicated HeLa whole chromatin (2); (---□---) : sonicated HeLa whole chromatin (2); (—△—) : unsonicated AF8 whole chromatin (2); and (---△---) : sonicated AF8 whole chromatin (2).

Figure 5 shows $[\theta]_{310}$, as a function of r , for RNase digested HeLa cell nucleoli, unsonicated chromatin, DNA from nucleoli and DNA from extra-nucleolar chromatin. In Fig.5 $[\theta]_{310}$ is expressed for all preparations in concentration

Fig.5: Variation of $[\theta]_{310}$ (expressed in concentration of added EB) as a function of r for various DNA preparations from HeLa cells. Data were taken from the same experiments shown in Figures 2,3, and 4. (—□—) : purified DNA from extra-nucleolar chromatin ; (—●—) : purified DNA from nucleoli; (—▲—): unsonicated chromatin; and (—○—) : RNase digested nucleoli.

of added EB, not of nucleotide-P. For all samples $[\theta]_{310}$ (per added dye molecule) varies with r (2,3,24). All curves exhibit an increase at low r values and then level off at high r values. The maximal peak occurs at $r = 0.25$ for both DNA preparations from extra-nucleolar chromatin ($[\theta]_{310} = 43,400$) and from nucleoli ($[\theta]_{310} = 35,000$), $r = 0.13 - 0.15$ for unsonicated whole chromatin ($[\theta]_{310} = 15,000$), and $r = 0.08 - 0.15$ for the RNase digested nucleoli

($[\theta]_{310} = 10,200$). Calf thymus DNA and sonicated whole HeLa chromatin (not shown) have a maximum at $r = 0.25$ ($[\theta]_{310} = 60,000$) and 0.15 ($[\theta]_{310} = 24,000$), respectively. It is interesting to note that despite the difference in maximal molar ellipticity, all three DNA preparations reach the maximal cooperativity at $r = 0.25$.

The binding of EB to nucleoli was also studied by spectrofluorometric titration. Results of the Scatchard plots are shown in Figure 6.

Fig.6: Scatchard plots obtained by spectrofluorometric titration for EB binding to purified extra-nucleolar DNA (—▲—), sonicated extra-nucleolar chromatin (—△—), nucleoli (—○—) or RNase digested nucleoli (---●---). All are from HeLa cells and are expressed in DNA-P concentration.

A straight line was obtained for the purified HeLa extra-nucleolar DNA and a biphasic line for the sonicated HeLa extra-nucleolar chromatin; these results are similar to those of calf thymus DNA and chromatin (27,28). The binding isotherms for the HeLa nucleoli and the RNase digested nucleoli were very similar and were largely biphasic. The values for the association constant of the primary (K_1) and the secondary (K_2) binding site and the number of dye bound

per DNA-P (n) obtained from Figure 6 are listed in Table II.

Table II Binding parameters of EB to various DNA complexes obtained by spectrofluorometric titration				
Samples	K_1	n_1	K_2	n_2
DNA	3.9×10^6	0.240		
Sonicated chromatin	7.5×10^6	0.088	1.60×10^6	0.140
Nucleoli	11.3×10^6	0.060	0.77×10^6	0.130
RNase digested nucleoli	10.7×10^6	0.058	0.75×10^6	0.130

The HeLa nucleoli have a higher K_1 and a lower n_1 than HeLa chromatin or DNA. As in the case of calf thymus chromatin (27,28), HeLa chromatin has a higher K_1 and a lower n_1 than DNA. RNase digestion of the HeLa nucleoli causes very little change in binding isotherms and thus in binding parameters such as K and n . Since the isotherm of the untreated nucleoli (RNA/DNA = 0.7 in this preparation) is expressed in DNA-P concentration, the contribution of the RNA component in the nucleoli to the fluorescence seems insignificant. The secondary binding site(s) at higher r values seem to be heterogeneous; the K_2 and n_2 in Table II are approximate values.

Data from binding studies by spectrofluorometry seem to support the conclusion from CD studies that DNA in nucleoli is less accessible to EB intercalation than that in free form or in chromatin, and that in HeLa nucleoli, most of the EB molecules intercalate with DNA rather than RNA.

DISCUSSION

We have studied the intercalating binding of EB to HeLa cell nucleolus by circular dichroism. This technique, pioneered by Dalglish *et al.* (2) for DNA, and by Williams *et al.* (3) for chromatin, is now well established and has been used in a number of laboratories (for review, see reference 1). It is generally accepted that the induced circular dichroic activity is mostly produced by intercalated EB molecules; the contribution from free EB and EB weakly bound to the outside phosphate is negligible (2,3,24,25).

Using this technique in combination with spectrofluorometric titration we have shown that (a) the DNA in HeLa cell nucleolus is less accessible to intercalating binding of EB than protein-free DNA; (b) the RNA in nucleolus is also

less accessible to EB intercalation when compared to purified r-RNA; and (c) most of the intercalating binding of EB to HeLa cell nucleolus is with DNA, nucleolar RNA contributing only a modest fraction. As a corollary one could use intercalating binding of EB to study changes occurring in nucleolar DNA under different physiological or pathological conditions, since changes in the amount of RNA, if not different from RNA already existing in the nucleolus, would have only small influence on $[\theta]_{310}$ of the EB-nucleolus complex.

Changes in intercalating binding of EB to isolated whole chromatin have been described in different stages of maturation of the avian erythrocyte (29), in resting cells stimulated to proliferate (30) and in various phases of the cell cycle in HeLa cells (31). This binding has also been postulated to reflect the transcriptional activity of the genome, both in bacteria (32,33) and in eukaryotes (28,29). Our data have shown that DNA in the nucleolus seems to be less accessible to EB than DNA in whole chromatin, a finding in agreement with the observation that the former has a lower positive circular dichroism than the latter in the 250-300 nm region (19).

The decreased accessibility of RNA in the nucleolus in comparison to r-RNA is also of interest. It is possible that in such a condensed structure like the nucleolus, RNA molecules, which are complexed with protein, may not be as free as purified r-RNA in solution to fold on themselves and form double-helical structures. Alternatively, the RNA molecules may have the same degree of base-pairing but with an altered conformation which does not favor the EB binding. We have observed in the 250-300 nm region a 2.5-fold decrease in positive CD ellipticity and a red shift in peak (from 265 nm to 269-270 nm) for RNA complexed with proteins in HeLa cell nucleoli, when compared to nucleolar RNA measured in the presence of 1% sodium dodecyl sulfate (19). We realize that the proper control for RNA component in nucleoli would have been the purified protein-free nucleolar RNA, but the difficulty of obtaining it in sufficient amounts and the fact that most nucleolar RNA is r-RNA precursor seem to justify our choice without invalidating our conclusion.

Digestions of nucleoli with DNase or RNase have been successfully used in electron microscopic studies of in vivo structures of DNA and RNA (4,34). Digestion with RNase was also used to study the in vivo conformations of DNA and RNA (by CD) in Physarum polycephalum chromatin (35), which contains as much RNA as DNA. In a previous study (19) we have used the DNase and RNase digestions to obtain the individual CD spectra (250-300 nm) of DNA and RNA in HeLa cell nucleoli. The spectra of the nucleolar RNA obtained after DNase digestion is similar to the difference spectra deduced from the spectral difference between

the untreated and the RNase digested nucleoli. Thus, DNase and RNase digestions seem to cause little change in conformation of the remaining RNA and DNA, respectively. A similar situation occurs in EB-induced CD ellipticity. As shown in Figure 2, the variation of $[\theta]_{310}$ with r for the binding of EB to the DNase digested nucleoli is similar to that deduced from the spectral difference at 310 nm between the untreated and the RNase digested nucleoli, indicating a preservation in EB binding capacity of the remaining polynucleotide after DNase or RNase digestion. Finally, the secondary structure of proteins was preserved in the digested residues since the molar ellipticity at 225 nm remains virtually unchanged after RNase or DNase digestion.

The low $[\theta]_{310}$ for both purified HeLa cell nucleolar and extra-nucleolar DNA preparations in comparison to calf thymus DNA is not clear. Because of the slightly lower A_{260}/A_{230} ratio of HeLa cell nucleolar DNA than that of calf thymus DNA, one would think that the difference in $[\theta]_{310}$ is due to more protein contamination in nucleolar DNA. However, it is then difficult to explain the low $[\theta]_{310}$ for extra-nucleolar HeLa DNA which has a higher A_{260}/A_{230} ratio than calf thymus DNA. The slight difference in base composition (43% C+G for calf thymus DNA and 40% for HeLa DNA) is probably not the reason since the magnitude of the induced ellipticity of EB-DNA has been shown to be independent of base composition (2). A difference in base sequence between these DNA preparations as an explanation cannot be ruled out since the magnitude of the induced CD band varied when EB was bound to synthetic polydeoxyribonucleic acids with different base sequences (36).

Due to the size and the particulate nature, CD measurements of the isolated nucleoli, like those of chromatin or nuclei, may be complicated by light scattering artifacts such as differential light scattering and absorption flattening (37,38,39). These artifacts, especially the latter (39) are usually maximal at the peak of optical absorption (below 300 nm for polynucleotides). The EB induced CD spectra were measured in the 300-360 nm region and were found almost independent of the distance between the cuvette and the CD photomultiplier (from 3 cm to 13 cm). Thus, the scattering artifacts would be minimal. The scattering artifacts should have little effect on the fluorometric studies since the excitation (540 nm) and the emission (590 nm) wavelengths used in these studies are far beyond the absorption range of both DNA and RNA. Under this condition, the scattering peaks are usually separated from the intrinsic fluorescence peak (40).

In summary, circular dichroism at 310 nm can be used to study the structure and function of the isolated nucleolus by determining its accessibility to

intercalating binding by ethidium bromide.

ACKNOWLEDGEMENTS

This work was supported by U.S. Public Health Service Research Grants CA-12923 and Wistar Contract HD-06323 from the National Health Institutes.

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