Rat hepatoma cells nucleolar DNA. I. Analysis of nucleolar DNA

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ABSTRACT

The analysis by CsCl density gradient of nucleolar DNA has revealed that the 1.700 g/cm³ main component can be subdivided in three subcomponents with buoyant densities of 1.707 g/cm³, 1.700 g/cm³, 1.690 g/cm³. The 1.707 g/cm³ and 1.690 g/cm³ components contain all the repetitive sequences which comprise 15 % of the total nucleolar DNA. The ribosomal cistrons are found in components having buoyant density between 1.707 g/cm³ and 1.725 g/cm³. Sodium-p-aminosalicylate-DNA interactions have revealed that only the 1.700 g/cm³ fraction has a destabilized secondary structure. The possible localization of these different fractions on peri and intranucleolar fractions is discussed.

INTRODUCTION

The direct approach to the study of the eukaryotic genome organization is made difficult by its complexity: excess DNA of unknown function (1), presence of reiterated sequences (2), numerous transcription products that, in general are not easily characterized. In an attempt to simplify a preliminary approach of this problem, we have tried to isolate a distinct part of the chromatin. The nucleolus has been chosen because of its functional specialization (ribosomal RNA biosynthesis) (3,4) and the possibility of isolating and purifying it in a satisfactory state of structural integrity, as defined by electron microscopy.

Nucleolar DNA comprises intranucleolar DNA that is actively transcribed and the DNA that remains associated at the periphery of purified nucleoli (5,6). We have shown that the amount of nucleolar associated DNA can vary according to the methods used for nucleoli isolation and that, by sonication (7,8,9) in presence of divalent ions at low ionic strength, the structural integrity of nucleolar associated chromatin is maintained.

Few results have been reported concerning the organization of total nucleolar DNA, but a detailed analysis of the part of nucleolar DNA containing ribosomal cistrons (rDNA) has been carried out in *Xenopus laevis*

(8,10). Further, a higher relative amount of repetitious sequence DNA has been found in nucleolar DNA from different cell lines (11-13).

In this work, we have undertaken an analysis of rat hepatoma nucleolar DNA (5) with the aim of establishing the organizational mode of nucleolar DNA in the neighbourhood of ribosomal genes. An analysis of nucleolar DNA extracted from structurally defined chromatin systems is presented in this paper. This demonstrates the heterogeneous nature of these DNA sequences and has enabled the characterization of different fractions (ribosomal cistrons, repetitious sequences, sequences with a destabilized secondary structure). Using different techniques of nucleolar isolation, the interdependence of the presence of different DNA fractions, the type of nucleolar preparation, and the structural integrity of nucleolar chromatin have been established.

These data lead us to propose a model of nucleolar DNA organization that is presented in the following paper.

MATERIAL AND METHODS

a) Zajdela rat hepatoma cells labelled with ^3H thymidine:

approximately 2.10^7 Zajdela rat hepatoma cells were inoculated intraperitoneally into Wistar female rats (200 g). Four days later, the ascites of about 3.10^8 cells was collected (5). The labelling of DNA was performed (12) by intraperitoneal injection of 150 μCi of Thymidine ^3H (25 Ci/mmole, CEA) for various time just before ascites samples take out.

b) Isolation of nucleoli:

Nuclei are isolated according to a previously described technique (14). Purified nucleoli were isolated either at low ionic strength (7) by sonication of nuclei in presence of 3 mM Mg^{2+} , or at high ionic strength by shearing nuclei in the presence of 0.33 M Mg^{2+} (15). Highly purified nucleoli were routinely obtained and electron microscopy showed these were not contaminated by chromatin.

c) Extraction of nucleolar DNA:

Extraction of nucleolar DNA was carried out according to Kirby's procedure (16). About 10^8 nucleoli were resuspended in 1 ml of distilled water, then 1 ml of 2 % SDS, and 0.004 M EDTA was added. After 30 min. at room temperature, 2 ml of phenol-cresol (88 % - 12 % v/v) containing 1 mg/ml of hydroxyquinoline were added and the mixture shaken for another 30 min. After centrifugation the aqueous layer was exhaustively dialysed against a solution of 0.01 M EDTA in 0.02 M Tris-HCl (pH 7), then treated for 30 min.

at 37°C by beef pancreatic ribonuclease (RNase A) (20 µg/ml, preheated 10 min. at 80°C). An equal volume of phenol-cresol was added and the mixture was shaken for 30 min. at room temperature. After centrifugation, the aqueous phase was dialysed for two days against 0.7 mM phosphate (pH 6.9) buffer with several changes. For some experiments the DNA was purified by chromatography on hydroxylapatite (17).

d) Analysis of DNA by sucrose gradient centrifugation: The McGrath and Williams technique of sucrose gradient analysis (18) is used. Nuclei or nucleoli labelled with ³H-thymidine suspensions were layered on a 5ml of sucrose gradient (5% - 20%) at pH 12, and overlain by 0.2ml of a 2% SDS solution. After 10 min., the tubes were centrifuged in a Spinco SW 50 rotor for 60 min. at 20,000 rpm. Fractions of 5 drops were collected and 3 ml of cold 10% TCA added. The precipitate collected and washed on glass fiber filter discs (Whatman GFB) by 20 ml of 5 % TCA and counted in a Packard Tricarb scintillation counter.

e) Analysis of DNA by CsCl gradient centrifugation: To 5.385 g of CsCl, 4.2 ml of a solution containing DNA in 1 mM sodium citrate, 10 mM Tris-HCl (pH 7.4) were added to give a solution with a density of 1.700 g/cm³ (19). After 60 hours at 35,000 rpm in the Spinco 50 rotor, 2 drops fractions were collected from the bottom of the tube and radioactivity measurements were carried out as described above. On some fractions, the density was determined from refractive index measurements (20).

f) RNA-DNA hybridization: Cytoplasmic ribosomal RNA was extracted from ribosomes (14) isolated from cells labelled in vitro for 20 hours with ³²P, and was purified by chromatography on a Sephadex G-100 column.

The ³H thymidine labelled DNA, purified by chromatography on an hydroxylapatite column, was denaturated and fixed on filters as previously described (12). Filters loaded with 25 µg of DNA were incubated 4 days at 40°C, in 1 ml of 1 x SSC containing 8 M urea (21) and saturating RNA amounts (Specific Activity: 10⁵ cpm/µg). The filters were then treated with RNase A (type I Sigma) 20 µg/ml for 60 min. at 20°C, washed extensively and counted.

g) Renaturation of DNA: Sonicated DNA was denaturated by heat (10min. at 100°C) in 0.1 x SSC. After addition of 10 x SSC to obtain a final concentration of 1 x SSC, renaturation is followed at 60°C by measuring the absorbance changes at 260 nm with time, with a specially equipped Cary 14 spectrophotometer. Calculation of DNA complexity was done according to Wetmur theory (22). $S_{20,w}^{ph 13}$ of DNA was determined by analytical ultracentrifugation.

h) Sodium Para-aminosalicylate-DNA interactions: To DNA in 0.7 mM phosphate buffer (pH 6.9), 6% final PAS was added. The mixture was frozen 60

min. at -20°C , according to the technique previously described (23), then after thawing analysed by chromatography on hydroxylapatite column at room temperature. Single and double stranded DNA were further analysed by techniques described above.

RESULTS

1) Nucleolar DNA: We define nucleolar DNA as the part of nuclear DNA that remains associated with purified nucleoli. DNA isolated with the nucleoli varies from 12% to 2% of the total amount of DNA depending on whether the low or high ionic strength techniques respectively, are used, (see Material and Methods). When nucleoli, isolated by the low ionic strength technique are washed with a high ionic strength solution, nucleolar DNA can be fractionated: 18% remains associated with nucleoli and 82% is solubilized.

In the course of the isolation of nucleoli, the structural integrity of nucleolar DNA is preserved to different extents, according to the homogenization techniques used. In fig.1, the sedimentation pattern of nucleolar DNA in alkaline sucrose gradients is shown for the centrifugation of SDS-nucleolar lysates for 60 min. at 48,000 g. More than 50% of the DNA,

Figure 1: Analysis of nuclear and nucleolar DNA on SDS - sucrose gradient. 10^6 nucleoli (1 μg of DNA) or 10^5 nuclei, isolated from cells labelled 60 min. with thymidine ^3H are suspended in 0.1 ml of 0.15 M NaCl and immediately layered on a 5 ml sucrose gradient (5%-20%) at pH 12, according to the McGrath and Williams technique (18). 0.2 ml of 2% SDS solution are added and the samples are centrifuged in a Spinco SW 50 rotor for 60 min. at 20 000 rpm. Fractions are collected and counted as described in Materials and Methods. Δ --- Δ Total nuclear DNA; $\circ$ --- $\circ$ DNA from nucleoli prepared at low ionic strength; $\bullet$ --- $\bullet$ DNA from nucleoli prepared at high ionic strength.

associated with low ionic strength prepared nucleoli is present in relatively fast sedimenting components. On the other hand, DNA that remains associated with nucleoli prepared at high ionic strength is present in a slow-sedimenting component. It is interesting to note that shearing used in this latter case for nuclei disruption, lowers the molecular weight of DNA in solution less than does sonication. In each case, the radioactivity profile of long time labelled nucleolar DNA shows heterogeneous components sedimenting slower than homogeneous unfractionated nuclear DNA.

2) Buoyant density of nucleolar DNA: The heterogeneity of nucleolar DNA sequences has been determined by analysis of purified nucleolar DNA in neutral CsCl gradients. The DNA extracted from low ionic strength prepared nucleoli forms a band at a density of about 1.700 g/cm^3 similar to that of unfractionated nuclear DNA. In some experiments a shoulder appears at $\rho=1.690$. In another experiment the nucleolar DNA was sonicated 30 sec. before analysis in order to produce a DNA of low and more homogenous molecular weight (5×10^5 daltons, as determined by analytical ultracentrifugation). Fig.2 (a,b) shows the density distribution in CsCl gradient of unsonicated

Figure 2 : Analysis of nucleolar DNA by CsCl gradient.

Cells are labelled 30 min. by thymidine ^3H in the ret. The DNA is extracted by the Kirby's procedure (16) from nucleoli prepared at low ionic strength (a,b) or at high ionic strength (c). In figure 2b, the DNA solution is sonicated 30 sec. with a Bronson sonifier. The centrifugation conditions are those described in Materials and Methods. The determination of densities are done by measure of refractive index with a Zeiss refractometer. In figure 2c the pattern of figure 2b (●—●) and of DNA extracted from nucleoli prepared at high ionic strength (▲—▲) are shown.

and sonicated nucleolar DNA. It appears that the sonicated DNA forms three bands at densities of about 1.707g/cm^3 , 1.700g/cm^3 , 1.690g/cm^3 . Further sonication of the DNA does not alter this pattern. Fig2c shows the density distribution in a CsCl gradient of the DNA extracted from nucleoli prepared at high ionic strength. Three fractions banding at densities of 1.707g/cm^3 , 1.700g/cm^3 , 1.690g/cm^3 respectively, are observed, but in relative proportions, differing from those in Fig2b since the relative amount of the fraction 1.707g/cm^3 is higher.

3) Localization of rDNA: As in other eukariotic cells rDNA in hepatoma cells is mainly localized in nucleoli (3,4). Labelled cytoplasmic rRNA hybridizes with DNA from nucleoli prepared at high or low ionic strength (fig.3), but there is a greater proportion of rRNA genes in the DNA

Figure 3 : Hybridization with nucleolar DNA of cytoplasmic rRNA. Various amounts of ribosomal RNA, extracted from isolated cytoplasmic ribosomes of cells labelled "in vitro" 20 hours with ^{32}P and purified by chromatography on a Sephadex G-100 column, are hybridized, according to Kourilsky's procedure (21), with nucleolar DNA (-----) and with nucleolar DNA extracted from nucleoli isolated at a low (—▲—) or at a high (—●—) ionic strength. Conditions of hybridization are described in Materials and Methods. Results are given as percentage of total DNA involved in hybrids.

extracted from the high ionic strength prepared nucleoli. Thus the solubilization of a part of nucleolar DNA by the high ionic strength solution is a relatively non random process, since it results in an increase of certain types of nucleolar DNA sequences remaining associated with nucleoli.

To localize the nucleolar DNA fraction containing the rDNA sonicated nucleolar DNA was fractionated in a CsCl gradient as shown above and the DNA of each fraction annealed with a saturating amount of cytoplasmic rRNA according to Kourilsky procedure (21). The representative profiles of fig.4

Figure 4 : Hybridization with different nucleolar DNA fractions of rRNA.

DNA extracted from nucleoli prepared at a low ionic strength is analysed by a CsCl gradient as described in Figure 2. (Δ — Δ). The fractions are dialysed against 2xSSC, then the DNA is denaturated by heating 10 min. at 100°C in sealed tubes and fast cooling. The hybridization experiments are carried out as described under Figure 3 at a saturating rRNA concentration. The results are given as rDNA percentage in each fraction (amount of DNA in hybride/amount of DNA in the fraction) ($\circ$ — $\circ$).

show that the DNA complementary to rRNA can be subdivided into two distinct classes. The first class consists of the three peaks associated with the 1.690g/cm³, 1.700g/cm³ and 1.707g/cm³ DNA fractions. In the latter two peaks the level of hybridization is near to that obtained with total unfractionated nucleolar DNA, but with the 1.690g/cm³ DNA fraction it is very low. In the second class (density greater than 1.707g/cm³) the hybridization percentage of rRNA is very high, especially with the 1.725 g/cm³ DNA. In this latter case, the amount of DNA is too low to allow the determination of the exact hybridization level. (1.725g/cm³ is the density expected for a DNA sequence coding for 45S rRNA precursor,(23)). It is interesting to note that these results do not agree with those of Steele on rat liver (11). Steele found that rDNA is essentially associated with an 1.707 g/cm³ satellite DNA and in part with the bulk DNA. But in his report he did not specify the molecular weight of the analyzed nucleolar DNA. So rDNA could remain associated with satellite DNA, while in our experiments the DNA is sonicated to such an extent that parts of rDNA are completely released from other nucleolar DNA.

4) Repetitive sequences: Fig. 5 shows that two kinds of repetitious sequences with a moderate degree of reiteration are present in nucleolar DNA, as determined by renaturation experiments (see Material and Methods). At present it is not known if the real complexities of these sequences are $3 \cdot 10^8$ and $2 \cdot 10^9$ nucleotides pairs as determined by this technique or lower. Each sequence represents 7.5% of total nucleolar DNA, the second one being present in nucleolar DNA in a higher relative amount (two fold) than in nuclear DNA.

Figure 5: Renaturation of DNA. Nucleolar (●—●) or nuclear DNA (○---○) purified by two steps of hydroxylapatite chromatography column, were denaturated in spectrophotometer cuvette and the temperature lowered in less than 30 sec. to 60°C. The renaturation was followed by optical density measurements using a Cary 14 spectrophotometer. The concentration of DNA was 15 µg/ml.

5) Nucleolar DNA with a destabilized secondary structure: In a preceding paper (12), we showed that sodium p-aminosalicylate, a deproteinising agent used in the course of DNA extraction, interacts with DNA. This interaction results in the isolation of a part of DNA in a single stranded form. This part is distinct and different from newly replicated DNA. This result can be also obtained by freezing a nucleolar DNA solution below -12°C in the presence of PAS (24). In all the following experiments this latter technique was used, and the single stranded DNA obtained is called "DNA ss". The relative amounts of such destabilized DNA are respectively 15% and 7% for DNA extracted from nucleoli prepared at low and high ionic strength (Fig. 6).

Figure 6 : Analysis by chromatography on hydroxylapatite of nucleolar DNA extracted from nucleoli isolated at low ionic strength (Δ — Δ) or at high ionic strength ($\circ$ — $\circ$). The DNA solution is frozen before analysis at -20°C in presence of PAS (6 %) then is deposited on on 1 ml packed hydroxylapatite column and the DNA is eluted at room temperature by a phosphate gradient (1 mM - 0,3 M). Fractions of 1 ml are collected and the radioactivity determined on each.

a- Buoyant density of "DNA ss": Low ionic strength nucleolar DNA is fractionned after freezing in the presence of PAS, in single and double stranded fractions by chromatography on hydroxylapatite. The two fractions are further analysed on a CsCl gradient. Fig. 7 shows that the single

Figure 7 : Analysis by CsCl gradient of single stranded and double stranded nucleolar DNA fractions.

DNA extracted from nucleoli prepared at low ionic strength is fractionated in single and double stranded forms as described under Figure 6; each fraction is analysed by CsCl gradients as described under Figure 2.

a) Single stranded fraction

b) Double stranded fraction.

stranded fraction gives major band having a buoyant density of 1.714 g/cm^3 and a shoulder with $\rho = 1.704 \text{ g/cm}^3$. The double stranded fraction gives three peaks with buoyant densities of 1.707 g/cm^3 , 1.700 g/cm^3 , 1.690 g/cm^3 . However their relative proportions differ from those found in total non PAS treated nucleolar DNA (Table I summarizes these results). It has been shown that in these experimental conditions, the shift in buoyant density between the single and the double stranded forms of this DNA is 0.014. The results suggest that the "DNA ss" comes from the 1.700 g/cm^3 nucleolar DNA fraction.

TABLE I

Percentage of DNA in different nucleolar DNA fractions

DNA Fraction	Buoyant densities (g/cm^3)		
	1.707	1.700	1.690
Total Nucleolar			
DNA	26.9	41.6	31.5
DNA treated by PAS			
{ DS	32.8	31.6	35.5
{ SS	-	78.	22
DS Theoretical values	31.6	31.3	37
DNA is extracted from nucleoli isolated at a low ionic strength and analysed in a CsCl gradient. In another experiment nucleolar DNA is treated by PAS and separated in single stranded and double stranded fractions before CsCl gradient. The theoretical percentage is calculated after assuming that the origin of the whole single stranded DNA is the 1.700 fraction.			

b- Hybridization of "DNA ss" with rRNA: In Table 2, the results of hybridization between saturating amounts of rRNA and the single and double stranded nucleolar DNA fractions (separated by fractionation on hydroxylapatite) are summarized. Cytoplasmic rRNA hybridizes twice as much with total nucleolar DNA as with the DNA of the single stranded fraction. It is interesting to note

that this difference in hybridization level is greater when destabilized DNA is obtained in a single stranded form by extraction in presence of PAS at a high temperature than by freezing-thawing (12). More controls were carried out to show that treatment of DNA by PAS does not interfere with the hybridization reaction. On the other hand sonication of DNA results in a lower hybridization level (Table 2).

TABLE 2

Hybridization with ribosomal RNA of nucleolar DNA

Nucleolar DNA		Hybridization percentage
Total		0.35 %
Total Sonicated		0.27 %
Treated	By {	
By		DS 0.26 %
PAS		SS 0.17 %

c- Repetitive sequences and "DNA ss": Figure 8 shows that "DNA ss" does not contain repetitious sequences and that the renaturation pattern of the double stranded fraction DNA is similar to the one of total nucleolar DNA. Additional experiments were carried out to show that treatment of DNA

Figure 8 : Renaturation of single and double stranded nucleolar DNA.

Nucleolar DNA extracted from nucleoli prepared at low ionic strength is fractionated as described under Figure 6 and the two fractions are submitted to renaturation experiments as described under Figure 5. o----o Single stranded fraction
●—● Double stranded fraction.

with PAS does not change the renaturation process of repetitious sequences.

Nucleolar DNA is extracted and purified from nucleoli prepared at a low ionic strength. After chromatography on hydroxylapatite the DNA in 0.1 SSC is denatured 10 min. at 90°C, then fixed on filters (Sartorius SM 11 306), 10 μ g per filter; each filter is incubated with a saturating amount of 32 P labelled cytoplasmic rRNA in 0.5 ml of 2xSSC containing 8M urea, for 4 days at 60°C. The filters are then washed with 2xSSC, treated by RNase A (20 μ g/ml), and counted for radioactivity. Total sonicated DNA is nucleolar DNA submitted to ultrasonic oscillations for 30 sec. (Branson sonifier), "treated by PAS nucleolar DNA" is DNA frozen in the presence of PAS, thawed and fractionated into single and double stranded forms by chromatography on a hydroxylapatite column.

DISCUSSION

The present work describes the isolation and the characterization of a part of rat hepatoma cell DNA that is associated with the nucleolar structure. The analysis of such DNA has shown a heterogeneity in its primary sequence that makes possible its fractionation into discrete components. The results give an idea of the linear arrangement of definite nucleolar DNA sequences.

a) Isolation of nucleolar DNA: Nucleolar DNA is the DNA associated with isolated highly purified nucleoli. The purity of preparations and the nucleoli structural integrity are tested by electron microscopy observations (7). The two techniques of nucleoli preparation result into the isolation of two different nucleolar structures, the difference being essentially localized in associated chromatin (7,15), therefore in nucleolar DNA. By the low ionic strength technique (3 mM Mg^{2+}) there is no solubilization of nucleolar chromatin and the contamination by extranucleolar chromatin is very low (none detectable by electron microscopy (7)).

The observation by electron microscopy of intra- and peri-nucleolar chromatin of isolated nucleoli treated by pepsin and RNase (5) gives information about the preservation of these structures during nucleoli isolation procedures. It shows that during the low ionic strength isolation procedure the organization of intra- and peri-nucleolar chromatin is preserved and close to the one observed in-situ. In the high ionic strength technique (0.33 M Mg^{2+}), 85 % of nucleolar chromatin is solubilized but it appears that an equilibrium is reached since the value of DNA amount remaining associated with nucleolus is very reproducible in the course of different experiments. We can conclude from these facts that the fraction

of the DNA that remains associated with nucleolar structure is a definite one and does not result from a random fractionation of nucleolar DNA. Further the use of high ionic strength did not permit the visualization of intranucleolar chromatin and its resultant location is unknown (periphery of nucleoli, or extracted from nucleoli). By extracting DNA from nucleoli prepared by the two different techniques, we obtain information on the localization of DNA fractions in peri and intra nucleolar chromatin.

In relation to these observations we must consider the results obtained in the experiments of nucleolar DNA analysis by sucrose gradients. In nucleoli prepared in low ionic strength close to 50 % of the DNA is protected from sonication to the same extent as intranucleolar chromatin structure, while the 50 % remaining is fractionated to the extent as extranucleolar DNA. This behaviour leads us to suggest that the intra-nucleolar chromatin protected by nucleolar body contained DNA is insensitive to sonication while perinucleolar chromatin contains the other part of nucleolar DNA. On the other hand in high ionic strength prepared nucleoli the disorganization of nucleolar chromatin results in the susceptibility of the whole DNA to homogenization damage. This technique allows two fractions of nuclear DNA to be studied and the preliminary results obtained are in agreement with localization hypothesis.

b) Analysis of nucleolar DNA :

1- Fractionation by CsCl gradient. The results obtained by CsCl gradient analysis of purified nucleolar DNA prove that there is a great heterogeneity in its primary sequences and lend force to the hypothesis of a definite linear arrangement since different satellite DNA's appear only for a definite length of base sequences. Knowing the heterogeneity in molecular weight of nucleolar DNA before sonication (fig. 1), the results suggest that the sequences with buoyant densities of 1.707 g/cm^3 and 1.690 g/cm^3 are essentially localized in high molecular weight DNA of intranucleolar chromatin, while the 1.700 g/cm^3 fraction contains perinucleolar chromatin. The fact that DNA extracted from nucleoli prepared at high ionic strength contain a lower amount of perinucleolar chromatin and also of 1.700 g/cm^3 DNA, agree with this hypothesis.

2- rDNA in nucleolar DNA, If we use for rDNA, the definition given by Birnstiel et al. (10) for *Xenopus laevis* (45 S RNA genes plus spacers) the amount of rDNA in our nucleolar DNA preparations are respectively 1.4 % and 2.5 % from low and high ionic strength preparations. The solubilization of nucleolar DNA by high ionic strength results in a relative enriching of rDNA

but also in a loss of a part of it. In any case rDNA represents in hepatoma cells only a small proportion of nucleolar DNA.

The nucleolar fraction which contains the rDNA has been localized by means of hybridization of CsCl fractionated nucleolar DNA with cytoplasmic rRNA. It is possible that the fractions with buoyant densities of 1.725 g/cm^3 and 1.717 g/cm^3 correspond to fractions of rDNA complementary to 28S and 18S rRNA. The sequences which can be hybridized and have a buoyant density 1.707 g/cm^3 should correspond to fragments of rRNA cistrons associated with AT rich sequences. A more detailed complementary study is being carried out in order to determine the nature of the ribosomal cistrons linear arrangement, which it will then be possible to compare with that established for *Xenopus laevis* (10).

3- Repetitive sequences. The presence of repetitive sequences in the eukariotic cell genome seems to be a general phenomenon (2). These have been demonstrated to be present in the heterochromatin and in particular in the nucleolus (13). In hepatoma cells two families of repetitive sequences are present in the nucleolar DNA in relatively higher amounts than in extranucleolar DNA. Their apparent relatively high complexities are perhaps due to an inadequate analysis technique. Further experiments must be done to clarify this point and to determine their buoyant densities. This first approach has essentially been done to know if these sequences are present or not in DNA which present a destabilized secondary structure.

4- DNA with a destabilized secondary structure. The possible origin of DNA with a destabilized secondary structure has been described in a preceding paper (24). The present results suggest that these DNA sequences are essentially localized in perinucleolar chromatin. The fact that they contain only few ribosomal genes and no repetitive sequences gives new information about the linear arrangement of the different nucleolar DNA fractions.

c) Organization of DNA in nucleolar chromatin. The results lead us to propose in a following paper (25) models of a linear organization of nucleolar DNA and of nucleolar DNA in chromatin which takes into account the existence and properties of two patterns of nucleolar chromatin in-situ. In these models DNA with a destabilized secondary structure results from the packing of DNA in perinucleolar chromatin. The intranucleolar chromatin, containing ribosomal genes presents is composed of a limited number of loops that results from the interaction in superhelical chromatin structure of repetitive sequences. The formation of these structures can be controlled by specific proteins and be the target of regulation of ribosomal gene transcription.

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rDNA : Ribosomal cistrons plus spacers.
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