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Genes
ribosome
trisomy

Quantitation of Human Ribosomal DNA: Hybridization of Human DNA with Ribosomal RNA for Quantitation and Fractionation

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Extract

Quantitation of human genes for ribosomal RNA (rRNA) was possible by molecular DNA/RNA hybridization on nitrocellulose discs. Labeled ribosomal RNA hybridizes with an average of 14×10^{-5} of the total human DNA. If we assume that the diploid cell contains 5.5×10^{12} daltons of DNA, the ribosomal RNA hybridizes with 7.7×10^{8} daltons of ribosomal DNA (rDNA), which is equal to about 320 genes. Hybridization of rRNA with DNA of three different diploid sources shows a rRNA/DNA ratio which varies from $12–15.4 \times 10^{-5}$. The rRNA/DNA ratio of DNA derived from trisomy 21 cells is not significantly different from the normal cells. The genes for rRNA were found to resist melting at temperatures 10° or more above the T_m. This property permits enrichment of these genes by thermal fractionation.

Speculation

Ribosomal RNA genes are probably present on several human chromosomes. The ability to enrich these genes in relation to the whole human DNA indicates that they are clustered.

Introduction

Human genetics has been the study of mutations which lead to phenotypic changes in the individual. Recent studies of DNA from humans reveal that a large portion of this consists of highly repetitious sequences which is probably not transcribed and does not have phenotypic expressions [10, 20, 24]. Some of the DNA which are known to be transcribed are highly redundant and consist of several hundred repeated copies of a single gene [3]. Knowledge of this component of human DNA is gained by quantitative as well as qualitative techniques. These include characterization of the DNA by physical means, reannealing experiments, and molecular hybridization. The quantitation of the human genes for ribosomal RNA is presented here.

The location, organization, and redundancy of the genes for ribosomal RNA have not been established in humans although certain features of ribosomal RNA genes seem to be characteristic of higher organisms. The genes for rRNA are associated with a small portion of the nucleolar organizer chromosomes [3, 6, 23]. Eucaryotic cells contain from 200–1000 exact copies of

the rRNA gene which are arranged tandemly on the nucleolar organizer chromosome [2, 7, 8]. The DNA which codes for rRNA has a higher specific gravity and melting point than the average DNA of a cell, and it is possible to separate it by physical means such as equilibrium centrifugation in CsCl [22] and differential melting [21].

Information pertinent to diploid cells from humans has been derived from heteroploid cells such as the HeLa cells of endometrial carcinoma. The DNA isolated from nucleoli of HeLa cells by McConkey and Hopkins [17] was enriched fivefold for ribosomal DNA. In HeLa cells, which contain approximately 2 times the normal diploid number of chromosomes, there are from 400–1200 DNA sequences to code for rRNA [1, 17]. That is, the ribosomal genes comprise about 0.02% of the total cell DNA. Huberman and Attardi [12] demonstrated by metaphase chromosome fractionation of HeLa cells that the two fractions which were enriched for the acrocentric chromosomes *(groups D* and *G)* were enriched for rDNA.

Molecular hybridization of DNA with ribosomal RNA has several advantages as a model for the quantitation of redundant human genes. Ribosomal RNA can be readily labeled, isolated, and highly purified in sufficient quantities. In eucaryotes, the genes which code for ribosomal RNA are present in several hundred repeated copies. The base composition of genes which code for ribosomal RNA is sufficiently different from the average base composition of the DNA of the cell to permit enrichment of this DNA. There is a large body of knowledge about the genes for rRNA in other cells which permits comparison with genes for rRNA in humans [3].

We chose the disc hybridization technique for the hybridization. This method is advantageous since DNA/DNA hybridization is prevented, although the DNA is present at a high concentration. The low concentration of ribosomal genes in the mammalian cell causes difficulty in achieving accurate quantitation by procedures satisfactory for other organisms. Through the use of nitrocellulose disc hybridization only 30 μg DNA can be fixed to each disc. Since the rDNA comprises only about 0.02% of the total DNA, we cannot expect more than 0.006 μg rRNA to hybridize with each disc. The technique which we used produces rRNA with a specific activity of about 20,000 cpm/μg. The reaction conditions require about 60,000 cpm rRNA to be used in each reaction to achieve saturation of the DNA sites, although only about 120 cpm can be expected to hybridize specifically with the DNA. Thus, low levels of a nonspecific reaction obscure the results, and the specific reaction is near background levels of activity. To obtain a more reliable level of hybridization counts one could either increase the specific activity of the rRNA or enrich the DNA for rDNA.

We have not been able to increase the specific activity of the rRNA, inasmuch as the cells do not tolerate higher levels of radioactivity and decreasing time of labeling decreases purity of the rRNA. High purity is necessary inasmuch as the hybridization conditions permit a low level of contaminating heterogeneous RNA to hybridize with the total DNA very efficiently. Therefore, we chose to increase the level of hybridization by enrichment of DNA for rDNA before hybridization. The level of enrichment employed allowed retention of all rDNA. This rationale had been employed by Brown and Weber [7] for hybridization of *Xenopus laevis* rDNA.

Materials and Methods

DNA Preparation

Spleens surgically obtained were the source of normal diploid DNA, whereas spleens from cadaver were used for the preparation of trisomy 21 DNA. A modification of the procedure by Marmur [16] was used for DNA extraction. Ten g of tissue stored at −60° were homogenized in 20 ml 0.25 M sucrose in 0.05 M Tris, pH 7.5, 0.025 M KCl, 0.005 M $MgCl_2$ (TKM buffer) in a Waring Blendor. The homogenate was filtered through five layers of cheesecloth and centrifuged at 800 × *g* in a refrigerated centrifuge. The resulting nuclei-containing pellet was then suspended in 50 ml lysing solution (2% sodium dodecyl sulfate, 0.1 M EDTA, 0.15 M NaCl, pH 8.0) and incubated at 60° for 10 min. The viscous lysate was then deproteinized twice with an equal volume of chloroform/amyl alcohol (20:1 v/v). After centrifugation for 10 min at 10,000 × *g*, the top aqueous layer was precipitated with an equal volume of ethanol at −15° and redissolved in 0.1× SSC 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0 (SSC buffer). This solution was digested with a mixture of 100 μg/ml α-amylase and 20 μg/ml pancreatic ribonuclease [25] for 45 min at 35°. Sodium dodecyl sulfate was added to the incubation mixture to a concentration of 0.5%, and the DNA was again extracted twice with chloroform/amyl alcohol. The DNA was precipitated with 2 volumes of ethanol and wound out on a glass rod. This precipitate was dissolved in 0.1× SSC. The solution was made 1 M with

respect of NaCl and deproteinized two more times with chloroform/amyl alcohol. The DNA was again precipitated with 2 volumes of ethanol and redissolved in 0.1× SSC at a concentration of 100 μg/ml. The yield at this stage was usually 10 mg DNA for 10 g starting tissue.

DNA was further purified by chromatography on a jacketed 35° methylated-albumin-kieselguhr (MAK) column or a nitrocellulose column. For MAK column chromatography, a 1-cm × 25-cm jacketed 35° MAK column was prepared by the method of Mandell and Hershey [15]. It was equilibrated with 0.2 M NaCl in a 0.05 M phosphate buffer (pH 7.6). Then 2 mg DNA in solution were absorbed to the column. The column was washed with 100 ml 0.4 M NaCl in phosphate buffer and eluted by a linear salt gradient formed with 150 ml 0.6 M NaCl and 150 ml 1.0 M NaCl in phosphate buffer. The DNA eluted in a peak at 0.75 M NaCl. At this point DNA was free of RNA as demonstrated by a negative orcinol reaction and by quantitative absorption to nitrocellulose after melting. For nitrocellulose chromatography, the column was prepared as described by Boezi and Armstrong [5]. A 4-cm × 25-cm packed column retained the protein bound and single stranded DNA. The double stranded, unbound DNA was not retarded by the column. This material did not contain detectable protein by the Lowry reaction.

Preparation of Ribosomal RNA

The preparation of labeled rRNA was a modification of the method of Blobel and Potter [4]. Labeling of rRNA was achieved by growing HeLa cells in Eagle's Minimal Essential Media with 10% fetal calf serum in Blake culture bottles. A 1 to 10 inoculum from a fully grown culture was used. The cultures were grown for 3 days, at which time the cells were approximately two doublings from complete growth. The media was replenished and 0.5 mCi of tritium-labeled uridine (5 Ci/mM) was added to the 100 ml of fresh media. The cells were grown for 36 hr, the media was then replaced with the same media containing 0.5 mM of cold uridine, and the cells were grown for 15 hr longer. This was done to permit decay of the less stable RNA's.

The labeled HeLa cells from two Blake bottles were used for each preparation. The trypsinized and centrifuged cells were suspended in 10 ml 0.01 M Tris-Cl, 0.01 M KCl, 0.0015 M $MgCl_2$, pH 7.4 (RSB solution) and broken gently with 10 strokes of a Dounce homogenizer. The nuclei and cell membranes were removed by centrifugation at 1000 × *g* for 10 min, and the supernatant was treated with 1.3% deoxycholate. Of this supernatant fluid, 5 ml were layered over a step gradient of sucrose consisting of an upper layer of 2.5 ml 1.0 M sucrose in TKM buffer and a 2.5-ml lower layer of 1.8 M sucrose in TKM buffer. The ribosomes were sedimented for 12 hr by centrifugation at 100,000 × *g* in a type 50 fixed angle Spinco rotor [26] at 4°.

The ribosomal pellet was dissolved in 1 ml dissociation media (0.05 M Tris, 0.5 M KCl, 0.002 M $MgCl_2$, pH 7.5) with 1 mM puromycin; then clarified by centrifugation at 10,000 × *g* for 10 min in a Sorvall centrifuge. The ribosome suspension was then incubated at 35° for 1 hr in order to separate the 60 S and 40 S ribosomal subunits and to dissociate the tRNA and mRNA. The dissociated ribosomes were layered over a 5 ml 5–20% linear sucrose gradient in a SW 50 rotor at 40,000 × *g* for 1.5 hr. Fractions of 0.5 ml were collected and counted for radioactivity to locate the dissociated subunits. The RNA was extracted from the subunits by incubating each subunit fraction in 0.5% sodium dodecyl sulfate for 2 min at 37° followed by shaking with an equal volume of 90% phenol. The RNA in the aqueous phase was removed and dialyzed for 4 hr against TKM buffer. The rRNA from each subunit fraction was then layered over a 5–20% linear sucrose gradient in an SW 25.1 Spinco rotor and centrifuged for 15 hr at 60,000 × *g* at 4°. Thirty fractions were collected and the 28 S and 18 S peaks were identified by cold trichloroacetic acid-precipitable radioactivity. The appropriate peaks were then dialyzed twice against 1,000 volumes 2× SSC. The specific activity was usually between 10,000 and 30,000 cpm/μg.

The same procedure was used for preparing unlabeled RNA except that the cytoplasm of 2 g human spleen was used as a source in order to obtain sufficient quantities of ribosomes. The ribosomes were initially pelleted three times.

Thermal Fractionation

DNA was fractionated by two methods to enrich for rDNA: it was fractionated according to specific gravity by CsCl equilibrium centrifugation as described by Brown and Weber [7] and according to differential melting temperature on a nitrocellulose column. For differential melting a column was prepared by the method of Boezi and Armstrong [5]. Fractionation by differential melting is based on the principle that a DNA solution is composed of a heterogeneous population of double stranded DNA fragments. These fragments vary in their base composition and, therefore, in

their melting temperatures. Differential melting was performed by heating a solution of DNA in 0.1× SSC to a predetermined temperature and quickly cooling. The solution was then adjusted to a 3.3× SSC concentration and chromatographed on a nitrocellulose column. The single stranded DNA was retained on the column, whereas the unmelted, double stranded DNA was unretarded.

Specific Gravity Determination

Density of normal and fractionated DNA was determined with ultraviolet optics in a model E centrifuge using the method and calculations outlined by Mandel *et al.* [14]. Standards used were DNA of bacteriophage SP2 and *Micrococcus lysodecticus* [27].

Thermal Denaturation

Thermal denaturation curves were performed with a Beckman waterjacketed cuvette chamber, cuvette thermistor probe [28], and ground glass-stoppered cuvettes.

Molecular Weight Determinations

The molecular weight of the DNA was determined by the method of Burgi and Hershey [9] using a 5–20% linear sucrose gradient and bacteriophage P-22 DNA with a molecular weight of 2.7×10^6 [29] as standard.

DNA Labeling

About 10^6 diploid cells which had been obtained by skin biopsy were labeled with 0.1 mCi thymidine methyl-H^3 (6.7 Ci/mM) [30] in Eagle's Minimal Essential Medium for 48 hr and the DNA was prepared as outlined.

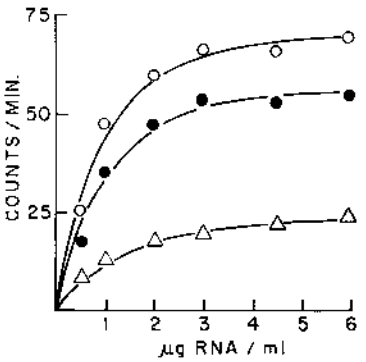

Fig. 1. DNA/RNA hybridization on nitrocellulose discs with increasing concentrations of labeled rRNA. Thirty micrograms DNA were attached to each disc. Background courts of 22 cpm were subtracted. Methods are described in text. ○——○: 28 + 18 S rRNA. ●——●: 28 S rRNA. △——△: 18 S rRNA.

Hybridization

Hybridization was performed by the nitrocellulose disc technique of Gillespie and Spiegelman [11]. This involved immobilizing DNA to nitrocellulose discs [31], incubating with labeled RNA in 2× SSC, eliminating unhybridized RNA by ribonuclease digestion, and counting the formed hybrids with a scintillation counter. Conditions were optimized for the highest counts and lowest background. The temperature of peak hybridization was seen at 65°, and a 12-hr incubation gave the highest counts with the lowest background. These conditions were used in all hybridization reactions.

Incubation was done with 1–2 ml labeled rRNA in a 2× SSC solution in a clean scintillation vial. Between 2 and 10 discs were used; 0.25 ml incubation mixture was used for each disc. Blank discs were used with each incubation to determine the background. The background was usually less than 35 cpm. The total amount of DNA on each disc was determined after scintillation counting by measuring the DNA which was hydrolyzed from the discs by 1.0 N HCl at 100° for 15 min [7]. Additionally tritiated DNA was prepared from cultured human fibroblasts and fixed to nitrocellulose discs. These discs were incubated in hybridization conditions. The amount of DNA detected by this method was in good agreement with the acid hydrolysis technique.

Results

Hybridization

Denatured DNA was hybridized with various concentrations of labeled rRNA. The extent of hybridization of labeled RNA with nitrocellulose fixed DNA was measured at each concentration. As seen in Figure 1, 28 S and 18 S rRNA tended to saturate the DNA when the concentration of RNA in solution was 4 µg/ml and the DNA fixed to each disc was 30 µg. The 28 S RNA plateaued at a lower ratio and more completely than 18 S RNA. At saturation, the RNA to DNA ratio was about 0.14×10^{-3}. The addition of 28 S to 18 S RNA was cumulative (Fig. 1).

Because the total counts were quite low at saturation and it was difficult to attain satisfactory saturation conditions, we explored means of increasing the level of hybridization radioactivity. In a preliminary experiment, DNA was prefractionated with CsCl equilibrium centrifugation according to the method of Brown and Weber [7]. The rRNA hybridized with the heavy

portion of the DNA band as previously shown for *Xenopus laevis* [7]. This indicated that the rDNA molecules were clustered and that they could be enriched. This method had limited application for enrichment inasmuch as only about 100 μg DNA could be processed in each preparative centrifuge tube. We then examined the properties of DNA melting as a means of rDNA enrichment.

Hyperchromicity and Nitrocellulose Absorption with Heating

The hyperchromic effect of heating DNA in 0.1 × SSC took place between 60° and 81° (Fig. 2). The absorbance at 260A° was increased 40% during this melting. The midpoint of the hyperchromic transition (T_m) was 69.5°. Figure 2 summarizes an experiment to determine the effect of heating on nitrocellulose absorption. DNA which had been labeled with tritiated thymidine was heated to the indicated temperatures and cooled. The solution was then adjusted to a salt concentration of 3.3× SSC and at each temperature the percentage of DNA which was absorbed to the nitrocellulose disc was calculated by scintillation counting. In comparing nitrocellulose absorption with hyperchromicity, the hyperchromic effect is nearly complete at 82°, whereas the absorption is not complete until 90°.

Hyperchromicity of the DNA solution as measured by ultraviolet absorption is an indication of the degree of dissociation of the complementary base pairs of the solution in general and does not reflect the degree of separation of any given double stranded fragment. The nitrocellulose filter assay was a means of detecting complete strand separation. If heating completely separated the two strands of a DNA molecule, no reannealing between the two strands could occur when they were quickly cooled, and they would absorb to the nitrocellulose discs. However, if the strands were incompletely separated by heating, some of the complementary strands could reanneal with cooling, return to their double stranded state, and not be reabsorbed to the nitrocellulose. We expected that DNA strands which melt completely at 75° would have a lower average guanosine + cytosine (G + C) content than the material which can reanneal and flow through the filter. This provides a rationale for the enrichment of strands with high G + C content.

Specific Gravity

We compared the specific gravity of whole DNA with thermally fractionated DNA, inasmuch as the specific gravity of DNA is an independent means of estimating G + C content. Analytical ultracentrifugation revealed that human DNA had a specific gravity of 1.699 and DNA fractionated on a nitrocellulose column at 82° had a broad band with a peak of about 1.720 (Fig. 3). This DNA was double stranded since it did not absorb to a nitrocellulose disc but it could be melted at 100° and retained on a nitrocellulose disc. We concluded that the DNA which was not absorbed to nitrocellulose after heating did have a higher G + C content.

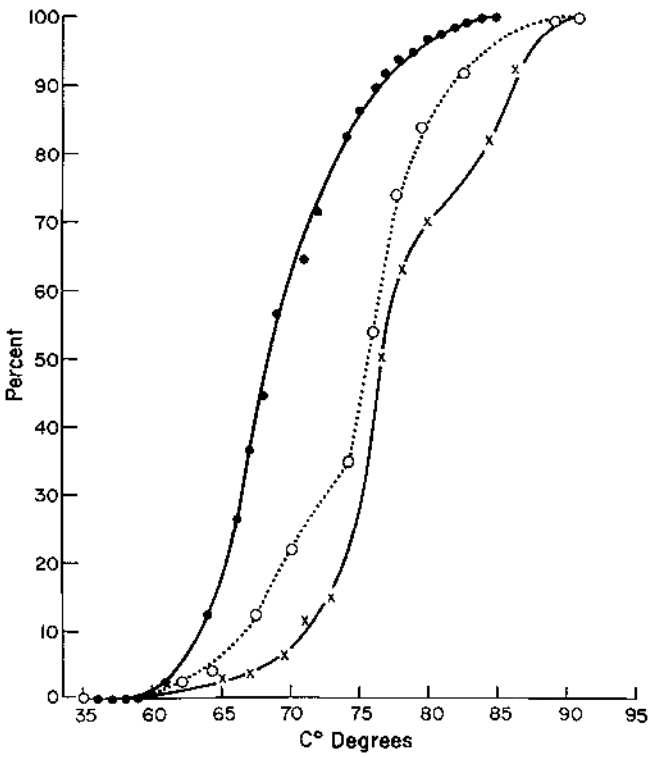

Fig. 2. Melting curve and nitrocellulose absorption of DNA in 0.1× SSC. ●——●: Ultraviolet absorption at 260A° as percentage of absorption at 82°. Fifteen milliliters DNA (10 μg/ml, 350 cpm/μg) were heated to the indicated temperatures in 0.1× SSC. One-milliliter samples were removed and cooled quickly in 1 ml 6.6× SSC. The percentage radioactivity which was absorbed after nitrocellulose disc filtration was measured by scintillation counting. ○····○ DNA of 10^6 molecular weight. ×——× DNA of 10^7 molecular weight.

Hybridization with Thermally Fractionated DNA

This experiment was based on the assumption that rDNA would have a higher melting temperature than average DNA. The G + C content of rDNA is about 60% as compared with 40% for the total DNA.

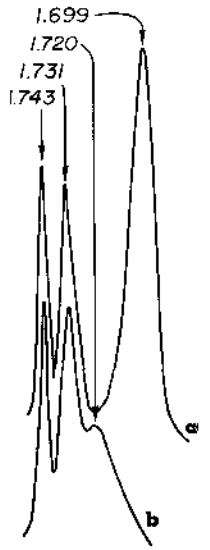

Fig. 3. Analytical ultracentrifuge analysis of human DNA. The above figure represents the densitometer tracings of an ultraviolet photograph of the analytical cell. *a:* Unfractionated DNA. *b:* DNA fractionated at 82° as described in *Materials and Methods.* The marker DNA's were from *Micrococcus lysodecticus* (specific gravity 1.731) and bacteriophage SP2 (specific gravity 1.743).

Table I shows a comparison of the hybridization of rRNA with DNA fractionated at various temperatures. When rDNA was enriched 7-fold–10-fold, there was no loss of rDNA. It was also noted that DNA which melted at 70° did not contain any detectable rDNA. The rDNA could be enriched almost 100-fold by melting at 80°, but with this degree of fractionation there was loss of some of the rDNA.

Approximately 4 mg purified DNA were heated to 74° in 0.1× SSC and passed over a nitrocellulose column in 3.3× SSC. The unabsorbed DNA was then dialyzed against 0.1× SSC and melted in a boiling water bath. The melted DNA was fixed to individual nitrocellulose discs in 0.75 OD unit fractions. The discs were then hybridized with increasing levels of rRNA in a 2 × SSC solution at 65° for 12 hr. The results of one experiment are shown in Figure 4. This shows that thermally fractionated DNA hybridizes with rRNA at levels which are well above background levels.

Using DNA from various spleens obtained surgically and using the thermally fractionated DNA, we compared the quantitation of rDNA. Three spleens were obtained from diploid persons whose spleens were removed because of thalassemia major (*subject 1*) spherocytosis (*subject 2*), and traumatic rupture (*subject 3*). Two spleens were removed from patients with trisomy 21 who died during surgery. Table II gives the results of these hybridizations. The degree of hybridization was determined with saturating levels of rRNA. The degree of fractionation of each sample was determined by measuring the optical density of the DNA before and after nitrocellulose chromatography. The degree of hybridization for normal diploid individuals and

Table I. Fractionation of DNA with subsequent hybridization of that DNA to rRNA[1]

Amount of DNA, μg	Fractionation temperature, °C	DNA remaining double stranded, μg	rRNA hybridized, cpm
30	25	30	80
30	80	3	78
320	80	30	705
320	84	5	500

[1] The starting material was heated to the indicated temperature and then passed through a 0.5- × 5-cm nitrocellulose column. The unabsorbed material was then prepared for disc hybridization as described in *Materials and Methods.*

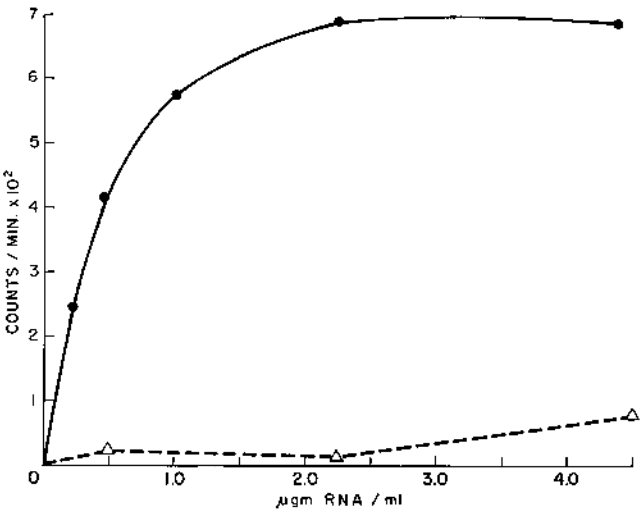

Fig. 4. Hybridization of rRNA with DNA which had been thermally fractionated. One hundred optical density units at 260A° of DNA were fractionated as described in the text. The unabsorbed DNA (17.5 OD) was then used for hybridization with increasing levels of rRNA. The hybridization was performed as described in *Materials and Methods.* Discs with fixed DNA (●——●) were compared with discs without fixed DNA △- - -△). The specific activity of the rRNA was 26,000 cpm/μg. Since the discs were saturated with 695 cpm the amount of rRNA hybridized was 695/26,000 or 26.8 ng rRNA. The DNA on the disc was calculated to be 30.4 μg by acid hydrolysis of the disc and measurement of ultraviolet absorption (7). We concluded that 26.8 × 10^{-3}/30.4 of the DNA was hybridized with DNA to give a rRNA/DNA ratio of 0.88 × 10^{-3}. In as much as 100/17.5 or 5.7 times as much DNA was present prior to fractionation we concluded that the rRNA hybridized with 0.88 × 10^{-3}/5.7 or 15.4 × 10^{-5} of the total DNA.

Table II. Comparison of hybridization of rRNA with fractionated DNA from various sources[1]

Source of DNA	I rRNA hybridized, mμg	II DNA per disc, μg	III Avg. RNA/DNA × 10^3	IV Fractionation of DNA	V rRNA/total DNA × 10^6
Normal spleen					
1	24.2±1.8	29.5±1.6	0.82	6.8	12.0
2	27.5±1.5	29.8±1.4	0.92	6.4	14.4
3	26.8±2.1	29.6±2.0	0.88	5.7	15.4
Mongol spleen					
1	28.2±1.1	28.6±1.6	0.96	5.9	16.2
2	27.3±1.3	29.4±0.8	0.95	6.2	15.3

[1] Calculations for columns I, II, and IV are described in Figure 4. Column III represents the ratio of column I to column II. Column IV represents the ratio of DNA which was absorbed to that which was not absorbed to a nitrocellulose column. Column V represents column III divided by the fractionation ratio (IV).

persons trisomic for a nucleolar chromosome is quite similar.

Discussion

The ribosomal RNA gene is a highly redundant gene in the human. If the DNA content of a single diploid cell is 9.2 pg or 5.5×10^{12} daltons and from our data we take 14×10^{-5} as the portion of human DNA which hybridizes with rRNA, then 7.7×10^8 daltons of rRNA hybridize with the DNA of a single cell. Since rRNA has a molecular weight of 2.4×10^6 [13], we conclude that at least 320 molecules of rRNA are complementary to the DNA of a single cell.

The ability to fractionate human rDNA by CsCl and partial melting indicate that it is clustered with other DNA of high G + C content. DNA is isolated in a size which is equal to about three transcriptional units of 4.4×10^6 daltons each. If the high rDNA sequences were intermixed randomly with genes of average G + C content, the fractionation would not be possible. The fractionation step does not purify the ribosomal DNA but merely concentrates it to a level (1×10^{-4}) which permits quantitation above background. The hybridization technique does provide an assay for the successive steps of purification.

It is of considerable interest to find that cells trisomic for a nucleolar organizer chromosome do not have a significantly increased amount of ribosomal DNA. Similar chromosomal changes in the *Xenopus laevis* [6] or *Drosophila melanogaster* had dramatic effects [19]. The factor which is probably responsible is that the nucleolar organizers are probably located on five pairs of chromosomes instead of a single pair as the other species [18]. However, if homologous stretches of rDNA are present on several human chromosomes, the opportunity for crossing over between nonhomologous chromosomes is present.

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29. Bacteriophage P-22 DNA was a gift of Myron Levine.
30. New England Nuclear Corp., Boston, Mass.
31. Schleicher & Schuell, Keene, N. H.
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