Determination of the Size of Rat Ribosomal Deoxyribonucleic Acid Repeating Units by Electron Microscopy†

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ABSTRACT: The employment of a novel method of affinity chromatography, which makes use of antibodies that specifically bind DNA/RNA hybrids, has made it possible to enrich for rat rDNA molecules which contain R loops formed with the 18S and 28S rRNAs. An approximately 150-fold enrichment of the rat rRNA coding sequences was obtained by this affinity chromatography procedure. This degree of enrichment made it possible to visualize these R loop containing molecules in the electron microscope and, thus, to obtain a map of the transcribed and spacer regions of rat rDNA. Eleven of the molecules that were observed contained either 3 or 4 R loops, or else 2 R loops separated by a long spacer. Thus, these molecules provided direct information in regard to the length of rat rDNA repeating units. The mean length of the repeating units was 37.2 kbp with a standard deviation of 1.3 kbp. Within the errors of the measurements, these could all represent repeating units of exactly the same length, although a certain degree of length heterogeneity, possibly up to 4 or 5 kbp, cannot be ruled out by the data. If significantly longer or shorter rDNA repeating units exist in the rat genome, they are probably much less common than the 37.2-kbp unit. These electron microscopic measurements provide the most definitive data yet available on the size of the repeating units of mammalian rRNA genes.

The study of the organization of the rRNA genes (rDNA) in eucaryotic organisms has been the subject of numerous investigations. Two general patterns of organization have emerged. In one, characteristic of lower eucaryotes such as *Tetrahymena*, *Dictyostelium*, *Acetabularia*, and *Physarum*, the many copies of the rRNA genes are inversely repeated around a central axis of symmetry (Berger et al., 1978) which also exist as dimers on extrachromosomal DNA fragments (Karrer & Gall, 1976; Taylor et al., 1977; Vogt & Braun, 1976; Stumph et al., 1978). In the second pattern of organization which is characteristic of all the higher eucaryotes studied, the multiple gene copies are linked together in tandem arrays in which each copy has the same orientation as its neighbors. The most extensively studied system exhibiting this organization is that of the amphibian *Xenopus laevis*. In this animal, the tandem repeating units exhibit a heterogeneity of lengths ranging from 10.5 to 17 kbp in length (Wellauer et al., 1976). Another tandemly repeated system which has been extensively characterized is that of *Drosophila melanogaster*. In this insect, the rDNA repeating units fall mainly into two size classes centered around 11.5 and 17 kbp which are characterized by the absence or presence of an intervening sequence which interrupts the 28S rRNA coding sequence (Glover & Hogness, 1977; Wellauer & Dawid, 1977; Pellegrini et al., 1977; Wellauer et al., 1978). Intermediate size repeats were also found which result primarily from differences in the lengths of the intervening sequences; however, a minor degree of length heterogeneity was also observed in the nontranscribed spacer regions as well.

The data which are available on mammalian rDNA organization are much more limited. Most of the information comes from blots (Southern, 1975). Arnheim & Southern (1977) reported repeating units of about 38 and 31 kbp for mouse and human rDNA, respectively. Cory & Adams (1977) assigned a similar (44 kbp) minimum length to the repeating unit of the mouse. Blin et al. (1976), employing related methods on rDNA enriched from calf thymus DNA, found that the bovine rRNA genes exist in repeating units of approximately 33 kbp. However, in the absence of additional evidence, such determinations should be regarded as setting a minimum length on the size of the repeating units since fragments in the nontranscribed spacer region may escape detection. Such studies have also revealed a heterogeneity among different repeats (Arnheim & Southern, 1977; Cory & Adams, 1977). This heterogeneity could be due either to differences in the lengths of the spacers or to heterogeneity in their sequences which could result in the presence or absence of certain restriction sites. Some evidence on the length of mammalian rDNA repeating units has also been obtained from the direct observation of rDNA transcription units after gentle cellular lysis and centrifugation of the cellular contents onto grids for electron microscopy. In spreads from HeLa cells (Miller & Bakken, 1972; Hamkalo & Miller, 1973), transcription units were observed which were separated from others by a distance approximately as large as the unit of transcription. If the assumption is made that the transcribed DNA and the spacer DNA are extended to the same degree, this would suggest a human rDNA repeating unit of around 30 kbp since the 45S rRNA precursor is about 14–15 kbp long.

The studies which are described in this report were undertaken to more definitively examine the lengths of rDNA repeating units in a mammal, in this case, the rat. Another purpose was to assess the extent of length heterogeneity in the repeating unit. In this study, molecules of rat rDNA which contained R loops (White & Hogness, 1977; Thomas et al., 1976) were observed in the electron microscope in order to obtain direct information in regard to the structure and organization of rat rDNA. This was only made possible by the greater than 100-fold enrichment of these molecules from whole genomic rat DNA by using a novel method of gene enrichment (Stumph et al., 1978). This procedure involved the R-looping of whole genomic rat DNA with the rRNAs followed by chromatography of the nucleic acids on an affinity column of antibodies which specifically bind DNA/RNA hybrids. This permitted the selection for DNA molecules containing rRNA R loops. These molecules could then be observed and studied in the electron microscope.

Experimental Procedures

Antibody and Affinity Column Preparation. Antiserum against DNA/RNA hybrids was prepared as described in

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Stumph et al. (1978). The IgG fraction was purified and passed over columns of DNA–Sepharose, oligo(dT)-cellulose, and poly(rA)–Sepharose. Antibodies which cross-react with nucleic acids other than DNA/RNA hybrids bind to and are left behind on the nucleic acid columns, whereas the bulk of the IgG and the antibodies specific to hybrids pass through the column without binding. The antibodies which react specifically with DNA/RNA hybrids were then purified from the bulk of the nonreacting IgG by affinity chromatography on a column of oligo(dT)-cellulose to which poly(rA) had been hybridized. The column, containing 10 g of type T-2 oligo(dT)-cellulose from Collaborative Research, was equilibrated with 0.5 M NaCl, 0.01 M sodium phosphate, pH 7.5 at 5 °C. Then 30 mg of poly(rA) was applied to the column in the same buffer. The column was next washed with 0.5 × PBS to elute loosely bound poly(rA), and then equilibrated with 0.8 × PBS (PBS is 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5.) Next, 2500 A_{280} units of IgG (containing DNA/RNA hybrid specific antibody) was loaded onto the column in 0.8 × PBS. Washing with the same buffer was continued until the A_{280} of the effluent was below 0.05. Then, bound antibody was eluted from the poly(rA)·oligo(dT)-cellulose column with 3 M NaSCN in PBS. The purified antibody (yield: 10 A_{280} units) was then dialyzed against PBS.

The specific activity of the purified antibody was compared with the IgG, from which it was purified, by means of glass-fiber filter assays (Lewis et al., 1973) exactly as described in Stumph et al. (1978). Briefly, 0.2-μg aliquots of [^{3}H]-poly(rA)·poly(dT) were incubated with various amounts of IgG or purified antibody for 45 min at 37 °C in 1.0 mL of PBS to allow the formation of antibody–antigen complexes. The mixtures were then poured over Whatman GF/F filters which were rinsed, dried, and counted.

The affinity resin was prepared by reacting 1.0 A_{280} unit of purified antibody with 0.5 g of CNBr-activated Sepharose (Pharmacia, lot 9429) and washed exactly as described in Stumph et al. (1978). The binding of the antibody to the resin was essentially quantitative. A one-third aliquot of this affinity resin was used for each of the affinity chromatography runs described below. In order to carry out the affinity chromatography experiments, the aliquot of resin was placed in a 5-mm diameter column and thoroughly washed with: 1 M NaCl, 0.01 M sodium phosphate (pH 7.5); 5 M NaSCN in PBS; and PBS.

DNA and RNA. Rat DNA labeled with [^{3}H]thymidine to a specific activity of 4000 cpm/μg was prepared from ascites cells by organic extraction and ribonuclease and Pronase digestion as described by Pearson et al. (1978). Unlabeled high molecular weight DNA was prepared from the liver of an individual Sprague-Dawley rat by the method of Blin & Stafford (1976). Ribosomal RNA was obtained from the cytoplasmic poly(A)-minus fraction of rat liver RNA which was a gift of J. Sala-Trepat (Sala-Trepat et al., 1978). The 18S and 28S rRNA species were further purified on 15–30% linear sucrose gradients containing 0.05% $NaDodSO_4$,[1] 0.1 M NaCl, 0.01 M Tris (pH 7.6) in a Beckman SW27 rotor at 24 krpm for 18 h at 22 °C. The 18S and 28S rRNA peaks were kept separate and re-run individually on the same type of gradients. The separated rRNAs were stored as ethanol precipitates, and aliquots were redissolved in sterile water immediately before use.

Ribosomal RNA labeled with ^{125}I was used to assay the relative amounts of rRNA genes in various DNA fractions. A typical reaction mixture for preparing the [^{125}I]rRNA contained 32 μg of rRNA (an equimolar mixture of 18S and 28S), 1–2 mCi of Na^{125}I (Amersham), and 10 μg of $TlCl_3$ in a total volume of 50 μL of 0.1 M sodium acetate (pH 4.7). The mixture was incubated for 20 min at 60 °C in a sealed glass tube. The solution was then passed over a Bio-Gel A-1.5m column equilibrated in 0.1 M sodium acetate (pH 4.7) to separate unincorporated iodine. The excluded peak was dialyzed against the same buffer for 2.5 h at 60 °C, and then against 2 × SSC at 4 °C. (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.) The [^{125}I]rRNA was stored at −20 °C for up to 2 weeks. Immediately before use, the [^{125}I]rRNA was passed through a Bio-Gel A-1.5m column equilibrated in 2.5 × PEN buffer. PEN buffer is 0.08 M monosodium Pipes (Calbiochem), 0.01 M Na_2EDTA, 0.37 M NaCl, 0.03 M NaOH (pH 7.4). The excluded peak of radioactivity was used for the filter hybridizations. Specific activities were approximately 0.5–1 × 10^7 cpm/μg.

Formation of R Loops and Enrichment for rDNA. Three separate experiments, each using slightly different procedures or materials, were performed.

Experiment 1. Prior to doing a preadsorption of the DNA over the affinity column, a mock R-loop reaction without RNA was first performed. Approximately 1.6 mg of ^{3}H-labeled DNA was dialyzed into 1.2 × PEN buffer, 84% formamide. All formamide stocks were deionized with Amberlite MB-3 (Mallinckrodt) before use (Pinder et al., 1974). The DNA solution was adjusted to 1 × PEN, 70% formamide giving a total volume of 4.0 mL. The DNA was heated at 59 °C for 3 h and then dialyzed thoroughly against PBS. The DNA was then loaded onto a column containing an aliquot of the affinity resin. The 98.2% of the DNA which did not bind to the column was dialyzed against 1/10 × PEN and lyophilized. In order to regenerate the resin, the small amount of bound DNA (1.8%) was eluted with 1 M NaCl and 5 M NaSCN and discarded. The unbound DNA was redissolved in 1.2 × PEN, 84% formamide, and dialyzed against the same buffer. Next, 18S and 28S rRNAs were each added to a final concentration of 100 μg/mL and the buffer concentration was adjusted to 1 × PEN, 70% formamide. The mixture was then heated at 59 °C for 3 h to form R loops. It was quick-cooled and dialyzed thoroughly against PBS at 4 °C. The DNA–RNA mixture was then applied to the affinity column containing the same aliquot of resin used above. The fractions containing bound DNA (eluted in 1 M NaCl and 5 M NaSCN) were pooled and dialyzed against PBS. This DNA was then reapplied to the affinity column now containing a fresh aliquot of the affinity resin, and bound DNA was eluted as above. The 1 M NaCl and 5 M NaSCN fractions of the DNA were kept separate and dialyzed against 100 mM Tris, 10 mM EDTA (pH 8.4) at 4 °C. The various fractions were then prepared either for electron microscopy or for filter hybridizations.

Experiment 2. This was carried out with ^{3}H-labeled DNA as in the first experiment with the following modifications. No mock R-loop reaction and no preadsorption over the affinity column were done. The R-loop reaction mixture was incubated at 54 °C for 10 h. (The rationale for the lower temperature was to try to maintain the DNA molecules at longer double-stranded lengths by minimizing partial denaturation of the DNA. The presence of single-strand nicks in combination with partial denaturation could result in significantly shorter double-stranded lengths after the incubation.) The affinity resin used for the initial passage in this experiment was the aliquot used for the second passage in experiment 1, and again

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

a fresh aliquot of resin was used for the second cycle of purification in this experiment. Unlike the first experiment, only the DNA eluted in 5 M NaSCN in the first cycle was reapplied to the affinity column for the second cycle.

Experiment 3. The primary purpose of this experiment was to obtain electron microscopic data on the length of the repeating unit of rat rDNA. Unlabeled rat DNA prepared by the method of Blin & Stafford (1976) was employed according to the experiment 2 procedure with the following modification. The R-loop reaction mixture was divided into two equal portions. One was incubated at 54 °C and the other at 50 °C, each for 10 h. After R-loop formation, the two portions were recombined and the gene enrichment procedure carried out as for experiment 2 by using, respectively, the same aliquots of affinity resin for the first and second cycles of purification as used in that experiment. The final 5 M NaSCN fraction was prepared for electron microscopy.

Filter Hybridizations. The relative amounts of rRNA in various DNA fractions from the affinity column were assayed by hybridization of [^{125}I]rRNA to [^{3}H]DNA immobilized on 25-mm diameter nitrocellulose filters (Gillespie & Spigelman, 1965; Manning et al., 1977). Filters were prepared containing 0.9–70 μg of whole genomic DNA or rDNA-depleted DNA, or between 0.14 and 3.5 μg of various DNA fractions potentially enriched for rDNA. Before loading onto the filters, all DNA fractions were incubated 1 h at 37 °C in 0.2 N NaOH to denature the DNA and to hydrolyze any RNA present. The hybridizations were carried out in siliconized glass scintillation vials in PEN buffer containing 50% deionized formamide with the [^{125}I]rRNA at a concentration of 0.2 μg/mL at 45 °C for 20 h. This is approximately the empirical $R_0t_{1/2}$ of the reaction under these hybridization conditions (unpublished data). Nonspecific background was measured by using filters containing no DNA. All DNA samples were assayed at least in duplicate. As many as 60 filters were incubated in a single vial.

After the completion of the incubation, the filters were rinsed and washed with two 45-min changes of PEN buffer, 50% formamide at 45 °C. The [^{125}I]rRNA hybridized to the filters was determined using a Beckman gamma counter. The amount of [^{3}H]DNA on the filters at the end of the incubation was estimated by one (or in some cases, both) of two methods which were found to give results consistent with each other. In some cases, following the γ counting, the total cpm was determined in a liquid scintillation counter and the ^{3}H cpm could be calculated by subtracting the ^{125}I cpm. If the amount of DNA on the filters was too small to make this method practical, other identical filters were incubated in hybridization buffer lacking [^{125}I]rRNA, washed as above, and then counted in the liquid scintillation counter to determine ^{3}H cpm. In all cases the amount of DNA on the filters at the end of the incubation was approximately 65–80% of the DNA orginally bound.

Electron Microscopy. DNA from the affinity column was spread for electron microscopy by the modified Kleinschmidt technique of Davis et al. (1971). The spreading mixtures contained 0.06–0.1 mg/mL cytochrome *c*, 40–45% formamide, 70–100 mM Tris, 7–10 mM EDTA (pH 8.4). Circular molecules of SV40 DNA were included in most spreads as an internal length standard. The hypophase consisted of 10–15% formamide in 10 mM Tris, 1 mM EDTA (pH 8.4). The DNA was picked up on Parlodion coated grids, stained for 20 s in 5×10^{-5} M uranyl acetate in 90% ethanol and rinsed in methylbutane. The grids were rotary shadowed with platinum and palladium (80/20) and viewed in a Phillips 300 electron microscope. A Hewlett-Packard digitizer was used to measure the DNA seen in the 35-mm film. Lengths were calculated based on the SV40 internal standards taken to have a contour length of 5224 base pairs (Fiers et al., 1978).

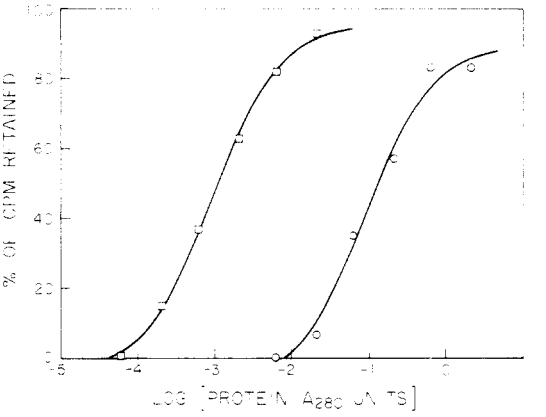

FIGURE 1: Specific activity of purified antibody compared with total IgG. In order to assay antibody activity, 0.2-μg aliquots of ^{3}H-labeled poly(rA)·poly(dT) were incubated with increasing amounts of IgG (isolated from antiserum against DNA–RNA hybrids and adsorbed to remove antibodies which cross-react with nonhybrid nucleic acids) or with specific antibody purified from that IgG. The formation of antibody–antigen complexes was measured by retention of the labeled poly(rA)·poly(dT) on glass fiber filters and plotted vs. the log of the total protein concentration. Each point represents the average of two separate determinations. (○) IgG; (□) purified antibody. The purified antibody exhibits a 100-fold increase in specific activity.

Results

Antibody Purification. Purified antibodies specific to DNA/RNA hybrids were used to construct an affinity resin to be used for enrichment of rat rDNA sequences. The preparation of antiserum against DNA/RNA hybrids, removal of cross-reacting antibody, and characterization of the IgG fraction have been described in Stumph et al. (1978). In that work, an affinity resin for gene enrichment was constructed by using the total IgG fraction (after prior removal of antibodies which cross-reacted with nonhybrid nucleic acids). The affinity resin used for the experiments described in this report was prepared by using antibodies which had been purified by an additional step. The IgG fraction (after removal of cross-reacting antibody) was applied to a column of poly(rA)·oligo(dT)-cellulose. The specific DNA/RNA hybrid antibody which bound to column was eluted with 3 M NaSCN. Figure 1 shows that a 100-fold purification of specific antibody was obtained by this procedure. The assay employed in Figure 1 measures the formation of antibody–antigen complexes. There is a difference of 2 log units in the amount of IgG required to reach the point of half-reaction. This indicates that the purified antibody has a 100-fold greater specific activity than the IgG from which it was purified. This purified antibody was then attached to CNBr-activated Sepharose to produce an affinity resin which specifically binds DNA/RNA hybrids.

Enrichment for Rat rDNA. The antibody affinity resin was used to enrich for rDNA sequences from whole genomic rat DNA by affinity column chromatography. The general procedure involves the formation of R loops by using the 18S and 28S rRNAs and total rat DNA. After R-loop formation, the bulk of the DNA and RNA passes through the affinity column without binding, whereas molecules containing R loops can bind to the column and be eluted by increasing the salt concentration. Two cycles of purification on the affinity column were employed to enrich for rat rDNA. This pro-

Table I: Enrichment for Rat rDNA by Affinity Chromatography

DNA sample	% of recovered DNA		fraction of DNA hybridized × 10^4		fold enrichment rel to whole DNA	
	expt 1	expt 2	expt 1	expt 2	expt 1	expt 2
whole unfractionated DNA			0.53	0.47	1.00	1.00
1st passage PBS flow-through	98.5	97.1	0.23	0.23	0.43	0.49
1st passage 1 M NaCl effluent[a]	1.0	1.9	*b*	1.3		2.8
1st passage 5 M NaSCN effluent[a]	0.5	0.9	*b*	24		51
2nd passage PBS flow-through	0.98	0.42	2.3	<1.8[c]	4.3	<4
2nd passage 1 M NaCl effluent	0.30	0.21	12.6	<3[c]	24	<7
2nd passage 5 M NaSCN effluent	0.22	0.26	72	84	136	179

[a] In experiment 1, the 1.5% of the DNA recovered in the 1 M NaCl and 5 M NaSCN fractions were pooled and passed over the affinity column a second time. In experiment 2, only the 0.9% of the DNA in the 5 M NaSCN fraction was applied to the column for the second cycle of purification. [b] Filter hybridizations were not carried out for these intermediate DNA samples in experiment 1. [c] Significant cpm above background were not obtained for these DNA samples because of the low degree of enrichment in combination with the limited amount of DNA available. Thus, only a maximum level of enrichment could be estimated.

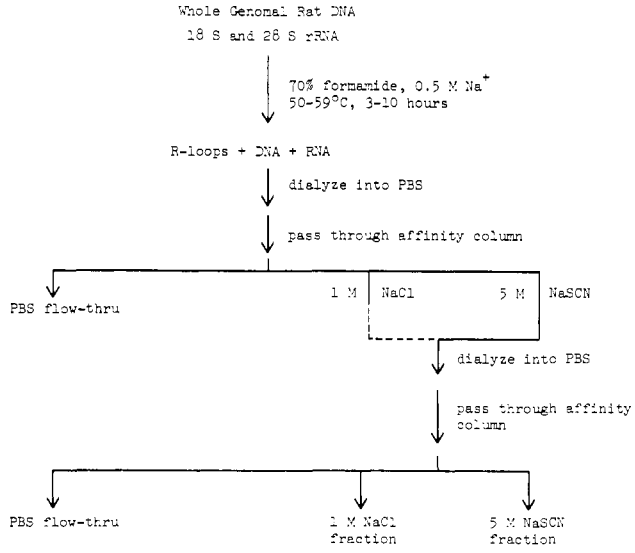

FIGURE 2: Method of rat rDNA enrichment. After the formation of R loops between total genomic rat DNA and 18S and 28S rRNAs, DNA molecules containing R loops were selected by two cycles of purification over an affinity column containing antibodies which specifically bind DNA–RNA hybrids. In experiment 1, the first cycle 1 M NaCl and 5 M NaSCN effluents were pooled and used for the second cycle of purification. In experiments 2 and 3, only the 5 M NaSCN fraction was used as the starting material for the second cycle.

cedure is outlined in the flow chart in Figure 2. Three separate gene-enrichment experiments were performed, and their individual differences are detailed in Experimental Procedures. One noteworthy difference is that in experiment 1 the 1 M NaCl and 5 M NaSCN fractions from the first cycle were pooled and together used for the second cycle of purification. Since analysis of this first experiment indicated that the greatest degree of enrichment was to be found in the 5 M NaSCN fraction alone, only this fraction was used in the subsequent experiments as the starting material for the second cycle of purification.

In the first two columns of Table I, the percent of DNA recovered in each fraction from the affinity column is shown for experiments 1 and 2. (An unlabeled preparation of DNA was used for experiment 3, so the amounts of DNA could not be determined in that case.) For both experiments, over 97% of the original DNA was eliminated as nonbinding DNA in the first cycle of purification. During the second cycle, a significant but smaller amount of DNA was eliminated as nonbinding DNA. It appears that further cycles would not result in much of a further purification of the rDNA. In each case, 0.2–0.3% of the original DNA was recovered in the second cycle 5 M NaSCN fraction.

The relative concentrations of rRNA coding sequences in the various samples of DNA from the affinity columns were estimated by RNA-driven filter hybridizations. Aliquots of DNA were immobilized on nitrocellulose filters and hybridized by [^{125}I]rRNA. The amount of DNA hybridized in these reactions is shown in the third and fourth columns of Table I. The degree of enrichment for rRNA coding sequences in the two experiments is shown in the fifth and sixth columns. This was calculated directly from the data in the third and fourth columns by normalizing the hybridization level of whole DNA to 1.00, and then correcting the subfractions from the affinity column accordingly. The greatest degree of enrichment was found in the 5 M NaSCN fractions. After two cycles of enrichment over the affinity columns, the 5 M NaSCN fractions were 136- and 179-fold enriched for rRNA coding sequences in experiments 1 and 2, respectively. Presumably the DNA in experiment 3 was similarly enriched.

The amount of DNA which is actually rDNA in these most highly enriched fractions can be estimated from the data in Table I (columns 3 and 4, bottom line). The fraction of DNA which was hybridized was 0.72% and 0.84% of the two second cycle 5 M NaSCN fractions in the two experiments. However, these numbers need to be multiplied three times by a factor of approximately 2 for the following reasons: (1) the hybridizations were carried out only to about the $R_0t_{1/2}$ of the reaction; (2) only the coding DNA strand is hybridized, so the numbers need to be corrected to take the noncoding strand into account; (3) the rDNA molecules contain DNA sequences adjacent to the coding cistrons which are not measured by the filter hybridizations. This last factor is dependent upon the average length of the enriched rDNA molecules relative to the length of the coding cistrons. If this last factor is assumed to be approximately 2, then it can be calculated that about 6% of the DNA in the most highly enriched fractions is actually rDNA. Because of the approximations involved and some uncertainty in the exact specific activity of the iodinated rRNA, this is not a hard number but is probably correct within a factor of two in either direction (3–12% rDNA). A similar calculation, based upon the hybridization of whole unfractionated DNA (Table I, top line), reveals that approximately 0.02% of the nuclear DNA codes for 18S and 28S rRNA, which indicates that there are approximately 80 copies of the rRNA genes per haploid rat genome. This number does not differ significantly from the 100 copies determined by similar techniques for the rRNA genes of the mouse (Gaubatz et al., 1976).

Electron Microscopic Mapping of the Transcribed Region of Rat rDNA. The greater than 100-fold enrichment for rat

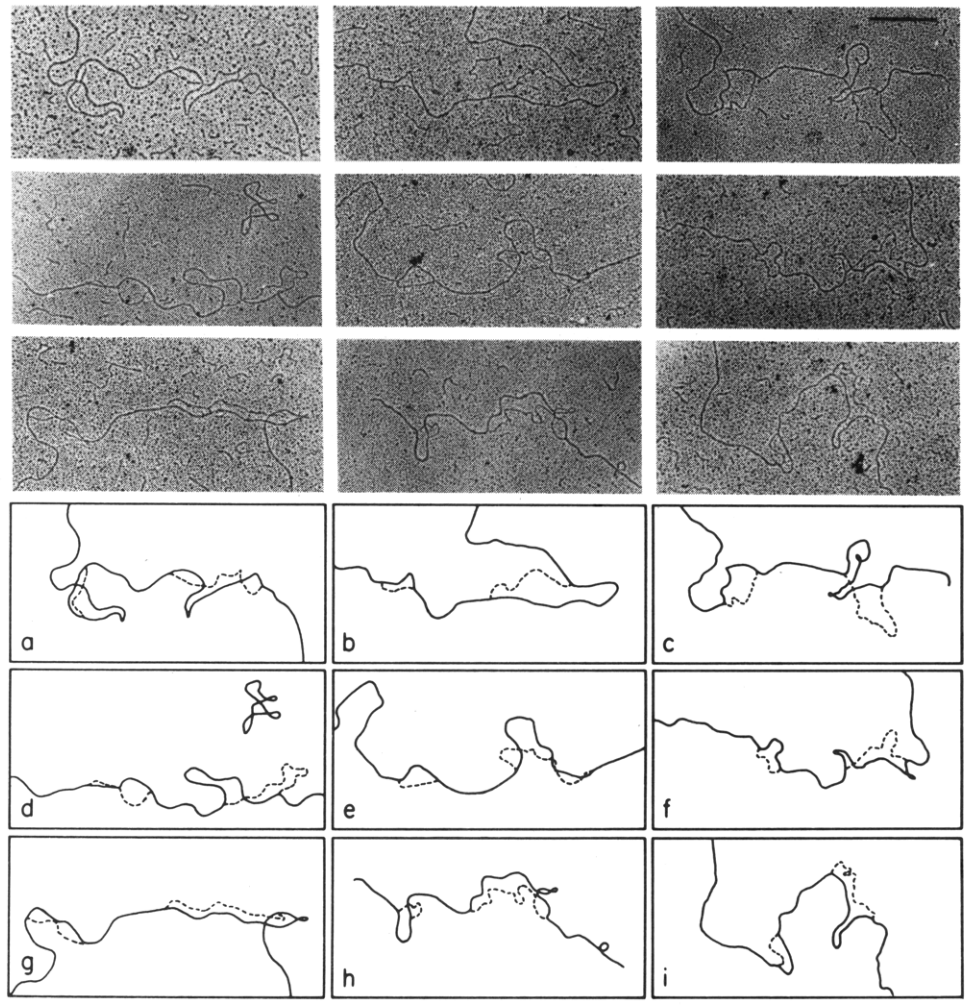

FIGURE 3: Molecules of rat rDNA containing 18S and 28S rRNA R loops. In the tracings at the bottom of the figure, solid lines indicate either double-stranded DNA or DNA/RNA hybrid. The dashed lines represent single-stranded DNA. In panel d, an SV40 molecule, included as an internal length standard, is shown. The bar at top right represents 0.5 μm.

rRNA genes obtained by affinity chromatography permitted the direct observation of these R-loop structures in the electron microscope. Figure 3 shows several examples of molecules which contain both 18S and 28S rRNA R loops. Figure 4 is a schematic diagram of 68 such molecules that were measured. All of these molecules extend for a distance of less than 35 kbp from the midpoint of the short between-loop spacer. (Longer molecules are shown in Figure 5.) The lengths of the DNA molecules and loops were calculated in kilobase pairs from the measured lengths of circular SV40 molecules which were included in the DNA solutions as internal standards. The results were as follows: 18S R loop, 1.38 ± 0.29 kbp; 28S R loop, 4.31 ± 0.44 kbp; and interloop (transcribed) spacer, 2.24 ± 0.22 kbp.

The measured lengths for the R loops are not as large as the actual lengths of the two rRNA species. Based upon the reported molecular weights of rat and of other mammalian rRNAs (Schibler et al., 1975; Wellauer et al., 1974; Wellauer & Dawid, 1973; Loening, 1968; Attardi & Amaldi, 1970), the 18S and 28S rRNAs would be expected to have lengths of approximately 2.1 and 5.4 kb, respectively (Cory & Adams, 1977). Thus, the average lengths of the R loops in these experiments are approximately 66% (18S) and 80% (28S) of the full lengths of the rRNAs. The shorter R-loop lengths are to be expected since this phenomenon has been observed consistently by other workers and has been discussed by White & Hogness (1977) and by Thomas et al. (1976). The lengths of the R loops depends upon such factors as the particular conditions used in spreading the molecules for electron microscopy, displacement of the ends of the RNA by branch migration, and the possibility that DNA/RNA hybrids may not exhibit the same contour length as DNA–DNA duplexes of equal length in terms of base pairs. Also, shorter loops result when the R-loop formation reaction is carried out at temperatures further below the T_m of the DNA. Indeed, in the experiments reported here, the molecules from expeiment 1 (incubated at 59 °C) did exhibit larger R loops than the molecules from the later experiments in which the reaction temperature was lowered. For these reasons, the largest R loops observed (1.98 kbp for the 18S and 5.07 kbp for the 28S) probably more accurately reflect the actual RNA lengths than do the overall averages.

The measured length of the short spacer between the loops was 2.24 kbp. This can be taken as a maximum distance due to the fact that the R loops themselves are shorter than the sequences which actually code for the mature rRNAs. If the assumption is made that the shortening of the R loops results in a proportional increase in the spacer length, then the transcribed spacer between the two genes could be as short as 1.34 kbp. However, the validity of such an assumption depends upon the actual mechanisms involved in the foreshortening of the R loops. Restriction digest mapping of mouse rDNA (Cory & Adams, 1977) would indicate the 2.24-kbp value as measured may be the more accurate figure.

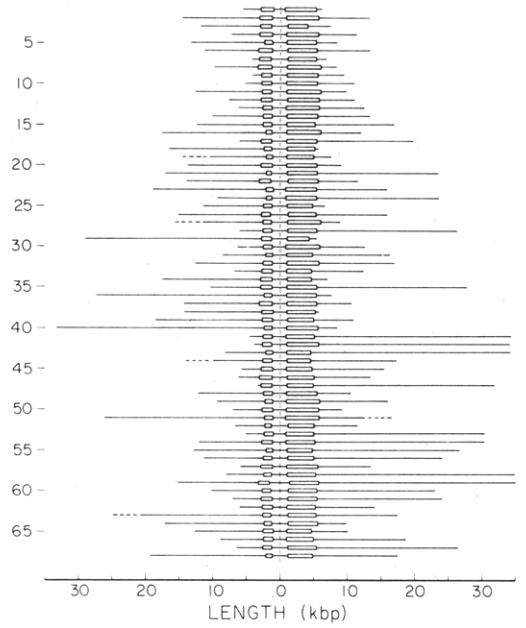

FIGURE 4: Length measurements of rat rDNA molecules. All molecules with 18S and 28S rRNA R loops both intact were measured. All of the molecules in this figure extend for 35 kbp or less from the midpoint of the interloop spacer; longer molecules are shown in Figure 5. Molecules which end in dashed lines either went off the grid mesh at that point or became entangled with other molecules. Molecules 1–12 are from experiment 1; molecules 13–48 are from experiment 2; and molecules 49–68 are from experiment 3. Four of the experiment 3 molecules shown in Figure 5 were found in the electron microscope during the same period of time in which the 20 experiment 3 molecules shown in this figure were found. After that point, short molecules were ignored in the electron microscope and only long molecules were measured.

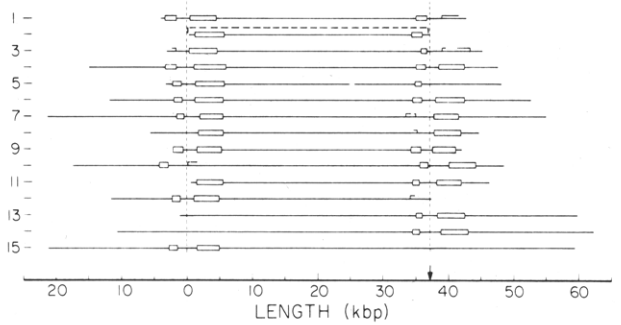

FIGURE 5: Schematic diagram of long rat rDNA molecules. All molecules containing at least two intact R loops were measured. The first 11 molecules each contain three of four R loops, or else two R loops separated by a long spacer. The mean repeating unit length calculated for these 11 molecules is 37.2 kbp. Because of the variation in measured lengths, each molecule is arranged symmetrically between the two vertical dashed lines at 0.0 and 37.2 kbp. Broken or incomplete R loops, seen as branch structures in the electron microscope, are shown schematically as branches above the main trunk lines of the rDNA molecules. Molecules 1 and 2 are from experiments 1 and 2, respectively. The remaining 13 molecules are from experiment 3. Molecule 2 existed in the electron microscope as a circle. For a further discussion of this molecule and also numbers 12–15, see the text.

Determination of the Rat rDNA Repeating Unit. Occasionally molecules were seen in the electron microscope which were longer than those presented in Figure 4. Figure 5 is a schematic representation of 15 molecules which extend for a distance greater than 35 kbp from the midpoint of the short transcribed spacer. Most of these molecules (numbers 1 through 11) contain either three or four R loops, or else two R loops separated by a long spacer. Thus they span a complete repeating unit of rDNA. These 11 molecules give direct

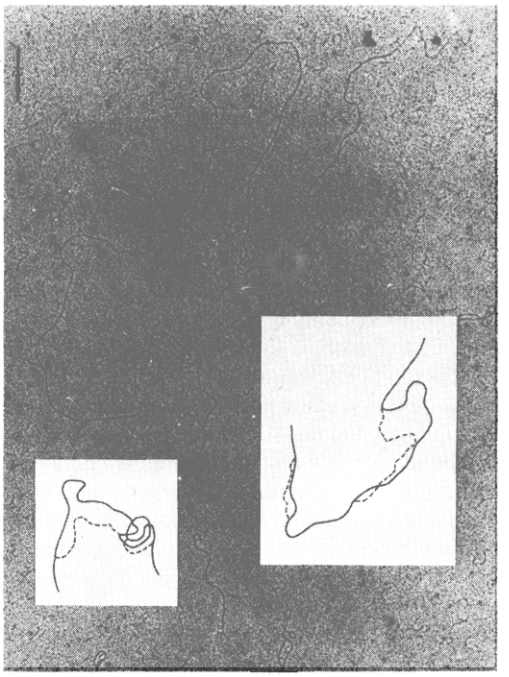

FIGURE 6: A long rat rDNA molecule containing two sets of R loops separated by a long spacer. Interpretations of the R loops are shown in the insets at a higher magnification. The bar represents 0.5 μm.

information regarding the size of rat rDNA repeating units. The longest measured unit was 39.9 kbp, and the shortest was 35.5 kbp. The average length and standard deviation of the measured repeating units for these 11 molecules is 37.2 ± 1.3 kbp. This standard deviation is 3.5% of the mean length. Populations of SV40 molecules, which are all presumbaly exactly the same length, had similar standard deviations from the mean (2–6%). Thus, it is certainly possible that the repeating units of molecules 1–11 in Figure 5 may represent repeating units of exactly the same length, and that is probably the preferred interpretation of the data. Nevertheless, a certain degree of length heterogeneity, possibly up to 4–5 kbp, cannot be positively ruled out. Figure 6 shows an example of a molecule which spans an entire repeating unit and has two sets of R loops separated by a long spacer.

Molecule 12 in Figure 5 is barely long enough to span a 37.2-kbp repeating unit. It has an R-loop doublet at one end of the molecule, and a branch near the other end which probably represents a broken 18S R loop. Thus, it is consistent with a 37.2-kbp repeating unit. Molecules 13, 14, and 15 are also long enough to span a repeating unit of this size. However, each contains only a single R-loop doublet. Thus, these molecules could be taken as evidence for the existence of longer repeating units. Indeed, because of the greater abundance of shorter molecules due to random breakage of the DNA preparations, shorter repeating units would be more likely to be found. However, it was also not uncommon to observe molecules in the electron microscope which contained only a single 18S or 28S R loop when both cistrons should have been present on the molecule. In such cases, the missing R loop may never have been formed to begin with, or it could have been destroyed during the subsequent steps of affinity chromatography or preparation for electron microscopy. A second possibility is that molecules without a second set of R loops may be the first or last repeating units within an array of tandem repeats. Since the rat haploid genome contains about 80 copies of the rRNA genes according to our estimation, and since mammalian rRNA genes are dispersed on several different chromosomes (Elsevier & Ruddle, 1975;

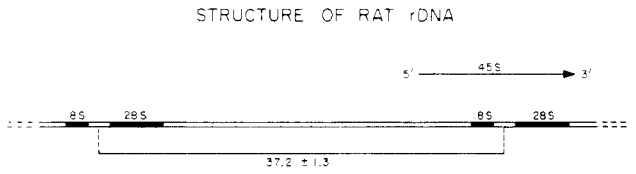

FIGURE 7: Model for the structure of rat rDNA. The length of the repeating unit is given in kilobase pairs with a standard deviation calculated from the data presented in Figure 5.

Henderson et al., 1972), one or two such repeating units would be expected to occur in a population of 15 randomly selected molecules. In any case, if there is any major heterogeneity in the lengths of rDNA repeating units, the 37.2-kbp unit is probably the most abundant. If significantly shorter repeating units exist in the genome, they must be very rare. This is demonstrated in Figure 4 in which absolutely no evidence for the existence of shorter repeating units is found.

Molecule 2 of Figure 5 possessed a unique structure in the electron microscope where it existed as a circular molecule. In Figure 5 it was split between the R loops and linearized to emphasize the length of the repeating unit. The circular structure observed in the electron microscope does not necessarily mean that this was an individual circular molecule in its native state. A reasonable explanation is that, due to single-strand nicks and potential partial denaturation over short stretches of the DNA during the incubation for R-loop formation, complementary single-stranded "sticky ends" could be generated at homologous locations which could later reanneal to produce a circular molecule. The total length of such a molecule would be the length of the repeating unit, which is what is observed for this molecule.

Discussion

The employment of a novel method of affinity chromatography, which makes use of antibodies that specifically bind DNA/RNA hybrids, has made it possible to enrich for rat rDNA molecules which contain R loops formed with the 18S and 28S rRNAs. In two separate experiments, the rat rRNA coding sequences were enriched 136- and 179-fold. With this degree of enrichment, it is estimated that the rDNA comprises approximately 3–12% of the DNA in these enriched fractions. This made it possible to directly observe these R loop containing molecules in the electron microscope. Based upon these observations, a model is proposed in Figure 7 for the structure of rat rDNA. From the published data for the rat (Schibler et al., 1975; Liau & Hurlbert, 1975, taking into account Dawid & Wellauer, 1976) and by analogy to other eucaryotic systems (for references, see Dawid & Wellauer, 1976), the transcription unit of the 45S rRNA precursor has been placed in the 5′ to 3′ direction at the location shown. Since the 45S precursor has a length of about 14 kb (Schibler et al., 1975), only about 40% of the DNA in the repeating unit is transcribed into 45S precursor RNA. The function of the remainder of the DNA is not known at the present time.

In *Drosophila melanogaster*, some of the repeating units of rDNA are interrupted in the coding region of the 28S rRNA by an intervening DNA region which apparently does not code for rRNA (Glover & Hogness, 1977; Pellegrini et al., 1977; Wellauer & Dawid, 1977; Wellauer et al., 1978). Such intervening sequences have been found in many single copy genes which code for proteins in the higher vertebrates. The possibility of such intervening sequences was kept in mind as the grids of rat rDNA were scanned for molecules containing R loops. Occasionally a 28S R loop was found which could possibly be interpreted as a structure resulting from the presence of an intervening sequence (see White & Hogness, 1977). An example of such a possibility is the bottom left set of R loops in the molecule shown in Figure 6. In this case, if the loops were interpreted as a single 28S R loop interrupted by an intervening sequence, the R loop would be too long, i.e., longer than the 28S rRNA. Thus, it is clearly apparent in the case of this molecule that the structure is actually composed of a 28S R loop and 18S R loop overlying each other. Another possibility for an intervening sequence would be in the molecule in panel h of Figure 3. In this case, the possibility cannot be discounted, but the R loop would be unusually small if interpreted in that way. Thus, no definitive evidence for the existence of intervening sequences within the rat rRNA coding regions could be found. If such sequences do exist in rat rDNA, they are either probably rare or too small to be detected by the R-loop method, or else they were not enriched for in the fractionation procedure.

The data presented in this report confirm by a totally independent method the observations of other investigators that mammalian rRNA genes consist of very long repeating units. From blots of *Hin*dIII digested nuclear DNA probed with radioactive RNA, Arnheim & Southern (1977) assigned repeating unit lengths of 38 and 31 kbp to mouse and human rDNA, respectively. By using the same enzyme, the value determined by Cory & Adams (1977) for mouse rDNA was 44 kbp. This slight discrepancy in the case of mouse rDNA probably results from differences in the calibration of the agarose gels. Based upon the 37-kbp repeating unit determined in this report for rat rDNA, it seems possible that the repeating units of mouse rDNA determined by blots of *Hin*dIII digested DNA probably do represent the complete repeating unit of mouse rDNA. Nevertheless, data from this laboratory (A. Reyes and R. B. Wallace, personal communication) indicate that the enzyme *Hin*dIII makes at least one additional cut in the spacer region of rat rDNA. Thus, not all of the rDNA fragments of the repeating unit can be detected by hybridization of rRNA (or complementary RNA or DNA) probes to *Hin*dIII digested rat DNA. Although the restriction enzyme sites in the coding sequences may be highly conserved throughout vertebrate evolution, it seems likely that there may be a great deal of restriction site heterogeneity in the spacer regions. Therefore, in the absence of additional confirmatory evidence, care should still be taken in assigning complete lengths to rDNA repeating units of other organisms when this is based solely on the Southern blot type of experiments probed only with rRNA sequences. For these reasons, the direct electron microscopic measurements of rat rDNA repeating units desecribed in this report represent a significant aid to the understanding of mammalian rDNA structure.

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Nucleosome Reconstitution: Effect of DNA Length on Nucleosome Structure†

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ABSTRACT: Core histones (H2A, H2B, H3, and H4) are reconstituted by salt gradient dialysis with DNA molecules ranging in length from 177 bp down to 50 bp. While reconstituted particles containing 125 bp are very similar to native particles, those particles containing a single piece of shorter DNA tend to aggregate. The aggregation depends on the ionic strength and DNA length. The DNA placement on the histone core is not random as determined by pancreatic DNase I digestions of particles containing ^{32}P 5′-end-labeled DNA. Rather, it is found that all DNA molecules, up to 161 bp in length, reassociate with core histones in such a way as to produce defined patterns of DNase I cutting with respect to the 5′ ends. Particles were made that contained two pieces of 65-bp DNA. These particles are very similar to native particles under most conditions but tended to dissociate at very low ionic strength. It is suggested that this dissociation results in the production of two half-nucleosomes (hemisomes).

As was shown in an earlier paper (Tatchell & Van Holde, 1977), nucleosomes can be reconstituted from isolated DNA and histones, reproducing most or all of the structural features found in vivo [see Felsenfeld (1978) for review]. Using physical and enzymatic probes as criteria for reconsititution, we have shown that nucleosomal core particles reconstituted from 144-bp DNA and histones are very similar, if not identical, to the native particle. In this paper we describe reconstitutions using core histones together with DNA molecules both shorter and longer that usually associated with the core particle. The resultant products have been characterized by a number of techniques.

The core histones in eukaryotic chromatin protect 140–170-bp DNA fragments from hydrolysis by micrococcal nuclease but there is evidence that DNA–histone interactions are not uniform along the DNA (Simpson & Whitlock, 1976; Mirzabekov et al., 1978; Weischet et al., 1978). Pancreatic DNase I digestion of core particles containing DNA ends labeled with ^{32}P indicates that some positions along the DNA are highly protected while others are much more susceptible

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