GENE ENRICHMENT USING ANTIBODIES TO DNA/RNA HYBRIDS: MAPPING THE RIBOSOMAL DNA OF SLIME MOLD AND RAT

Thesis by

William Edward Stumph

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To my parents

with love and gratitude

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Abstract

A novel method for gene enrichment has been developed and applied to mapping the rRNA genes of two eucaryotic organisms. The method makes use of antibodies to DNA/RNA hybrids prepared by injecting rabbits with the synthetic hybrid poly(rA)·poly(dT). Antibodies which cross-react with non-hybrid nucleic acids were removed from the purified IgG fraction by adsorption on columns of DNA-Sepharose, oligo(dT)-cellulose, and poly(rA)-Sepharose. Subsequent purification of the specific DNA/RNA hybrid antibody was carried out on a column of oligo(dT)-cellulose to which poly(rA) was hybridized. Attachment of these antibodies to CNBr-activated Sepharose produced an affinity resin which specifically binds DNA/RNA hybrids.

In order to map the rDNA of the slime mold Dictyostelium discoideum, R-loops were formed using unsheared nuclear DNA and the 17S and 26S rRNAs of this organism. This mixture was passed through a column containing the affinity resin, and bound molecules containing R-loops were eluted by high salt. This purified rDNA was observed directly in the electron microscope. Evidence was obtained that there is a physical end to Dictyostelium rDNA molecules approximately 10 kilobase pairs (kbp) from the region which codes for the 26S rRNA. This finding is consistent with reports of other investigators that the rRNA genes exist as inverse repeats on extra-chromosomal molecules of DNA unattached to the remainder of the nuclear DNA in this organism.

The same general procedure was used to map the rRNA genes of the rat. Molecules of DNA which contained R-loops formed with the 18S and 28S rRNAs were enriched approximately 150-fold from total genomal rat DNA by two cycles of purification on the affinity column. Electron microscopic measurements of

these molecules enabled the construction of an R-loop map of rat rDNA. Eleven of the observed molecules contained three or four R-loops or else two R-loops separated by a long spacer. These observations indicated that the rat rRNA genes are arranged as tandem repeats. The mean length of the repeating units was 37.2 kbp with a standard deviation of 1.3 kbp. These eleven molecules may represent repeating units of exactly the same length within the errors of the measurements, although a certain degree of length heterogeneity cannot be ruled out. If significantly shorter or longer repeating units exist, they are probably much less common than the 37.2 kbp unit.

The last section of the thesis describes the production of antibodies to non-histone chromosomal proteins which have been exposed to the ionic detergent sodium dodecyl sulfate (SDS). The presence of low concentrations of SDS did not seem to affect either production of antibodies or their general specificity. Also, a technique is described for the in situ immunofluorescent detection of protein antigens in polyacrylamide gels.

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CHAPTER I

Gene Enrichment Using Antibodies to DNA/RNA Hybrids: Purification and Mapping of Dictyostelium discoideum rDNA

Introduction

The study of the organization of eucaryotic DNA sequences has been a fertile area of recent biochemical research. It is expected that knowledge of the arrangement of DNA sequences will lead to a better understanding of how gene activity is regulated in higher eucaryotes. For many purposes, it is desirable or necessary to purify specific DNA sequences which code for RNA, and also those near-by sequences which are adjacent to the coding sequences and which may play a role in the expression of the gene. Various methods have been employed for such gene enrichment and purification with varying degrees of success (Manning et al., 1977; Tiemeier et al., 1977; Wellauer and Dawid, 1977; Woo et al., 1977; Anderson and Schimke, 1976; Dale and Ward, 1975; Georgiev et al., 1977). The cloning of fragments of higher organism DNA in procaryotic vectors permits the isolation of many copies of a single sequence by amplification of the recombinant vector following the isolation of clones containing the desired sequence. However, in the case of high complexity genomes as found in mammals, the detection and isolation of a clone containing a particular unique DNA sequence (which represents only about 1 part in 10^6) can become a formidable task. Often, some degree of gene enrichment is desirable to facilitate clonal detection and isolation (Tiemeier et al., 1977; Ohshima and Suzuki, 1977).

I have therefore explored the feasibility of using antibodies to DNA/RNA hybrids (Stollar, 1970; Rudkin and Stollar, 1977) as a method for gene enrichment. These antibodies, produced by injecting rabbits with the synthetic hybrid poly(rA)·poly(dT), react specifically with DNA/RNA hybrids following adsorption of the IgG fraction over columns of DNA-Sepharose, oligo(dT)-cellulose, and

poly(A)-Sepharose. The antibody-containing IgG fraction has been covalently attached to Sepharose to prepare an affinity column to be used for gene enrichment studies.

I describe here results with the rRNA genes of the slime mold Dictyostelium discoideum. This system was chosen because the rDNA comprises an unusually large percentage of the genome of this organism, and much is already known about its structure (Cockburn et al., 1976; Maizels, 1976). For these studies, DNA was isolated from nuclei and hybridized with the two major rRNA species under conditions to form R-loops (Thomas et al., 1976; Frankel et al., 1977). This mixture was then passed through the affinity column, and bound molecules containing DNA/RNA hybrids were recovered by high-salt elution. Examination of the recovered DNA in the electron microscope revealed that half of the molecules in this high-salt fraction contained intact R-loops, and over 80% appeared to contain DNA/RNA hybrid regions over a portion of their length. The molecules were typically 15-40 kbp in length, whereas the regions which code for the 17S and 26S rRNA molecules total only about 6 kbp. Thus, a considerable amount of adjacent DNA was co-purified with the coding sequences. Additional evidence has also been obtained that there is a physical end to the Dictyostelium rDNA molecule and that this is approximately 10 kbp beyond the region which codes for the 26S rRNA. This is consistent with the recently reported finding (Taylor et al., 1977) that Dictyostelium rDNA is extra-chromosomal.

Materials and Methods

Antiserum and IgG Preparation. Antibodies against DNA/RNA hybrids were prepared according to the method of Stollar (1970). New Zealand White

rabbits were each injected intradermally with a mixture containing 2.5 A_{260} units of poly(rA)·poly(dT) (Collaborative Research, Waltham, Massachusetts) and 125 μg of methylated bovine serum albumin (Calbiochem, San Diego, California) in complete Freunds adjuvant. The injection was repeated two weeks later in incomplete Freunds adjuvant, followed a week later and at monthly intervals thereafter by an intravenous injection, and the collected serum was heated at 56°C for 30 min to inactivate complement.

The IgG fraction was isolated by standard methods of ammonium sulfate precipitation and DEAE cellulose chromatography (Garvey et al., 1977). This purified IgG fraction was then adsorbed by two passages through columns of DNA-Sepharose, oligo(dT)-cellulose, and poly(rA)-Sepharose in order to remove antibodies which bind to DNA or the respective homopolymers. The columns were linked in tandem, and the entire adsorption procedure was carried out at 4°C in 2/3 X PBS solution. PBS is 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5. In order to regenerate the columns, bound protein was eluted with PBS containing 3 M NaSCN. Following the re-equilibration of the columns with 2/3 X PBS, the IgG which did not bind on the first passage was re-applied to the tandem columns. The IgG which did not bind on this second passage constitutes the IgG fraction after adsorption, and is devoid of antibodies which react with DNA, poly(rA), or oligo(dT) under the conditions of the adsorption procedure. It was concentrated by ammonium sulfate precipitation and then exhaustively dialyzed against PBS to be used in later experiments. The DNA-Sepharose and poly(rA)-Sepharose had been prepared according to the method of Wagner et al. (1971), using Sepharose freshly activated with cyanogen bromide. The DNA which was bound to the Sepharose was a mixture of calf thymus DNA (Sigma, St. Louis, Missouri) and ^{3}H-rat

DNA. It was first sheared to an average single-strand length of about 2000 nucleotides, and then heat-denatured in 0.2 M MES buffer (pH 6). The DNA probably contained some rapidly renatured double-stranded regions in addition to the predominantly single-stranded DNA. Tritium-labeled poly(rA) was mixed with unlabeled poly(rA) (both from Schwarz-Mann, Orangeburg, New York) and dissolved directly in the MES buffer. The nucleic acid was bound to the freshly-activated Sepharose by tumbling overnight at 4°C. Reactive groups on the resin were then blocked by washing with 1 M 2-aminoethanol (pH 8). About 130 mg of DNA were bound to 65 ml of Sepharose, and 15 mg of poly(rA) to 15 ml of Sepharose, estimated by scintillation counting aliquots of the resins. The oligo(dT)-cellulose used for the adsorption was 10 g of Type T-2 from Collaborative Research.

Filter Assays. The formation of antibody-nucleic acid complexes was measured by retention of the complexes on glass fiber filters (Lewis et al., 1973; Trilling et al., 1970). Briefly, 0.2 μg aliquots of tritium-labeled nucleic acid were incubated with various amounts of serum or IgG for 45 min at 37°C in 1.0 ml of PBS. The mixture was then poured over Whatman GF/F filters, which were rinsed, dried, and counted. Labeled poly(rA) was purchased from Schwarz-Mann, and labeled poly(dT) from Miles (Elkhart, Indiana). Tritium-labeled poly(rA)·poly(dT) was prepared by mixing equimolar quantities of ^{3}H-poly(rA) with unlabeled poly(dT) and incubated at 56°C for 1 hr in 0.6 M sodium phosphate, pH 6.8, and then dialyzed against PBS. Rat DNA labeled with ^{3}H-thymidine was prepared from ascites cells by organic extraction and ribonuclease and pronase digestion as described by Pearson et al. (1978). The rat DNA/RNA hybrid was prepared by means of an in vitro transcription reaction (Warner et al., 1963; Chamberlin and Berg, 1964) in which 200 μg of heat denatured ^{3}H-rat DNA were transcribed with 500 units

of E. coli RNA polymerase for a period of 90 min at 37°C, using 5'-ribonucleotide triphosphates in 10-fold molar excess over the amount of DNA. The RNA polymerase was prepared according to the method of Humphries et al. (1973). After the reaction, free RNA was removed by Cs_2SO_4 density gradient centrifugation.

Affinity Resin. The affinity resin was prepared by mixing 141 A_{280} units of adsorbed IgG with 0.5 g of washed CNBr-activated Sepharose (Pharmacia, Lot 9429) in 0.5 M NaCl, 0.02 M sodium phosphate, pH 7.5, and tumbling overnight at 4°C. A total of 118 A_{280} units of IgG bound to the Sepharose. The resin was placed into a column and thoroughly washed with 1 M 2-aminoethanol (pH 8), PBS, and PBS containing 5 M NaSCN. For analytical purposes, 2-5 μg of nucleic acid were loaded onto the column in PBS at 4°C and step elutions were carried out using 1 M NaCl, 0.01 M sodium phosphate, pH 7.5; 3 M NaSCN in PBS; and 5 M NaSCN in PBS. Radioactivity was determined by scintillation counting in Aquasol-2 with corrections made for quench due to thiocyanate. After washing with 5 M NaSCN, the column could be reused with no noticeable loss of activity. Overall recoveries from the column were 80-105%. In the tables, the recoveries are normalized to 100% for direct comparison.

Isolation of Dictyostelium rDNA. Nuclear DNA was isolated from ^3H-thymidine labeled Dictyostelium discoideum cells, strain AX-3, grown in liquid culture, using a combination of published methods (Bakke and Bonner, 1978; Firtel et al., 1976; Firtel and Bonner, 1972) The washed cells were lysed in 0.5% NP-40, and the nuclei pelleted at low speed. The nuclei were lysed in 4% Sarcosyl at 65°, and the DNA was purified by two cycles of centrifugation in CsCl gradients containing ethidium bromide.

The rRNA was prepared by phenol-chloroform extraction of the supernatant of a cellular lysate centrifuged at 20,000 x g for 10 min. The RNA was ethanol precipitated, redissolved in water, heated at 60°C for 1 min to denature 26S-5.8S rRNA complexes, and centrifuged on 15-30% linear sucrose gradients containing 0.05% SDS, 0.1 M NaCl, 0.01 M Tris (pH 7.6) in a Beckman SW27 rotor at 26.5 krpm for 15 hr at 22°C (Firtel et al., 1972; Maizels, 1976). The 17S and 26S rRNA peaks were kept separate and re-run individually on the same type of gradients. The separated rRNAs were stored in water at -20°C.

Prior to isolation of the rDNA, 100 µg of the total nuclear DNA were loaded onto the affinity column in PBS at 4°C. After removing the bound DNA (which accounted for 4.4% of the total) with three high-salt step elutions, the column was re-equilibrated with PBS. The DNA which did not bind on the first passage was then re-applied to the column. This time only 0.9% of the DNA bound to the column. The DNA which flowed through without binding on this second passage was then used for the isolation of the rDNA.

After dialysis to remove the PBS, the DNA was precipitated with ethanol and redissolved at a concentration of 1080 µg/ml in 2.5 M NaCl, 0.25 M PIPES, 0.125 M EDTA (pH 6.8) in preparation for the formation of R-loops according to the conditions of Frankel et al. (1977). The total reaction volume of 160 µl contained nuclear DNA at 216 µg/ml, 17S and 26S rRNA each at 20 µg/ml, 67% recrystallized formamide, 0.5 M NaCl, 0.05 M PIPES, 0.025 M EDTA (pH 6.8). A second reaction mixture (control) was set up in an identical manner except that the RNA was omitted. Both reaction mixtures were sealed in capillaries and incubated 45 min at 50°C. The samples were quick-frozen and stored overnight at -20°C. Prior to loading onto the affinity column, each sample was diluted

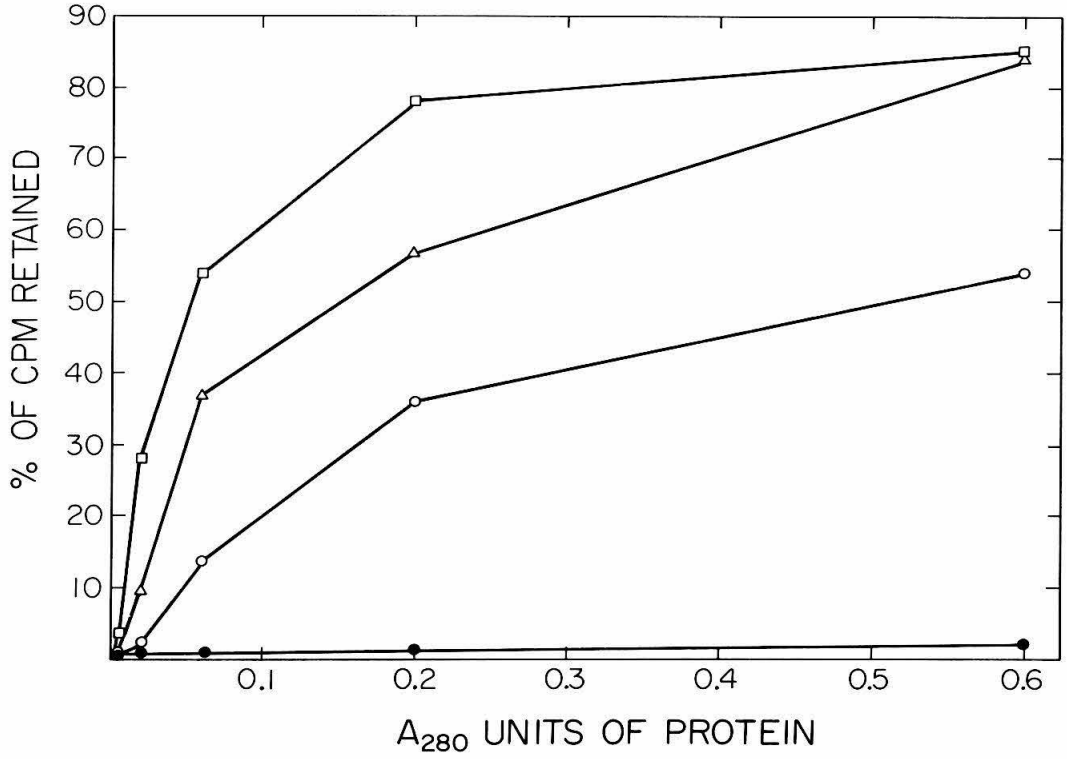
30-fold with 2 volumes of water and 27 volumes of PBS. The samples were separately passed over the affinity resin at 4°C and bound DNA was eluted as described above.

Electron Microscopy. DNA from the affinity column was dialyzed against 0.1 M Tris, 0.01 M EDTA (pH 8.4) and spread for electron microscopy by the modified Kleinschmidt technique of Davis et al. (1971). The spreading mixture contained 40% formamide, 0.05 mg/ml cytochrome c, 0.07 M Tris, and 0.007 M EDTA (pH 8.4), with a final DNA concentration of approximately 0.4 μg/ml. The mixture was spread on a hypophase containing 10% formamide, 0.01 M Tris, 0.001 M EDTA (pH 8.4). Parlodion coated grids were picked and stained for 20 sec 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. The DNA seen in the 35 mm film was measured using a Hewlett-Packard digitizer.

Results

Characterization of the Antibodies. Of six rabbits immunized for the present studies, five produced significant titers of antibodies as measured by the ability of their sera to retain poly(rA)·poly(dT) on glass fiber filters (data not shown). The antisera from these five rabbits were pooled. Figure 1 demonstrates the ability of this pooled antiserum to retain an increasing percentage of ^{3}H-poly(rA)·poly(dT) on filters as the concentration of whole antiserum is increased. It also shows that under identical conditions less than 1% of the synthetic hybrid was retained by normal rabbit serum. The IgG fraction was next purified from the pooled antiserum by ammonium sulfate precipitation and DEAE cellulose

FIGURE 1: Antibody activity assayed using poly(rA)·poly(dT). Antibody activity is measured by incubating 0.2 μg aliquots of tritium-labeled poly(rA)·poly(dT) with increasing amounts of IgG or serum. Antibody-antigen complexes which are formed are retained on glass fiber filters. Each point represents the average of two separate determinations. (O) whole antiserum; (□) purified IgG; (Δ) purified IgG after adsorption over DNA-Sepharose, oligo(dT)-cellulose, and poly(A)-Sepharose; (●) whole normal serum.


90
80
70
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% OF CPM RETAINED
0.1
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0.4
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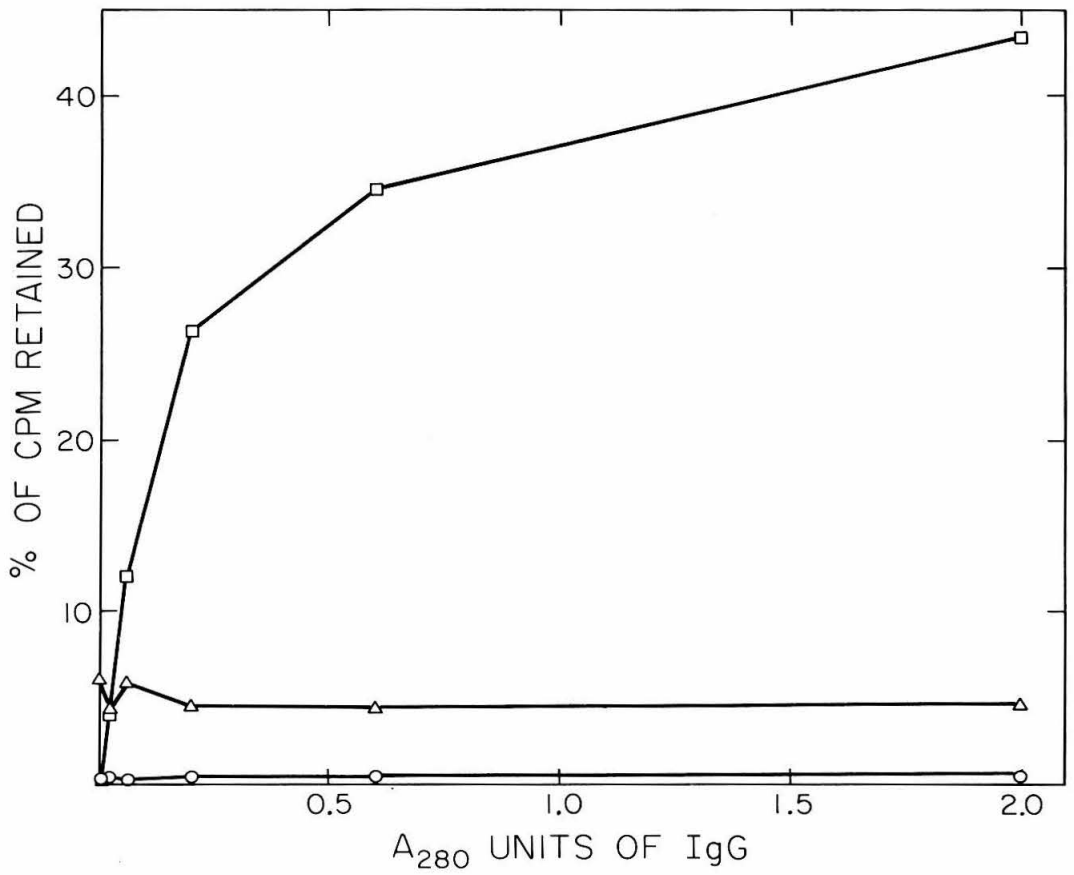
A280 UNITS OF PROTEIN

chromatography. The purified IgG was subsequently adsorbed over columns of DNA-Sepharose, oligo(dT)-cellulose, and poly(A)-Sepharose in order to remove antibodies in the IgG fraction which cross-react with non-hybrid nucleic acids. The antibody activities in the purified IgG both prior to and after adsorption were measured by filter assays using ^{3}H-poly(rA)·poly(dT) as the antigen, also shown in Figure 1. As expected, the purified IgG fraction had a 4- to 7-fold higher specific activity than the whole serum from which it was purified. (The specific activity of the assayed sample is greater when less total protein is required to retain the same percent of antigen.) After adsorption to remove antibodies which cross-react with DNA, poly(dT), and poly(rA), the resulting adsorbed IgG fraction exibited only about half the specific activity found in the purified IgG prior to adsorption. Thus, under the conditions of the adsorption, about half of the antibody activity cross-reacted with non-hybrid nucleic acids and was eliminated by the adsorption procedure.

The resultant product which did not bind to the DNA, poly(A), and oligo(dT) columns is hereinafter referred to as adsorbed IgG. A starting volume of 690 ml of pooled antiserum yielded 3100 A_{280} units of adsorbed IgG. This adsorbed IgG is comprised mostly of IgG molecules which do not bind to nucleic acids of any kind, and a small amount of antibody which binds specifically to DNA/RNA hybrids. Figure 2 shows the results obtained from filter assays with native and denatured rat DNA, and with a rat DNA/RNA hybrid, each incubated with increasing amounts of the adsorbed IgG. The DNA/RNA hybrid was prepared by transcribing denatured rat DNA with E. coli RNA polymerase. Such a reaction produces DNA/RNA hybrids (Warner et al., 1963; Chamberlin and Berg, 1964), and about 70% of the DNA molecules in the preparation were believed to contain regions of DNA/RNA hybrid

FIGURE 2: Specificity of the IgG after adsorption. In order to assay the antibody specificity, 0.2 μg aliquots of various tritium-labeled nucleic acids were incubated with increasing amounts of the adsorbed, purified IgG fraction. The formation of nucleic acid-antibody complexes was assayed by retention on filters. Each point is the average of two separate determinations. (□) rat DNA/RNA hybrid; (Δ) native rat DNA; (O) denatured rat DNA. Assays were also done using poly(dT) and poly(rA), and the results were essentially the same as those measured for the denatured DNA (always less than 1% retention).


40
30
20
10
% OF CPM RETAINED
0.5
1.0
1.5
2.0
A_{280} UNITS OF IgG

duplexes (see below). Figure 2 shows that these hybrids are retained on filters by the antibodies in the adsorbed IgG fraction. In contrast, no retention above the background levels was observed with either native or denatured DNA, even when the amount of antibody used was 100 times greater (2.0 A_{280} units) than that which produced measurable retention of hybrid (0.02 A_{280} units). Control assays were also done with poly(dT) and with poly(rA), and the level of retention was always less than 1% (not shown). Thus, the adsorbed IgG contains antibodies which bind to both the synthetic hybrid poly(rA)·poly(dT) and to rat DNA/RNA hybrids. The same IgG fails to exhibit any detectable cross-reactivity with non-hybrid nucleic acids under the conditions of the filter assays.

Affinity Resin Characterization. Since the adsorbed IgG exhibited a high degree of specificity for DNA/RNA hybrids, it seemed feasible to construct an affinity column which would specifically bind such hybrids. Ideally, such an affinity column should bind molecules which contain regions of DNA/RNA duplex, whereas nucleic acids which are purely DNA or RNA should pass through the column without binding. For this purpose, an affinity resin was prepared by covalently attaching an aliquot of the adsorbed IgG to CNBr-activated Sepharose. When poly(rA)·poly(dT) or the rat DNA/RNA hybrid were passed over the column in PBS, a total of 97% and 70% bound to the column, respectively, and were recovered in the high-salt elutions (Table I). In the latter case, the 30% which did not bind is probably comprised mostly of DNA molecules which were not transcribed into hybrid during the course of the polymerase reaction, and would therefore not be expected to bind to the column. Although both hybrids bind to the column in PBS, the affinity for poly(rA)·poly(dT) appears to be greater since most of it

TABLE I: Nucleic Acid Binding Properties of the Affinity Column[a]

Type of Nucleic Acid	% Eluted in: PBS	1 M NaCl	3 M NaSCN	5 M NaSCN	High Salt Total
Poly(rA)·poly(dT)	2.8	0.1	4.1	92.6	96.8
DNA/RNA hybrid prepared by *in vitro* transcription	29.6	38.0	28.0	4.2	70.2
Dictyostelium DNA (initial passage)	94.0	0.4	1.0	4.5	5.9
Dictyostelium DNA (unbound from initial passage re-applied to column)	98.7	0.2	0.1	0.8	1.1

[a]Two to five micrograms of ^{3}H-labeled nucleic acid were loaded onto the affinity column consisting of antibodies against DNA/RNA hybrids covalently linked to Sepharose. Bound nucleic acid was eluted in three steps of increasing salt concentration, and the percent recovered in each fraction was determined.

was not eluted until the 5 M NaSCN wash, whereas the rat DNA/RNA hybrid was mostly eluted by 1 M NaCl and 3 M NaSCN.

When nuclear DNA, isolated from Dictyostelium discoideum, was passed through the column, about 94% of the DNA flowed through without binding (Table I). However, a significant amount (5.9%) was retained, and most of this amount was eluted only by 5 M NaSCN. In three separate experiments, approximately 5% of the slime mold DNA was consistently recovered in the 5 M NaSCN fraction whenever a sample of the DNA preparation was passed through the column for the first time. This number was independent of the absolute amount of DNA loaded onto the column, and the nature of its binding is uncertain. It was possible, since the slime mold DNA was isolated without ribonuclease treatment, that this fraction of the DNA was retained because it contained regions of DNA/RNA hybrids which were present in vivo. The existence of such hybrids has been reported in the case of Drosophila polytene chromosomes (Rudkin, 1977). However, two lines of reasoning indicate that such hybrids may not be the cause of the binding which occurred during the first passage over the affinity column: 1) Most of this bound DNA was eluted only by 5 M NaSCN. All other natural hybrids [i.e., not poly(rA)·poly(dT)] are eluted for the most part by 1 M NaCl and 3 M NaSCN. 2) When an aliquot of the DNA eluted by 5 M NaSCN was dialyzed back into PBS, and then re-applied to the affinity column, less than 5% of it re-bound. This is unlike the behavior observed with authentic hybrids. When a sample of the rat DNA/RNA hybrid was eluted from the column in 5 M NaSCN, dialyzed back into PBS, and again passed through the affinity column, about 85% of it re-bound on this second passage (data not shown). Thus, the 5% of the slime mold DNA which bound on the

initial passage probably does not represent hybrids present in vivo. The reasons behind the observed binding and behavior remain unclear.

Nevertheless, when the 94% of the DNA which did not bind on the first passage was re-applied to the affinity column, only 0.8% was recovered in the 5 M NaSCN fraction on this second passage (Table I). When the contributions due to the 1 M NaCl and 3 M NaSCN elutions are included, the overall background level of binding to the column was 1.1%. With this relatively low level of background binding, it seemed reasonable to expect that the affinity resin could be used to enrich for DNA sequences which are transcribed into RNA.

Enrichment for Dictyostelium rDNA. The rRNA genes of Dictyostelium discoideum were chosen for initial studies on gene enrichment because the rDNA of this organism comprises an unusually large percentage of the genome. In Dictyostelium, the actual coding cistrons for the 17S and 26S rRNAs comprise 2.2% of the total nuclear DNA, and together with adjacent spacer regions, comprise about 18% of the nuclear DNA (Firtel and Bonner, 1972; Cockburn et al., 1976; Taylor et al., 1977).

In order to isolate the rRNA genes in double-stranded form with as much of the adjacent non-transcribed spacer region as possible, Dictyostelium nuclear DNA, which had been previously passed twice through the antibody-Sepharose affinity column, was incubated with purified 17S and 26S rRNA under conditions to form R-loops. In such a reaction, the RNA can displace one of the DNA strands and form a more stable DNA/RNA duplex over its complementary region (Thomas et al., 1976). When such a reaction was carried out and the products applied to the affinity column, the results in the top line of Table II were obtained. A substantial amount of DNA bound to the column in PBS and was eluted in higher salt,

TABLE II: Dictyostelium rDNA Enrichment by Affinity Chromatography[a]

DNA Sample	% of DNA Eluted In: PBS	1 M NaCl	3 M NaSCN	5 M NaSCN	High Salt Total
R-Loop Reaction	90.6	5.9	2.5	0.6	9.0
Control (Mock R-loop reaction--no RNA)	98.8	0.4	0.5	0.2	1.1

Molecules Containing:	% Observed In: High Salt Elution	PBS Run-off[b]
R-loops (single)[c]	43.5	4.7
R-loops (doublets)	6.5	0.3
Branches (R-loops with single-strand nicks)	32.5	10.3[d]
No hybrid regions (linears)	17.5	84.7

[a]After R-loop formation (or a control reaction carried out without RNA), approximately 34 µg of DNA were loaded onto the affinity column, and elution carried out as described in Table I and

Materials and Methods. These results are shown in the upper section of the table. The DNA in the high salt elutions and in the PBS run-off from the R-looped material was viewed in the electron microscope. Two hundred molecules in the high salt elution and 300 molecules in the PBS run-off were randomly scored, and these results are tabulated in the lower section of the table.

[b]In the PBS run-off, approximately 20-40% of the DNA was in the form of completely single-stranded molecules. Such molecules were not included in the tabulation; only double-stranded molecules were scored.

[c]This category includes molecules with a single R-loop plus a branch.

[d]In the case of about half the molecules in the PBS run-off scored as having branches, the branches were too long to be considered as broken R-loops. Nevertheless, they were still included in this category. These long branches could be due to partial denaturation and reannealing. Such long branches were not observed in the high salt fraction.

particularly by 1 M NaCl and 3 M NaSCN. As a control, an identical aliquot of DNA was put through the same procedures of the R-loop reaction except for the absence of RNA. When this sample was applied to the affinity column, only background levels of DNA were obtained in the corresponding high salt fractions (Table II).

The DNA from the R-loop reaction was then spread for electron microscopy. Aliquots of both the bound and run-off fractions were spread, and at least 200 molecules from each fraction were scored in the electron microscope. The results are shown in the lower section of Table II. In the bound fraction, the largest class of molecules (43.5%) contained a single R-loop, and in some cases, an additional branch structure. A smaller class of molecules (6.5%) contained R-loop doublets, where both a 26S and 17S rRNA had hybridized to neighboring DNA sequences. A third class (32.5%) consisted of long DNA trunk molecules with one or two shorter branches. A branch structure represents a broken R-loop which would be the result of a single-strand nick in that region of the DNA. A fairly high density of single-strand nicks in the DNA would not be unexpected due to the extremely high endonuclease content of Dictyostelium cells, which interferes with the isolation of high molecular weight DNA from these cells (Bakke and Bonner, 1978; Firtel et al., 1976). A total of 17.5% of the molecules appeared as linear duplexes which were apparently devoid of any DNA/RNA hybrid regions. These molecules most likely represent the background level of non-specific binding to the column, although some degradation of R-loops could also have occurred after elution and prior to the spreading.

The main point is that greater than 80% of the molecules eluted from the column in high salt appear to contain regions of DNA/RNA hybridization.

In contrast to the DNA which bound, only a very small percentage of the double-stranded DNA molecules in the run-off exhibited any evidence of hybridization (Table II). In addition, a considerable amount (an estimated 20-40%) of the DNA in the PBS run-off appeared to exist as completely single-stranded molecules, and such molecules are not included in the tabulation shown in Table II. Thus, it is clear that the bound fraction represents a substantial enrichment for DNA molecules which were hybridized by the rRNA. These molecules, as seen in the electron microscope, were typically 15-40 kbp in length, and thus contained a considerable amount of adjacent spacer DNA sequences along with the 8 kbp region which codes for the 37S rRNA precursor (Batts-Young et al., 1977).

Mapping Dictyostelium rDNA by Electron Microscopy. Some examples of rDNA molecules containing R-loops are shown in Figures 3 and 4. In Figure 3, sections of molecules are shown which contain either a single R-loop, an R-loop doublet, or an R-loop with a nearby branch. Figure 4 shows a 36 kbp long molecule with a large 26S rRNA R-loop and a shorter branch resulting from a 17S rRNA broken R-loop. No additional regions of hybridization are observed in the remaining part of the molecule, which is consistent with the restriction enzyme digest maps of Maizels (1976) and Cockburn et al. (1976) which indicate that the repeated units of Dictyostelium rDNA are at least 38 to 42 kbp long. Evidence has also been reported that the rRNA genes of Dictyostelium discoideum occur in the nucleus as dimers localized toward opposite ends of discrete 88 kbp extrachromosomal fragments of DNA (Taylor et al., 1977). According to their model, the rDNA forms an inverse repeat with a center of symmetry at the middle of the molecule. Also, there should be a physical end to the rDNA molecules approximately 9-10 kbp beyond the region which codes for the 26S rRNA. This

FIGURE 3: Coding regions of Dictyostelium discoideum rDNA molecules purified by affinity chromatography. Examples are shown of DNA molecules which contain one or two R-loops, or an R-loop plus a branch. The bar represents 0.1 μm.

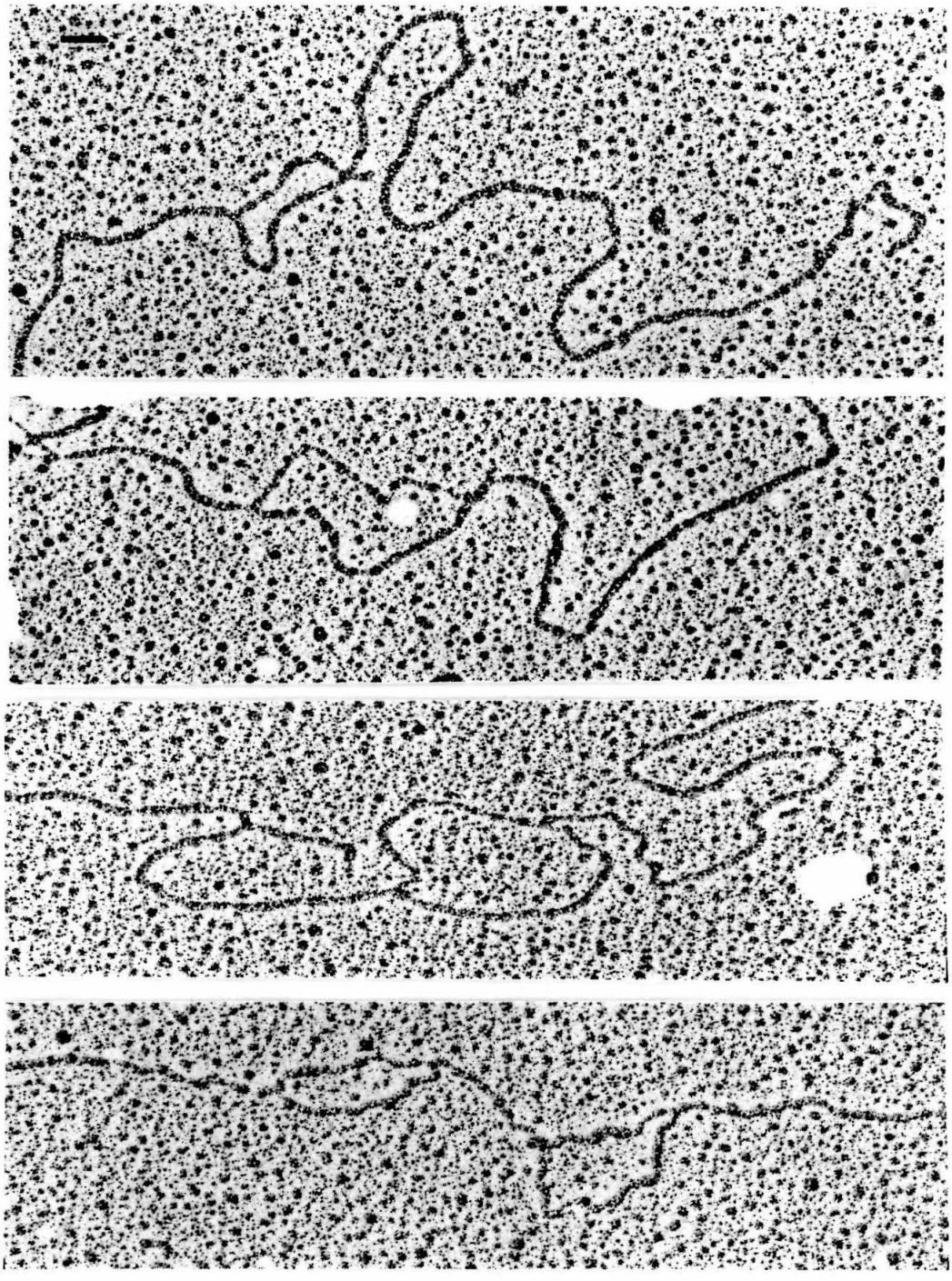
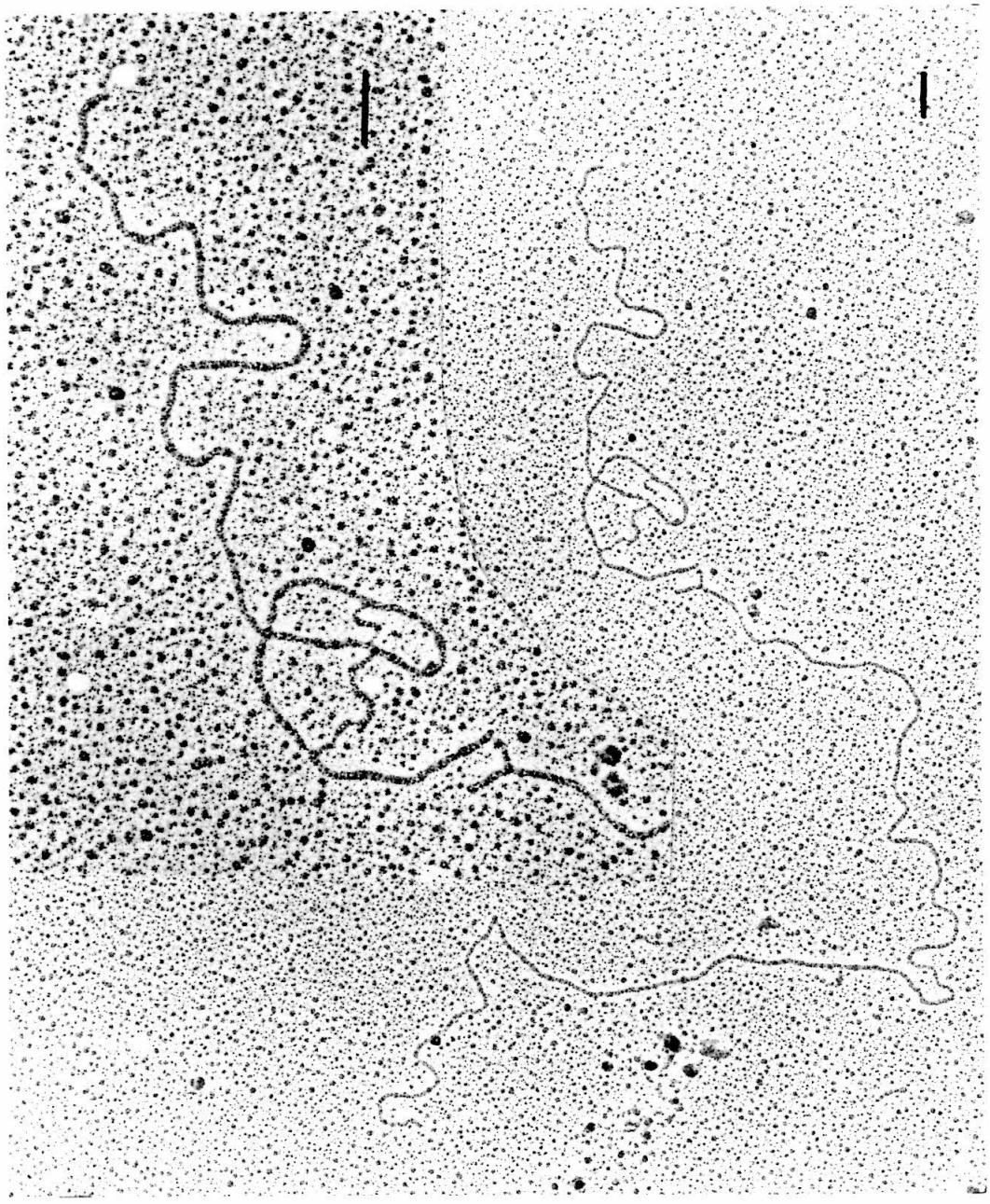


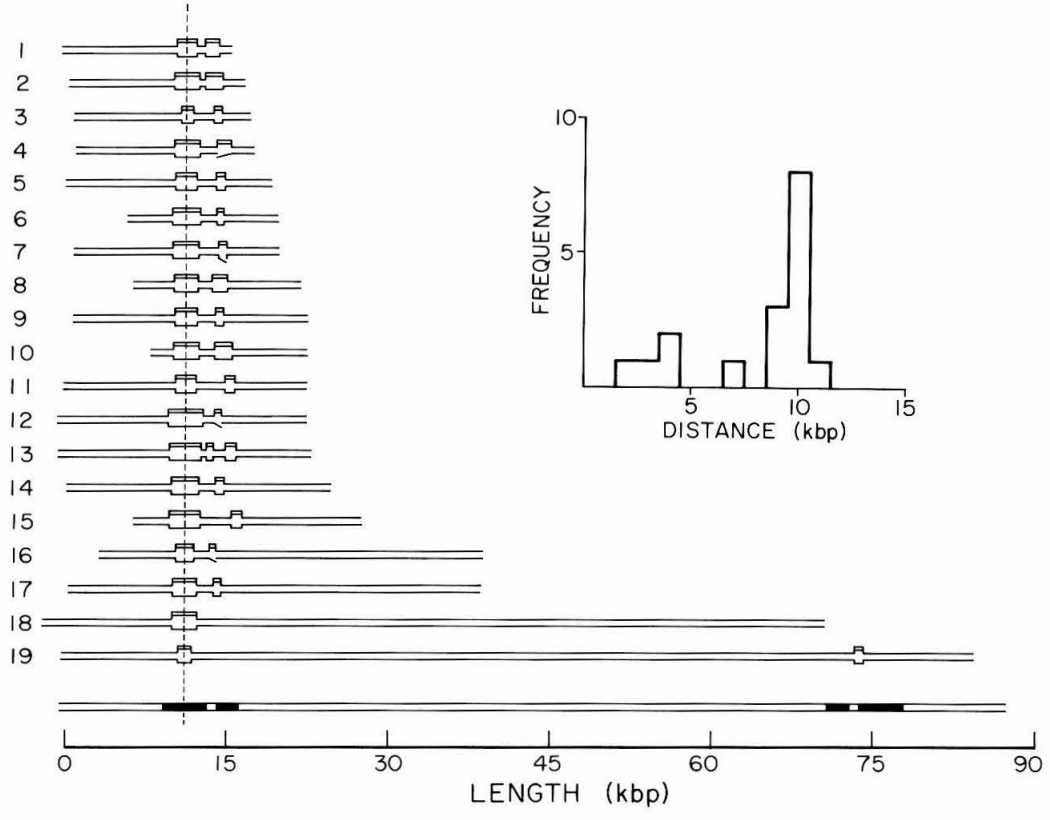
FIGURE 4: A 36 kbp long rDNA molecule containing an R-loop plus a short branch. The insert shows the R-loop region at a higher magnification. The bars represent 0.2 μm.



question has been examined in some detail by electron microscopic mapping of the rDNA isolated by affinity chromatography. Figure 5 is a schematic diagram of 19 molecules seen in the electron microscope. The first 17 molecules were chosen at random. The only bases of selection were that they each contained a 26S rRNA R-loop which could be identified by size and its orientation determined by the proximity of a neighboring 17S rRNA R-loop or branch. There is an obvious pattern which emerges: 70% of the molecules (12 out of the 17) end at a point which is 10.0 $\pm$ 0.5 kbp (insert in Figure 5) from the end of the 26S rRNA R-loop. In the remaining five molecules, the lengths were all shorter, presumably due to cleavage or breakage of the molecules during isolation and handling. In no cases were any molecules observed which extended for a greater distance. In contrast, the lengths on the 17S side of the molecules exhibited a great deal of heterogeneity. Thus, these data strongly suggest that there is a physical end to the molecules in vivo approximately 10 kbp past the region coding for the 26S rRNA, and supports the model that most, if not all, of the rDNA in Dictyostelium discoideum exists as discrete molecules not covalently attached to the chromosomes.

The 18th and 19th molecules shown in Figure 5 were not selected at random, but are included on the merits of their lengths. The 19th molecule contains a single R-loop near each end, and may represent an intact full-length extra-chromosomal molecule. The last position represents an idealized molecule which would be predicted by the inverse-repeat model proposed by Taylor et al. (1977). The molecules which we observed are certainly consistent with such a model, and thus provide additional evidence in support of it. Such patterns of inverse repetition have been observed in the extra-chromosomal rDNA of at least two

FIGURE 5: Map of Dictyostelium discoideum rDNA molecules which were isolated by affinity chromatography. The molecules are arranged by aligning the centers of the large R-loop at a common vertical line. An idealized molecule predicted by the inverse-repeat model of Taylor et al. (1977) is represented at the bottom of the figure. The insert in the figure is a histogram showing the distribution of lengths measured from the left end of the 17 randomly selected molecules to the beginning points of their respective 26S rRNA R-loops.


1
2
3
4
5
6
7
8
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10
11
12
13
14
15
16
17
18
19
0
15
30
45
60
75
90
LENGTH (kbp)
10
5
FREQUENCY
5
10
15
DISTANCE (kbp)

other lower eucaryotes, Tetrahymena (Karrer and Gall, 1976) and Physarum (Vogt and Braun, 1976).

Discussion

The immunization of rabbits with the synthetic hybrid poly(rA)·poly(dT) complexed with methylated bovine serum albumin has been found to be an efficient method for the production of antibodies which react with DNA/RNA hybrids (Stollar, 1970; this report). Such antibodies have been used by others to localize DNA/RNA hybrids on Drosophila polytene chromosomes using indirect immunofluorescence (Rudkin and Stollar, 1977; Rudkin, 1977). To increase the specificity for DNA/RNA hybrids, I have removed cross-reacting antibodies by adsorption over columns of DNA-Sepharose, oligo(dT)-cellulose and poly(A)-Sepharose. This adsorption is more efficient if carried out at a lower salt concentration than that used in subsequent experiments (my unpublished results; Rudkin and Stollar, 1977). The specific activity of antibody in the IgG preparation was decreased to about half of its original value following such an adsorption, due to the removal of the cross-reacting antibody population. This adsorbed IgG does not exhibit any measurable reactivity in filter assays with native or denatured DNA, or with poly(dT) or poly(rA) even at the highest concentration of antibody tested. In contrast, 1/100 of this maximum antibody concentration is sufficient to retain on filters measurable amounts of either poly(rA)·poly(dT) or a rat DNA/RNA hybrid preparation.

When the antibodies are covalently attached to Sepharose, they retain their ability to bind DNA/RNA hybrids. Table I reveals, however, that the resin has an apparently higher affinity for poly(rA)·poly(dT) than for the rat hybrid.

This is not surprising since poly(rA)·poly(dT) was the immunogen. It has also been noted (unpublished work) that the overall affinity of the resin for hybrids is proportional to the density of antibody on the Sepharose, which indicates that a hybrid molecule is being bound to the affinity resin by more than one antibody molecule. Thus, the affinity with which a hybrid binds to the column is also probably proportional to the length of the hybrid; a longer hybrid would be bound by more antibodies and thus exhibit a greater affinity for the resin.

The fact that the affinity column has the property of binding DNA/RNA hybrids while exhibiting only a low level of background binding for DNA has made it possible to enrich for the rRNA genes of the slime mold Dictyostelium discoideum. When native nuclear DNA was hybridized by 17S and 26S rRNA under conditions to form R-loops, a total of 9.0% of the DNA bound to the column. Greater than 80% of the molecules in this fraction represented rDNA sequences as based upon the electron microscopic observations in Table II. If all of the rDNA molecules were intact and contained R-loops, about 17% of the DNA would be expected to bind to the column. However, since the molecules were not full-length repeat units, the loss of some spacer DNA would be expected. Thus, the 9.0% of the DNA which did bind to the column probably represents a substantially higher recovery of rRNA cistrons in this fraction.

Of this 9.0% of the DNA which bound, approximately 1.1% was non-specific background based upon the amount recovered in this fraction in the control sample. Thus, about 12% (1.1/9.0) of the molecules in the bound, high salt eluted fraction would not be expected to contain regions of DNA/RNA hybridization. This number agrees fairly well with the 17.5% observed in the electron microscope. In addition, at least two other mechanisms could account for the presence

in the high salt fraction of molecules without R-loops or hybrid regions: 1) degradation of the RNA after elution with subsequent loss of the R-loops prior to spreading; 2) breakage of DNA molecules during elution from the column or preparation for electron microscopy, thus generating fragments without hybrid regions. However, these latter two processes probably do not occur to any great extent since non-specific background binding could account for most of the DNA molecules in the bound fraction on which hybrid regions were not observed.

The DNA sequences which code for the mature 17S and 26S rRNA molecules total approximately 6 kbp in length (Frankel et al., 1977). The average length of the randomly-selected rDNA molecules in Figure 5 was 21 kbp, or about 3-4 times that of the coding cistrons which were hybridized by the mature RNAs as the basis of the isolation. Molecules ranging up to 40 kbp in length were not uncommonly observed. Out of the hundreds of molecules observed in the electron microscope, one molecule was found which may represent a complete inverse repeat dimer. The scarcity of such very long DNA molecules is not surprising considering the high endonuclease content of Dictyostelium cells, and the extensive handling of the DNA during its isolation, affinity chromatography, and spreading for visualization in the electron microscope, all of which could contribute to the breakage of very long DNA. In addition, since the non-transcribed spacer regions of Dictyostelium rDNA are known to be A-T rich (Firtel et al., 1976; Firtel and Bonner, 1972), and since the R-loop hybridization reaction is carried out near the melting point of the relatively G-C rich rRNA cistrons, it is quite likely that the central regions of the palindromic dimer are extensively melted out under the conditions of R-loop formation. In this case, the presence of a pair of nicks in opposite strands of the DNA, even if relatively far apart, would result in complete

separation of the two halves of the rDNA dimer. As a result, the data presented here contribute little direct information on the structure of the intact palindromic rDNA dimer. However, Figure 5 does strongly support the finding that most, if not all, of the rDNA molecules are not covalently attached to the nuclear chromosomes since they possess a physical end approximately 10 kbp beyond the 26S rRNA cistron. No common end point has been observed on the 17S side of the molecule; this fact also is consistent with the inverse repeat model.

The rRNA genes of Dictyostelium discoideum were chosen as an initial system for studying the feasibility of gene enrichment using the antibody affinity column technique. This was done because the rDNA represents an unusually large percentage of the nuclear DNA of this organism, which simplifies its isolation. In other systems, where it may be possible to prepare DNA more free of nicks and where one is not dealing with relatively G-C rich genes bounded by near-by A-T rich regions, it may be possible to enrich for DNA which is even longer than that observed here in the case of Dictyostelium rDNA. Further experiments with other systems will be required in order to determine whether the antibodies can be successfully used to enrich for gene sequences which are present as a much smaller fraction of the total DNA. Since the background level of binding to the antibody column is about 1-2% of the DNA which is applied, re-cycling of the bound DNA over the column would be required to purify sequences present in less than this amount, or alternatively, the technique could be combined with other methods of gene enrichment which are available. The main advantages of the antibody affinity column seem to be that long double-stranded DNA can be isolated which is enriched both for coding sequences and for adjacent DNA sequences which may play a role in the expression of the gene. Also, the resolution

of the method should not be affected in any way by the ratio of the total DNA length to that of the hybrid, as long as the hybrid region is long enough to bind to the column. Finally, there is no need for any modification of the DNA or RNA, nor for the introduction of any special substituents, since the specificity resides solely in the existence of the DNA/RNA hybrid.

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CHAPTER II

Enrichment for and Mapping of Rat Ribosomal DNA

Introduction

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, and Physarum, the many copies of the rRNA genes exist as dimers on extra-chromosomal DNA fragments which are inversely repeated around a central axis of symmetry (Karrer and Gall, 1976; Taylor et al., 1977; Vogt and Brown, 1976; Chapter I of this dissertation). 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 gene regions which code for the rRNAs are separated by spacer regions a part of which is transcribed into the high molecular weight precursor of the rRNAs. However, a large part of the spacer is not transcribed and serves a presently unknown function. 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). This length heterogeneity is due to two variable-length regions within the non-transcribed spacer region which are themselves composed of variable numbers of short "subrepeats" (Wellauer et al., 1976).

A second 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 presence or absence of an intervening sequence which splits the 28S rRNA coding sequence (Glover and Hogness, 1977; Wellauer and 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 non-transcribed spacer regions.

The data which are available on mammalian rDNA organization are much more limited. Nevertheless, it is clear that the size of the repeating unit is much larger in mammals than that observed in either Drosophila or Xenopus. Most of the information comes from blots (Southern, 1975) in which restriction enzyme digests of total nuclear DNA are fractionated electrophoretically by size on agarose gels. Bands containing rDNA can be detected by hybridization with radioactive rRNA (or complementary RNA or DNA) probes. Using such methods, Arnheim and Southern (1977) reported repeating units of about 38 and 31 kbp for mouse and human rDNA respectively. Cory and 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 non-transcribed spacer regions may escape detection. Such studies have also revealed a heterogeneity among different repeats (Arnheim and Southern, 1977; Cory and 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. In at least one case, it is probably sequence differences, rather than length differences, which are responsible for the heterogeneity observed (Cory and Adams, 1977).

Some evidence on the length of mammalian rDNA repeating units has also been obtained from the results of Miller and his co-workers. Their technique allows the direct observation of rDNA transcription units after gentle cellular lysis and centrifugation of the contents onto grids for electron microscopy. In spreads from HeLa cells (Miller and Bakken, 1972; Hamkalo and 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 argue for 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 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 containing R-loops (White and Hogness, 1977; Thomas et al., 1976) were directly observed in the electron microscope. This was made possible by the greater than 100-fold enrichment of these molecules from whole genomal rat DNA using the novel method of gene enrichment described in the preceding chapter. This procedure involves chromatography on an affinity column containing antibodies which bind to DNA/RNA hybrids. This permits the selection for DNA molecules which contain R-loops. These molecules can then be observed and studied in the electron microscope.

Materials and Methods

Antibody and Affinity Column Preparation. Antiserum against DNA/RNA hybrids was prepared as described in Chapter I. 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 non-reacting 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. Next, 30 mg of poly(rA) were applied to the column in the same buffer. The column was next washed with 0.5 x PBS to elute loosely bound poly(rA), and then equilibrated with 0.8 x 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) were loaded onto the column in 0.8 x 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 was then dialyzed against PBS.

The specific activity of the purified antibody was compared to the IgG, from which it was purified, by means of glass fiber filter assays (Lewis et al., 1973) exactly as described in Chapter I. 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 Chapter I. The binding of the antibody to the resin was essentially quantitative. A 1/3 aliquot of this affinity resin was used for each of the affinity chromatography runs described below. 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 a modification of the method of Blin and Stafford (1976). A freshly removed liver was diced into liquid nitrogen and ground to a powder with a pre-cooled mortar and pestle. The proteinase K and ribonuclease digestions and the phenol extractions were carried out essentially as described by Blin and Stafford (1976). After the final dialysis, the DNA was precipitated with ethanol, wound out on a glass rod, and re-dissolved in 10 mM Tris, 1 mM EDTA, 10 mM NaCl (pH 8.4).

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% SDS, 0.1 M NaCl, 0.01 M Tris (pH 7.6) in a Beckman SW27 rotor at 24 krpm for 18 hr 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 re-dissolved 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 hr at 60°C, and then against 2 x 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 two weeks. Immediately before use the ^{125}I-rRNA was passed through a Bio-Gel A-1.5m column equilibrated in 2.5 x 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 x 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 pre-adsorption of the DNA over the affinity column, a mock R-loop reaction without RNA was first performed. Approximately 1.6 mg of ^3H-labeled DNA was dialyzed into 1.2 x 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 x PEN, 70%

formamide giving a total volume of 4.0 ml. The DNA was heated at 59°C for 3 hr 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 x PEN and lyophilized. The small amount of bound DNA (1.8%) was eluted with 1 M NaCl and 5 M NaSCN in order to regenerate the resin. The unbound DNA was re-dissolved in 1.2 x PEN, 84% formamide, and dialyzed against the same buffer. Next, 18S and 28S rRNAs (dissolved in water) were each added to a final concentration of 100 μg/ml along with additional water to adjust the buffer concentration to 1 x PEN, 70% formamide. The mixture was then heated at 59°C for 3 hr to form R-loops. It was quick-cooled and then 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 re-applied to the affinity column now containing a fresh aliquot of the affinity resin. 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 pre-adsorption over the affinity column were done. The R-loop reaction mixture was incubated at 54°C for 10 hr. (The rationale for the lower temperature was to try to maintain the DNA molecules at longer double-stranded lengths by minimizing 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 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 re-applied to the affinity column for the second cycle. The 1 M NaCl effluent from the first cycle was not included.

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 and 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 hr. After R-loop formation, the two portions were recombined and the gene enrichment procedure carried out as for Experiment 2 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 and Spiegelman, 1965; Manning et al., 1977). Filters were prepared containing 0.9 to 70 μg of whole genomal 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 hr 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. The incubations were done at 45°C for 20 hr. This is approximately the empirical $Rot_{1/2}$ of the reaction under these hybridization conditions (unpublished data). The conditions were chosen to maximize the degree of hybridization while minimizing non-specific background binding of ^{125}I-cpm and loss of DNA from the filters. Non-specific background was measured 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.

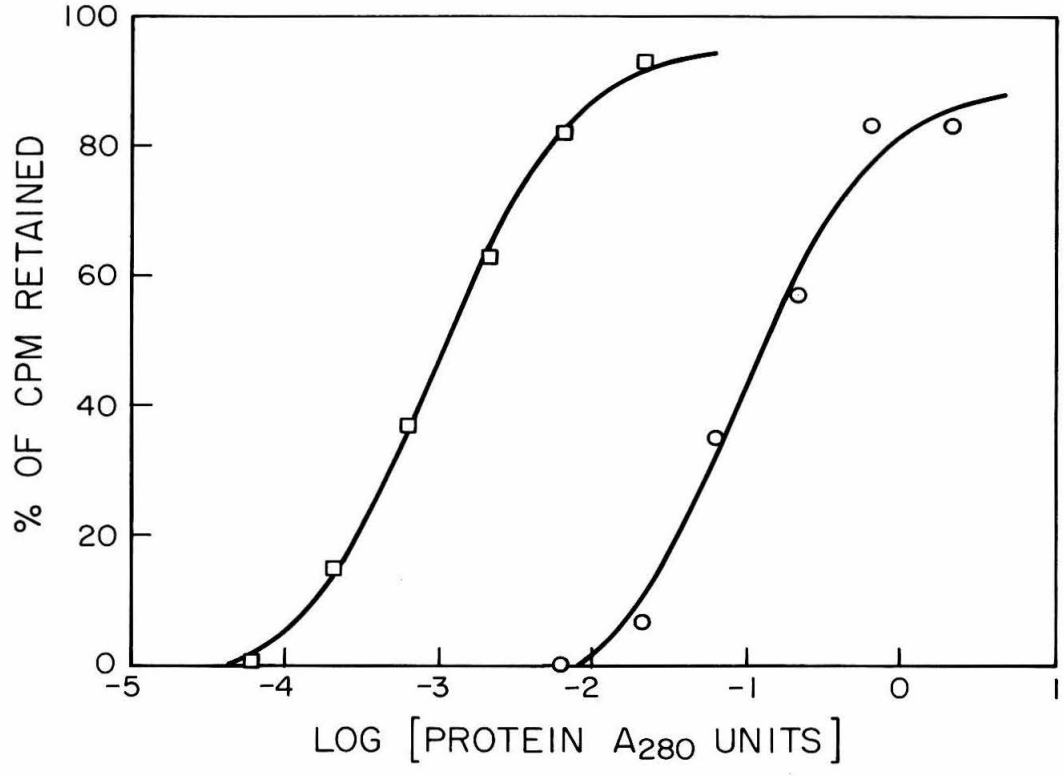
After the completion of the incubation the filters were rinsed and washed with two 45 min changes of PEN buffer, 50% formamide at 45°C. The ^{125}I-rRNA hybridized to the filters was determined using a Beckman gamma counter. The amount of ^{3}H-DNA on the filters at the end of the incubation was estimated by one (or in some cases, both) of two methods which were found to give results consistent with each other. In some cases (primarily those filters containing whole genomal DNA or rDNA-depleted DNA), the ^{125}I-cpm on the filters represented only a small fraction of the ^{3}H-DNA cpm. In these cases, following the gamma counting, the total cpm could be determined in a liquid scintillation counter and the ^{3}H-cpm could be calculated by subtracting the ^{125}I-cpm. When 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 measure ^{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 originally 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 and stained for 20 sec 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).

Results

Antibody Purification. The preparation of antiserum against DNA/RNA hybrids, removal of cross-reacting antibody, and characterization of the IgG fraction has been described in Chapter I. In that work, an affinity resin for gene enrichment was constructed using the total IgG fraction (after prior removal of antibodies which cross-reacted with non-hybrid nucleic acids). The affinity resin used for the experiments described in this report was prepared 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 this experiment measures the formation of antibody-antigen complexes. There is a difference of two log

FIGURE 1: Specific activity of purified antibody compared to total IgG. In this experiment, 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 non-hybrid 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 versus the log of the total protein concentration. (O) IgG; (□) purified antibody. The purified antibody exhibits a 100-fold increase in specific activity.


100
80
60
40
20
0
% OF CPM RETAINED
-5
-4
-3
-2
-1
0
1
LOG [PROTEIN A_{280} UNITS]

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 genomal rat DNA by affinity column chromatography. The general procedure involves the formation of R-loops 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 procedure is outlined in the flow-chart in Figure 2. Three separate gene-enrichment experiments were performed. Individual differences between the three experiments are detailed in the Materials and Methods section. 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

FIGURE 2: Method of rat rDNA enrichment. After the formation of R-loops between total genomal 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.

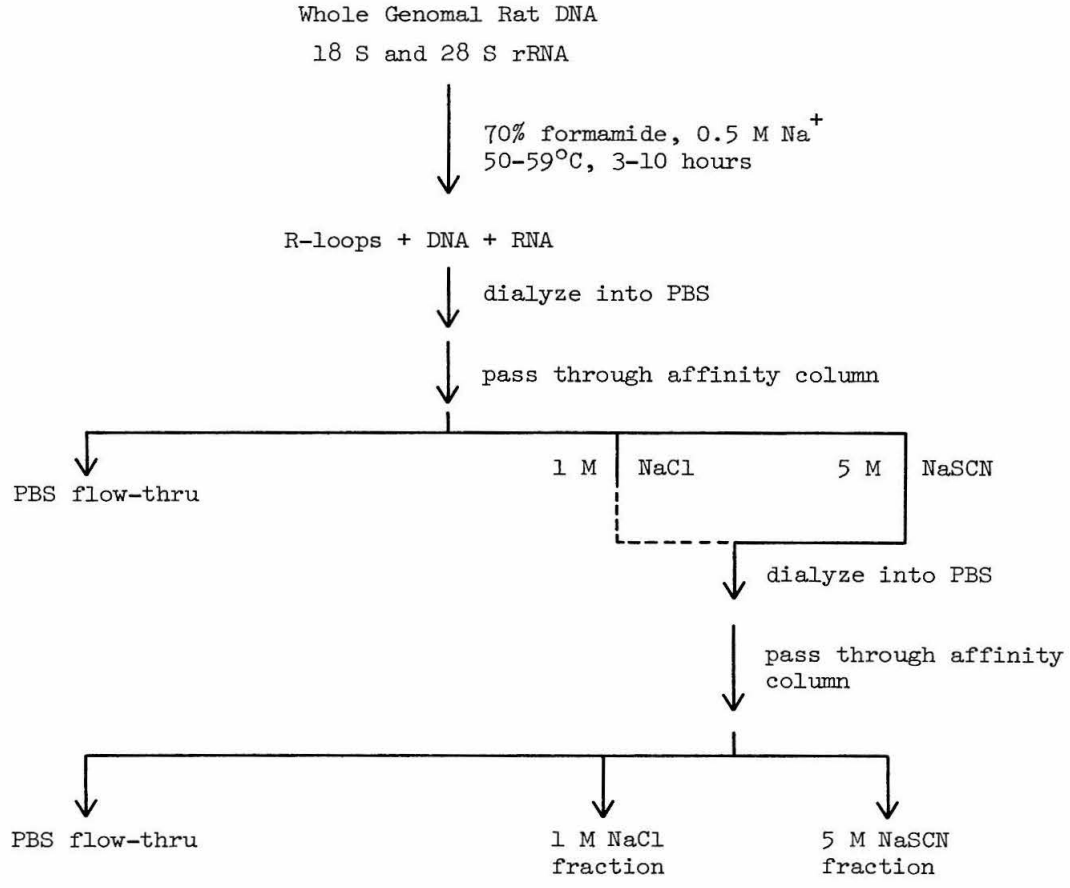

Whole Genomal Rat DNA
18 S and 28 S rRNA
70% formamide, 0.5 M Na^+
50-59°C, 3-10 hours
R-loops + DNA + RNA
dialyze into PBS
pass through affinity column
PBS flow-thru
1 M NaCl
5 M NaSCN
dialyze into PBS
pass through affinity column
PBS flow-thru
1 M NaCl fraction
5 M NaSCN fraction

TABLE I: Enrichment for Rat rDNA by Affinity Chromatography

DNA Sample	% of Recovered DNA		Fraction of DNA Hybridized x 10^4		Fold Enrichment Relative to Whole DNA	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Whole unfractionated DNA	-	-	0.53	0.47	1.00	1.00
1st passage PBS flow-thru	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-thru	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.

original DNA was eliminated as non-binding DNA in the first cycle of purification. During the second cycle, a significant but smaller amount of DNA was eliminated as non-binding 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 sub-fractions 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 $Rot_{1/2}$ of the reaction; 2) only the coding DNA strand is hybridized, so the numbers

need to be corrected to take the non-coding 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, 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 is consistent with 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 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 mean lengths and standard deviations were calculated for the two loops and for the spacer between the loops based upon the data used in the construction of Figure 4.

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 represents 0.5 μm.

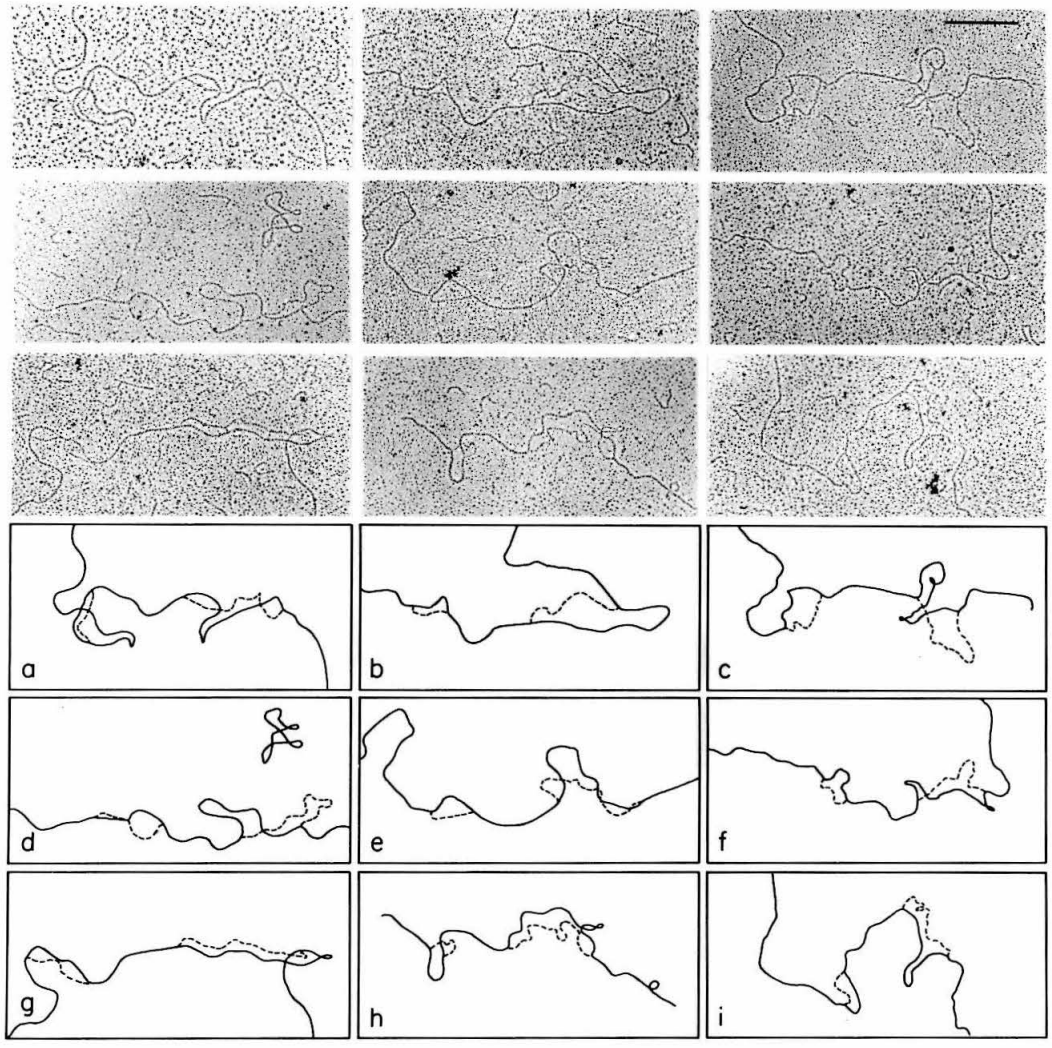
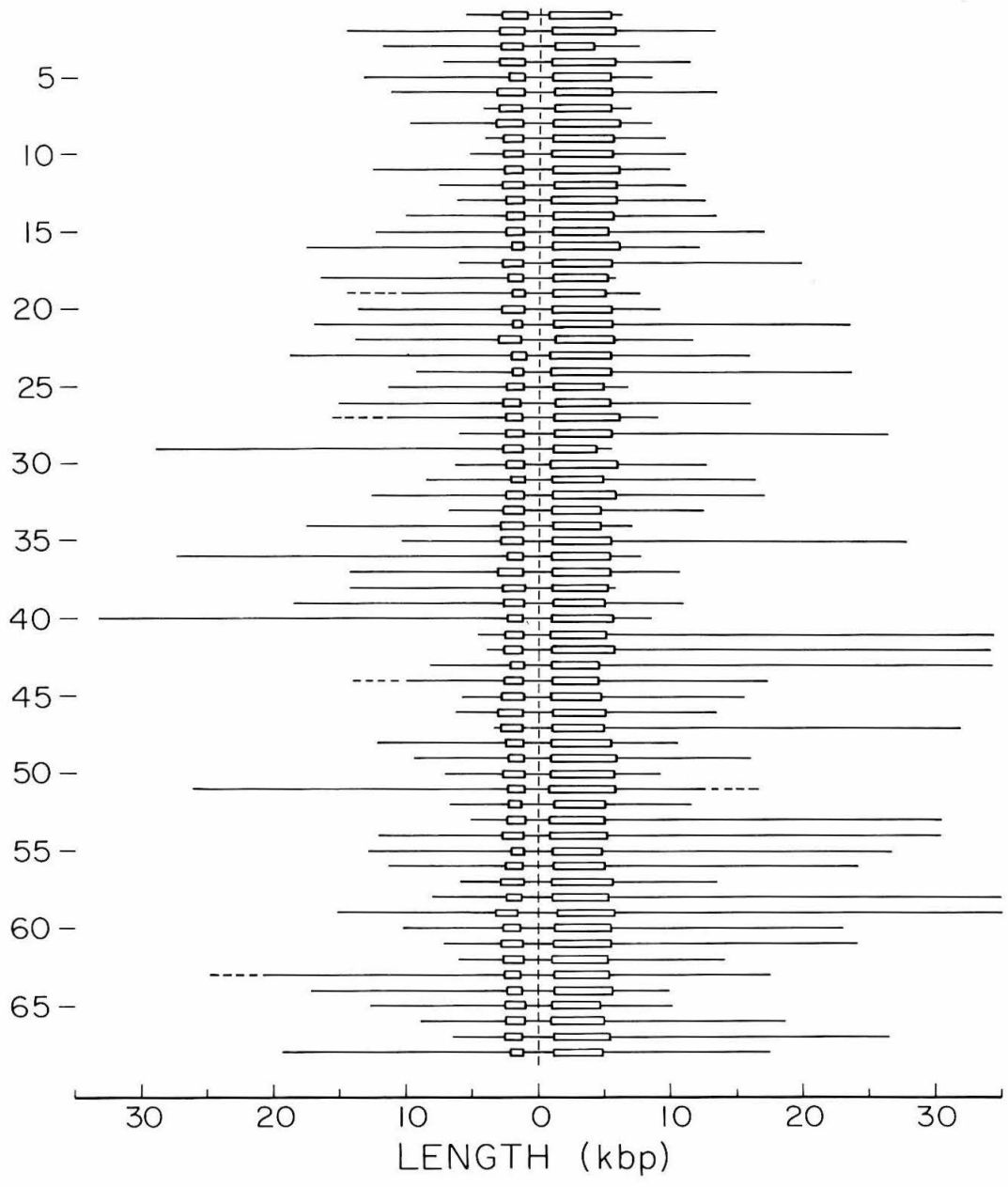

a
b
c
d
e
f
g
h
i

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 mid-point of the inter-loop 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 twenty 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.


5
10
15
20
25
30
35
40
45
50
55
60
65
30
20
10
0
10
20
30
LENGTH (kbp)

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 and Dawid, 1973; Loening, 1968; Attardi and Amaldi, 1970), the 18S and 28S rRNAs would be expected to have lengths of approximately 2.1 and 5.4 kb respectively (Cory and 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 and Hogness (1977) and by Thomas, White and Davis (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 Experiment 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 and Adams, 1977) would indicate the 2.24 kbp value may be the more accurate figure.

Determination of the Rat rDNA Repeating Unit. Occasionally molecules were seen in the electron micrsocope 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 mid-point 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 eleven molecules give direct 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 eleven molecules is 37.2 $\pm$ 1.3 kbp. This standard deviation is 3.5% of the mean length. Populations of SV40 molecules, which are all presumably 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 kbp, cannot be positively ruled out. In Figures 6 and 7, two examples are shown of molecules which span an entire repeating unit and have R-loops near each end.

FIGURE 5: Schematic diagram of long rat rDNA molecules. All molecules containing at least two intact R-loops were measured. The first eleven molecules each contain three or four R-loops, or else two R-loops separated by a long spacer. The mean repeating unit length calculated for these eleven 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.

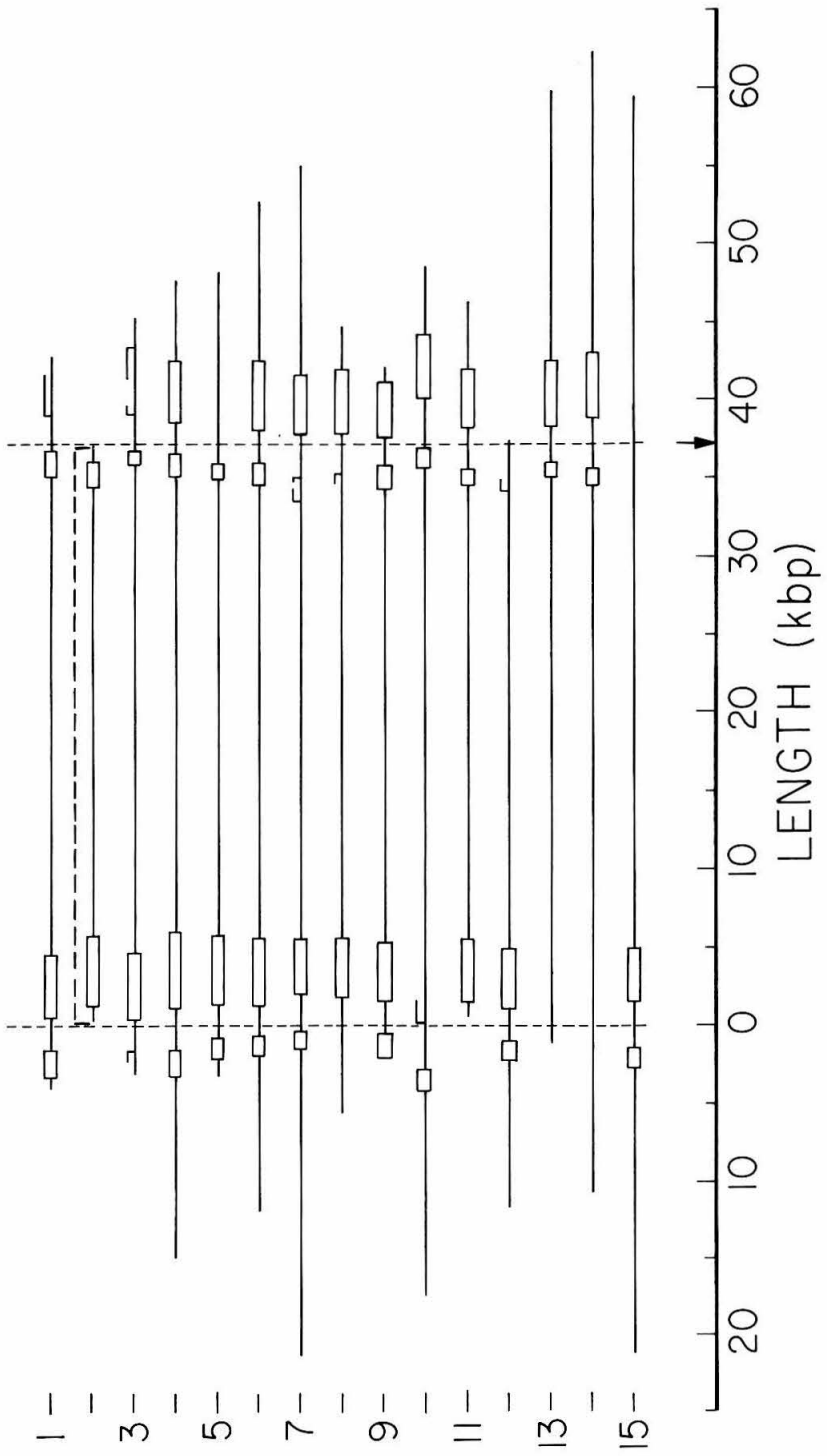

1
3
5
7
9
11
13
15
20
10
0
10
20
30
40
50
60
LENGTH (kbp)

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.

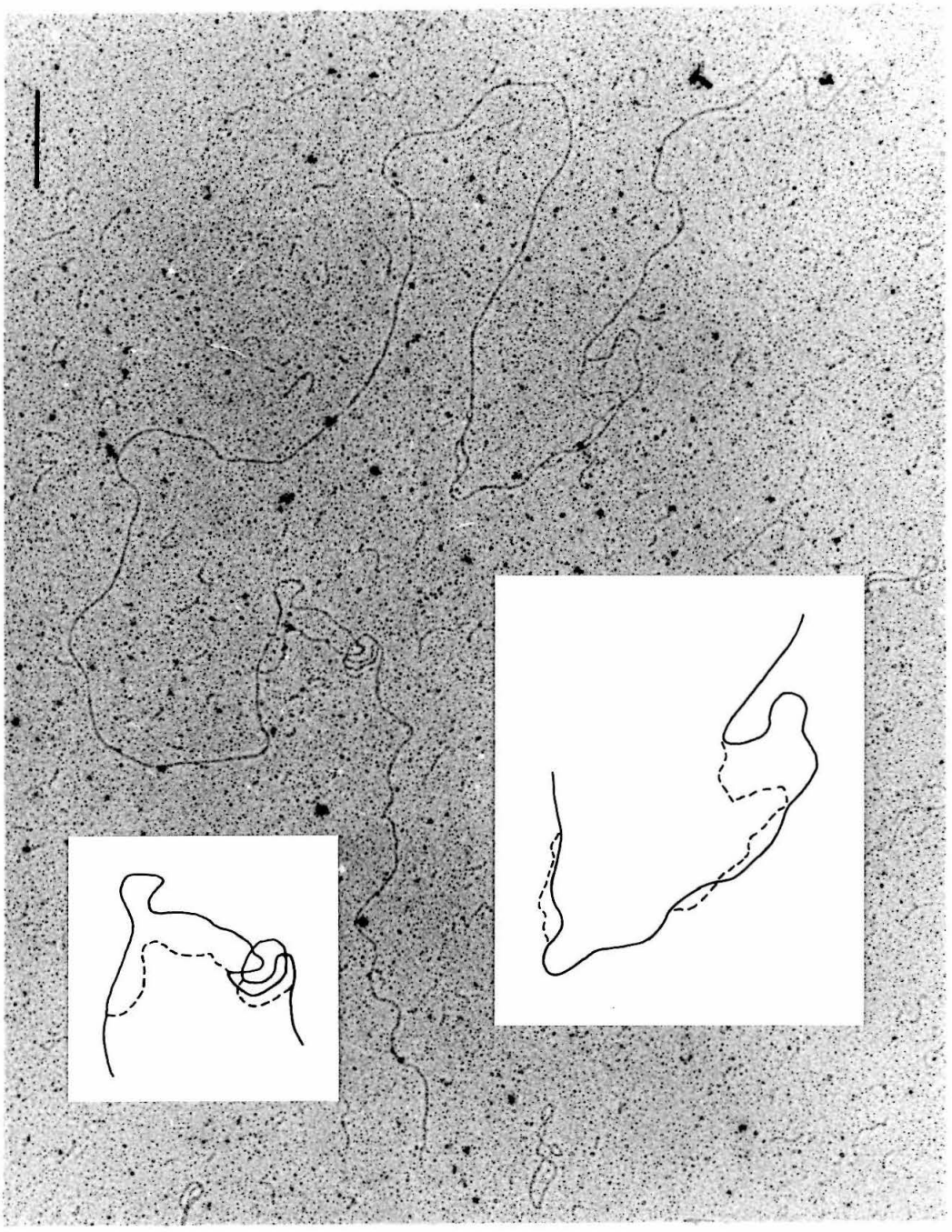
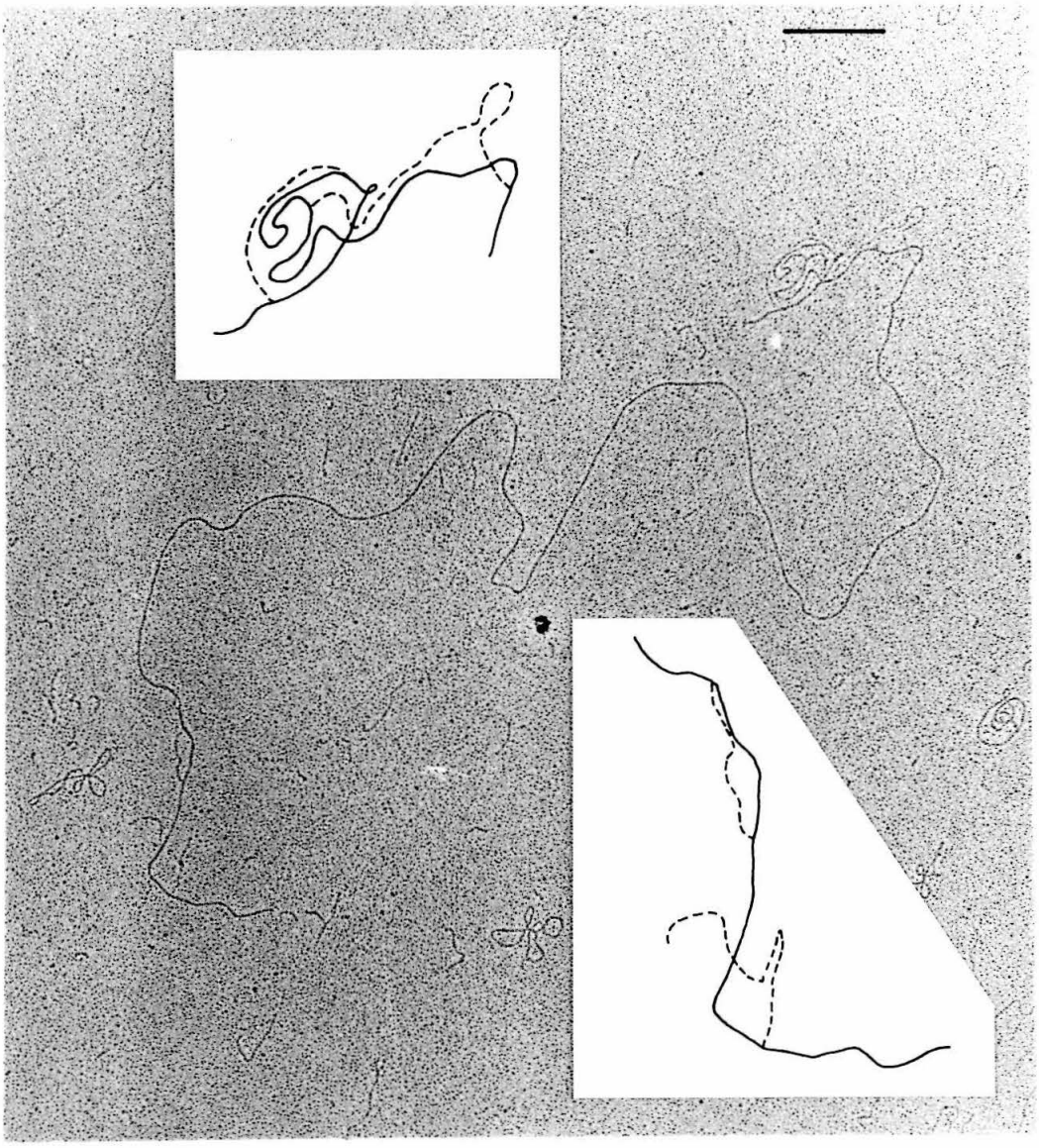


FIGURE 7: A long rat rDNA molecule containing an R-loop doublet, a long spacer, an 18S R-loop, and an incomplete 28S R-loop. Interpretations of the R-loops are shown in the insets at a higher magnification. The bar represents 0.5 μm.



Molecule 12 in Figure 5 is barely long enough to span a 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. This could be the case for molecules 13, 14, and 15. A second possibility is that the 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, and since mammalian rRNA genes are dispersed on several different chromosomes (Elsevier and Ruddle, 1975; Henderson et al., 1972), one or two such repeating units would be expected to occur in a population of fifteen randomly selected molecules. Thus the absence of R-loops on these molecules is not firm evidence for the existence of longer repeating units.

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 longer repeating units were common, a larger number of molecules like 13, 14, and 15 probably

would have been observed. 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.

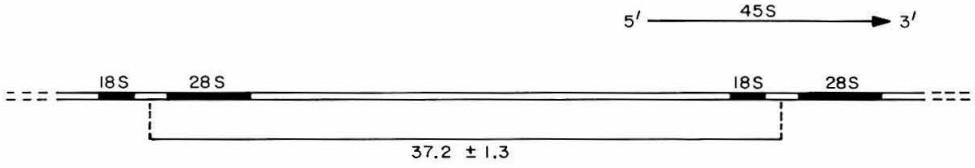
Molecule 2 of Figure 5 possessed a unique structure in the electron microscope where it existed as a circular molecule. In Figure 5 it was split between the R-loops and linearized to emphasize the length of the repeating unit. The circular structure observed in the electron microscope does not necessarily mean that this was an individual circular molecule in its native state. Due to single-strand nicks and potential complete denaturation over short stretches of the DNA during the incubation for R-loop formation, complementary single-stranded "sticky ends" could be generated 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, I propose the model presented in Figure 8 for the structure of rat rDNA. From the published data for the rat (Schibler et al., 1975; Liau and Hurlbert, 1975, taking

FIGURE 8: 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.

STRUCTURE OF RAT rDNA



into account Dawid and Wellauer, 1976) and by analogy to other eucaryotic systems (for references see Dawid and Wellauer, 1976), I have placed the transcription unit of the 45S rRNA precursor 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 for the 28S rRNA by an intervening DNA region which apparently does not code for rRNA (Glover and Hogness, 1977; Pellegrini et al., 1977; Wellauer and 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 and Hogness, 1977). An example of such a possibility is the set of R-loops furthest to the left 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 apparent 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 4. In this case, the possibility cannot be entirely discounted, but the R-loop would be unusually small if interpreted in that way. Thus, no firm evidence for intervening sequences within the rRNA coding regions could be found.

If such sequences do exist in rat rDNA, they are either probably rare or 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 Hind III digested nuclear DNA probed with radioactive RNA, Arnheim and Southern (1978) assigned repeating unit lengths of 38 and 31 kbp to mouse and human rDNA respectively. Using the same enzyme, the value determined by Cory and Adams (1978) 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 Hind III 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 Hind III 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 Hind III 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, care should still be taken in assigning complete lengths to rDNA repeating units of other organisms based solely on rRNA hybridizations to blots of restriction enzyme digested DNA in the absence of additional confirmatory evidence. For these reasons, the direct electron microscopic measurements of rat rDNA repeating units described in this report contribute significantly to our understanding of mammalian rDNA structure.

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CHAPTER III

Antibodies to Proteins Dissolved in Sodium Dodecyl Sulfate

Introduction

In recent years there has been a considerable increase in the successful use of antibody techniques to study the distribution of proteins of interest in situ. Studies have been done both at the light microscope level, utilizing immunofluorescence, and at the electron microscope level, using conjugated ferritin or peroxidase. Examples include the localization of muscle proteins within the sarcomere in thin sections of muscle (Levine et al., 1972), and the localization of spectrin on the inner surface of red blood cell membranes (Nicolson et al., 1971). The technique should lend itself to studies of complex structures, such as membranes, viruses, and chromosomes, where further information on the distribution of protein components is needed. In many cases these complex structures have been dissociated and the proteins isolated only in the presence of sodium dodecyl sulfate (SDS) or other strongly denaturing solvents (Marushige et al., 1968; Elgin and Bonner, 1970; Fairbanks et al., 1971; Laemmli, 1970). It would therefore be very useful to be able to prepare antibodies to proteins dissolved in SDS. I have explored this possibility.

Materials and Methods

Antigens. Chromatin was prepared from 6 to 18 hr old wild-type Drosophila melanogaster embryos according to the method of Elgin and Hood (1973), an adaptation of the general method of Bonner et al. (1968). Sheared chromatin in 0.01 M Tris, pH 8, was used directly as an immunogen at 0.5 to 1.0 mg DNA/ml. The nonhistone chromosomal protein (NHC protein) fraction was prepared from sheared chromatin as follows. The histones were extracted in 1.6 N NaCl-0.2 N HCl. The precipitated DNA-NHC protein complex was redissolved in 0.05 M Tris,

pH 8, 1% SDS at a concentration of 1.5 mg DNA/ml, dialyzed against three changes of 0.01 M Tris, pH 8, 0.1% SDS, and the DNA removed by centrifugation (Elgin and Bonner, 1970). The NHC proteins were used to immunize rabbits either directly or following 3- to 8-fold concentration and re-dialysis to 0.01 M Tris, pH 8, 0.1% SDS. Samples were concentrated under nitrogen in an Organomation Association N-Evap at 37°C. Crystallized and lyophilized BSA (Sigma Chemical Company, St. Louis, Mo.) was used as an immunogen after dissolving in 0.01 M Tris, pH 8, 0.1% SDS. Conalbumin was also from Sigma Chemical Co. SDS (Sipon WD) was purchased from Alcolac Chemical Corp. (Baltimore, Md.).

Immunization. Rabbits were immunized by multiple injections first into the toe pads with complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan) and then intraperitoneally and intravenously without adjuvant. Two rabbits (designated B-1 and B-2) were each injected with 4 mg BSA, three rabbits (N-1, N-2, and N-3) with 9-26 mg NHC proteins, and two rabbits (C-1 and C-2) each with whole chromatin containing approximately 10 mg NHC proteins. NHC proteins and BSA were dissolved in 0.01 M Tris, pH 8, 0.1% SDS. Chromatin was in 0.01 M Tris, pH 8. The quantities of NHC proteins were calculated from the OD_{260} of the chromatin and based on an NHC protein to DNA ratio of 1.19 (Elgin and Hood, 1973). Rabbits were boosted periodically with additional IP and IV injections, and the antiserum from subsequent bleedings was pooled with earlier samples taken from the same rabbit.

Antibody Assays. Agar gel immunoplates, pattern B, for Ouchterlony double-immunodiffusion tests were purchased from Hyland Laboratories, Costa Mesa, California, and used to examine the antisera for precipitating antibodies.

A single-diffusion test combined with polyacrylamide gel electrophoresis was developed whereby reacting antigens could be detected and localized within SDS phosphate polyacrylamide gels. Following electrophoresis of NHC proteins or BSA on gels (as described in Elgin and Bonner, 1970) some gels were stained with Coomassie Blue, and others were immediately placed in tubes to incubate for two days with enough antiserum to completely surround the gel (ca. 1 ml). Gels were then washed for two days in 30 ml of 0.01 M sodium phosphate buffer, pH 7.2, 0.9% NaCl, with a single change after 24 hr. Gels were then incubated for 2 to 3 days in ca. 1 ml of a 10-fold dilution of Pentex FITC-conjugated IgG fraction of goat antiserum directed against rabbit IgG (Miles Laboratories, Kankakee, Ill., Lot No. 16). The gels were then washed for 2 days in phosphate saline, and pictures taken by time exposure under UV light. Coomassie Blue stained gels and the negatives from pictures of gels treated with FITC-conjugated anti-rabbit IgG were scanned on a Gilford spectrophotometer. Scanning of both gels and negatives was done at 600 nm. For optimal results, the amount of sample loaded on gels to be stained with Coomassie Blue was about one half that loaded on gels to be treated with antiserum.

Results

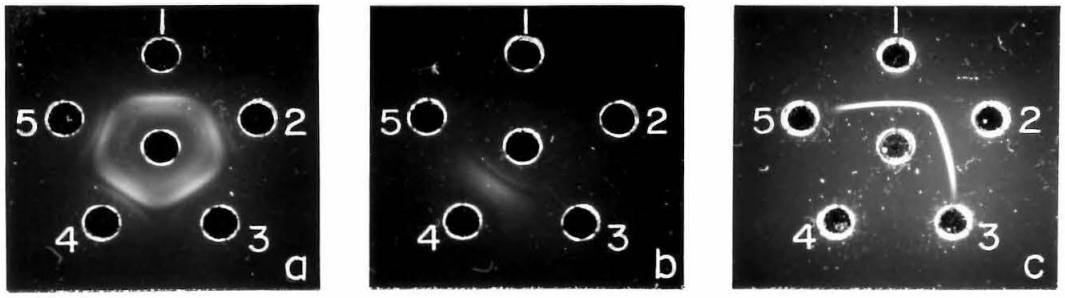
All of the rabbits immunized with either NHC proteins in 0.1% SDS or chromatin in 0.01 M Tris, pH 8, produced antibodies which react with NHC proteins in 0.1% SDS. The two rabbits immunized with BSA in 0.1% SDS produced antibodies which react with BSA in either 0.1% SDS or buffered saline. Figure 1-a shows the results of a double-diffusion experiment in which total NHC proteins in the center well were challenged with antisera from the three rabbits immunized

FIGURE 1: Immunodiffusion demonstrating the specific reaction between NHC proteins or BSA with antibodies raised in rabbits against chromatin in 0.01 M Tris, against NHC proteins in 0.1% SDS, or against BSA in 0.1% SDS.

a) Center well: NHC proteins in 0.01 M Tris, pH 8, 0.1% SDS. (1) Antiserum from rabbit N-1 to NHC proteins. (2) Antiserum from rabbit N-2 to NHC proteins. (3) Antiserum from rabbit N-3 to NHC proteins. (4) Antiserum from rabbit C-1 to chromatin. (5) Antiserum from rabbit C-2 to chromatin.

b) Center well: Antiserum from rabbit N-2 to NHC proteins. (1) and (5) BSA, 1 mg/ml in 0.01 M Tris, pH 8, 0.1% SDS. (2) and (3) BSA, 1 mg/ml in 0.01 M sodium phosphate, pH 7.2, 0.9% NaCl. (4) NHC proteins.

c) Center well: Antiserum from rabbit B-1 immunized with BSA in 0.1% SDS. (1) BSA, 1 mg/ml in 0.01 M Tris, pH 8, 0.1% SDS. (2) BSA in 0.01 M sodium phosphate, pH 7.2, 0.9% NaCl. (3) Conalbumin, 1 mg/ml in 0.01 M sodium phosphate, pH 7.2, 0.9% NaCl. (4) Conalbumin, 1 mg/ml in 0.01 M Tris, pH 8, 0.1% SDS. (5) NHC proteins.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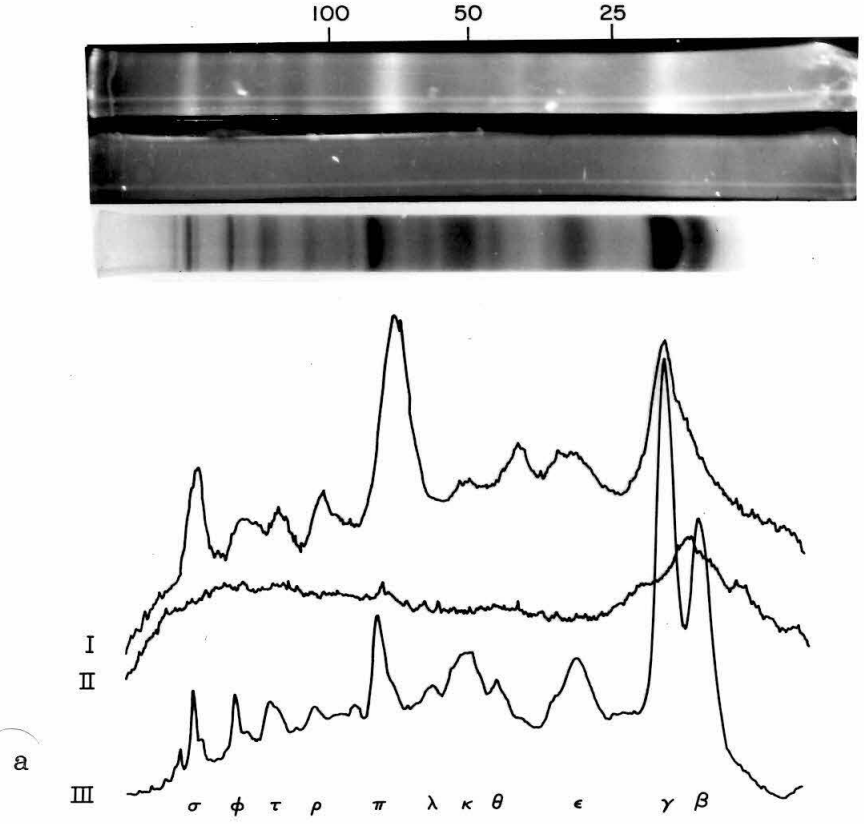
with NHC proteins and the two rabbits immunized with chromatin. Both methods of immunization seem to yield antisera which react approximately equally as well with total NHC proteins in Tris-SDS. The specificity of the reaction is shown in Figures 1-b and 1-c. In Figure 1-b it can be seen that the antiserum in the center well made against NHC proteins reacts with NHC proteins, but not with BSA dissolved in either phosphate-saline or Tris-SDS. Finally, Figure 1-c shows that antiserum made against BSA in Tris-SDS reacts with BSA dissolved in either Tris-SDS or buffered saline, but not with conalbumin in either solvent, nor with NHC proteins in Tris-SDS. This demonstrates that the antibodies are specific and not directed against a random denatured conformation of an SDS-protein complex.

The antisera to NHC proteins and to chromatin react with many of the protein components of this complex mixture. This can be seen from the experiments in which NHC proteins were subjected to SDS polyacrylamide disc-gel electrophoresis, followed by immersion of the gel in antiserum. After about 4 hr of incubation or longer, precipitin bands began to form in selected areas of the gel, indicating major reacting components. The gel could then be washed free of unbound antiserum. In many cases a band of nonspecific precipitation was observed at the running front of the gel. This nonspecific precipitin band was also observed in gels incubated in normal rabbit serum. The basis for this nonspecific precipitation at the running front is not understood. Subsequent incubation of gels with FITC-conjugated anti-rabbit IgG resulted in the specific staining of the precipitin bands, and also resulted in most cases in the appearance of new minor precipitin bands which had not been visible previously. Following removal of nonreacted FITC-conjugated anti-rabbit IgG, the stained gels could be observed under UV

light. The nonspecific precipitin bands at the running fronts were observed to take up only a minor amount of stain.

Results from this type of analysis are shown in Figure 2. Figure 2-a compares three gels, all of which were loaded with NHC proteins. Three major and six minor bands are resolved when an NHC protein gel is placed in antiserum from rabbit N-1 followed by treatment with the FITC-conjugated anti-IgG. This pattern can be compared to that of the gel stained with Coomassie Blue. When gels were incubated with antisera from other rabbits immunized with either Drosophila NHC proteins or chromatin, the results were similar. However, frequently one or several of the minor bands were missing. The three major bands, one at very high molecular weight, one in the middle region, and one of low molecular weight were always present but varied in intensity. When NHC protein gels were placed in antiserum made to BSA in SDS, only the nonspecific precipitin band at the running front of the gel was observed, and this was not as intense as that in the specifically stained gels. Further evidence of the specificity is presented in Figure 2-b. In this case, the gels were loaded with BSA, and again specific staining occurred when the gels are treated with the BSA-directed antiserum. No staining is observed with antiserum directed against NHC proteins. In further experiments (not shown) with the BSA system it was observed that the intensity of the specific precipitin band was decreased by either decreasing the sample load, or by diluting the antiserum. In this case, no band developed when less than 0.33 μg of BSA was loaded on the gel, or when the antiserum was diluted more than 64:1.

FIGURE 2: Antibody antigen reactions in SDS polyacrylamide gels. NHC proteins (a) or BSA (b) were subjected to SDS polyacrylamide gel electrophoresis. All gels run left to right. Gels III and VI were stained with Coomassie Blue, and scanned. Gels I and V were immersed in antiserum from rabbit N-1 which had been immunized with NHC proteins. Gels II and IV were immersed in antiserum from rabbit B-1, which had been immunized with BSA. Gels I, II, IV, and V were all treated with FITC-conjugated anti-rabbit IgG. Pictures of these gels were taken under UV light, and the negatives of the pictures were scanned. Fluorescent bands appear only when gels are treated with homologously-directed antiserum.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III
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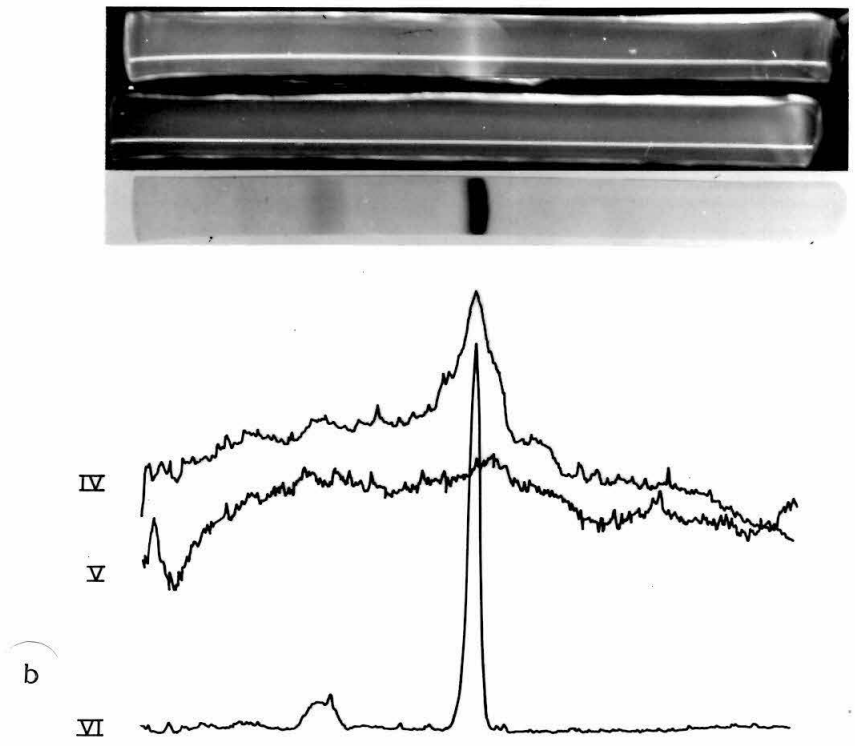
a


IV
V
VI
b

Discussion

These results indicate that antibodies can be raised against isolated proteins and complex protein mixtures dissolved in low concentrations (0.1%) of SDS. In the case of the antibodies made against BSA in 0.1% SDS, these were found to react equally as well by Ouchterlony double-diffusion with either native BSA or BSA in 0.1% SDS. The same type of direct test could not be carried out for the NHC proteins, as they were characteristically isolated by techniques involving exposure to SDS. However, it was shown that antibodies made either to native chromatin or to isolated NHC proteins in SDS react with the isolated NHC proteins in SDS. In all cases, the antibodies were specific to the protein used for immunization and not to a random SDS protein complex, as they failed to react with any other proteins dissolved in SDS that were tested. In summary, immunization with antigen in a 0.1% SDS solution seems neither to interfere with the production of antibodies nor with their general specificity. Indeed, no interference was detected in their ability to react with native antigen. These observations are in agreement with the report of Shanmugam et al. (1972) of the preparation of specific antibodies to viral antigen complexes disrupted with 0.1% SDS.

A technique involving the incubation of SDS polyacrylamide gels in antiserum was developed for assessing the diversity of the antibody response against a heterogeneous mixture of antigens. The results indicated that when total NHC proteins were used as antigen, antibodies were produced against at least several of the components. In work where it is desirable to have antibodies against a single NHC protein, this technique will be useful in confirming that the antibodies are directed against that single component. Similar analyses of protein populations separated on gels have been used for other purposes; for example, to assess the

heterogeneity of the antibody population produced against a single hapten one may "stain" isoelectric focusing gels with radioisotope-labeled hapten (Williamson, 1971). In some instances it has been found necessary to fix the proteins in the gel before the inward diffusion of other macromolecules (Keck et al., 1973). Fixation was not found to be required in the present case; the use of such a process could possibly increase the sensitivity, but was not investigated in this work. The experiments reported here demonstrate specific antibody-antigen interactions in polyacrylamide gels containing SDS; the technique should be useful in working with complex protein mixtures commonly analyzed on these gels (viruses, membranes, etc.).

This report shows that it is possible to raise antibodies against Drosophila NHC proteins which have been exposed to 1% SDS, and then dialyzed against 0.1% SDS. The use of these immunologic reagents should contribute to our understanding of the role of these proteins in chromosome structure and function.

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