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

William E. Stumph, Jung-Rung Wu, and James Bonner*

ABSTRACT: Antibodies, shown to be specific for DNA/RNA hybrids, have been covalently attached to CNBr-activated Sepharose. The resultant affinity resin specifically binds DNA/RNA hybrids and has been used to enrich for the DNA which codes for rRNA in the slime mold *Dictyostelium discoideum*. By utilizing the technique of R-loop formation, DNA molecules containing the rRNA genes were isolated from total nuclear DNA in a double-stranded form. These rDNA molecules, which were recovered by high salt elution from the affinity resin, were typically 15–40 kbp in length, and thus contained DNA sequences adjacent to the selected sequences coding for the 17S and 26S rRNAs. In addition, evidence has been obtained concerning the structure of *Dictyostelium* rDNA which agrees with the finding (Taylor et al. (1977) *ICN-UCLA Symp. Mol. Cell. Biol. 8,* 309–313) that the rDNA molecules are not covalently attached to the chromosomes of this organism.

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 nearby 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 & Dawid, 1977; Woo et al., 1977; Anderson & Schimke, 1976; Dale & 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 & Suzuki, 1977).

We have therefore explored the feasibility of using antibodies to DNA/RNA hybrids (Stollar, 1970; Rudkin & 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.

We describe here our results with the rRNA genes of the slime mold *Dictyostelium discoideum* as an initial model system for demonstrating the enrichment of specific genes using the antibody affinity column. This system was chosen because the rDNA[1] 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 kpb. Thus, a considerable amount of adjacent DNA was copurified with the coding sequences. We have also obtained additional evidence 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 extrachromosomal.

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, Mass.) and 125 μg of methylated bovine serum albumin (Calbiochem, San Diego, Calif.) in complete Freund's adjuvant. The injection was repeated 2 weeks later in incomplete Freund's adjuvant, followed a week later and at monthly intervals thereafter by an intravenous injection without adjuvant. The rabbits were bled 3–10 days after each 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 am-

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[1] Abbreviations used: rDNA, DNA, including adjacent spacer regions, which codes for rRNA; kbp, kilobase pairs; PBS, phosphate-buffered saline; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid.

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monium 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 × 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 reequilibration of the columns with 2/3 × PBS, the IgG which did not bind on the first passage was reapplied 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, Mo.) and ^{3}H-labeled 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, N.Y.) 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 was 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 incubating at 56 °C for 1 h 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 & Berg, 1964) in which 200 μg of heat-denatured ^{3}H-labeled rat DNA was transcribed with 500 units of *Escherichia coli* RNA polymerase for a period of 90 min at 37 °C, using 5′-ribonucleotide triphosphates in tenfold 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 was 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 [^{3}H]thymidine-labeled *Dictyostelium discoideum* cells, strain AX-3, grown in liquid culture, using a combination of published methods (Bakke, 1978; Firtel et al., 1976; Firtel & 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 °C, 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*g* for 10 min. The RNA was ethanol precipitated, redissolved in water, heated at 60 °C for 1 min to denature 26–5.8S rRNA complexes, and centrifuged on 15–30% linear sucrose gradients containing 0.05% $NaDodSO_4$, 0.1 M NaCl, 0.01 M Tris (pH 7.6) in a Beckman SW27 rotor at 26.5 krpm for 15 h 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 was 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 reequilibrated with PBS. The DNA which did not bind on the first passage was then reapplied 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

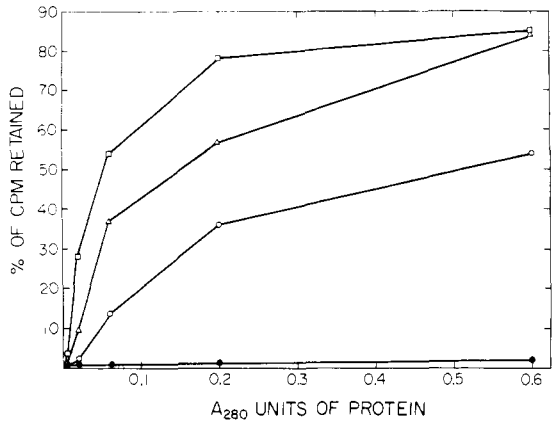

FIGURE 1: Antibody activity assayed using poly(rA)·poly(dT). Antibody activity is measured by incubating 0.2-μg aliquots of tritium-labeled poly(rA)·poly(dT) with increasing amounts of IgG or serum. Antibody–antigen complexes which are formed are retained on glass fiber filters. Each point represents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

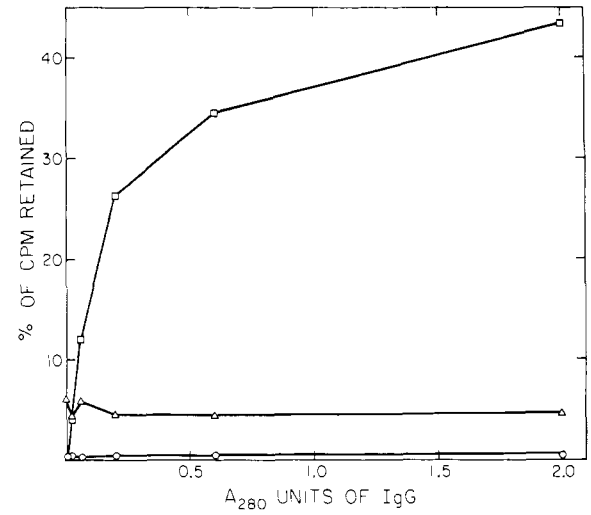

FIGURE 2: Specificity of the IgG after adsorption. In order to assay the antibody specificity, 0.2-μg aliquots of various tritium-labeled nucleic acids were incubated with increasing amounts of the adsorbed, purified IgG fraction. The formation of nucleic acid–antibody complexes was assayed by retention on filters. Each point is the average of two separate determinations. (□) Rat DNA/RNA hybrid; (△) native rat DNA; (○) 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).

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 s in 5×10^{-5} M uranyl acetate in 90% ethanol and rinsed in methylbutane. The grids were rotary shadowed with platinum and palladium (80/20) and viewed in a Phillips 300 electron microscope. 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 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 nonhybrid 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 four- to sevenfold 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 exhibited 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 nonhybrid 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 & Berg, 1964), and about 70% of the DNA molecules in the preparation were believed to contain regions of DNA/RNA hybrid 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} unit). 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 nonhybrid 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 seem 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 was 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

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

type of nucleic acid	% eluted in: PBS	1 M NaCl	NaSCN 3 M	NaSCN 5 M	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 reapplied to column)	98.7	0.2	0.1	0.8	1.1

[a] Two to five micrograms of ^{3}H-labeled nucleic acid was 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.

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 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 reapplied to the affinity column, less than 5% of it rebound. 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 rebound 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 reapplied 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

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:	% obsd 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 was 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.

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 & 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 nontranscribed 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 were 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, 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, 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 nonspecific 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 kpb 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. We have examined this question 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 ± 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.

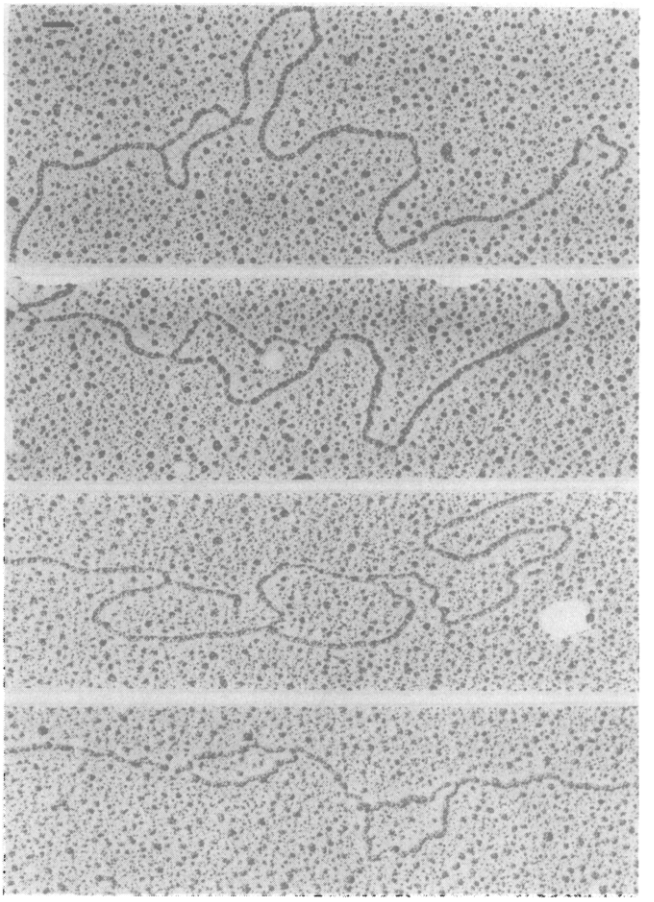

FIGURE 3: Coding regions of *Dicytostelium discoideum* rDNA molecules purified by affinity chromatography. Examples are shown of DNA molecules which contain one or two R loops, or a R loop plus a branch. The bar represents 0.1 μm.

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 extrachromosomal 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 extrachromosomal rDNA of at least two other lower eucaryotes, *Tetrahymena* (Karrer & Gall, 1976) and *Physarum* (Vogt & Braun, 1976).

Discussion

The immunization of rabbits with the synthetic hybrid

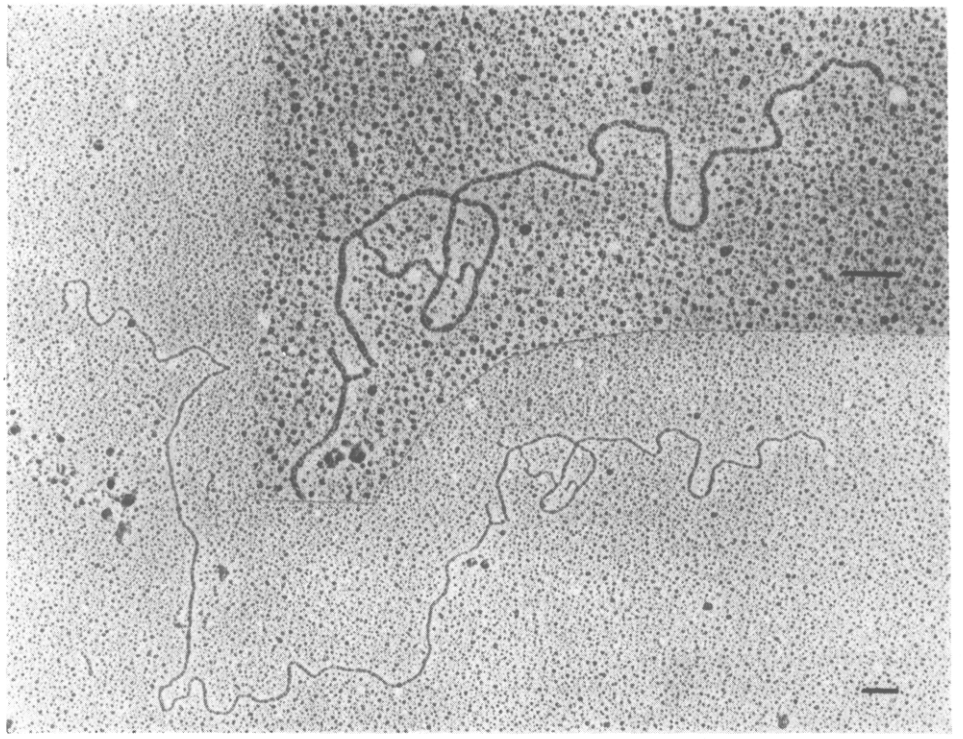

FIGURE 4: A 36-kbp long rDNA molecule containing an R loop plus a short branch. The insert shows the R-loop region at a higher magnification. The bars represent 0.2 μm.

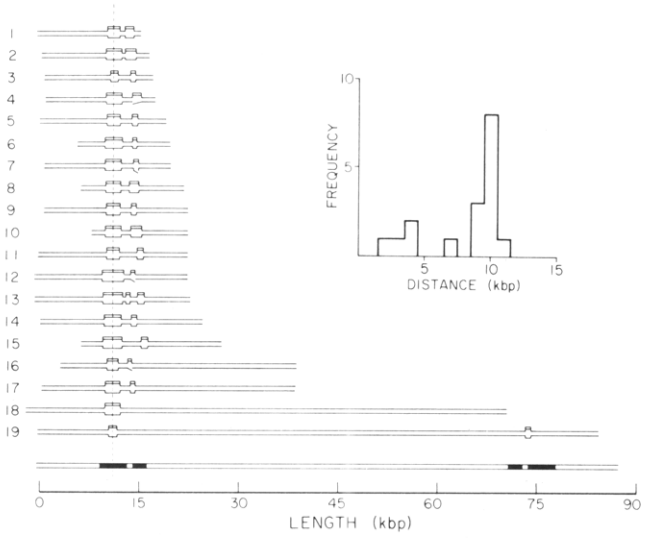

FIGURE 5: Map of *Dictyostelium discoideum* rDNA molecules which were isolated by affinity chromatography. The molecules are arranged by aligning the centers of the large R loop at a common vertical line. An idealized molecule predicted by the inverse-repeat model of Taylor et al. (1977) is represented 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.

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 & Stollar, 1977; Rudkin, 1977). To increase the specificity for DNA/RNA hybrids, we 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 (our unpublished results; Rudkin & Stollar, 1977). The specific activity of antibody in our 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 nonspecific 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 nonspecific 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 three to four 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 nontranscribed spacer regions of *Dictyostelium* rDNA are known to be A-T rich (Firtel et al., 1976; Firtel & 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, our data 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 data presented in Table II demonstrate the successful enrichment of *Dictyostelium discoideum* rRNA genes from total nuclear DNA. The rRNA genes of this slime mold were chosen as the initial system for studying the feasibility of gene enrichment using the antibody affinity column technique because they represent an unusually large percentage of the nuclear DNA of this organism. This permitted the rDNA sequences to be enriched to greater than 80% purity by a single passage through the affinity column. 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, recycling 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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Escherichia coli RNA Polymerase–Rifampicin Complexes Bound at Promoter Sites Block RNA Chain Elongation by *Escherichia coli* RNA Polymerase and T7-Specific RNA Polymerase†

George A. Kassavetis, Kathleen M. Kaya, and Michael J. Chamberlin*

ABSTRACT: *Escherichia coli* RNA polymerase that has been complexed with the drug rifampicin is not able to initiate a long RNA chain but still retains its ability to bind at promoter sites on T7 DNA. When conditions are such that the inactive enzyme forms "open" promoter complexes on T7 DNA, that enzyme serves as a barrier to transcription through the promoter site by RNA polymerase molecules which have initiated transcription on the same DNA strand from distal upstream promoters. Both *E. coli* RNA polymerase and the T7 phage-specific RNA polymerase are sensitive to blockage by these *E. coli* RNA polymerase–rifampicin open complexes. When inert bacterial RNA polymerase is not present on T7 DNA, there is no detectable termination by RNA polymerase molecules which traverse a promoter site in the course of transcription. The ability of *E. coli* RNA polymerase–rifampicin complexes to block chain elongation by RNA polymerase transcribing from distal promoters probably accounts for the coincidence of minor promoter and "terminator" sites on T7 DNA in the studies of Minkley and Pribnow [Minkley, E. G., and Pribnow, D. (1973), *J. Mol. Biol. 77,* 255–277] and can lead to some difficulty in the interpretation of in vitro transcription patterns where overlapping transcription units are present. Since bacteriophage T7 induces inhibitors which bind to and inactivate the bacterial RNA polymerase, it is possible that a similar kind of blocking phenomenon involving binding of inert *E. coli* RNA polymerase at T7 minor promoters plays a regulatory role in the turn off of class I and II T7 RNA synthesis in vivo.

The antibiotic rifampicin is an inhibitor of *E. coli* RNA polymerase which prevents the productive initiation of RNA synthesis but not the elongation of RNA chains (Sippel and Hartmann, 1968). The inhibition of *E. coli* RNA polymerase by rifampicin results from the binding of the drug to the β subunit (Rabussay and Zillig, 1969). This interaction can occur when the RNA polymerase is either free in solution or bound to DNA (Hinkle et al., 1972) but not when the RNA polymerase is in an actively transcribing ternary complex (Eilen and Krakow, 1973).

RNA polymerase that is complexed with rifampicin retains its ability to recognize promoters and to form a stable "open" complex at these sites (Hinkle et al., 1972; Bordier and Dubochet, 1974). This property of RNA polymerase–rifampicin complexes has been used to explain the dominance of rifampicin-sensitive alleles over rifampicin-resistant alleles in *E. coli* strains that are heterodiploid for the *rpoB* gene (Khesin et al., 1971; Ilyina et al., 1971; Austin et al., 1971). It has been shown that rifampicin-inactivated RNA polymerase can bind to promoters and thereby make them unavailable to rifampicin-resistant RNA polymerase (Bordier, 1974).

Recent studies on the in vitro transcription of ϕX174 DNA (Axelrod, 1976) and T7 DNA (Stahl and Chamberlin, 1977) indicate that, when *E. coli* RNA polymerase and rifampicin are employed in RNA synthesis reactions at elevated molar ratios of enzyme to DNA, termination events occur at sites that normally function as promoters which are located downstream from where transcription was initiated. These termination events are believed to result from blockage of RNA chain elongation by RNA polymerase–rifampicin complexes bound at downstream promoters.

In this paper, we examine more precisely the ability of the *E. coli* RNA polymerase–rifampicin complex to block transcription of RNA polymerases which have initiated RNA chains at distal promoters.

Materials and Methods

RNA Polymerases. E. coli RNA polymerase was purified

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received August 14, 1978.* This work was supported by Grant GM12010 from the National Institute of General Medical Sciences.

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