A Method for Gene Enrichment Based on the Avidin–Biotin Interaction. Application to the *Drosophila* Ribosomal RNA Genes†

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ABSTRACT: A method of enriching, from the total DNA of an organism, for long DNA strands carrying a particular gene is described. The purified RNA corresponding to the gene is covalently attached to biotin via a cytochrome *c* bridge. This modified RNA is hybridized to the total DNA. Those DNA strands which hybridize are separated from all the other DNA, using the avidin–biotin interaction, by one of two methods. Avidin is covalently attached to submicroscopic polymer spheres; the complexes of avidin spheres with the DNA:RNA–biotin hybrids band in CsCl at a much lower buoyant density than does free DNA. Alternatively, the DNA:RNA–biotin hybrids are isolated by affinity chromatography on an avidin–solid support column. These methods have been used to prepare long single strands of *Drosophila* ribosomal DNA (rDNA) in high yield and 42 to 80% pure.

The preparation of highly enriched fractions of specific DNA segments from the eukaryotic genome is useful in the study of sequence organization and its relation to gene expression. Many interesting problems require that the DNA segments be long, so that the relation of a gene, or other specific sequence, to its flanking sequences can be studied.

In a number of cases, for clustered, reiterated genes such as the rRNA genes of *Xenopus* and the histone genes of the sea urchin (Wallace and Birnstiel, 1966; Brown and Weber, 1968; Kedes and Birnstiel, 1971; Brown et al., 1971) enrichment is possible by buoyant banding. These procedures require that the DNA segment which is to be selected have a different base composition than the average for the genomic DNA and/or that it contain a repeated sequence with a special affinity for a heavy metal ion or a dye, thus shifting the buoyant density.

In principle, if the RNA coded for by the gene of interest is available in reasonable purity and in sufficient quantity, the straightforward way to enrich for the gene is to make an RNA:DNA hybrid and to devise a procedure in which the RNA:DNA hybrid is separated from all the unhybridized DNA. Several interesting experimental realizations of this general strategy have been described (Shih and Martin, 1973; Anderson and Schimke, 1976; Miller et al., 1974). Because of the recent discovery of the phenomenon of R-loop formation (Thomas et al., 1976), effective techniques for the separation of RNA:DNA hybrids from pure DNA will be useful, at least in some cases, for gene enrichment with duplex DNA as well as with single-stranded DNA.

The isolation of a single copy gene and its flanking sequences in pure form from a typical eukaryotic genome requires enrichment by a factor of 10^5 to 10^6. None of the procedures described so far has been demonstrated to be effective for such a high degree of enrichment with reasonable yield for long DNA. Our goal is to develop such a method. In the present paper, we describe our initial tests on a much easier system: the *Drosophilia* ribosomal RNA (rRNA)[1] genes. In this case, because of the relatively small size of the genome and because the genes are approximately 185-fold reiterated (Tartof and Perry, 1970), the genes plus reiterated spacer sequences constitute about $1/150$ of the genome.

The rRNA genes of *Xenopus laevis* differ sufficiently in base composition from the average for the entire genome so that enrichment by buoyant banding is effective (Birnstiel et al., 1968; Brown and Weber, 1968). In contrast to *Xenopus*, the G + C content of rRNA in *Drosophila* is only slightly higher (42.7%) than the G + C content (41%) of the total DNA (Ritossa et al., 1966; Hastings and Kirby, 1966). Thus, gene enrichment by DNA buoyant methods would probably be difficult. While our work was in progress, Wellauer and Dawid (1977) showed that it is possible to form R-loops with *Drosophila* rRNA and long duplex *Drosophila* rDNA and then purify the DNA with R-loops from the rest of the DNA by buoyant banding, based on the density shift due to the RNA. The density shift for an RNA:DNA hybrid is proportional to the ratio of the length of RNA:DNA hybrid to the length of the flanking DNA sequences; thus, this method is probably limited to long or tandemly repeated genes. It is our hope that the method described below will ultimately be applicable for gene enrichment even for short, nonreiterated genes.

The method is based on the affinity of the small molecule biotin to the protein avidin. Biotin reacts rapidly with avidin to form a very stable complex ($K_{diss} \simeq 10^{-15}$ M; Green, 1963). The overall procedures are summarized as follows.

(a) Biotin is covalently coupled to cytochrome *c* at a ratio of several biotins per cytochrome *c* by an *N*-hydroxysuccinimide ester acylation. The modified cytochrome *c* is then covalently attached to the RNA at a ratio of about 1 cytochrome to 130 nucleotides by formaldehyde cross-links, all as previously described (Manning et al., 1975a).

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[1] Abbreviations used are: rRNA, ribosomal ribonucleic acid; SSC, standard saline citrate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(b) This rRNA-cytochrome *c*-biotin is hybridized to long single strands of *Drosophila* DNA. The problem now is to separate the DNA:RNA-biotin hybrids from the rest of the DNA.

(c) One separation procedure is based on the covalent attachment of avidin to submicroscopic, water-soluble, poly(methyl methacrylate) spheres by a carbodiimide condensation. These polymer spheres, which are made by emulsion polymerization, have a diameter of 58 nm, an estimated molecular weight of about 7.6×10^7, and a buoyant density in CsCl of 1.27 g/cm^3 (Manning et al., 1975a). When DNA:RNA-biotin hybrids are incubated with avidin-spheres, some of the biotin sites on the hybrids bind spheres. The mixture is banded in CsCl. The spheres function as massive floats for those DNA strands (mol wt ca. 2×10^7) to which they are attached. These strands band at a low density relative to the bare DNA strands at $\rho = 1.71$ g/cm^3.

(d) An alternative to step c is conventional affinity chromatography. Avidin is attached to a solid support and the mixture of DNA:RNA-biotin plus unhybridized DNA is slowly passed through a column of this material. We have found that it is convenient and effective to attach the avidin to *N*-hydroxysuccinimide activated Corning controlled pore glass beads as the solid support.

(e) Removal of the DNA from either the DNA:RNA-avidin-sphere complex or the DNA:RNA-avidin-bead column is accomplished by denaturation of the hybrid with sodium hydroxide.

Our electron microscope studies of the arrangement of rRNA genes on the long single strands of rDNA enriched by the above procedure are described in a separate paper (Pellegrini et al., 1977).

Experimental Procedures

DNA and RNA. High molecular weight unlabeled *Drosophila melanogaster* DNA was extracted from a crude nuclear pellet prepared from organisms 16 to 24 h after puparium formation (Manning et al., 1975b).

^{3}H-Labeled *D. melanogaster* DNA was prepared from homogenized embryos cultured in the presence of [^{3}H]thymidine (C. Laird, personal communication). One gram of 0-8-h embryos was collected, washed in sterile H_2O, and dechorionated in 50% Clorox for 1 to 2 min. The embryos were thoroughly rinsed with sterile double distilled H_2O, dispersed in 5 mL of Schneider's medium containing 15% fetal calf serum, and homogenized using 8 strokes with a Type A Dounce homogenizer. The suspension was clarified by centrifugation at 100 rpm in a Sorval SS34 rotor for 1 min at 25 °C. The supernatant was removed and further centrifuged at 900 rpm for 7 min at 25 °C. The resultant pellet was gently resuspended in 1 mL of Schneider's medium–15% fetal calf serum containing 100 μL of [^{3}H]thymidine (67 Ci/mmol) and incubated at 25 °C for 24 h. The solution was adjusted to 0.5% sodium dodecyl sulfate–0.5 M $NaClO_4$–0.15 M NaCl–0.005 M Tris–0.05 M EDTA (pH 8.5) by adding appropriate volumes of 20% sodium dodecyl sulfate–5.0 M $NaClO_4$ and 0.45 M NaCl–0.015 M Tris–0.15 M EDTA (pH 8.5). DNA was then extracted as previously described (Manning et al., 1975b). The specific activity of a typical preparation of ^{3}H-labeled DNA was 2.5×10^5 cpm/μg under our standard counting conditions. ^{3}H-Labeled *E. coli* and pSC101 DNA were generous gifts from Dr. Mark Guyer.

The preparation of covalently closed pDm 103 plasmid DNA from *E. coli* K12 strain HB101 (pDm 103) was according to Glover et al. (1975). Closed circular pDm 103 DNA was converted to open circular DNA by exposure to x radiation (Sharp et al., 1970).

^{3}H-Labeled *E. coli* DNA, coupled with cytochrome *c*-biotin, was prepared as described previously (Manning et al., 1975a) with the following modification: prior to dialysis against 0.01 M triethanolamine (pH 7.8), the DNA was sheared in 0.12 M sodium phosphate (pH 6.8) in a Virtis 60 blender at 14 krpm for 30 min at 4 °C. After shearing the single-strand weight average length of the DNA was measured as 2.2 kb by zone sedimentation through an alkaline 5–20% sucrose gradient.

The preparation of the *Drosophila* rRNA and its modification with cytochrome *c*-biotin have been described previously (Manning et al., 1975a).

Covalent Coupling of Avidin to a Solid Support. The solid support material used is the *N*-hydroxysuccinimidyl ester of glycophase controlled pore glass beads (Pierce Chemical Co.). One gram of the support material was added to 15 mL of 0.1 M $NaHCO_3$ containing 20 mg of avidin (Sigma Chemical Co.) at 4 °C. The mixture was degassed in vacuo, gently agitated for 12 to 16 h at room temperature, and stored at 4 °C. The number of biotin binding sites per cubic centimeter of avidin-glass beads was assayed by the addition of [^{14}C]biotin, followed by repeated washes with 1.0 M NaCl–0.005 M Tris–0.001 M EDTA (pH 8.5). One cubic centimeter of avidin-glass beads bound ca. 3×10^{-2} μmol of biotin.

Hybridization of E. coli DNA. The effect of the cytochrome *c*-biotin modification on the reassociation of a polynucleotide was studied with *E. coli* DNA. Nonradioactive *E. coli* DNA in 0.1 × SSC was denatured by the addition of 0.2 vol of 1.0 N NaOH. After 20 min at 25 °C the DNA was neutralized by the addition of 0.4 vol of 1.0 M Hepes (protonated form, Calbiochem). ^{3}H-Labeled DNA-cytochrome *c*-biotin in 1.0 M NaCl–0.001 M Tris–0.0002 M EDTA (pH 8.5) was added to a final [^{3}H]DNA to nonradioactive DNA ratio of 1:200. NaCl (5.0 M)–Tris (0.005 M)–EDTA (0.001 M) (pH 8.5) and twice recrystallized formamide were added to give a final concentration of 0.7 M Na^+ and 55% formamide. This solution was incubated at 35 °C for 0.5 to 1.5 h.

The extent of DNA reassociation was monitored by removing aliquots of the DNA sample at appropriate C_0t values. The aliquots were dialyzed against 0.12 M sodium phosphate (pH 6.8) at 4 °C for 24 h. The final volume of the aliquot was adjusted to 2.0 mL and the solution was fractionated on hydroxylapatite as described previously (Manning et al., 1975b). Recovery of DNA after dialysis was generally greater than 88%.

Hybridization of rRNA-Cytochrome c-Biotin to D. melanogaster DNA. [^{3}H]DNA (either relaxed plasmid DNA or total nuclear DNA) in 0.1 × SSC was denatured by the addition of 0.2 vol of 1.0 N NaOH. After 30 min at 25 °C the DNA was neutralized by the addition of 0.4 vol of 1.0 M Hepes. Ribosomal RNA-cytochrome *c*-biotin in 1.0 M NaCl–0.001 M Tris–0.002 M EDTA (pH 8.5) was added to a final DNA:RNA ratio of either 15:1 or 5:1. NaCl (5.0 M)–Tris(0.005 M)–EDTA (0.001 M) (pH 8.5) and twice recrystallized formamide were added to give a final concentration of 0.7 M Na^+ and 55% formamide. The solution was incubated at 35 °C for 0.5–1.5 h.

The reassociated sample was dialyzed against 1.0 M NaCl–0.005 M Tris–0.001 M EDTA (pH 8.5) at 4 °C with rocking for 5 h. The sample was then concentrated and fractionated on a 2 cm × 30 cm column of Sepharose 2B in 1.0 M NaCl–0.005 M Tris–0.001 M EDTA (pH 8.5). The elution of the DNA was monitored by radioactivity. This step removes

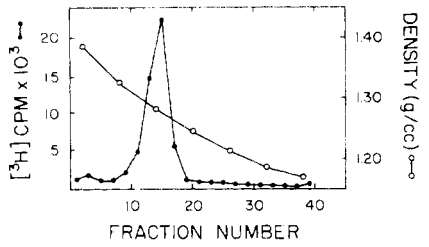

FIGURE 1: Buoyant banding experiment in CsCl of avidin-spheres labeled with [^{14}C]biotin.

much of the unhybridized rRNA. The peak fractions were pooled and further fractionated by use of avidin-spheres or avidin-glass beads.

Separation of rDNA:RNA Hybrid Molecules by Avidin-Spheres. The preparation and properties of the covalent conjugates of avidin to poly(methyl methacrylate) spheres have been described (Manning et al., 1975a). After hybridization to rRNA-biotin and fractionation on Sepharose 2B, the pooled DNA fractions were concentrated by vacuum evaporation to a volume of ca. 1.0 mL. Avidin-spheres (200 μL) (15 mg/mL in 1.0 M NaCl-0.005 M Tris-0.001 M EDTA (pH 8.5)) were added and the mixture was incubated at 4 °C for 16–20 h. Tris (0.05 M)-EDTA (0.005 M) (pH 8.5) was added to a final volume of 4.0 mL followed by CsCl to a final density of 1.284 g/cm^3. The mixture was centrifuged at 35 krpm for 24 h at 15 °C in a Spinco SW 50.1 rotor. The avidin-spheres formed a sharp band about $\frac{1}{3}$ of the distance from the top of the tube at a density of 1.27 g/cm^3. The sphere band plus the region slightly above and below was gently removed with a broken tip Pasteur pipet. The remainder of the gradient was removed and the DNA pellet at the bottom of the tube was resuspended in 1.0 mL of SSC. Quantitation of the amount of DNA in the sphere band, the remaining gradient, and the pellet was based on the radioactivity present in each fraction.

DNA was separated from spheres by treatment with 1 M NaOH for 30 min at 25 °C. The solution was neutralized with 2 M Tris-HCl and the spheres pelleted by centrifugation for 30 min at 15 krpm in the Sorvall SS34 rotor.

Separation of rDNA:rRNA Hybrid Molecules by Avidin-Glass Bead Affinity Chromatography. rDNA:rRNA hybrid molecules formed with either total *Drosophila* nuclear [^{3}H]DNA or ^{3}H-labeled pDm 103 DNA were purified from nonhybrid molecules by passage of the pooled DNA fractions from the Sepharose 2B column over a 1-mL avidin-glass bead column in 1.0 M NaCl-0.005 M Tris-0.001 M EDTA (pH 8.5). The flow rate was regulated to 0.5 mL/min. The column was then washed with three 2.0-mL washes of 1.0 M NaCl-0.005 M Tris-0.001 M EDTA (pH 8.5) to elute the nonbound DNA fragments. DNA fragments containing a hybrid region were eluted by dissociating the hybrid region with either three 1.0-mL washes of 99% formamide or two 2.0-mL washes of 1.0 N NaOH. Quantitation of the amount of DNA in the 1.0 M NaCl-0.005 M Tris-0.001 M EDTA (pH 8.5) fractions, the formamide, and the NaOH fractions was based on the radioactivity present. Prior to hybridization with [^{125}I]rRNA, the fractionated DNA was gently deproteinized with an equal volume of water-saturated phenol.

Preparation of [^{125}I]rRNA. ^{125}I-Labeled rRNA was used to assay for the amount of rDNA in the several fractions. Radioactively labeled [^{125}I]rRNA was prepared essentially according to Orosz and Wetmur (1974). rRNA (an equimolar mixture of 18S + 28S) was dialyzed extensively vs. 0.01 M NaOAc (pH 5.0). Typically, a 30-μL reaction contained 2 μg of rRNA, 1 mCi of Na^{125}I (carrier free, Amersham Searle), and 2×10^{-4} M $TlCl_3$ in 0.1 M NaOAc (pH 5.0). The mixture was incubated for 30 min at 60 °C in a sealed glass tube and treated as described by Orosz and Wetmur (1974). The [^{125}I]rRNA was further purified by buoyant density banding in a sodium iothalamate (Mallinckrodt Pharmaceuticals) gradient (Serwer, 1975). Iothalamate was removed by extensive dialysis against 1.0 M NaCl-0.01 M Tris-0.001 M EDTA (pH 8.5). The RNA was stored in 2 × SSC at −20 °C. Specific activities of RNA preparations ranged from 5×10^6 to 8×10^7 cpm per μg.

Filter Hybridization of [^{125}I]rRNA to DNA. Quantitation of rRNA coding sequences in both fractionated and unfractionated DNA was achieved by filter hybridization with [^{125}I]rRNA. Membrane filter hybridization of [^{125}I]rRNA to DNA was carried out by the mini-filter technique exactly according to Kourilsky et al. (1974). Nitrocellulose filters (13 mm) containing 0.01 to 1 μg of denatured DNA were prepared. To these a 10-μL solution of [^{125}I]rRNA (0.8 μg/mL) in 2 × SSC was added and the filters were immediately immersed in mineral oil. They were incubated at 65 °C for 18 h and then treated with RNase as described by Kourilsky et al. (1974).

Solution Hybridization of [^{125}I]rRNA to pDm 103. The rate and extent of hybridization of [^{125}I]rRNA to the rDNA of pDm 103 were determined as follows: the hybridization solution contained 0.7 M NaCl, 0.001 M Tris, 0.0002 M EDTA (pH 8.5), 55% formamide, 0.025 μg/mL DNA, and 0.13 μg/mL [^{125}I]rRNA. Aliquots (100 μL) were sealed in small glass tubes and incubated at 35 °C to the appropriate R_0t values. The solution was then diluted to 1 mL with SSC and RNase A was added to a final concentration of 50 μg/mL. The mixture was at room temperature for 30 min. Carrier DNA (75 μg) was added and the nucleic acid molecules were precipitated by addition of 1.0 mL of cold 10% trichloroacetic acid and the precipitate was collected on glass fiber filters. The extent of hybridization of [^{125}I]rRNA to the plasmid was determined as RNase resistant counts.

Results and Discussion

CsCl Equilibrium Density Centrifugation of Avidin-Spheres. Figure 1 shows the results of the centrifugation of a solution containing 1 mg (1.8×10^{-5} μmol) of avidin-labeled poly(methyl methacrylate) spheres (avidin-spheres) and 1 μmol of [^{14}C]biotin (45 Ci/mol) in 5.0 mL of CsCl solution of average density 1.313 g/cm^3 for 48 h at 35 krpm in a Spinco SW 50.1 rotor. Fractions were collected by puncturing the bottom of the centrifuge tube. The density profile of the CsCl solution was determined from the refractive index of selected samples. The buoyant position of the avidin-spheres, as determined by the radioactivity present in the fractions, corresponded to a density of 1.27 g/cm^3.

The buoyant band of avidin-spheres in a CsCl density gradient can be identified readily because of its turbidity (Figure 2). This identification facilitates the removal of high molecular weight DNA:avidin-sphere complexes with minimum shear to the DNA.

The observation that avidin-spheres will specifically attach to DNA molecules which contain covalently bound cytochrome *c*-biotin has been previously reported (Manning et al., 1975a). The density at which the DNA:avidin-sphere complex bands in a CsCl equilibrium density gradient was determined as follows. A solution containing 1 mg of avidin-spheres and 0.35 μg of ^{3}H-labeled *E. coli* DNA (4×10^4 cpm per μg, 3.5 kb fragment length) with an average of one covalently bound cytochrome *c*-biotin per 130 nucleotides was incubated for 16

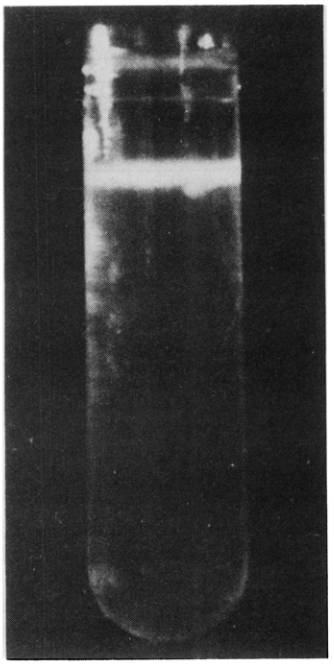

FIGURE 2: Photograph of a buoyant band of avidin spheres in a CsCl density gradient.

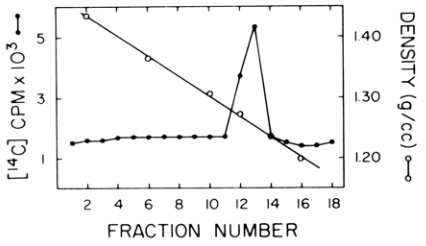

FIGURE 3: Banding of ^{3}H-labeled *E. coli* DNA covalently linked to cytochrome *c*-biotin and mixed with avidin-spheres in a CsCl density gradient; 100 μL of each fraction was spotted on a GFA filter and counted in toluene. The uniformly high background across the gradient represents Cl_3CCOOH soluble material.

h at 25 °C. Solid CsCl was added to the DNA–sphere solution to give an average density of 1.260 g/cm^3 and centrifuged for 48 h at 35 krpm at 25 °C in a Spinco SW 50.1 rotor. The gradient was fractionated into 10-drop fractions by puncturing the bottom of the tube. The position of the sphere band was determined by turbidity and the position of the DNA by radioactivity. As shown in Figure 3, the position of both the sphere band and the DNA band corresponded to a density of 1.27 g/cm^3. The DNA band at ρ = 1.27 g/cm^3 represented 70% of the total Cl_3CCOOH precipitable input counts, while the remainder of the DNA pelleted. Total recovery of the DNA by this procedure was 97%. Examination of the DNA found at ρ = 1.27 g/cm^3 in the electron microscope showed that 90% of all DNA fragments contained one or more attached avidin–spheres.

We believe that the DNA fragments which were removed with the sphere band but did not contain an attached sphere by electron microscopic observation are not due to nonspecific weak association of DNA with avidin–spheres but rather to breakage of large DNA molecules (one part of which initially contained an attached sphere) during gradient fractionation or spreading for electron microscopy.

When a CsCl density gradient experiment was performed with ^{3}H-labeled *E. coli* DNA with no attached cytochrome

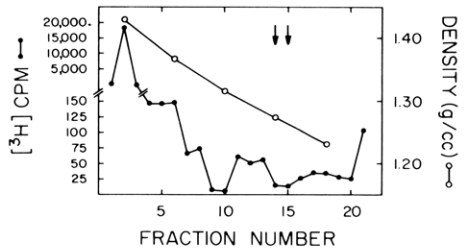

FIGURE 4: A mixture of avidin spheres and ^{3}H-labeled *E. coli* DNA which is not modified with cytochrome *c*-biotin is banded in CsCl. The position of the sphere band is indicated by arrows. Note the change of scale for the amount of [^{3}H]DNA.

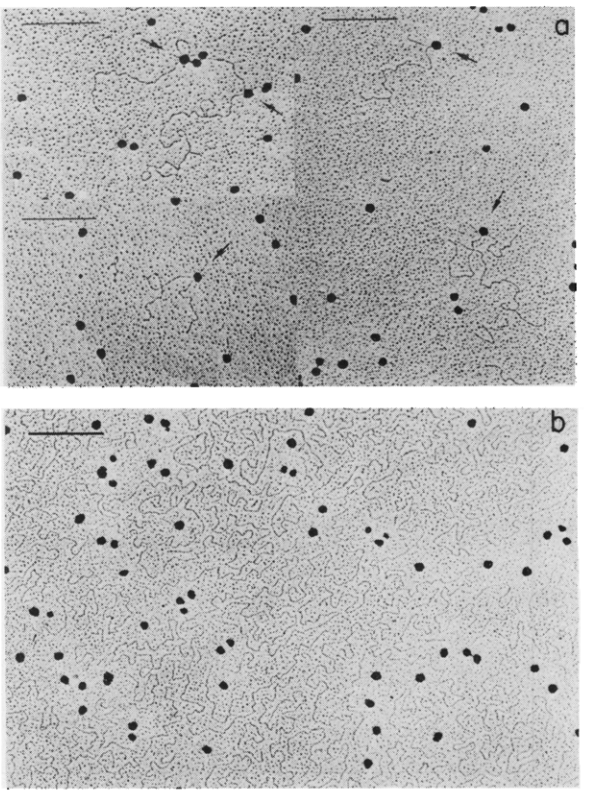

FIGURE 5: Electron micrographs of single-stranded *E. coli* DNA coupled to cytochrome *c*-biotin mixed with avidin-spheres and spread by the formamide–cytochrome *c* method (a) and of unmodified DNA spread with avidin-spheres (b). In a, arrows identify spheres attached to DNA. The calibration marker is 1 kb in length.

c-biotin and avidin–spheres, less than 0.05% of the DNA was present in the avidin–sphere band (Figure 4). Thus, there is only a very low level of nonspecific binding of unmodified DNA to the spheres.

This low level of nonspecific binding is shown in an electron micrograph (Figure 5) of unmodified and cytochrome *c*-biotin modified DNA spread with excess spheres in a formamide–cytochrome *c* spread. In the former case, no spheres are attached to DNA; in the latter case, as shown by the arrow, some spheres are attached to the DNA.

In conclusion, the CsCl density gradient and the electron microscope experiments show that the avidin–spheres, CsCl density gradient procedure is effective for separating DNA strands coupled to cytochrome *c*-biotin from those which are not.

Hybridization of Cytochrome–Biotin Labeled DNA with Unlabeled DNA. The gene enrichment procedure requires that nucleic acid molecules containing attached cytochrome *c*-

TABLE I: Gene Enrichment Tests on pDm 103.[a]

DNA	Method of Enrichment	% of rRNA Coding Sequences in "Enriched" Fraction	% of rRNA Coding Sequences in "Depleted" Fraction	% of Total DNA in "Enriched" Fraction	% of Total DNA in "Depleted" Fraction
pSC 101	Avidin-sphere, CsCl			<0.01	>99.99
pDm 103 theor.		100	0	50	50
pDm 103	Avidin-sphere, CsCl	66	34	42	58
pDm 103	Avidin-sphere, CsCl	69	31	36	64
pSC 101	Avidin-glass bead column			<0.01	>99.99
pDm 103	Avidin-glass bead column	90	10	44	56

[a] Open circular [^{3}H]plasmid DNA was denatured and hybridized to rRNA-cytochrome *c*-biotin (130 nucleotides/cytochrome *c*-biotin). Hybridizations were carried out in 0.5-mL volumes in 55% formamide-0.7 M Na^+ at 35 °C with rRNA-biotin and plasmid DNA at concentrations of 30 and 2 μg/mL, respectively. The percent of rRNA coding sequences in each fraction was determined by membrane filter hybridization with [^{125}I]rRNA as described under Experimental Procedures. The percentage of total DNA in the "enriched" or "depleted" fraction was determined as Cl_3CCOOH precipitable counts and normalized to 100%. Recovery of total Cl_3CCOOH precipitable input counts was greater than 97%.

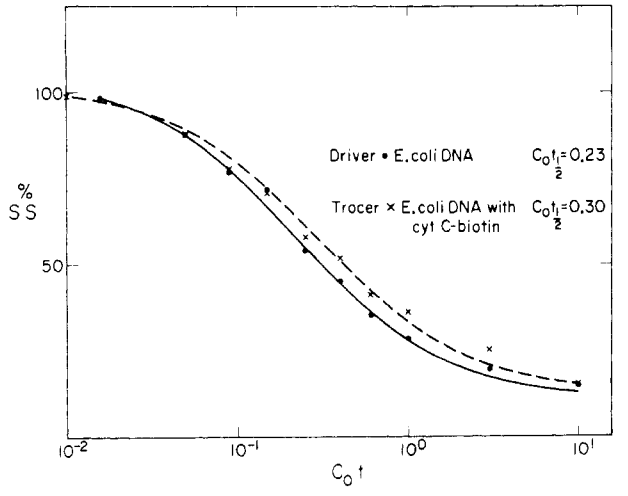

FIGURE 6: Reassociation rate, as measured by hydroxylapatite chromatography of excess unmodified unlabeled DNA with itself and of cytochrome *c*-biotin modified [^{3}H]DNA with the unmodified driver DNA.

biotin be capable of hybridizing with unlabeled nucleic acid molecules. We have compared the hybridization rate of cytochrome *c*-biotin labeled DNA with unlabeled DNA to the reassociation rate for unlabeled DNA. Cytochrome *c*-biotin was attached to single-stranded ^{3}H-labeled *E. coli* DNA of average fragment length 2.2 kb to give a final ratio of 130 nucleotides per attached cytochrome *c*-biotin. Nonradioactive *E. coli* DNA of average fragment length 2.2 kb was denatured and added in 200-fold excess to the cytochrome *c*-biotin-[^{3}H]DNA. Samples were reassociated to a variable set of C_0t values and fractionated by hydroxylapatite chromatography.

As shown in Figure 6, the reaction rate of the modified DNA with unmodified DNA is only 0.23/0.30 slower than the reassociation rate of unmodified strands. We do not know whether the small difference in rate is real or is experimental error, but the main point is that the rates are almost the same. The effect of the modification reaction on the thermal stability of the hybrids was not measured. We can only say that the hybrids remain duplex at 60 °C in 0.12 M sodium phosphate (pH 6.8), which are the hydroxylapatite fractionation conditions used.

Gene Enrichment Tests on pDm 103. The recombinant plasmid pDm 103 consists of the procaryotic vector, pSC 101, of length 9.2 kb and a *Drosophila* fragment of length 17.1 kb which contains one copy of the 18S and 28S rRNA coding sequences (Glover et al., 1975).

The lengths of the 18S and 28S rRNA genes of *Drosophila* have been measured as 2.2 and 4.3 kb, respectively (Pellegrini et al., 1977; Wellauer and Dawid, 1977; White and Hogness, 1977). Thus, the coding sequences constitute 12.3% (6.5/52.6) of the plasmid.

The rate and extent of hybridization of an excess of ^{125}I-labeled 18S and 28S rRNA with pDm 103 were measured as described under Experimental Procedures. The saturation hybridization value was 11.5 ± 2.5% of the total plasmid DNA, in agreement with the calculation above. The observed $R_0t_{1/2}$ value was 5.8×10^{-3} mol s L^{-1} in a 0.7 M Na^+-55% formamide solvent at 35 °C. This value is approximately as expected for an RNA driven RNA:DNA reaction rate for an RNA of this complexity (J. W. Casey, personal communication).

Since only one of the strands of pDm 103 contains the coding sequences for the rRNAs (Glover et al., 1975), this DNA is an excellent test system for the proposed gene isolation procedures. The results of such tests, which are presented in Table I, show the following. (a) The binding of the control DNA (pSC 101) by avidin-spheres and by avidin-glass beads is less than 0.01% in both cases. (b) Of the rRNA genes 65 to 70% are selected in the "enriched" fraction by the avidin-sphere procedure; 30 to 35% are not. If all of the DNA strands were full length, 32 to 35% of the total DNA should then be in the "enriched" fraction, in approximate agreement with observation. The slightly higher value (42%) observed in one of the two experiments may be due to some DNA reassociation. (c) Ninety percent of the genes and 44% of the DNA are selected by the avidin-glass bead affinity column, thus indicating efficient isolation of the genes with little strand breakage.

TABLE II: Gene Enrichment Experiments on Total *Drosophila* DNA.[a]

DNA	Method of Enrichment	% of rRNA Coding Sequences in "Enriched" Fraction	% of rRNA Coding Sequences in "Depleted" Fraction	% of Total DNA in "Enriched" Fraction	% of Total DNA in "Depleted" Fraction	Enrichment Factor (% of Total rRNA + Spacers)
Total nuclear DNA, theor.		100	0	0.64	99.36	156 (100)
Total nuclear DNA	Avidin-sphere,[b] CsCl	20	80	0.30	99.70	66 (42)
Total nuclear DNA	Avidin-sphere,[b] CsCl	25	75	0.20	99.80	124 (80)
Total nuclear DNA	Avidin-glass bead[c] column	57	43	0.77	99.23	74 (47)

[a] The calculation that rDNAs (genes plus spacers on the coding strand) make up $1/156$ of the total *Drosophila* DNA is based on the following. The average total length of the spacer sequences in *Drosophila* rDNA has been measured as 7.25 kb (Pellegrini et al., 1977). About 37% of the 28S sequences contain an insertion of average length 4.57 kb. Thus, the average total length of a rDNA repeat unit is 15.46 kb. The average length of 18S plus 28S gene sequences is 6.5 kb. The gene sequences make up 0.27% of the genome. The total rDNA, therefore, makes up 0.0027 $(15.46/6.5) = 1/156$ of the genome. [b] $R_0t = 0.016$. [c] $R_0t = 0.13$. Hybridizations were performed at RNA-biotin and DNA concentrations of either 6 and 90 μg/mL, respectively, or 7.5 and 35 μL, respectively. The percent of rRNA coding sequences in each DNA fraction was determined by membrane filter hybridization with [^{125}I]rRNA.

Isolation of rRNA Coding Sequences from D. melanogaster Nuclear DNA. The experiments for the isolation of DNA fragments which contain the rRNA coding sequences from total *D. melanogaster* nuclear DNA are identical, in principle, with those described for the isolation of the rRNA coding strand in pDm 103 DNA. They differ, however, in the initial amount of DNA required. Since in *Drosophila* only 0.27% of the nuclear DNA is rRNA coding sequences, the amount of DNA required for an accurate measurement of the degree of enrichment is between 1 and 2 mg per experiment. Using this amount of starting material, the DNA is denatured and hybridized with an excess of cytochrome *c*-biotin coupled rRNA. The ratio of RNA to DNA in the hybridization mixture was either 1:5 or 1:15. This ratio represents a 74- to 24-fold excess of rRNA over rDNA. Following hybridization to saturation ($R_0t = 0.13$ or 0.016 mol s L^{-1}, which is approximately 400 or 30 times the observed $R_0t_{1/2}$ of 5.3×10^{-3} mol s L^{-1}), the RNA:DNA hybrid molecules were selectively removed by either binding the hybrids to avidin-spheres and banding the complex in a CsCl equilibrium gradient or by binding the hybrids to an avidin-glass bead column. The results of the enrichment of DNA strands containing rRNA coding sequences by the above selection procedures are shown in Table II.

The 185-fold reiterated 18S and 28S rRNA genes make up 0.27% of the *Drosophila* genome (Tartof and Perry, 1970). The several spacer sequences between the tandemly repeated genes are, on the average, slightly greater in length than the genes for the mature rRNAs, so that the total amount of gene plus spacer on the coding strand is 0.64% or $1/156$ of the total DNA. The details of this calculation are given in the legend to Table II.

An enrichment factor of 156 would mean that the rDNA was 100% pure. In the several experiments reported in Table II, enrichment factors of 66 to 124 were achieved, giving long rDNA strands which are 42 to 80% pure.

In the avidin-sphere enrichment experiments, 20 and 25% of the total rDNA was recovered in the enriched fraction. In the avidin-glass bead experiment, 57% was recovered.

Electron microscope observations showed that the single-strand length of the DNA starting material was primarily in the range of 70 to 100 kb. The lengths of the molecules of enriched DNA recovered from the avidin-sphere experiments were mostly in the range of 50 to 70 kb for the avidin-glass bead experiments they were 20 to 30 kb. Thus, in this respect, the sphere banding procedure is better. It may be, however, that with further attention to conditions for very gentle passage of the DNA over the solid support, it will be possible to avoid breakage of the strands in this procedure.

Further Discussion

The experiments described here demonstrate that rRNA:rDNA hybrid structures can be selectively isolated from total nuclear DNA in sufficient quantity, of sufficient purity, and of sufficient single-strand length for an electron microscope study of the sequence organization of the genes (Pellegrini et al., 1977). Although this approach has been successful in the isolation of repetitive genes, we believe that further tests will be necessary to determine its usefulness in the isolation of single copy genes and their flanking sequences.

Acknowledgments

We are grateful to our colleagues L. Angerer, T. Broker, N. D. Hershey, and P. Yen who have generally contributed to the development of avidin-biotin methodology, to N. D. Hersey who developed the method of preparing spheres, and to M. Grinder for the preparation of nucleic acids.

References

Anderson, J. N., and Schimke, R. T. (1976), *Cell 7,* 331.

Birnstiel, M. L., Speirs, J., Purdom, I., Jones, K., and Loening, U. E. (1968), *Nature (London) 219,* 454.

Brown, D. D., and Weber, C. S. (1968), *J. Mol. Biol. 34,* 661.

Brown, D. D., Wensink, P. C., and Jordan, E. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3175.

Glover, D. M., White, R. L., Finnegan, D. J., and Hogness, D. S. (1975), *Cell 5,* 149.

Green, N. M. (1963), *Biochem. J. 89,* 585.

Hastings, I. R. B., and Kirby, K. S. (1966), *Biochem. J. 11,* 532.

Kedes, L., and Birnstiel, M. L. (1971), *Nature (London), New Biol. 230,* 165.

Kourilsky, p., Mercereau, O., Gros, D., and Trembaly, G. (1974), *Biochimie 56,* 1215.

Manning, J. E., Hershey, N. A., Broker, T. R., Pellegrini, M., Mitchell, H. K., and Davidson, N. (1975a), *Chromosoma 53,* 197.
Manning, J. E., Schmid, C. W., and Davidson, N. (1975b), *Cell 4,* 141.
Miller, W. L., Brenner, D. J., and Doctor, B. P. (1974), *Biochim. Biophys. Acta 366,* 188.
Orosz, J. M., and Wetmur, J. G. (1974), *Biochemistry 13,* 5467.
Pellegrini, M., Manning, J., and Davidson, N. (1977), *Cell* (in press).
Ritossa, F. M., Atwood, K. C., Lindsley, D. L., and Spiegelman, S. (1965), *Natl. Cancer Inst. Monogr. 25,* 449.
Ritossa, F. M., Atwood, K. C., and Spiegelman, S. (1966), *Genetics 54,* 663.
Serwer, P. (1975), *J. Mol. Biol. 92,* 433.
Sharp, P. A., Hsu, M., Ohtsubo, E., and Davidson, N. (1970), *J. Mol. Biol. 71,* 471.
Shih, T. Y., and Martin, M. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1976.
Tartof, K. D., and Perry, R. P. (1970), *J. Mol. Biol. 51,* 171.
Thomas, M., White, R. L., and Davis, R. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2294.
Wallace, H., and Birnstiel, M. L. (1966), *Biochim. Biophys. Acta 114,* 296.
Wellauer, P. K., and Dawid, I. B. (1977), *Cell* (in press).
White, R. L., and Hogness, D. S. (1977), *Cell* (in press).

An Endonuclease from Calf Liver Specific for Apurinic Sites in DNA†

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ABSTRACT: An endonuclease specific for apurinic sites in double-stranded DNA has been partially purified from calf liver extracts. The enzyme has a pH optimum of 9.5, is only slightly stimulated by low concentrations of Mg^{2+}, and has a molecular weight of 28 000. Inhibitors of the endonuclease include Ca^{2+}, EDTA, *p*-HOHgBzO, NaCl, and tRNA. The enzyme introduces single- and double-stranded breaks in depurinated DNA. High concentrations of the enzyme preparation degrade untreated single-stranded DNA, but not ultraviolet (UV) irradiated DNA or DNA treated with methylmethanesulfonate or 7-bromomethyl-12-methylbenz[*a*]anthracene. Enzymatic incisions produce 3′-hydroxyl and 5′-phosphate end groups. Some of the properties of the calf liver apurinic endonuclease differ from those of a similar endonuclease obtained from calf thymus by S. Ljungquist and T. Lindahl [(1974), *J. Biol. Chem. 249,* 1530] and in this laboratory. The data suggest that these are isozymes.

Deoxyribonucleic acid in cells may undergo depurination damage by various pathways. Base release by either chemical or enzymatic reactions can occur after the bases are altered by specific alkylating agents or γ irradiation. Lindahl and Nyberg (1972) demonstrated the slow spontaneous hydrolysis of purines under physiological conditions and calculated that up to 3% of the total purines could be lost during the lifetime of cells such as human neurones if there was no replacement. An endonuclease active only at depurinated sites was isolated from *Escherichia coli* by Paquette et al. (1972), while a preparation active on depurinated sites and alkylated DNA (Hadi and Goldthwait, 1971) has now been resolved into an enzyme active on depurinated sites and one active on alkylated DNA (endonuclease II, Kirtikar et al., 1976a). Evidence for the existence in mammalian tissues of endonucleases capable of recognizing depurinated sites was obtained by Verly and Paquette (1973). Ljungquist and Lindahl (1974) purified an endonuclease 830-fold from calf thymus and reported many of its properties. Subsequently, an enzyme active on apurinic sites in DNA was demonstrated in a number of human cell lines (Teebor and Duker, 1975) and an altered specific endonuclease for depurinated sites has been found in extracts of two cell lines from xeroderma pigmentosum patients (Kuhnlein et al., 1976). This paper reports the isolation and partial purification from calf liver of an endonuclease active on depurinated DNA which possesses some properties that differ from those observed with the enzyme obtained from calf thymus. The possibility of apurinic endonuclease isozymes is discussed.

Materials and Methods

Reagents. [^{3}H]Thymidine was obtained from the New England Nuclear Corp., Boston, Mass. Sodium borohydride (98% pure) was purchased from the Fisher Scientific Co., Fairlawn, N.J. Dithiothreitol and 2-mercaptoethanol were both from Sigma Chemical Co., St. Louis, Mo. Toluenesulfonyl fluoride, 97%, was obtained from Aldrich Chemical Corp., Milwaukee, Wis. 1,3-Bis[tris(hydroxymethyl)methylamino]propane (BTP buffer),[1] A grade (pK_{a_1} = 9.0, pK_{a_2} = 6.8), was purchased from Calbiochem, Los Angeles, Calif. DNase I was also obtained from this source. Bovine spleen and snake venom phosphodiesterases were obtained from the Worthington Co.

DNA. The procedure for preparing [^{3}H]thymine-labeled *Bacillus subtilis* or *Escherichia coli* DNA of Smith (1967) was employed. Specific activity of the DNA was approximately

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received October 13, 1976.* This work was supported by a grant from the National Institutes of Health (CA 11322) and a contract with ERDA (11-1) 2725. D.A.G. was supported by National Institutes of Health Research Career Award No. KO-6-GM-21444.

[1] Abbreviations used are: BTP buffer, 1,3-bis[tris(hydroxymethyl)-methylamino]propane-hydrochloric acid buffer; EDTA, ethylenediaminetetraacetic acid, sodium salt; *p*-HOHgBzO, *p*-hydroxymercuribenzoate; AP, alkaline phosphatase.