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Application of the avidin-biotin method of gene enrichment to the isolation of long double-stranded DNA containing specific gene sequences

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ABSTRACT

A method of enriching for long double-stranded segments of eukaryotic DNA carrying particular genes is described. A purified RNA coded for by the gene is covalently attached to biotin via the protein, cytochrome c. This modified RNA is hybridized to total nuclear, double-stranded DNA under conditions that allow the formation of R-loops. Avidin, which has a high affinity for biotin, is covalently attached to polymer spheres. The complexes of avidin-spheres with DNA:RNA-biotin R-loop hybrids band in CsCl at a much lower bouyant density than does free DNA. This density is a function of the length of DNA coupled per avidin-sphere. This method was used to prepare very long double-strands of DNA highly enriched in the coding sequences for the large rRNAs of D. melanogaster and L. donovani and the histone mRNAs of S. purpuratus.

INTRODUCTION

The isolation of long fragments of eukaryotic DNA containing specific genes is especially useful to the study of the structure and sequence organization of such genes. Preparation of specific segments of double-stranded DNA are particularly valuable because such DNA is amenable to further study with the powerful techniques of restriction enzymology, cloning, and electron microscopy. In addition, the isolation of very long (50 to 150 kilobases) DNA fragments allows the study of the sequential arrangement of sets of repeated genes on a single piece of DNA.

We have recently described a method for the isolation of specific single-stranded fragments of eukaryotic DNA based on the biotin-avidin interaction (3). We now wish to report the extension of this methodology to the isolation of long segments of double-stranded DNA which contain sequences complementary to a specific RNA. This technique makes use of the formation of R-loops, that is, the hybridization of an RNA molecule to double-stranded DNA by displacement of the identical DNA strand (4). These techniques were used to isolate double-stranded DNA complementary to the histone mRNAs of the sea urchin, Strongylocentrotus purpuratus, and the ribosomal RNAs (rRNAs) of the fruit fly, Drosophila melanogaster, and the parasitic hemoflagellate,

Leishmania donovani. These several RNAs vary both in their G+C content, their complexity, and the percent of the total genomic DNA to which they are complementary. The isolation of highly enriched segments of DNA containing these specific RNA-complementary sequences illustrates the usefulness of this technique for the study of the DNA of multigene families.

MATERIALS AND METHODS

DNA: High molecular weight unlabeled D. melanogaster DNA was extracted from a crude nuclear pellet prepared from organisms 16 to 24 hours after puparium formation as described previously (5). [^{3}H] labeled D. melanogaster DNA was prepared in the same manner from homogenized embryos cultured in the presence of [^{3}H] thymidine (3). [^{3}H] and unlabeled DNA were pooled giving a final specific activity of 3 x 10^6 cpm/µg.

Culture form (promastigote) cells of L. donovani malakal area sudan strain were the generous gift of Dr. S. Krassner. High molecular weight DNA was extracted from whole cells by the same procedures as used to prepare D. melanogaster DNA. Nuclear DNA was then purified from these preparations of total cellular DNA (nuclear and kinetoplast DNA) by repeated sedimentation through a 20% sucrose solution as described previously (6).

DNA was isolated from fresh S. purpuratus sperm. Sperm was added dropwise to 0.2 M Tris pH 9.0, 0.3 M EDTA at 65° to a DNA concentration of about 100 µg/ml (sperm contains about 18 mg/ml DNA). The solution was gently swirled after the addition of each drop to ensure mixing. 20% SDS was added to a final concentration of 0.5% and the mixture was incubated at 50° overnight with 200 µg/ml Proteinase K (E. M. Labs). Nucleic acids were extracted at room temperature with an equal mixture of phenol and chloroform and dialysed against 20 mM Na borate pH 9.2. DNA was separated from RNA by bouyant density centrifugation in CsCl (7), dialysed against 5 mM Na borate pH 9.2 and stored at 4°C.

The preparation of covalently closed pDm 103 plasmid DNA from E. coli K12 strain Hb101 (pDm103) was according to Glover et al. (8). Closed circular pDm 103 DNA was converted to open circular DNA by exposure to x-radiation (9). pSC101 DNA was the generous gift of Dr. Mark Guyer.

RNA: The preparation of D. melanogaster 18S and 28S rRNAs and L. donovani 16S and 26S rRNAs have been previously described (10,11). The term rRNA used below refers to an equimolar mixture of both large species of rRNA, i.e., for D. melanogaster 18S + 28S and for L. donovani 16S + 26S. Total histone mRNA was a gift from Drs. R. Cohn and L. Kedes. It was prepared as described previously (12). ^{3}H-cRNA was prepared from the chimeric plasmids pSp2 and

pSp17 essentially by the procedure described by Kedes et al. (13).

Coupling of RNA and cytochrome c-biotin: The modification of these RNAs with cytochrome c-biotin is described elsewhere (10). In the case of the histone mRNA coupling, only 2 µg of mRNA were available; therefore, 20 µg of E. coli 16S rRNA was added to the mRNA as carrier. A sufficient amount of cytochrome c-biotin was added to this mixture of RNAs to ensure covalent coupling of both RNAs to cytochrome c-biotin to a final ratio of 130 nucleotides per cytochrome c-biotin.

R-loop hybridizations with nuclear DNA: Double-stranded nuclear DNA in 0.1 X SSC or 20 mM Na borate pH 9.2 was mixed with cytochrome c-biotin modified RNA in 1.0 M NaCl, 0.001 M EDTA, 0.05 M triethanolamine (pH 8.0) and dialysed for 2.5 hr at 4°C against 100 volumes of 90% formamide, 0.15 M NaCl, 0.005 M EDTA, 0.05 M triethanolamine (pH 8.0). In some reactions, 0.02 M sodium borate was used in place of triethanolamine. Following dialysis, the reaction volume was approximately half of the original. In each reaction 1.0 mg of DNA was used. The final DNA and RNA concentrations for each system were: for L. donovani, 400 µg/ml DNA, 34.5 µg/ml rRNA; for D. melanogaster, 350 µg/ml DNA, 14.7 µg/ml rRNA; and for S. purpuratus 333 µg/ml DNA, 0.67 µg/ml histone mRNA. These RNA concentrations represent an RNA excess over their DNA coding sequences of 8.4-fold (L. donovani),15-fold (D. melanogaster) and 1.5-fold (S. purpuratus). The rRNA reaction mixturewith L. donovani and D. melanogaster DNA were incubated for 1.5 hr at 35°C. The $R_o t$'s attained were 0.56 M sec and 0.48 M sec, respectively. In order to reach a comparable $R_o t$ for the histone mRNA-S. purpuratus DNA reaction, where the RNA concentration was substantially less than for the rRNA reactions, the mixture was incubated at 35°C for 24 hr. The $R_o t$ attained was 0.17 M sec. The exact nature of the reaction kinetics for the formation of R-loops of different sizes and base compositions has not been determined. However, the $R_o t$'s reached in each of these three experiments would correspond to 90% or greater hybridization of RNA to complementary DNA present if that DNA were single-stranded.

Circular, covalently open pDm 103 DNA and either unmodified or cytochrome c-biotin modified D. melanogaster rRNA were incubated under R-loop forming conditions for 1.5 hr as described for D. melanogaster nuclear DNA. The final concentrations of DNA and RNA were 10 µg/ml and 12 µg/ml, respectively. pDm 103 contains one copy each of the 18S and 28S D. melanogaster rRNA coding sequences which account for 12.3% of the total plasmid DNA (8). Therefore, the 1.2-fold mass excess of rRNA over plasmid DNA represents a 10-fold excess of RNA over DNA coding sequences as was the case for the D. melanogaster

nuclear DNA reactions.

Separation of R-loop:DNA hybrid molecules with avidin-spheres: The preparation and properties of the covalent conjugates of avidin to polymethacrylate spheres (avidin-spheres) has been described (10). Following R-loop hybridization of DNA, the reaction mixtures were dialysed versus 100 volumes of 1.0 M NaCl, 0.005 M EDTA, 0.02 M Na borate (or 0.05 M triethanolamine) pH 8.0, for 2 hr at 4°C. Avidin-spheres (200 µl) at 20 mg/ml in 1.0 M NaCl, 0.005 M EDTA, 0.02 M Na borate pH 8.0 were added and the dialysis was continued for 2 hr at 4°C. This solution was then added slowly and with gentle mixing to a solution of CsCl in 0.005 M EDTA, 0.05 M triethanolamine, pH 8.0, to give a final volume of approximately 5.0 ml and a density of 1.45 g/ml. The mixture was centrifuged at 35,000 rpm for 48 hr at 15°C in a Spinco SW 50.1 rotor. The avidin-spheres formed a sharp band near the top of the gradient at a density of 1.27 g/ml. The gradient was fractionated into ten 0.5 ml aliquots using a broken tip pasteur pipet. The DNA which pelleted at the bottom of the tube was resuspended in 1.0 ml of SSC. Quantitation of the amount of DNA in each fraction and the pellet was based on either the radioactivity in each fraction (for [^{3}H] D. melanogaster DNA) or by electron microscope analysis (for non-radioactive L. donovani or S. purpuratus DNA) as described below. Concentrations of pelleted DNA were also determined by absorbance at 260 nm.

In any particular reaction the majority of the enriched DNA was found in only a small portion of the gradient, i.e., one or two of the 0.5 ml fractions. This will be discussed further in Results and Discussion.

Preparation of [^{125}I] rRNA: [^{125}I] labeled rRNA was used to assay the amount of rDNA in the several gradient fractions. [^{125}I] labeled rRNA was prepared as described previously (3) and purified by buoyant density banding in sodium iothalamate (14).

Filter hybridization of radioactive RNA to DNA: Quantitation of the rRNA or histone mRNA coding sequences in both fractionated (i.e., enriched and depleted DNA) and unfractionated DNA was achieved by filter hybridization of [^{125}I] rRNA or [^{3}H] histone cRNA using the mini-filter technique of Kourilsky et al. (15).

Electron microscopy: Samples of pDm 103 DNA which had been hybridized under R-loop forming conditions with D. melanogaster rRNA (either coupled to cytochrome c-biotin or unmodified) were examined in the electron microscope. 2 µl aliquots of the R-loop reaction mixture were prepared for electron microscopy by the formamide modification of the Kleinschmidt procedure (16). This mounting procedure was performed as quickly as possible following the R-loop

incubation since the formamide mounting conditions are not optimal for retention of such structures (4).

Aliquots of CsCl gradient fractions containing D. melanogaster R-loop rDNA and avidin-spheres were also prepared for electron microscopy using the formamide modification of the Kleinschmidt procedure (16).

The amount of S. purpuratus or L. donovani DNA in CsCl gradient fractions was determined by adding a known concentration of pSC 101 circular double-stranded DNA to a specific volume of the gradient DNA and preparing the mixed sample for electron microscopy as above. By comparing amounts of the circular DNA standard with the linear DNA extracted from the gradient it is possible to accurately determine the concentration of the unknown sample (17).

RESULTS AND DISCUSSION

Formation of R-loops with rRNA modified with cytochrome c-biotin: The recombinant plasmid pDm 103 consists of the prokaryotic vector pSC 101 and a segment of D. melanogaster DNA which contains one copy of the 18S and 28S rRNA coding sequences. The physical arrangement of the two rDNA sequences in this clone have been observed by R-loop mapping (18) and gene-32-ethidium bromide mapping (19). This DNA is, therefore, an excellent test system for determining the efficiency of R-loop formation with cytochrome c-biotin modified rRNA. Either modified (1 cytochrome c-biotin/130 nucleotides) or unmodified rRNA was incubated with pDm 103 DNA under conditions that favor the formation of R-loops. The resulting hybrid molecules were examined in the electron microscope. With unmodified rRNA 81% of the pDm 103 molecules observed (n = 150) contained R-loop structures. With cytochrome c-biotin modified rRNA, the number of pDm 103 molecules (n = 155) containing R-loop structures was 67%. In addition to the quantitative decrease in the number of DNA molecules containing R-loop structures with modified rRNA, a small decrease in the size of R-loops was also observed.

The effect of R-loop hybridization conditions on the stability of R-loops containing the cytochrome c-biotin modified rRNA was also studied with pDm 103. Following the formation of R-loops with modified rRNA, the pDm 103 reaction mixture was dialysed for 2 hrs at 4°C against 1.0 M NaCl, 0.005 M EDTA and 0.05 M triethanolamine (pH 8.0) and aliquots were examined in the electron microscope. After dialysis 54% of the DNA molecules were observed to contain R-loops. Similar results were observed for R-loops formed with unmodified rRNA.

Following the addition of avidin-spheres to the sample of pDm 103 DNA containing R-loops with cytochrome c-biotin modified rRNA, 44% of the DNA

molecules (n = 95) were observed to bind one or more avidin-spheres. Such a molecule is shown in Figure 1b. Therefore, of the DNA molecules containing R-loops, 81% were observed to bind avidin-spheres. In the control experiment with unmodified rRNA, no molecules of the 450 examined showed an avidin-sphere bound to it. An example of such a molecule is also shown in Figure 1a. We have previously determined that the non-specific binding of avidin-spheres to unmodified single- or double-stranded DNA is less than 1 in 5 x 10^4 strands of DNA of average length 3500 nucleotides (3).

It is clear from these data that these R-loop hybridization conditions allow the formation of stable R-loops with cytochrome c-biotin modified RNA and that the unique topography of R-loops do not sterically inhibit the reaction of avidin-spheres with hybrids containing cytochrome c-biotin modified RNA.

Isolation of rRNA coding sequences from D. melanogaster and L. donovani nuclear DNA and histone mRNA coding sequence from S. purpuratus nuclear DNA: The DNA complementary to rRNA from D. melanogaster and L. donovani was isolated by forming rDNA:rRNA R-loop hybrids with cytochrome c-biotin modified rRNA and selecting these hybrid molecules by buoyant banding with avidin-spheres in a CsCl density gradient as described in Materials and Methods. The results of these experiments are presented in Table I. Forty-four and 60% of the rDNA was recovered for L. donovani and D. melanogaster, respectively, and it was 28% and 80% pure. The DNA complementary to histone mRNAs of the sea urchin, S. purpuratus, was isolated exactly as described for the preparation of rDNA. As presented in Table I, 22% of these mRNA coding sequences were isolated and they were 21% pure.

Under the conditions used for CsCl buoyant density centrifugation, free nuclear double- or single-stranded DNA from any of these three organisms pellets. Therefore, any DNA found in the gradient presumably arises from the attachment of one or more avidin-spheres. As stated above one or two of the 0.5 ml gradient fractions contained > 90% of this avidin-sphere coupled DNA. These fractions, therefore, contained DNA putatively enriched for RNA coding sequences, while the pelleted DNA was putatively depleted of these sequences. This was confirmed by measuring the RNA coding sequence content of each of the enriched and depleted DNA samples by mini-filter hybridization with radio-active RNA as described in Materials and Methods.

Electron microscope observations showed that the double strand length of the DNA starting material for the Drosophila and Leishmania experiments was in the range of 70 to 100 Kb (L. donovani) and 100 to 150 Kb (D. melanogaster).

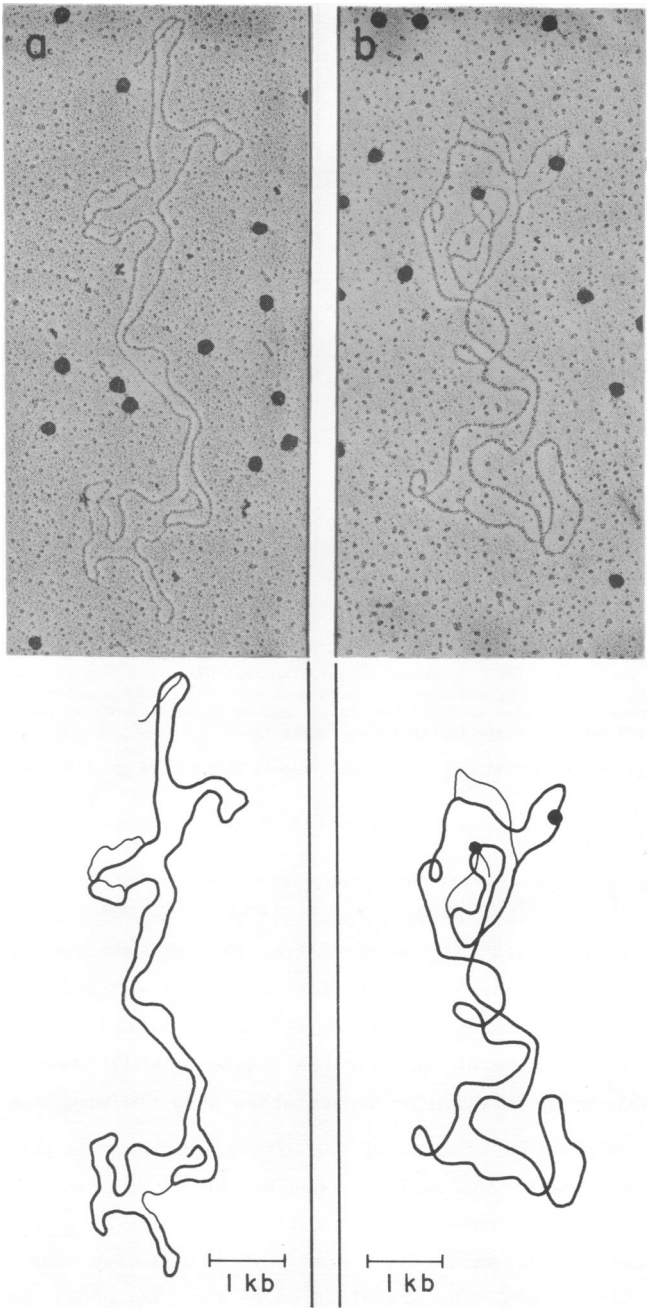

Figure 1. **Electron micrographs of two double-stranded pDm 103: rRNA hybrid** molecules. In (a) unmodified rRNA was hybridized to pDM 103 followed by the additional of avidin-spheres. In (b) cytochome c-biotin modified rRNA was used in the same reaction.

TABLE I: GENE ENRICHMENT EXPERIMENTS

System	% of Total DNA in "enriched" fraction	% of Total DNA in "depleted" fraction	% of RNA Coding Sequences "enriched" fraction	% of RNA Coding Sequences "depleted" fraction	% Purity of "enriched" fraction (coding sequences + spacers)
rRNA of D. melanogaster theoretical	1.28[a,b]	98.72	100	0	100
rRNA of D. melanogaster total nuclear DNA	9.96	99.04	60	40	80
rRNA of L. donovani theoretical	3.44[a,c]	96.56	100	0	100
rRNA of L. donovani total nuclear DNA	5.40	94.60	44	56	28
histone mRNA of S. purpuratus theoretical	0.40[a,d]	99.60	100	0	100
histone mRNA of S. purpuratus total nuclear DNA	1.85	98.15	22	78	21

[a] The calculation for the percent of total nuclear DNA containing the tandemly repeated RNA coding sequences includes both gene and flanking spacer sequences. It also includes the DNA of the non-coding as well as the coding strand since both are isolated simultaneously.

[b] D. melanogaster rDNA (gene + spacers) = 15.46 Kb repeated ca. 110 times per haploid genome (18, 19, 24).

[c] L. donovani rDNA (genes + spacers) = 13.45 Kb repeated ca. 166 times per haploid genome (11).

[d] S. purpuratus histone DNA (genes + spacers) = 6.54 Kb repeated ca. 500 times per haploid genome (13).

The molecules of enriched DNA recovered from the CsCl gradient had a double strand weight average length of 90 Kb for both samples and a single strand length of 29 Kb (L. donovani) and 40 Kb (D. melanogaster). Thus, this procedure allows the selection of specific DNA fragments while maintaining them as very long double strands which on denaturation show few single strand nicks.

Electron microscope studies of the arrangement of rRNA genes on long strands of rDNA from L. donovani enriched by the above procedure have been described in a separate communication (11). rDNA from D. melanogaster obtained by these methods was denatured and hybridized with rRNA and studied in the electron microscope. The organization of the rRNA genes observed in this DNA agreed exactly with our previous results obtained from electron microscopy mapping of rDNA isolated in single-stranded form with similar techniques (19).

Determination of the buoyant density of long double strands of DNA with

an attached avidin-sphere: The physical separation of a DNA strand attached to an avidin sphere(s) from total DNA relies on the ability of the sphere(s) to shift the buoyant density of the DNA strand to which it is attached. The amount of this shift should be proportional to the ratio of the weight of the attached sphere to the weight of the DNA strand. The weight fraction of spheres on any DNA strand can be calculated, i.e., $\frac{\text{weight of avidin-spheres}}{\text{weight of avidin-spheres + weight of attached DNA}}$, assuming an average molecular weight of 6.60×10^2 per nucleotide pair of DNA and 7.60×10^7 for an avidin-sphere (10). Since both the weight of randomly sheared DNA strands and the weight of the spheres are distributions rather than uniform weights, the DNA:sphere particles should exhibit a range of buoyant densities.

The buoyant density in CsCl of long double strands of DNA containing different numbers of attached avidin-spheres was determined by electron microscope observation of long fragments of Drosophila rDNA. The total Drosophila nuclear DNA material was randomly sheared during its isolation. It was treated with cytochrome c-biotin modified rRNA to form R-loops, incubated with avidin-spheres and the mixture centrifuged to equilibrium in CsCl. The resulting gradient was fractionated into ten - 0.5 ml aliquots and the density of each fraction was determined by its refractive index. Each fraction was then examined in the electron microscope and the length of double-stranded DNA and the number of avidin-spheres attached to the DNA strands were quantitated. Only six of the ten fractions contained DNA attached to avidin-spheres. The free avidin-spheres formed a compact band at a mean density of 1.27 g/ml and the free DNA pelleted in this gradient. In Figure 2 the weight fraction of avidin-spheres attached to DNA molecules is plotted as a function of CsCl density. These points show a linear relationship. Extrapolation of this linear relationship to a weight fraction of avidin-spheres of 0 gives a density of 1.7 g/ml, the average density of free Drosophila DNA. Extrapolation to a weight fraction of 1.0 gives a density of 1.25 g/ml approximately equal to the density observed for free avidin-spheres, 1.27 g/ml.

In this experiment, and in the isolation of L. donovani rDNA, over 90% of the enriched DNA was found at a density of 1.45 g/ml. This represented very long (90 Kb fragments of DNA) containing one avidin-sphere, although much longer strands containing more than one sphere were also observed. Examples of such molecules is shown in Figure 3. In the isolation of S. purpuratus histone mRNA sequences the enriched DNA was observed in and just below the free avidin-sphere band at a density of 1.27 to 1.30 g/ml. Electron

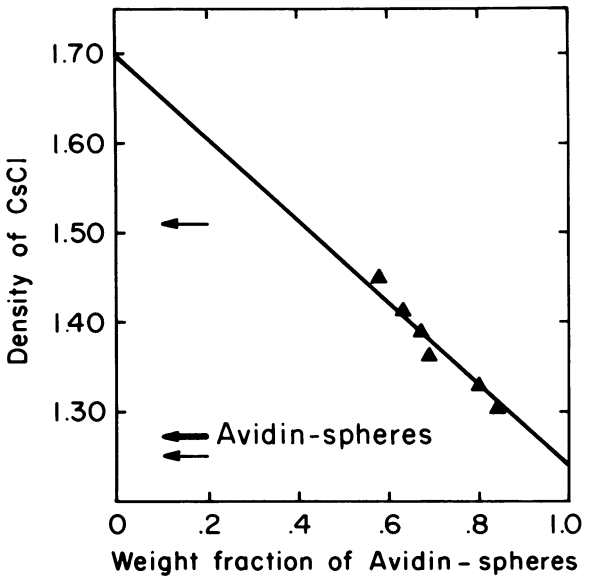

Figure 2. Plot of the buoyant density in CsCl of avidin-sphere containing D. melanogaster DNA. The density of the top and bottom of the gradient are indicated by the arrows, as is the position of the tight band of free avidin-spheres.

microscope observation of this DNA showed the molecules had an average length of 20 Kb with one attached sphere per 7 Kb on the average, or a weight fraction of spheres of 0.94. This DNA, therefore, banded at a density consistent with the relationship depicted in Figure 2.

FURTHER DISCUSSION

The experiments presented in this paper provide a significant addition to the methodology we have previously described (3) for the physical isolation of long single-stranded DNA fragments containing particular gene sequences, i.e., the isolation of specific long double-stranded DNA fragments. Modified conditions are described for the formation of stable R-loop structures using cytochrome-c-biotin-RNA. These conditions allow the integrity of the cytochrome-c-biotin-RNA complex to be maintained for several hours, while previously reported conditions for the formation of R-loops (14,18,24) result in a cleavage of the linkage between the cytochrome c and the RNA. Cytochrome-c modified RNA present in the R-loop structures formed using this modified procedure also remain reactive to avidin-spheres. This allows the RNA (both ribosomal and messenger):double stranded DNA hybrids to be selectively

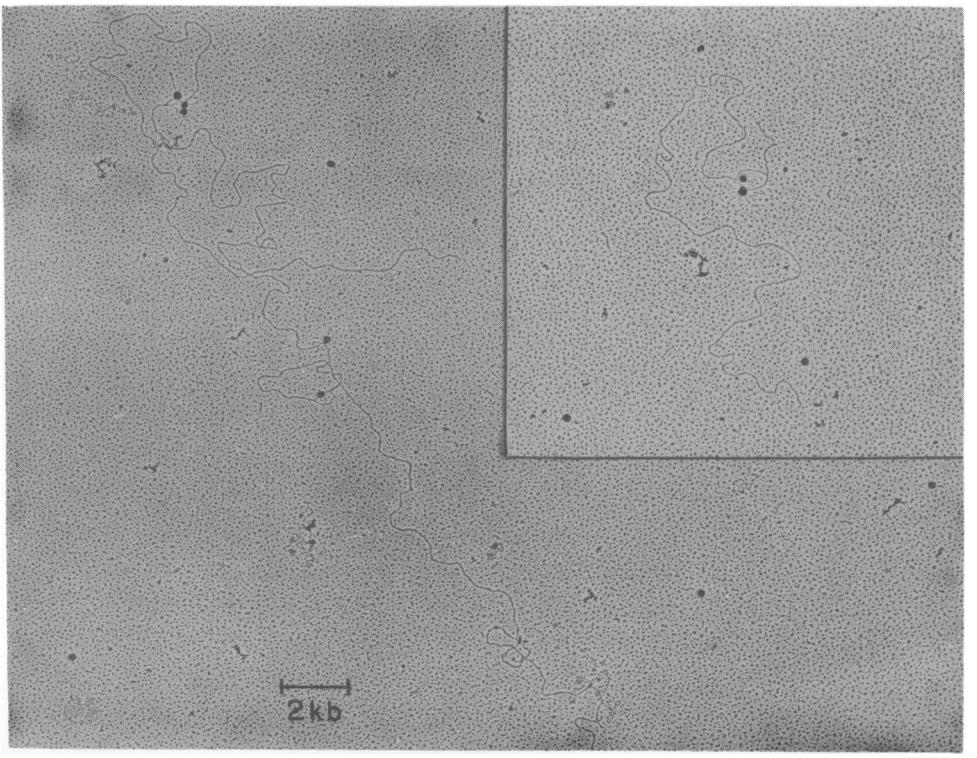

Figure 3. Electron micrographs of two double-stranded rDNA molecules containing attached avidin-spheres. The molecules were isolated from total D. melanogaster nuclear DNA by buoyant banding in CsCl after attachment of avidin-spheres to rRNA:rDNA R-loops.

isolated from total nuclear DNA in both a good yield (22 to 60%) and a relatively pure form (21 to 81%) by a single CsCl gradient centrifugation step.

Most importantly, the DNA strands obtained by this selection procedure are of sufficient length (i.e., 10 Kb to 150 Kb; <L> = 90 Kb) to allow the study of the organization of specific sequences over short (1-5 Kb) as well as long (5-50 Kb) distances. The desirability of examining such long range gene structural patterns is clearly evidenced in the study of the interspersion of the two classes of rRNA gene sequence arrangements in D. melanogaster (19). It is anticipated that the observation of long range patterns of gene organization will also be advantageous in the study of multigene families and coordinately expressed sets of genes. In addition, long DNA strands containing specific coding sequences should allow the study of both directly adjacent and more distant gene flanking sequences that may play a role in genetic regulation.

It is now possible to determine the approximate size of DNA fragments containing a specific gene sequence following restriction enzyme digest of total genomic DNA (20). The results of our study show that the density of a sphere:DNA complex containing a DNA of known length can be effectively predicted. Thus it is inviting to suggest the combined use of these two procedures for the selection of specific DNA strands for subsequent construction of recombinant DNA molecules.

Double-stranded DNA containing complementary sequences to the rRNA of X. laevis (21,22) and the histone mRNAs of the sea urchins S. purpuratus (13) and P. milaris (23) have been isolated by buoyant banding. However, this is possible only because the base composition of these tandemly reiterated genes differs significantly from the average for the entire genome. Thus, gene enrichment by DNA buoyant methods would be difficult for non-reiterated genes or genes whose base composition does not vary substantially from that of the total nuclear DNA. While our work was in progress, Wellauer and Dawid (24) showed that it is possible to isolate long duplex D. melanogaster rDNA by the formation of R-loops followed by buoyant banding. In this case, separation of the R-loop containing fragments from the rest of the DNA is based on the density shift in Cs_2SO_4 due to the RNA present in R-loop hybrids. The density shift for an RNA:DNA hybrid is proportional to the ratio of the length of RNA:DNA hybrid to the length of flanking DNA; thus, this method is probably limited to long or tandemly repeated genes, such that the RNA hybrid regions are at least half as long as the flanking DNA.

The results reported here show that the polymer sphere labeling procedure is effective for the isolation of repetitive gene sequences. It is in some ways more convenient as well as efficient than previously reported methods for the isolation of double strand DNA molecules containing specific gene sequences (13,21-24). In principle, the method can be applied to any polynucleotide and, therefore, may be useful for the isolation of mRNA sequences coded for by non-reiterated genes. Further experiments are being carried out to determine the effectiveness of the procedure for the isolation of non-reiterated gene sequences in Drosophila.

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