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MOLECULAR CLONING OF PART OF THE MITOCHONDRIAL DNA OF DROSOPHILA MELANOGASTER

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SUMMARY

We report the construction of recombinant plasmids containing part of the mitochondrial DNA of Drosophila melanogaster. Of the four fragments of this DNA generated by the restriction endonuclease HindIII, two were successfully cloned into the HindIII site of the plasmid pCM2. Unexpectedly the other two fragments could not be isolated by cloning into the HindIII site of either pCM2 or pBR322. Part of a third fragment, containing the gene for the large ribosomal RNA, was incorporated into the PstI site of pBR322. We show that this recombinant plasmid contains sequences complementary to an abundant RNA species which is present in Drosophila embryos and which binds to oligo-dT-cellulose.

INTRODUCTION

We are interested in applying recombinant DNA technology to a study of developmentally regulated genes of Drosophila melanogaster. A first step in isolating structural genes is the construction of double stranded cDNA copies of poly(A) RNA and the incorporation of these molecules into bacterial plasmids (1, 2). The major component of poly(A) RNA from Drosophila has been shown to be the 14S large mitochondrial ribosomal RNA (3 - 6). In order to develop a convenient assay for mitochondrial sequences we attempted to clone the corresponding mitochondrial gene. Klukas and Dawid (3) showed that the circular mitochondrial DNA of Drosophila melanogaster is split into four fragments by the restriction enzyme HindIII and that the gene for the large ribosomal RNA is contained within the largest of these fragments. Our initial aim, therefore, was to clone these fragments in the HindIII site of plasmid pCM2 (7) and select those recombinant plasmids containing the largest mitochondrial fragment.

Abbreviations used. poly(A) RNA = polyadenylated RNA. Tc = tetracycline Cm = chloramphenicol. Ap = ampicillin. ^{R} resistant. ^{S} sensitive.

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MATERIALS AND METHODS

Preparation of Mitochondrial DNA. Mitochondrial DNA was prepared from highly purified mitochondria from D. melanogaster (Oregon R) embryos as described by Bultmann and Laird (8).

Bacteria and Plasmids. HB101 recAr^- m^- was the host in transformation experiments and for the growth of plasmids. The properties of the plasmid cloning vectors pCM2(7) and pBR322(9) have been described. The conditions used for the transformation of bacteria and the preparation of plasmid DNA were as previously described (7).

Enzymes. The conditions for the preparation and use of restriction endonucleases, T4 DNA ligase, S1 nuclease (7) and terminal transferase (10) were as described previously.

Preparation of RNA and cDNA. Preparation of poly(A) RNA from Drosophila embryos was carried out as described previously (6). Poly(A) RNA was centrifuged through sucrose-SDS gradients (15-30% sucrose in 5mM Tris (pH 7.5), 100mM NaCl, 1mM EDTA, 0.5% SDS) at 25°C in the Spinco Sw40 rotor. The peak fractions sedimenting at about 14S were pooled and the RNA collected by ethanol precipitation. Using this 14S RNA as a template, cDNA labelled with [^{3}H]dCMP to about 10^7 cpm/μg was prepared as described by Bishop et al (11).

Hybridization. Plasmids were first linearized by sonication (4 x 30 seconds) in 0.3M NaCl, 10mM CH_3COONa (pH 5), followed by passage through a 1ml Chelex-100 column equilibrated with the same buffer. The DNA was then concentrated by ethanol precipitation. The plasmid DNA was mixed with about 10^4 cpm of tritiated cDNA in a 10 fold molar excess of plasmid DNA in 10mM Tris (pH 7.5). The solution was heated at 100°C for 3 minutes, transferred to a 70°C bath and made 0.36M NaCl, 30mM Tris (pH 7.5). Incubation was carried out until the Cot value was about 40 times the $Cot_{\frac{1}{2}}$ of the plasmid DNA. Two equal volume aliquots were then taken; one was treated with S1 nuclease and the TCA precipitable radioactivity in each was measured.

Agarose gel electrophoresis. Vertical slab agarose gels were run using 90mM Tris, 90mM boric acid, 3mM EDTA (pH 8.3), stained with 1μg/ml ethidium bromide and photographed under ultra violet light. The sizes of restriction fragments were estimated by comparison with EcoRI digested phage λ DNA or EcoRI and BamI digested pCM2 DNA (7) run on the same gels.

Single colony gels, for estimation of the sizes of plasmids in crude bacterial lysates were as described by Barnes (12).

RESULTS

Mitochondrial DNA was prepared from purified mitochondria from D. melanogaster embryos (8). Initially we used the restriction enzymes EcoRI, HindIII and HaeIII, to derive a restriction map of the circular DNA. The map, shown in Figure 1, is in good agreement with previously published maps (3, 13-15). The sizes of some fragments appear to be underestimated from our electrophoresis data compared with electron microscope studies. This is not surprising since D. melanogaster mitochondrial DNA has an unusually high (A + T) content.

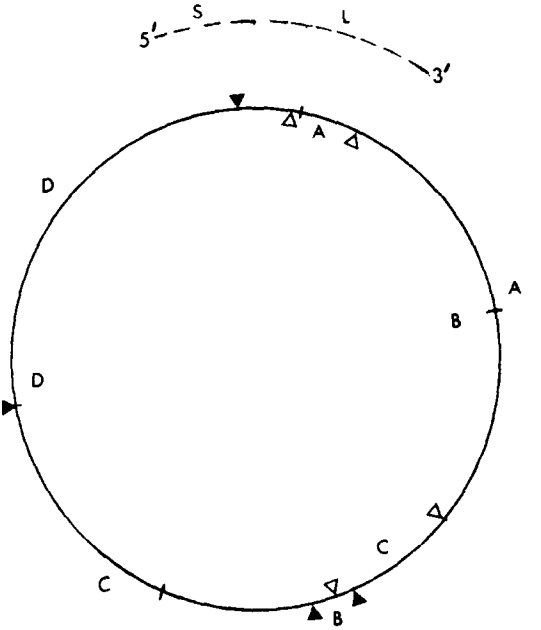

Figure 1. A restriction map of mitochondrial DNA derived from agarose gel electrophoresis of restriction fragments. The restriction enzyme sites are △ EcoRI ▲ HindIII | HaeIII. The dotted lines represent ribosomal RNA genes and their map positions are taken from the data of Klukas and Dawid (3). S, small ribosomal RNA; L, large ribosomal RNA. The sizes in kilobase pairs of the various fragments are HindIII A, 7.8; B, 0.45; C, 4.35; D, 4.65 and EcoRI A, 0.75, B, 5.1; C, 1.5; D, 9.9.

Plasmid pCM2 DNA(7), linearlized by treatment with HindIII, was mixed with HindIII-generated fragments of mitochondrial DNA in a 5-fold molar excess of mitochondrial DNA in the presence of T4 DNA ligase. The mixture, after ligation was used to transform E. coli cells and recombinant plasmids were selected by their $Cm^{R}Tc^{S}$ phenotype. Plasmid DNA was isolated from these colonies, restricted with HindIII and the resulting fragments examined by agarose gel electrophoresis. Only three different types of $Cm^{R}Tc^{S}$ plasmids were isolated using this approach. Of 24 plasmids examined, 12 contained the 4.35 kb mitochondrial DNA fragment, 6 contained the 0.45 kb mitochondrial DNA fragment and 6 resulted from a deletion of part of the pCM2 DNA. Figure 2 shows restriction analysis of each of these three classes of molecules. The experiment was repeated using pBR322; again only the 4.35kb and 0.45kb fragments were cloned.

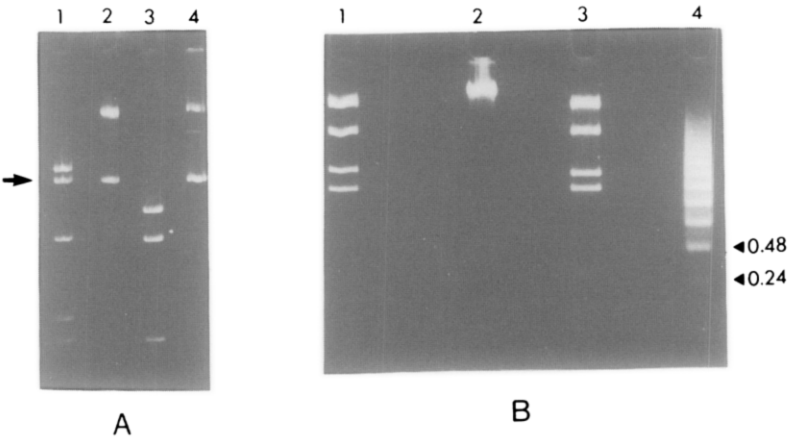

Figure 2. Restriction enzyme analysis of plasmid DNA. DNAs were restricted and the fragments subjected to electrophoresis in a 1%(A) or 2%(B) agarose gel. A. pMD107 treated with HindIII and EcoRI(1) and HindIII(2). pMD108 treated with HindIII and EcoRI(3) and HindIII(4). The arrow indicates the position of the 4.35kb mitochondrial DNA fragment cleaved out of pMD107 by HindIII. Comparison of tracks 1 and 3 shows that pMD108 is the result of a deletion. The bands in track 4 are the supercoiled, linear, and open circular forms of plasmid pMD108 which does not contain a site for HindIII. B. pMD112 treated with EcoRI(1), HindIII(2), EcoRI and HindIII(3), mouse satellite DNA treated with EcoRII(4). The size of the two smallest fragments in kilobase-pairs is indicated. The gel shows that pMD112 contains a 0.45kb mitochondrial DNA fragment which is cleaved out by HindIII and contains a site for EcoRI.

As an alternative cloning strategy (10) the HindIII mitochondrial DNA fragments were extended by the addition of blocks of cytosine residues to their 3' termini using the enzyme terminal transferase. The fragments were then annealed with pBR322 DNA which had been cleaved at its single PstI site and extended at the resulting 3' termini with blocks of guanosine residues. After annealing, the mixture was used to transform *E. coli* cells and colonies harbouring recombinant plasmids ($Tc^R Ap^S$) were selected. 17 such colonies were examined on single colony gels (12) to screen for any recombinant plasmids large enough to contain the 7.8 kb HindIII fragment. No such plasmids were generated using this approach. However, two plasmids were unusual in that they appeared to contain a length of mitochondrial DNA which did not correspond to any of the HindIII fragments. Restriction analysis of one of these plasmids, pMD417 (Figures 3 and 4) shows that it contains about 3.7kb of mitochondrial DNA derived from the part of the

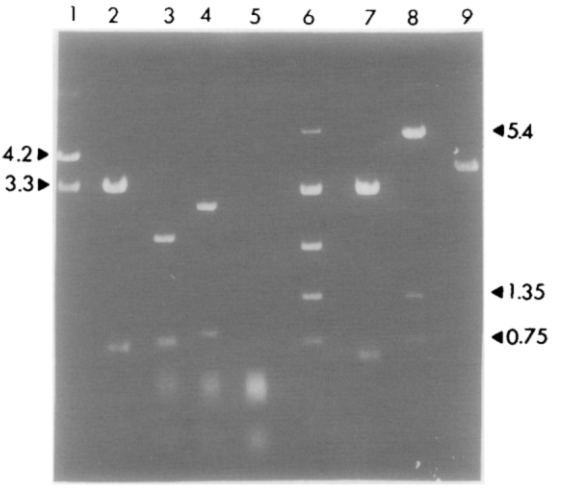

Figure 3. Restriction enzyme analysis of pMD417 DNA. pMD417 DNA (tracks 1, 3, 4, 6 and 8) and pBR322 DNA (2,5,7,9) were restricted and subjected to electrophoresis in a 1% agarose gel. The restriction enzymes used were PstI and HindIII (1,2), EcoRI and HaeIII(3), HaeIII(4,5), EcoRI and PstI(6,7), EcoRI(8,9). The fragment sizes shown are in kilobase pairs.

7.8kb HindIII fragment which was shown (3) to contain the gene for the large ribosomal RNA.

To demonstrate the presence of mitochondrial ribosomal RNA sequences within pMD417, plasmid DNA was sonicated to a length of about 400 base-pairs, denatured, and annealed with a trace of tritiated cDNA prepared from the 14S peak of an embryo poly(A) RNA preparation. The proportion of cDNA which became double stranded was assayed using nuclease S1. Table 1 shows that sequences in plasmid pMD417 are complementary to an abundant species in this RNA preparation. It has been established previously that the large mitochondrial ribosomal RNA is an abundant, 14S, poly(A) RNA (3 - 6).

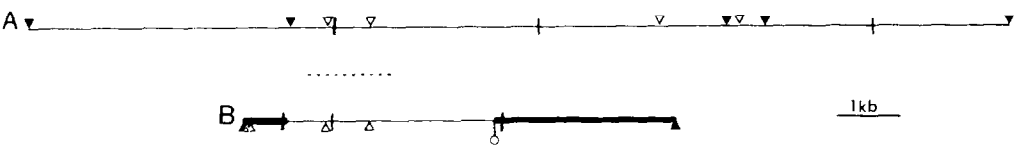

Figure 4. A. A restriction map of mitochondrial DNA as shown in Figure 1. B. A restriction map of plasmid pMD417. The thick line represents pBR322 DNA, the thin line mitochondrial DNA. The only HaeIII sites shown in pBR322 DNA are those immediately flanking the PstI site. Restriction enzyme sites are ▲ HindIII △ EcoRI | HaeIII ⚲ PstI. The dotted line represents the position of the gene coding for the large mitochondrial ribosomal RNA(3).

Table 1. Nuclease S1 resistance of tritiated cDNA made against 14S poly(A) embryonic RNA after annealing with plasmid DNAs as described in Materials and Methods. The results shown are the means of 3 separate experiments.

Plasmid	cpm - S1	cpm + S1	S1 resistance
pBR322	4700	234	5%
pMD417	4240	2160	51%

DISCUSSION

This paper demonstrates the construction of bacterial plasmids containing two of four HindIII fragments of D. melanogaster mitochondrial DNA and of a plasmid containing part of a third fragment. The 4.65kb fragment, none of which we have cloned, consists almost entirely of a region of very high (A+T) content (3). The length of this region varies widely within the genus Drosophila whereas the rest of the mitochondrial DNA is more highly conserved (13, 15). It is likely, therefore, that few biologically important functions are encoded in this region.

The mitochondrial DNA sequence in the plasmid pMD417 consists of only a part of the 7.8kb HindIII fragment. It may have arisen because the terminal transferase initiated homopolymer synthesis at a nick within this fragment. This plasmid is shown here to contain sequences complementary to an abundant 14S poly(A) RNA and detailed restriction mapping of this plasmid, of mitochondrial DNA, considering the data of Klukas and Dawid (3), shows that it contains the entire gene coding for the large mitochondrial ribosomal RNA. Therefore this plasmid may be used in the construction of DNA cellulose columns (16) to specifically remove 14S RNA from mRNA preparations or it can be labelled in vitro and used to probe for the presence of mitochondrial sequences in cDNA clones (17).

Finally the plasmids described here may be useful in a study of the organization and expression of the D. melanogaster mitochondrial genome.

ACKNOWLEDGEMENTS

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