Organization and Cloning of Mitochondrial Deoxyribonucleic Acid from *Paramecium tetraaurelia* and *Paramecium primaurelia*

DONALD J. CUMMINGS* AND JANE L. LAPING

Department of Microbiology and Immunology, University of Colorado School of Medicine, Denver, Colorado 80262

Previously we showed that the mitochondrial deoxyribonucleic acid (DNA) from *Paramecium aurelia* consists of a linear genome and that replication of this genome is initiated at one terminus and proceeds unidirectionally to the other terminus. Analyses of mitochondria from four closely related species (1, 4, 5, and 7) indicated that the species 1, 5, and 7 DNAs are essentially completely homologous but that the species 4 mitochondrial DNA is only 40 to 50% homologous with that from species 1. The major regions of homology are those containing the genes for ribosomal ribonucleic acid (RNA). To understand the replication and organization of the linear mitochondrial genome better, we compared species 1 (*Paramecium primaurelia*) and 4 (*Paramecium tetraaurelia*) DNAs with regard to restriction fragment mapping and homology between initiation regions; we also identified the sites of the genes for ribosomal RNA. In general, the structures of the species 1 and 4 mitochondrial genomes were quite similar. Each ribosomal RNA gene was present in one copy per genome, with the large ribosomal RNA gene located near the terminal region of replication and the small ribosomal RNA gene located more centrally. These two genes were separated by about 10 kilobases in the species 1 genome and by about 12 kilobases in the species 4 genome. In contrast to our previous findings, by using nonstringent hybridization conditions we detected homology between the species 1 and 4 DNA fragments containing the initiation regions. We constructed recombinant DNA clones for many fragments, especially those containing the initiation region and the ribosomal RNA genes. We also constructed restriction enzyme maps for six enzymes for both *P. primaurelia* and *P. tetraaurelia*.

In contrast to the mitochondrial deoxyribonucleic acids (mtDNA's) of most other organisms, the mtDNA's of *Paramecium* species are linear molecules that are 13 to 14 μm long, corresponding to about 40 kilobases (kb) (8, 9). So far, linear mtDNA genomes appear to be a unique feature of ciliates, since linear structures have been reported only in *Paramecium* and *Tetrahymena* (10, 21). However, unlike *Tetrahymena* mtDNA, in which replication proceeds bidirectionally (10), *Paramecium* mtDNA replication is initiated at a unique end and proceeds unidirectionally, producing lariat and head-to-head dimer-length molecules as intermediates (9). These dimer-length molecules have been used to clone dimer fragments of the initiation regions (16). This unusual type of replication may also occur in viral genomes, such as the vaccinia virus genome (24), and in yeast nuclear DNA (7), thus making it important to clarify the structure and function of *Paramecium* mtDNA.

From another viewpoint, *Paramecium* has been used to study mitochondrial-nuclear interactions. For example, Beale and Knowles showed that mitochondria from antibiotic-resistant cells can be used to transform sensitive cells by direct microinjection (2). The most interesting feature of this study was the demonstration that in mitochondrial transfers between different species of *Paramecium* only certain combinations were successful. Species 1 and 5 mitochondria were essentially interchangeable regardless of which species was used as donor and which was used as recipient. However, species 1 and 5 mitochondria were expressed in a species 7 background, but the reverse was not true. In addition, species 4 mitochondria were not compatible with species 1, 5, or 7. There are also species 4 mutants which affect mitochondrial function; these include the Cl_1 mutant (18), which is a double nuclear-mitochondrial mutant, and several stock and antibiotic-resistant mito-

chondrial mutants. Thus, a detailed study of the molecular nature of the mitochondrial linear genome would be valuable.

Previous studies in our laboratory showed that the DNAs from species 1, 5, and 7 *Paramecium aurelia* mitochondria are essentially completely homologous but that species 4 mtDNA is only 40 to 50% homologous with the mtDNA's of these related species (5, 6). The regions in species 1 and 4 mtDNA's with the highest degree of homology are the fragments containing the ribosomal ribonucleic acid (rRNA) genes. The least homology occurs in the fragments containing the initiation regions. Restriction enzyme mapping studies with a limited number of endonucleases showed that species 1 and 5 mtDNA's have essentially the same structure. Evolutionary divergence calculations also agreed with these homologies (4). The extent of the biological and molecular differences between species 1 and 4 mitochondria caused us to examine these mtDNA's more closely. In this paper we present detailed restriction enzyme maps which demonstrate that although there are differences between these two mtDNA's, the general structures of the two genomes are quite similar. The large rRNA gene is located near the termination end of the molecule in both species, whereas the small rRNA gene is located more centrally. These genes are separated by about 10 kb in species 1 DNA and by 12 kb in species 4 DNA. In addition, by utilizing nonstringent hybridization conditions, we demonstrated that the initiation region in species 4 DNA is homologous with the same region in species 1 DNA.

MATERIALS AND METHODS

Stocks and culture conditions. We used *Paramecium* species 1 (*Paramecium primaurelia* stock 513) and the following stocks and mutants of species 4 (*Paramecium tetraaurelia*): stocks 51, 51 mating type 7, and 172; mutant 51(7) Cl_1 M^{Cl}, a slow-growing (croissance-lente) strain containing the nuclear mutation Cl_1 and the mitochondrial suppressor M^{Cl}; and mutant 51(7) ERNG 5-6, a slow-growing erythromycin-resistant strain carrying a double mitochondrial mutation (1). All stock cultures were grown on an infusion of bacterized Scotch grass (7) containing stigmasterol at a concentration of 1 mg/liter to assist in growth. The mitochondria were isolated and the DNAs were extracted and purified on 4',6-diamidino-2-phenylindol-dihydrochloride–CsCl gradients as previously described (5, 6).

Restriction enzyme analysis. The reaction conditions used for digesting clones and mtDNA's were as described previously (12) or as specified by the enzyme supplier. The DNA fragments were separated by agarose gel electrophoresis (1.2, 1.5, or 1.8% agarose [Bethesda Research Laboratories]), stained with ethidium bromide, and photographed under a short-

wavelength ultraviolet transilluminator by using a Polaroid camera and type 665 positive/negative film.

Extractions of DNAs from agarose or acrylamide gels were performed by electroelution. The DNA fragments were visualized by using a long-wavelength transilluminator. Ethidium bromide was removed by butanol extraction and the DNA was precipitated as described previously (5). The methods used to determine the molecular weights of species 1 and 4 mtDNA restriction enzyme digest fragments have been described previously (5, 14).

Cloning of isolated DNA fragments. Depending on the endonuclease used, the mtDNA fragments were cloned into the *Eco*RI, *Bam*HI, *Pst*I, or *Hind*III sites of plasmid pBR325. Transformants were selected for by using tetracycline, chloramphenicol, or ampicillin, as appropriate, and these transformants were amplified in liquid cultures containing spectinomycin (12). The mtDNA insertions were isolated by electrophoresis and electroelution. These insertions were nick-translated with a ^{32}P Nick Translation System (New England Nuclear Corp.) (17). ^{32}P-end-labeling of isolated DNA fragments was also performed by using the New England Nuclear Corp. kit (15).

Southern hybridizations (20) were performed on nitrocellulose paper filters as described previously (23). Each hybridization mixture contained 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2.5× Denhardt solution, 60 mM phosphate, 600 μg of sonicated herring sperm DNA per ml (23), and 5 × 10^5 to 10 × 10^5 cpm of ^{32}P probe. Hybridization was allowed to proceed for 24 h at 42°C unless otherwise specified, and each strip was washed four times in 50% formamide–2× SSC–1% sodium lauryl sulfate at 42°C and then two times at room temperature in 2× SSC. The strips were dried, placed on type XRP-5 X-ray film over a Lightning-Plus intensifying screen, and exposed for 15 to 72 h at −70°C.

RESULTS

Restriction maps for species 1 mtDNA. As reported previously (5, 6), species 1 and 5 mtDNA's are quite similar both with regard to their restriction maps and with regard to the positions of their rRNA genes. In both of these genomes, each rRNA gene (large and small) is represented once, and these genes are separated by several thousand base pairs. Therefore, the data which provided *Eco*RI, *Bam*HI, and *Sma*I restriction maps for species 1 mtDNA are not shown here. In this study our main purpose was to make a detailed comparison between species 1 and 4 mtDNA's since the greatest evolutionary divergence has occurred between these two species (4); moreover, these two species of mitochondria cannot be exchanged successfully by microinjection (2).

We used the restriction enzyme maps published previously (5) to generate new *Pst*I and *Hind*III maps. In addition, while this work was in progress, we received the species 1 mtDNA

*Pst*I, *Sal*I, and *Hpa*I maps of Beale and Tait (G. Beale and A. Tait, Int. Rev. Cytol., in press). The *Pst*I map of Tait and his co-worker completely agreed with our map, and we did not construct maps for the other two enzymes. Since we had begun a *Sal*I map of species 4 mtDNA, we include the *Sal*I map of Beale and Tait for species 1 mtDNA here (Fig. 1) for reference. The technique which we used to construct the *Pst*I and *Hind*III maps was Southern blot hybridization between fragments, using nick-translated probes. For example, *Eco*RI fragment 2, which was located at the initiation end of species 1 mtDNA, hybridized with *Bam*HI-4, *Bam*HI-3, *Pst*I-3, *Pst*I-7, and *Pst*I-2. *Bam*HI-4, the *Bam*HI fragment at the initiation end, hybridized only with *Pst*I-3, whereas *Bam*HI-2 hybridized with *Pst*I-2 and *Pst*I-5. From this, we concluded that the order of fragments in this region of the *Pst*I map was *Pst*I-3, *Pst*I-7, *Pst*I-2, *Pst*I-5. Similarly, we used ^{32}P-labeled *Hind*III fragments 2 and 4 to show that *Pst*I-2 and *Pst*I-5 were linked and that *Pst*I-5 and *Pst*I-4 were linked. *Pst*I-6 hybridized with *Eco*RI-1, *Bam*HI-1, *Hind*III-6, and *Hind*III-10. The *Sal*I map of Beale and Tait was useful in that it led us to isolate *Sal*I-1, the largest fragment. This fragment was used in two ways. First, an *Eco*RI digest yielded *Eco*RI fragments 3, 4, and 5 and the part of *Eco*RI fragment 1 which was located on the right half of the map (fragment pE1) (Fig. 1). Hybridization with *Hind*III-3 showed homology with *Eco*RI fragments 4, 5, and pE1, indicating that this fragment completely encompassed *Eco*RI-5 and overlapped *Eco*RI fragments 4 and pE1. In addition, ^{32}P-end-labeling of *Sal*I-1 followed by digestion with *Eco*RI showed unambigously that *Eco*RI fragments 3 and pE1 were at the ends. *Hind*III digestion demonstrated that *Hind*III fragment 6 was at one end of *Sal*I-1; no obvious fragment for the other end was detected. With the *Sal*I-1 end-labeled fragments, the most label found was in *Eco*RI fragment pE1. *Eco*RI-3 and the *Hind*III fragment located terminally did not have restriction sites at their terminal ends, and hence these ends were least available for labeling. Figure 2 shows these results, along with other restriction digest fragment patterns.

The results described above and other similar results allowed us to construct the maps shown in Fig. 1. The exact locations of the rRNA genes were of particular interest. Previous studies (5)
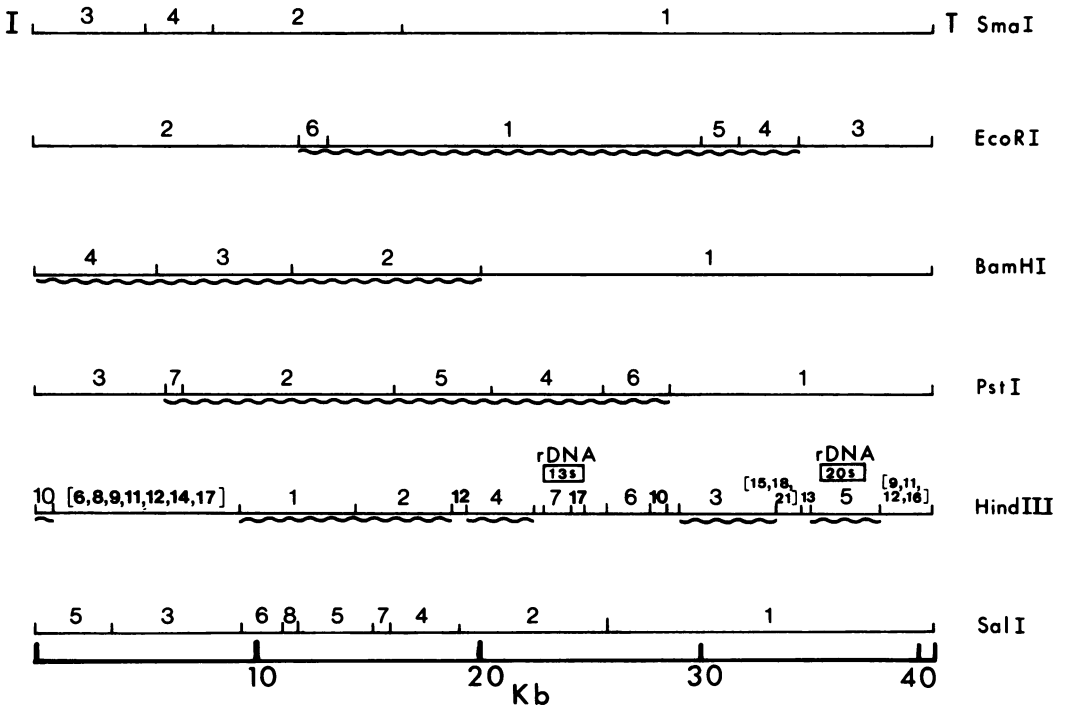

FIG. 1. *Restriction enzyme maps of species 1 stock 513 mtDNA. I, Initiation end of the molecule; T, termination end. The locations of the rDNA fragments are indicated. For the HindIII map, fragments whose order is still uncertain are in brackets. The calibration scale was constructed from data obtained in this study, as well as in previous reports (5, 14). The wavy lines indicate the fragments which have been cloned; the lines under the monomer (M) regions represent dimer initiation region clones.*
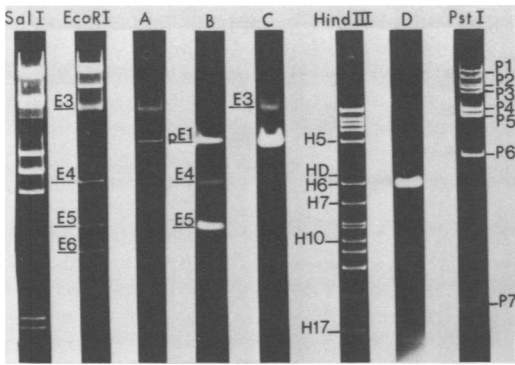
FIG. 2. *Enzyme digestion patterns and* ^{32}*P-end-labeling involving species 1 stock 513 mtDNA. The tracks show ethidium bromide-stained gels of digests of the complete genome, as labeled. Track A, Ethidium bromide-stained gel of an EcoRI digest of isolated SalI fragment 1; track B, autoradiograph obtained from a Southern blot by using* ^{32}*P-labeled species 1 mtDNA HindIII-3 as a probe; track C, autoradiograph of an EcoRI digest of* ^{32}*P-end-labeled SalI-1; track D, autoradiograph of a HindIII digest of* ^{32}*P-end-labeled SalI-1. Band HD in the HindIII digest pattern is the dimer fragment isolated and identified as described previously (16).*

showed that the large rRNA subunit (20S) hybridized with EcoRI-3 and HindIII-5, and we also observed this. The small subunit (12S to 14S) hybridized with EcoRI-1, HindIII-7, and HindIII-17; in addition, it hybridized with PstI-4. J. Seilhamer has done a fine structure analysis, including DNA sequencing of HindIII-5 and PstI-4, and his findings will be published elsewhere. His data allowed fairly precise positioning of HindIII-7 and HindIII-17, from which we calculated that the large and small rRNA genes were separated by about 10 kb.

Clones of several fragments of species 1 mtDNA were constructed, and these are shown in Fig. 1. As expected, we were not able to clone the fragments located at the ends of this linear genome. Some attempts have been made to guanine-cytosine or adenine-thymine tail these end fragments, but these attempts have not been successful. However, we have cloned the HindIII dimer initiation fragment (twice the molecular weight of HindIII-10) (16). Most of species 1 mtDNA has been cloned.

Restriction maps for species 4 stock 51 mtDNA. In our previous studies with species 1 and 5 mtDNA's (5), we encountered little difficulty with the localization of the rDNA fragments. Because of comigrating fragments, in species 4 mtDNA the exact locations of some of the fragments containing the rDNA genes were not determined. This was true particularly for the

large rRNA subunit which hybridized with HindIII-3; these fragments contained perhaps three comigrating species. In addition, technical difficulties (nonspecific hybridization) in using ^{125}I-labeled rRNA as a probe confounded this identification. Rather than pursue this line of investigation, since the rRNA genes were the genes which were most conserved in species 1 and 4 mtDNA's (4–6), we used the fragments containing these genes from species 1 mtDNA as probes (HindIII-5 for the large subunit and HindIII-7 and HindIII-17 for the small subunit). These were available as cloned fragments and hence were quite pure. Figure 3 shows the results when these fragments were used as probes, and Fig. 4 shows the corresponding DNA fragment patterns for the different enzymes. As these figures show, species 1 mtDNA HindIII-5 hybridized with species 4 mtDNA EcoRI-3, BamHI-2, PstI-1, HindIII-3, and SalI-1. Species 1 mtDNA HindIII fragment 7 was homologous to species 4 mtDNA EcoRI-2, BamHI-1, PstI-3, PstI-6, HindIII-8, and SalI-1. Species 1 mtDNA HindIII-17, the other part of the small rRNA gene, hybridized with EcoRI-2, BamHI-1, PstI-3, and HindIII-8 but not PstI-6. These results allowed us to identify with certainty which species 4 mtDNA fragments contained genes for rRNA.

As indicated above, one difficulty which we encountered with the HindIII fragment pattern was that HindIII-3, the fragment which was homologous to the large rRNA subunit, consisted of possibly three comigrating fragments (Fig. 4). There were approximately 3,000 base pairs in species 1 mtDNA HindIII fragment 5, more than enough to account for one copy of the large rRNA subunit. However, there were only about 2,300 base pairs in species 4 mtDNA HindIII fragment 3, raising the possibility that more than one of these comigrating fragments contained information for the large rRNA subunit. To clarify this, we prepared a preparative gel of an HindIII digest of species 4 mtDNA, isolated the entire HindIII-3 complex, and constructed clones. We found 50 clones, and 19 of these were subsequently amplified and examined on gels. A total of 12 of these migrated in the HindIII-3 region, and 6 migrated at the HindIII-5 position. Another HindIII-3 clone was also isolated when the DNA from the region in the gel above HindIII-1 (i.e., partially digested DNA) was cloned. This proved to be a double clone of HindIII-3 and HindIII-8, the fragment containing the small rRNA gene. HaeIII digestion (Fig. 5) showed that three general types of fragments were present; these were designated HindIII-3A, HindIII-3B, and HindIII-3R. The last of these was designated HindIII-3R only
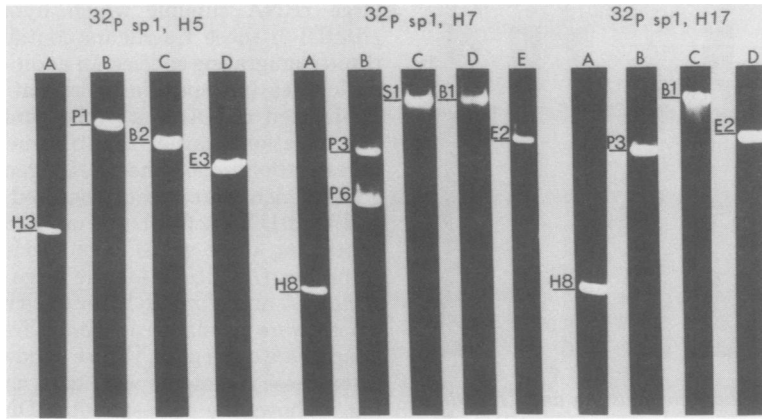
FIG. 3. *Identification of species 4 stock 51 mtDNA restriction enzyme digest fragments containing rRNA genes by using ³²P-labeled species 1 stock 513 mtDNA HindIII-5 (³²P sp 1, H5) (large rRNA subunit gene) and HindIII-7 (³²P sp 1, H7) and HindIII-17 (³²P sp 1, H17) (small rRNA subunit gene). All tracks are autoradiographs of Southern blots constructed by using HindIII (H), PstI (P), BamHI (B), SalI (S), and EcoRI (E) digests of species 4 stock 51 mtDNA. Tracks A show the hybridization with a HindIII digest, and the other tracks are as indicated.*
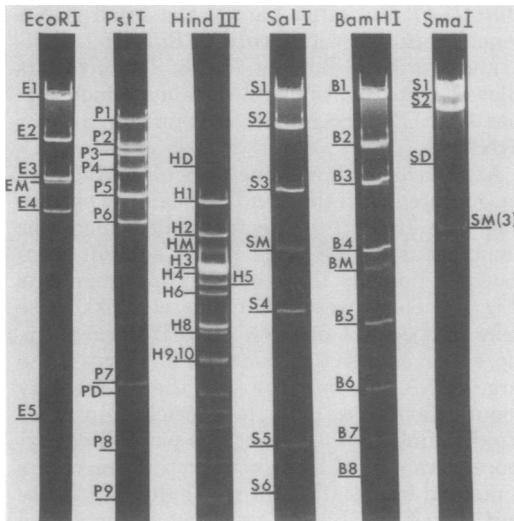

FIG. 4. *Species 4 stock 51 mtDNA restriction enzyme digest patterns. The fragments labeled M and D are the initiation monomer and dimer fragments, respectively, and are discussed in the text. Note that these initiation fragments (M) are present in less than molar amounts.*

FIG. 5. *HaeIII restriction digest patterns of isolated insertions from HindIII-3B (lane H3B), HindIII-3R (lane H3R), and HindIII-3A (lane H3A) clones of species 4 stock 51 mtDNA (lane 4,51). Species 1 stock 513 mtDNA HindIII-5 hybridized with only HindIII-3R (data not shown).*

after we found that this was the only HindIII-3 fragment which hybridized with species 1 mtDNA HindIII-5. These three fragments, as well as HindIII-1, HindIII-2, and HindIII-5 cloned insertions, were used as probes for mapping. The HindIII-3R insertion probe gave the same hybridization pattern as species 1 mtDNA HindIII-5, namely, EcoRI-3, BamHI-2, PstI-1, SalI-1, and SmaI-1. ³²P-labeled probes of
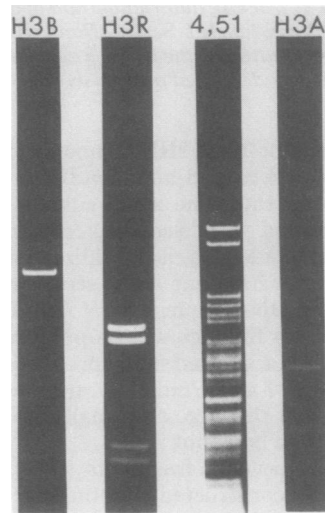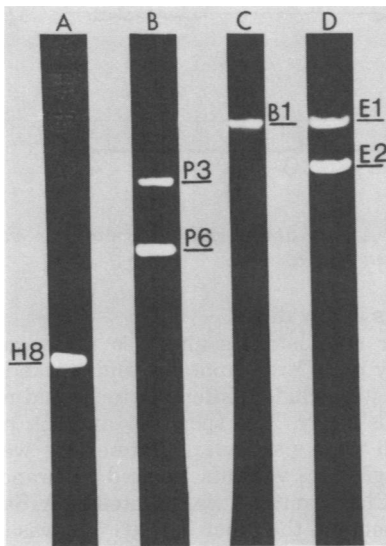
HindIII-3A and HindIII-3B gave different homology patterns, and these are summarized in Table 1. Table 1 also shows the homologies demonstrated by using species 4 mtDNA HindIII-8 (the small rRNA locus) as a probe. The autoradiograph for this experiment is shown in Fig. 6. These results were interesting because they showed the power of using small fragments as probes for mapping studies, as well as for

TABLE 1. *Homologies of species 4 mtDNA, as determined by using species 4 mtDNA HindIII fragments 1, 2, 3A, 3B, 3R, 5, and 8 and species 1 mtDNA HindIII fragments 5, 7, and 17 as probes*

HindIII probe		Homologous fragments				
mtDNA species	Fragment	EcoRI	BamHI	PstI	SalI	SmaI
1	5	3	2	1	1	
1	7	2	1	3,6	1	
1	17	2	1	3		
4	8	2,1	1	3,6		
4	3A	1	1	4	2,4,6	2
4	3B	2	1	3		1
4	3R	3	2	1	1	
4	1	1	1	4,5,7	2	2
4	2	1	1	5	2	
4	5	4,5	2,3	1		

FIG. 6. *Autoradiographs of ³²P-labeled species 4 mtDNA HindIII-8 hybridized to species 4 stock 51 mtDNA digests of HindIII (lane A), PstI (lane B), BamHI (lane C), and EcoRI (lane D).*

demonstrating homologous hybridization (species 4 to species 4) versus heterologous (species 1 to species 4) hybridization. When species 1 mtDNA HindIII-7 and HindIII-17 were used as probes, only species 4 mtDNA EcoRI-2 showed hybridization. However, when species 4 mtDNA HindIII-8 was used, EcoRI-1 and EcoRI-2 displayed homology, indicating that there were additional base pairs on HindIII-8 which were not concerned with the small rRNA gene. The fact that species 1 mtDNA HindIII-17 did not hybridize with PstI-6 whereas HindIII-8 did also helped position this small fragment on the map, as did the EcoRI-1 and EcoRI-2 hybridizations with species 4 mtDNA HindIII-8. The results obtained when ³²P-labeled HindIII-1, HindIII-2,

and HindIII-5 were used as probes are also shown in Table 1.

These analyses of homology with the rRNA gene fragments were used to construct maps. Additional probes were also useful. For example, BamHI-3 hybridized with SmaI-1, EcoRI-2, EcoRI-4, PstI-1, PstI-3, SalI-1, HindIII-5, HindIII-7, HindIII-12, HindIII-13 and HindIII-14. HindIII-1, HindIII-2, HindIII-5, BamHI-3, BamHI-5, BamHI-6, BamHI-7, BamHI-8, EcoRI-1, EcoRI-2, EcoRI-3, EcoRI-4, EcoRI-5, PstI-2, PstI-3, PstI-4, PstI-5, PstI-6, PstI-7, PstI-8, and PstI-9 were all used as probes. The data obtained with these probes, as well as the data obtained by using dimer molecules (see below), were used to construct the maps shown in Fig. 7. Although some of these hybridizations were redundant, they did confirm the assigned positions. For example, hybridization with ³²P-labeled PstI-5 was particularly nice since this fragment hybridized mostly with HindIII-2 but also with HindIII-1 and HindIII-6, thus fixing the positions of these fragments and confirming the results of the HindIII-1 and HindIII-2 hybridizations. ³²P-end-labeling of PstI-1 confirmed that BamHI-2 was located at the termination end of the genome. Finally, to complete the mapping results, PstI-2 hybridized to BamHI-M, BamHI-4, BamHI-5, BamHI-6, EcoRI-M, EcoRI-1, SmaI-M, SmaI-2, HindIII-M, SalI-M, and SalI-3, thus fixing the initiation end of the molecule. As Fig. 1 and 7 show, the general structures of the species 1 and 4 mtDNA genomes were quite similar. In particular, the termination ends of the EcoRI maps had much in common. The large rRNA gene was located here, and some restriction sites overlapped. The small rRNA gene was also located in a more central position for both species, with the separation being about 12 kb in species 4 mtDNA and about 10 kb in species 1 mtDNA. This was also true in species 5 mtDNA (5). Some of the
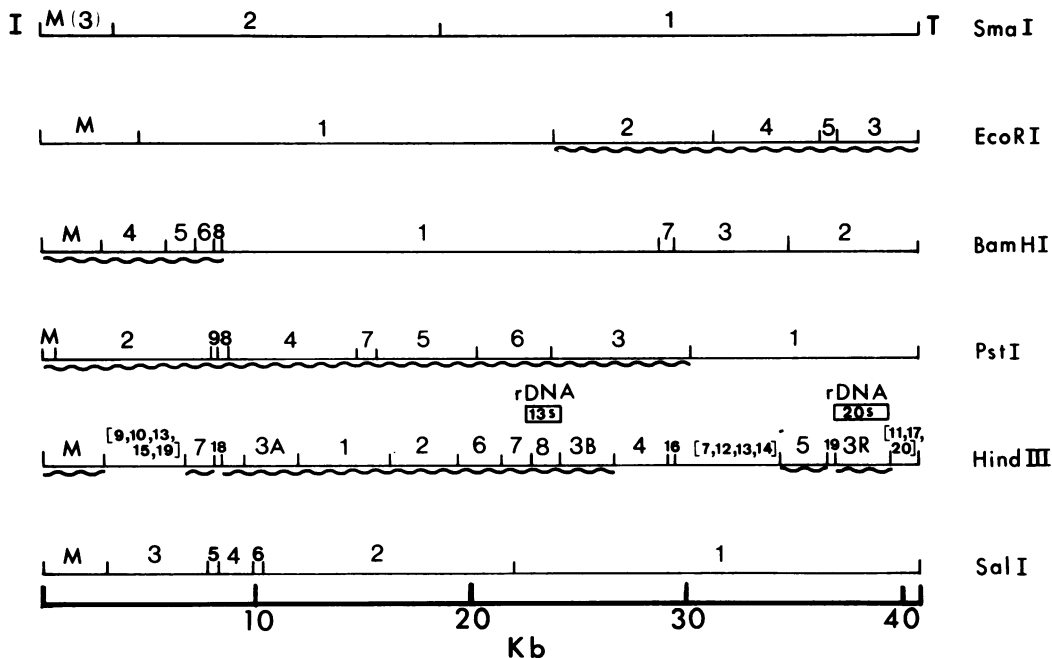
FIG. 7. *Restriction enzyme maps of species 4 stock 51 mtDNA. I, Initiation end; T, termination end of the genome. The wavy lines indicate the fragments which have been cloned.*

locations of the *Hind*III sites in both species 1 mtDNA and species 4 mtDNA may have to be adjusted slightly in future studies, and clearly not all fragments, especially the smallest, have been mapped. Some of these positions will be clarified when the fine structure maps of the rRNA genes are completed.

As with species 1 mtDNA, many fragments of species 4 mtDNA were cloned, and these are shown in Fig. 7. In general, we were not able to clone the terminal fragments. However, *Eco*RI-3 was clearly at the termination end of species 4 mtDNA; yet we obtained several clones with this insertion. There may well be another *Eco*RI site very close to the end of this molecule, which would account for the successful cloning of *Eco*RI-3. Examinations with gels containing higher percentages of agarose (or acrylamide) have not revealed another fragment, but this does not rule out the possibility of a fragment less than 50 base pairs long.

Studies of the DNA initiation region. As indicated above, it was not difficult to detect the initiation regions of species 1 and 5 mtDNA's on gels. Each of these regions was present in essentially stoichiometric quantities. For species 4 mtDNA, this was not the case (Fig. 4), and this complicated early attempts at constucting a map. The ability to clone the dimer fragment from one endonuclease digest (16) enabled us to identify the monomer and dimer initiation frag-

ments in any digest.

For completeness and also to indicate the utility of different mutants and stocks in mapping, we included different stocks and mutants in this study. The species 4 mtDNA mutants which were particularly interesting were two slow-growing variants isolated in France. One (the Cl M[Cl] mutant) was isolated by A. Sainsard-Chanet, and the other (ERNG 5-6) was isolated by A. Adoutte. Figure 8 shows the *Pst*I and *Bam*HI digest patterns of these variants. Species 4 mtDNA from strain 51(7), the parent of the two mutants, was also examined, and this DNA gave the expected pattern. Several things should be noted here. First, the mutant ERNG 5-6 DNA lacked *Pst*I-3, and the *Pst*I-1 of this mutant had a higher molecular weight, indicating that in the wild-type mtDNA *Pst*I-1 and *Pst*I-3 were linked. This represents the first demonstration of an erythromycin-resistant mitochondrial mutant of *Paramecium* in which genomic change has been found in a gene other than the rRNA gene. As expected for this mutant, *Hind*III-8 from species 4 stock 51 mtDNA hybridized with *Pst*I-1 and *Pst*I-6. Similarly, in the *Bam*HI digest, *Bam*HI-7 was absent in species 4 stock 172 mtDNA, and *Bam*HI-3 had a higher molecular weight, indicating that *Bam*HI-3 and *Bam*HI-7 were linked. Moveover, ^{32}P-labeled *Pst*I-3 from species 4 stock 51 mtDNA hybridized with *Bam*HI fragments 1, 3, and 7, indicat-
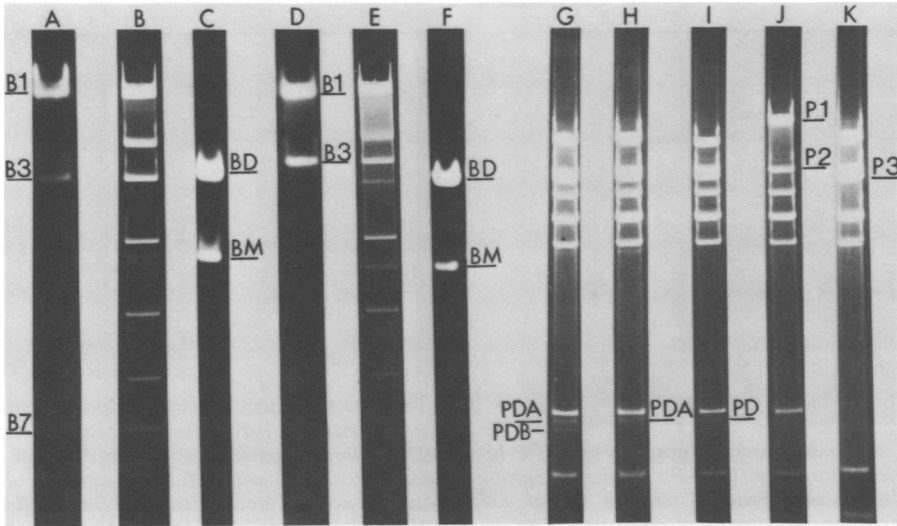
FIG. 8. *Restriction enzyme digests of different stocks and mutants containing species 4 mtDNA. Tracks A and D, Autoradiographs obtained by using ^{32}P-labeled species 4 stock 51 mtDNA PstI-3 hybridized with a species 4 stock 51 mtDNA BamHI digest (track B) and a species 4 stock 172 mtDNA BamHI digest (track E); tracks C and F, autoradiographs obtained by using ^{32}P-labeled species 4 stock 51 mtDNA PstI dimer hybridized with identical digests in tracks B and E, respectively. Note the difference in the motilities of the BamHI monomers and dimers in the two stocks. Tracks G through K, PstI digests of mutant Cl M^{Cl} mtDNA (track G), species 4 stock 51(7) mtDNA (track H), species 4 stock 51 mtDNA (track I), species 4 mutant 51(7) ERNG 5-6 mtDNA (track J), and species 4 stock 172 mtDNA (track K). Not all tracks were from the same gel. Hence, PstI-9 is not always visible, but it was present in each case. The only major difference noted in the species 4 mutant Cl M^{Cl} mtDNA was the presence of two dimer species, labeled PDA and PDB.*

ing that these fragments were linked in the order *Bam*HI-1, *Bam*HI-7, *Bam*HI-3. This was demonstrated by using either *Pst*I-3 or *Eco*RI-2 as a probe. More importantly, another change was observed in the *Bam*HI digests of species 4 stock 51 mtDNA and species 4 stock 172 mtDNA. Fragments previously thought to be artifacts were identified as the initiation end fragments (designated M and D for monomer and dimer, respectively). Thus, two *Bam*HI sites were different in species 4 stock 172 mtDNA (the link between *Bam*HI fragments 3 and 7), and the initiation fragment was smaller. In the *Pst*I digests of the so-called Cl mutant mtDNA two dimer fragments were evident, both of which hybridized with the initiation region. Right now, we do not know whether this polymorphism of the dimer initiation region was due to the nuclear mutation (Cl), to the mitochondrial suppressor (M^{Cl}), or to some as-yet-unspecified mechanism. We are currently performing DNA sequence analyses of these fragments to ascertain what molecular alterations have occurred. Species 4 stock 172 mtDNA also contained two altered fragments in the *Pst*I digest; *Pst*I fragments 4 and 7 were linked to form a new fragment which comigrated with *Pst*I-3, and the *Pst*I dimer fragment appeared to be absent. Au-

toradiography showed that this dimer fragment had a lower molecular weight than usual, approximately equal to the molecular weights of the monomer fragments of the other stocks. It should be emphasized that most of these *Pst*I dimer fragments have been cloned, indicating they are indeed dimer fragments, and all hybridized quite well with our dimer fragment isolate (Fig. 9).

Hybridization of species 4 mtDNA initiation region with species 1 mtDNA. A disturbing feature of our previous findings was the hybridization of species 1 mtDNA *Hind*III dimer with species 4 mtDNA (5, 6). Although excellent hybridization was obtained with species 1, 5, and 7 mtDNA's, very little homology was detected with species 4 mtDNA. Since the initiation region of the mitochondrial genome is so critical to normal function, we felt that some hybridization should be demonstrable. Based on our previous assertion that this region was rich in adenine and thymine, we decided to perform hybridizations at lower temperatures, thus favoring possible adenine-thymine-rich regions. Figure 10 shows unmistakably that as described previously, hybridization between species 1 and 4 mtDNA initiation regions was very poor at temperatures above 37°C, but that good homol-
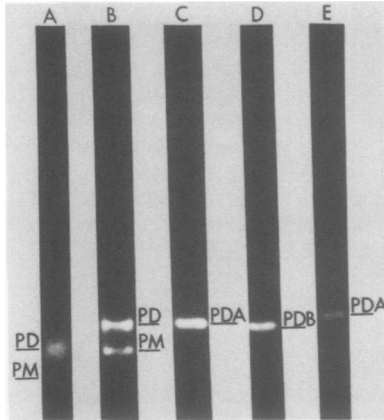
FIG. 9. *Autoradiographs obtained by using ^{32}P-labeled species 4 stock 51 mtDNA PstI dimer as a probe against PstI digests. Track A, species 4 stock 172 mtDNA (note the decreased molecular weight of the dimer and the diffuseness of this band); track B, species 4 stock 51 mtDNA; track C, clone of species 4 stock 51 mtDNA PstI dimer; track D, clone of species 4 mutant 51(7) Cl M^{Cl} PstI dimer fragment B; and track E, clone of species 4 mutant 51(7) Cl M^{Cl} mtDNA PstI dimer fragment A. Figure 8 shows the positions of these initiation fragments relative to the complete digest.*

FIG. 10. *Hybridization of ^{32}P-labeled species 4 stock 51 mtDNA PstI dimer to species 1 stock 513 mtDNA under nonstringent hybridization conditions. Tracks A, B and C, Autoradiographs obtained by using species 4 stock 51 mtDNA BamHI (track A), PstI (track B), and HindIII (track C) digests at 30°C, showing the monomer and dimer homologies. Tracks D through H were obtained by using species 1 stock 513 mtDNA. Track D was hybridized at 30°C, and track F was hybridized at 37°C, both against the gel pattern of species 1 stock 513 mtDNA shown in track E. In this experiment the cells containing species 1 stock 513 mtDNA were grown in the presence of chloramphenicol to enrich for dimer molecules. Hence, EcoRI fragment D (ED) showed enhanced hybridization relative to EcoRI-2, the monomer for species 1 mtDNA EcoRI. Tracks G and H were obtained by using normal species 1 stock 513 mtDNA (track G, PstI digest; and track H, HindIII digest). Both track G and track H were hybrided at 30°C. Heterologous nonstringent hybridization was not as prominent as homologous hybridization but was clearly demonstrable.*

ogy was demonstrated at 30°C for *Eco*RI, *Pst*I, and *Hin*dIII digests. Also included for analysis are species 4-species 4 hybridization results, which showed the monomer-dimer relationships for *Bam*HI, *Pst*I, and *Hin*dIII. This type of approach to homology can be termed nonstringent and has been used to show homologies among polyoma virus, simian virus 40, human papova virus (13), and different BK viruses (25). As much as 33% mismatch can be overcome by reducing the stringency of the hybridization conditions (13). These results show the need for sequence data to assess the actual degree of homology since hybridization can yield only qualitative information. However, it should be emphasized that some specificity is necessary, since we failed to demonstrate homology of the species 4 mtDNA *Pst*I dimer with either simian virus 40, ϕX-174, or *Neurospora* mtDNA.

DISCUSSION

Using this and other reports, we determined the physical organizations of the mtDNA's from three closely related species of *P. aurelia* mitochondria. Previously, we showed that there was little or no difference in contour length or guanine-plus-cytosine content (14) and that the rRNA genes were highly conserved (5, 6). In this paper we present detailed restriction maps of
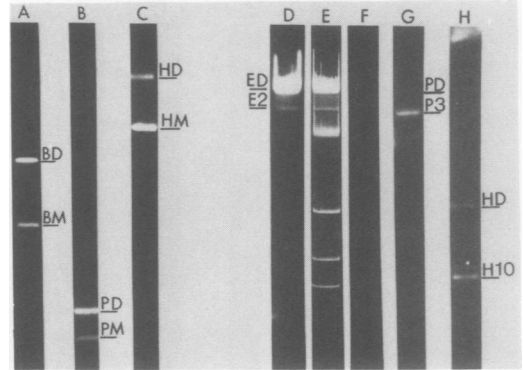
the least-related mtDNA's, species 1 and 4 mtDNA's, and show that the general structures of these mtDNA's are quite similar. In light of the degree of homology between species 1 and 4 mtDNA (5, 6), this similarity in structure is quite satisfying since it would be somewhat disturbing to have two members of the same biological complex be so dissimilar. Like yeast (19) and *Tetrahymena* (11) mtDNA's, but unlike mammalian (22) mtDNA's, the two rRNA genes are far apart and are located in about the same position in species 1, 4, and 5 mtDNA's. What this implies with regard to function is not clear, but the polycistronic message control which has been found in mammalian mtDNA's (22) may not be operative in these mtDNA's. Although we did not investigate the presence of intervening sequences in species 4 rDNA, we did investigate such sequences in species 1 and 5 rDNA's (5) and found none. The *Hin*dIII fragment containing the large rRNA gene (*Hin*dIII-3) is at most 2,300 base pairs long. Based on sequence

data for species 1 mtDNA *Hind*III-5 (J. J. Seil-hamer and D. J. Cummings, unpublished data), the large rRNA gene is about 2,100 base pairs long, so if there is an intervening sequence in species 4 mtDNA, which is doubtful, it cannot be very large. In the future we plan to compare the fine structure maps and the DNA sequences of both large and small rRNA genes, and this issue will be settled; we will also examine the role of the flanking transfer RNA sequences, which have been found in mammalian rRNA genes (22).

Even though we had to use nonstringent hy-bridization conditions, we demonstrated homol-ogy between the initiation regions of species 1 and 4 mtDNA's. We have no direct information on the extent of the homologies, but preliminary sequence studies (A. E. Pritchard and D. J. Cummings, unpublished data) have shown that for both species 1 and 4 mtDNA's there are very long stretches of adenines and thymines, which are homologous. Computer analyses of the data have not been performed yet, but these apparent homologies could easily account for the hybrid-ization found when nonstringent conditions were used. However, it is clear that the initiation regions in these two DNA species are not iden-tical. This may have some bearing on the in-ability to transform paramecia containing these DNAs in interspecies exchanges of antibiotic-resistant mitochondria. The genetic information carried by the mitochondrial genome is limited, and hence nuclear interaction may be necessary for correct expression of the initiation region. This may be the explanation for the finding that the C1 mutant apparently has two types of *Pst*I dimer molecules. This could affect the growth rate since initiation of replication might be im-paired. Sequence and computer analyses of these initiation regions may provide the necessary in-formation to understand these observations.

Finally, with regard to the nonmolar amounts of the species 4 mtDNA monomer initiation region, preliminary sequence data have indi-cated that this region consists of direct repeats of an almost pure adenine-thymine-rich nonpal-indromic unit. It may well be that in the mon-omer this region is labile, leading to its loss during isolation. Although no systematic study has been done, we have observed that cultures obtained from new exautogamous clones have more of this monomer, so perhaps autolytic en-zymes accumulate as a culture ages. Varying culture conditions (growth in Cerophyll, log-phase growth versus stationary growth, etc.) had no lasting effect on the amount of monomer. Of course, dimer-length fragments could be en-riched for by growth in the presence of chlor-

amphenicol (8, 9). The apparent equimolar amounts of monomer fragments in species 1 mtDNA can be explained on this same basis since in species 1 mtDNA this nonpalindromic unit is different in sequence and has many fewer repeats. Cells containing species 1 mtDNA have a much longer life span and may be subject to less accumulation of autolytic enzymes. Data concerning the sequences of dimer initiation fragments in both species 1 mtDNA and species 4 mtDNA will be described elsewhere.

LITERATURE CITED

1. **Adoutte, A., J. K. Knowles, and A. Sainsard-Chanet.** 1979. Absence of detectable mitochondrial recombina-tion in *Paramecium tetraaurelia*. Genetics **93:**797–831.
2. **Beale, G. H., and J. K. C. Knowles.** 1976. Interspecies transfer of mitochondria in *Paramecium aurelia*. Mol. Gen. Genet. **143:**197–201.
3. **Bos, J. L., C. Heyting, P. Borst, A. C. Arnberg, and E. F. J. VanBruggen.** 1978. An insert in the single gene for the large ribosomal RNA in yeast mitochon-drial DNA. Nature (London) **275:**336–338.
4. **Cummings, D. J.** 1980. Evolutionary divergence of mi-tochondrial DNA from *Paramecium aurelia*. Mol. Gen. Genet. **180:**77–84.
5. **Cummings, D. J., R. A. Maki, P. J. Conlon, and J. L. Laping.** 1980. Anatomy of mitochondrial DNA from *Paramecium aurelia*. Mol. Gen. Genet. **178:**499–510.
6. **Cummings, D. J., A. E. Pritchard, and R. A. Maki.** 1979. Restriction enzyme analysis of mitochondrial DNA from closely related species of *Paramecium*, p. 35–51. *In* D. J. Cummings, P. Borst, I. B. Dawid, S. M. Weissman, and C. F. Fox (ed.), ICN-UCLA Symposium on Extrachromosomal DNA. Academic Press, Inc., New York.
7. **Forte, M. A., and W. L. Fangman.** 1979. Yeast chro-mosomal DNA molecules have strands which are cross-linked at their termini. Chromosoma **72:**131–150.
8. **Goddard, J. M., and D. J. Cummings.** 1975. Structure and replication of mitochondrial DNA from *Parame-cium aurelia*. J. Mol. Biol. **97:**593–609.
9. **Goddard, J. M., and D. J. Cummings.** 1977. Mitochon-drial DNA replication in *Paramecium aurelia*. Cross linking of the initiation end. J. Mol. Biol. **109:**327–344.
10. **Goldbach, R. W., J. E. Bollen DeBoer, E. F. J. VanBruggen, and P. Borst.** 1978. Replication of the linear mitochondrial DNA of *Tetrahymena pyriformis*. Biochim. Biophys. Acta **521:**187–197.
11. **Goldbach, R. W., P. Borst, J. E. Bollen DeBoer, and E. F. J. VanBruggen.** 1978. The organization of ribo-somal RNA genes in the mitochondrial DNA of *Tetra-hymena pyriformis* strain ST. Biochim. Biophys. Acta **521:**169–186.
12. **Greene, P. J., M. C. Betach, H. M. Goodman, and H. W. Boyer.** 1974. The EcoRI restriction endonuclease *In* R. B. Wickner (ed.), DNA replication. Methods Mol. Biol. **7:**87–105.
13. **Howley, P. M., M. A. Israel, M.-F. Law, and M. A. Martin.** 1979. A rapid method for detecting and map-ping homology between heterologous DNAs. J. Biol. Chem. **254:**4876–4883.

14. **Maki, R. A., and D. J. Cummings.** 1977. Characteriza-
    tion of mitochondrial DNA from *Paramecium aurelia*
    with *Eco*RI and *Hae*II restriction endonucleases. Plas-
    mid 1:106–114.
15. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-
    labeled DNA with base specific chemical cleavages.
    Methods Enzymol. 65:449–560.
16. **Pritchard, A. E., L. M. Herron, and D. J. Cummings.**
    1980. Cloning and characterization of *Paramecium* mi-
    tochondrial DNA replication initiation regions. Gene
    11:43–52.
17. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P.
    Berg.** 1977. Labeling deoxyribonucleic acid to high
    specific activity *in vitro* by nick translation with DNA
    polymerase I. J. Mol. Biol. 113:237–251.
18. **Sainsard-Chanet, A.** 1978. A new type of mitochondrial
    mutation in *Paramecium*. Mol. Gen. Genet. 159:117–
    123.
19. **Sanders, J. P. M., C. Heyting, and P. Borst.** 1975. The
    organization of genes in yeast mitochondrial DNA. I.
    The genes for large and small ribosomal RNA are far
    apart. Biochem. Biophys. Res. Commun. 65:699–707.
20. **Southern, E. M.** 1975. Detection of specific sequences
    among DNA fragments separated by gel electrophore-
    sis. J. Mol. Biol. 98:503–517.
21. **Suyama, Y., and K. Muira.** 1968. Size and structural
    variations of mitochondrial DNA. Proc. Natl. Acad. Sci.
    U.S.A. 60:235–242.
22. **VanEtten, R. A., M. W. Walberg, and D. A. Clayton.**
    1980. Precise localization and nucleotide sequence of
    the two mouse mitochondrial rRNA genes and three
    immediately adjacent novel tRNA genes. Cell 22:157–
    170.
23. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient
    transfer of large DNA fragments from agarose gels to
    diazobenzyloxymethyl-paper and rapid hybridization by
    using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. 76:
    3683–3687.
24. **Wittek, R., and B. Moss.** 1980. Tandem repeats within
    the inverted terminal repetition of vaccinia virus DNA.
    Cell 21:277–284.
25. **Yang, R. C. A., and R. Wu.** 1979. BK virus DNA
    sequence: extent of homology with simian virus 40
    DNA. Proc. Natl. Acad. Sci. U.S.A. 76:1179–1183.