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IDENTIFICATION OF MAXICIRCLE DNA SEQUENCES IN *LEISHMANIA TARENTOLAE* THAT ARE HOMOLOGOUS TO SEQUENCES OF SPECIFIC YEAST MITOCHONDRIAL STRUCTURAL GENES

LARRY SIMPSON, TERENCE W. SPITHILL* and AGDA M. SIMPSON

Biology Department and Molecular Biology Institute, Los Angeles, CA 90024, U.S.A.

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Sequences homologous to the yeast mitochondrial structural genes for cytochrome oxidase subunits I and II, ATPase 6 and cytochrome *b* were identified on the kinetoplast DNA maxicircle molecule by low stringency hybridization of maxicircle blots with heterologous probes derived from mitochondrial DNA of yeast petite mutants. No hybridization was observed with the yeast ATPase 9 gene probe. The relative extent of base sequence mismatch was determined by melting of the heterologous hybrids. Candidates for the transcripts of these presumptive structural genes were proposed with reference to the transcriptional map of the maxicircle of *Leishmania tarentolae.* These results provide the first indication that maxicircle DNA specifies information for a limited number of conserved mitochondrial gene products similar to those already described for other eukaryotic cells.

Key words: *Leishmania tarentolae,* maxicircle DNA, kinetoplast DNA, heterologous probes.

INTRODUCTION

The genetic systems of mitochondria have the interesting characteristics that there are a limited number of specific gene products whose amino acid sequences and to a lesser extent nucleotide sequences are basically conserved in different species, but that the genomic organization of these structural genes in the mitochondrial DNA is not conserved. The best studied cases are the mitochondrial genomes of animal cells [1] and yeast [2–8] in which the genomic organization differs in terms of the relative positions of the ribosomal genes and structural genes, the presence or absence of intervening sequences, and the presence of untranslated spacer sequences. The maxicircle component of the kinetoplast DNA of hemoflagellate protozoa represents another type of mito-

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* *Present address:* Walter and Eliza Hall Institute, Royal Melbourne Hospital, Victoria 3050, Australia.
Abbreviations: SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7; HHM-5, 1 M NaCl, 10 mM Tris-HCl (pH 7.4), 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrolidone, 5% formamide; HMA, 5×SSC, 0.2% sodium dodecyl sulfate (SDS), 0.5 mg/ml sonicated denatured salmon sperm DNA, 0.5 mg/ml poly(rA), 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrolidone, 50% formamide.

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chondrial genomic organization in terms of the orientation of the ribosomal genes and the presence of high AT, possibly untranslated, spacers [9, 10]. The conservation of the nucleotide sequences of specific mitochondrial genes has been utilized to localize structural genes in the mitochondrial genomes of *Aspergillus nidulans* [11], *Neurospora crassa* [12, 13], and *Zea mays* [14]. In all three cases yeast mitochondrial DNA probes were employed. We have found that there is sufficient sequence homology between the mitochondrial structural genes of yeast and hemoflagellate protozoa to use the same procedure to identify homologous sequences on the maxicircle DNA of *Leishmania tarentolae.*

The results establish clearly that the maxicircle DNA represents the informational molecule in the kinetoplast DNA network and point the way for a direct analysis of specific mitochondrial structural genes in a hemoflagellate protozoan and the determination of the hemoflagellate mitochondrial genetic code.

MATERIALS AND METHODS

Cells. L. tarentolae cells were grown in Difco Brain Heart Infusion medium at 27°C as described previously [15]. The yeast petite strains were obtained from Dr. A. Tzagoloff and were grown in YEPD medium at 30°C (YEPD = 1% yeast extract, 2% Bactopeptone, 2% dextrose).

Isolation of kinetoplast DNA, maxicircle DNA and the pLt120 insert. Kinetoplast DNA was isolated from stationary phase cells as described [15]. Maxicircle DNA linearized at the single EcoRI site (RI Maxi DNA) was isolated by Hoechst dye-CsCl gradient centrifugation as described [15]. pLt120 plasmid DNA was isolated from chloramphenicol amplified *Escherichia coli* RR1 cells by ethidium bromide CsCl centrifugation, and the 120 insert isolated from the EcoRI/BamHI digested plasmid DNA by Hoechst dye-CsCl gradient centrifugation as described [16].

Isolation of petite mitochondrial DNA and the gene probes. The yeast cells were grown overnight in YEPD medium at 30°C. Cells were harvested and resuspended in 0.1 M EDTA (pH 9.0), 0.3 M β-mercaptoethanol, incubated for 15 min at 25°C and collected by centrifugation. The cells were suspended in 1.2 M sorbitol, 0.1 M EDTA (pH 7.5) and digested for 60 min with 500 μg/ml zymolyase at 37°C. The spheroplasts were then washed with 1.2 M sorbitol and lysed with 3% sarkosyl in 0.1 M NaCl, 0.05 M EDTA, 0.05 M Tris (pH 7.8) at 60°C for 1 h. The lysate was cleared at 17 kg for 40 min and the supernate was removed and taken to a refractive index (25°C) of 1.3970 with CsCl. Hoechst 33258 dye was added to a concentration of 200 μg/ml and the solution was centrifuged for 40 h at 40 000 rpm in the Ti60 rotor ([17] and Hyman B., personal communication). The minor upper fluorescent bands representing the mitochondrial DNAs were recovered and the dye removed by isopropanol extraction. The DNA was precipitated with ethanol after dialysis versus 10 mM Tris HCl, 1mM EDTA (pH 7.9) to remove CsCl and the pellets were resuspended in 10 mM Tris HCl (pH 7.9), 1 mM EDTA.

In all cases but DS302, only a single minor upper band in the Hoechst gradient in addition to the major lower band of nuclear DNA was visible. DS302 DNA showed two upper bands, of which only the lower was restrictable with PvuII and TaqI.

The petite DNAs were digested as shown in Table I and the fragments separated on 1% agarose. The indicated gene fragments were electroeluted and labeled by nick translation with [α-^{32}P]dATP and [α-^{32}P]dCTP.

Blot hybridization. The RI Maxi DNA and the 120 insert DNA were digested with various restriction enzymes and the fragments separated on agarose and blotted onto nitrocellulose (Schleicher & Schüll BA 83). The blots were prehybridized with 3×SSC (SSC= 0.15 M NaCl/0.015 M sodium citrate, pH 7), 0.2% bovine serum albumin, 0.2% polyvinylpyrolidone, 0.2% Ficoll at 60°C for 4 h and then hybridized with the labeled heterologous probes in HHM-5 (5% formamide, 1 M NaCl, 10 mM Tris HCl (pH 7.4), 0.2% bovine serum albumin, 0.2% polyvinylpyrolidone, 0.2% Ficoll) at 37°C for 48 h [18]. The blots were then washed for 2 h in 6×SSC at 41°C and exposed to preflashed Kodak XAR-5 film with a Quanta II intensifying screen at -70°C for appropriate times (usually several hours to overnight). The blots were then rewashed in 6×SSC at increasing temperatures and reexposed to determine the relative thermal stability of the hybrids. Often identical "diblots" were made of the same gel lane which were then washed at different temperatures [19].

Control hybridizations were performed in HMA (50% formamide, 5×SSC, 0.2% SDS, 0.5 mg/ml sonicated denatured salmon sperm DNA, 0.5 mg/ml poly(rA), 0.02% bovine serum albumin, 0.02% polyvinylpyrolidone, 0.02% Ficoll) at 37°C for 12 h. The blots were hybridized with the RI Maxi probe as a control for homologous hybridization. Blots were washed in 0.1×SSC at 45°C for 2 h and exposed as above.

Controls for the ATPase 9 hybridization experiment were performed as follows. First the labeled probe was shown to hybridize in HMA to a blot of the homologous petite mitochondrial DNA from strain DS400/A3 as expected. Secondly the presence of maxicircle DNA on the filter that showed no hybridization with the yeast ATPase 9 probe was verified by rehybridization of this filter with the homologous maxicircle probe (data not shown).

Additional control experiments showed that homologous yeast petite DNA hybrids (i.e., yeast probes versus yeast petite DNA blots) were stable under stringent hybridization and wash conditions (data not shown).

RESULTS

Hybridization probes. Purified RI Maxi DNA and cloned 120 insert DNA were digested with several enzymes and the fragments separated in agarose and blotted onto nitrocellulose. The blots were hybridized with nick translated heterologous probes derived from the mitochondrial DNA of yeast petite mutants characterized extensively by Tzagoloff (Table I). Originally we attempted to used the entire petite mitochondrial DNA as

TABLE I

Yeast petite probes for mitochondrial structural genes[a]

Yeast strain	Gene	Reference	Gene size in yeast (bp)	Digest	Fragment isolated[b] (bp)
1. DS6/A407	COX I (Exon A5)	[2]	1518	Hinf I/Taq I	≈1000, ≈840, 280 , 240, 210
2. DS302	COX II	[4]	756	Pvu II/Taq I	≈2800, 272 , 168
3. DS40	COX III	[5]	810	Hph I/Pvu II	1948, 1580, 478 , 170
4. DS14	ATPase 6	[7]	780	Mbo I/Eco RI	1747, 1205, 405 , 254
5. DS400/A3	ATPase 9	[6]	231	Alu I/Hpa II	611, 388, 359, 222, 84 , 75
6. DS400/M8	CYb (Exon b1)	[8]	1155	Hae III/Hinf I	807, 477 , 233

[a]Obtained from A. Tzagoloff.

[b]The fragments used as probes are underlined. These represent the structural gene portions of the petite DNA molecules.

probe after labeling by nick translation with [α-^{32}P]dCTP and controlled fragmentation by acid depurination to avoid nonspecific hybridization with the extensive AT-rich non-gene regions in these petite mitochondrial DNAs [2, 4, 8]. This approach was unsuccessful because of extensive non-specific hybridization. Therefore the specific fragments indicated in Table I were isolated from gels and labeled as probes. The COXI and CYb probes contained single exons of the genes, and the probes for COXII, COXIII, ATPase 6 and ATPase 9 contained the entire or nearly entire genes (Table I).

Hybridization to maxicircle blots was examined at varying formamide concentrations. Best results were obtained by hybridization in 5% formamide in 1 M NaCl at 37°C (HHM-5) for 48 h [18]. Increased specificity of hybridization could be obtained either by hybridizing in somewhat higher formamide concentrations (6–15%) or by washing the HHM-5 hybrids at increasingly higher temperatures in 6×SSC.

Identification of homologous sequences on the maxicircle DNA. Hybridization was observed with the yeast probes for COXI, COXII, COXIII, ATPase 6 and CYb (Figs. 1–6). No hybridization was observed with the probe for ATPase 9 although control experiments showed that the probe would hybridize with the homologous petite DNA and that the non-hybridizing filters did contain maxicircle DNA fragments. In most cases several maxicircle bands in non adjacent portions of the molecule lit up after hybridization at the low stringency conditions of "HHM-5, 37°C". Apparently nonspecific hybridization was eliminated by washing the blots at increasingly higher temperatures in 6×SSC, leaving one or two contiguous fragments labeled. One exception was the COXIII hybridization (Fig. 4) in which several non adjacent fragments showed hybridization; however all of these hybrids melted at a relatively low temperature (45–48°C) (Fig. 1b) implying extensive mismatch. In only one case (COXIII probe, Fig. 1b) was any hybridization observed with ϕXRF or λ fragments and these hybrids melted at a relatively low temperature (45°C).

The most specific localizations were the COXI, COXII and CYb hybrids. The COXI probe (Fig. 2) hybridized mainly to a 1.9 kb HhaI/MspI fragment and the COXII (Fig. 3) probe hybridized to an adjacent 2.6 kb MspI/HhaI fragment. Both hybrids melted at 55–60°C in 6×SSC (Fig. 1a, 1b). The CYb probe (Fig. 6) hybridized to a 2–3 kb region within the cloned 120 fragment of the maxicircle and the hybrid melted at 70–80°C (not shown). The ATPase 6 (Fig. 5) probe hybridized to a fairly large region of the maxicircle which included a portion of the 120 region and the hybrids melted at 65–70°C (Fig. 1c).

DISCUSSION

The detection of sequence homology of specific maxicircle fragments with yeast mitochondrial structural genes implies that the maxicircle contains these genes, but definitive identification of the maxicircle structural genes requires direct sequence analysis. However, there are several candidates for transcripts of these presumptive structural genes in view of the known sizes of these genes (Table I) and their transcripts in yeast and

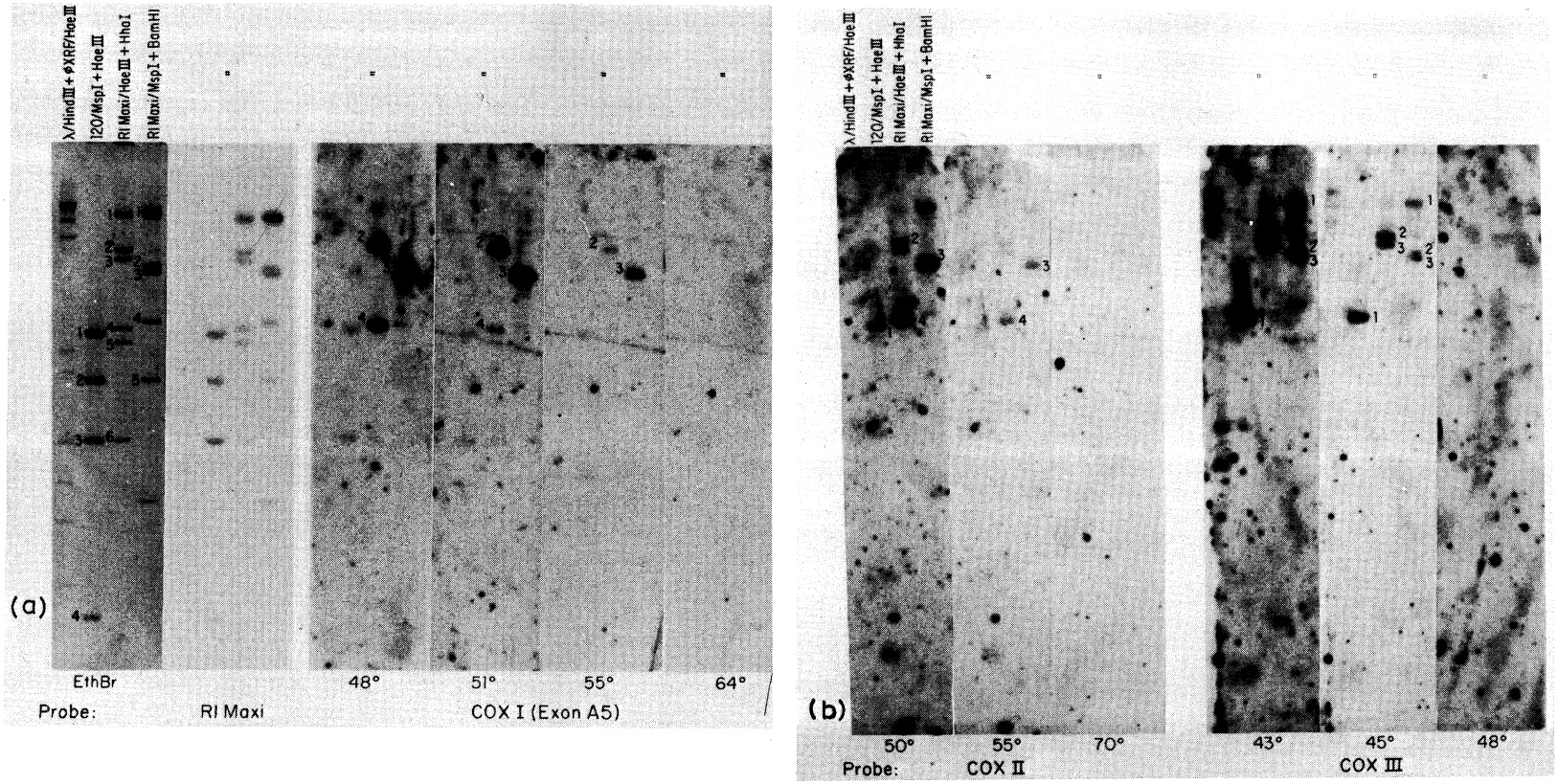
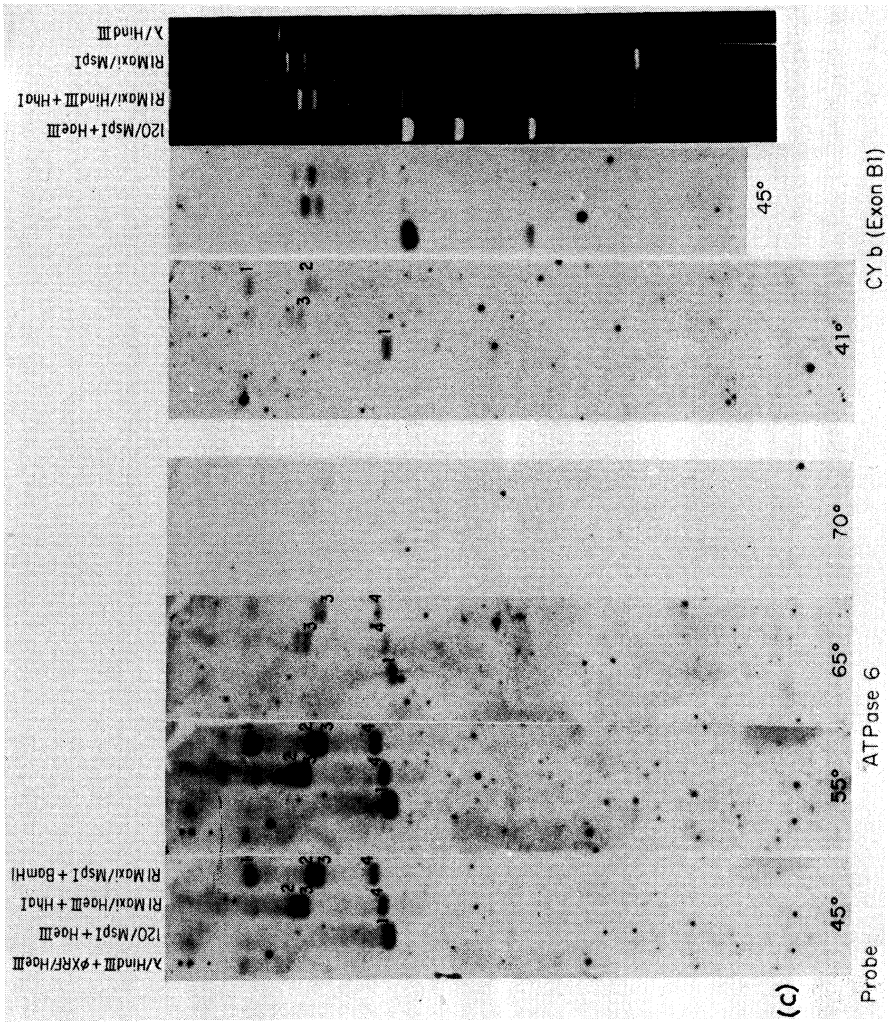


Fig. 1. Blot hybridization of maxicircle DNA with yeast probes. The indicated RI Maxi fragments and pLt120 insert fragments were separated in 1% agarose and blotted onto nitrocellulose. The blots were hybridized with the indicated DNA probes and the blots were washed in 6×SSC at the indicated temperatures and exposed for equivalent times. Probes used were: (a) RI Maxi probe as a control hybridization and the yeast COXI probe, (b) yeast COXII and COXIII probes, and (c) yeast ATPase 6 and CYb probes. Note that the CYb experiments shown represent two different blot series. Note also that only the 41°C-6×SSC washed blot of the CYb hybridization is shown for gel (a). The autoradiograph labeled 45°C represents hybridization of a blot of the ethidium stained gel (c) with the CYb probe. This blot was washed at 45°C in 6×SSC.


(c)
Probe
ATPase 6
45°
55°
65°
70°
λ/HindIII + φXRF/HaeIII
120/MspI + HaeIII
RIMaxi/HaeIII + HhaI
RIMaxi/MspI + BamHI
CYb (Exon B1)
45°
41°
120/MspI + HaeIII
RIMaxi/HindIII + HhaI
RIMaxi/MspI
λ/HindIII

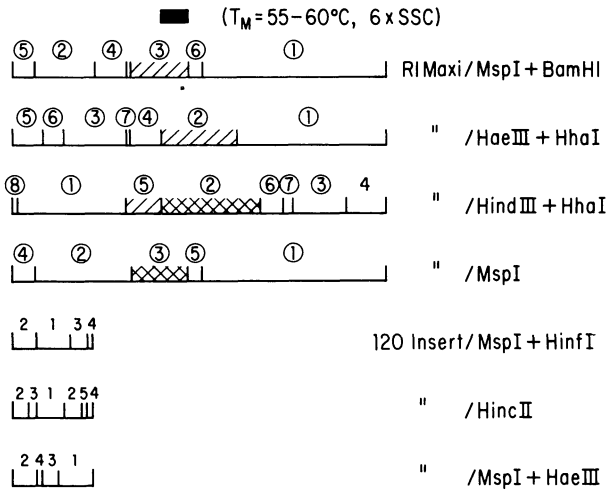


Fig. 2. Diagram of blot hybridization results using the COXI (Exon A5) (DS6/A407) probe. This figure schematically summarizes all the blot hybridizations performed using different digestions of RI Maxi DNA, some of which are presented in Fig. 1. The approximate melting temperature of the heterologous hybrids is given in parentheses. The approximate intensity of hybridization is indicated in the diagrams by cross-hatching: ▨ > ▨ . The fragments produced by each digestion, are indicated by circled numbers in terms of decreasing size. The consensus regions of hybridization are indicated by the solid boxes above the maps. For details of the maxicircle DNA restriction map see [9].

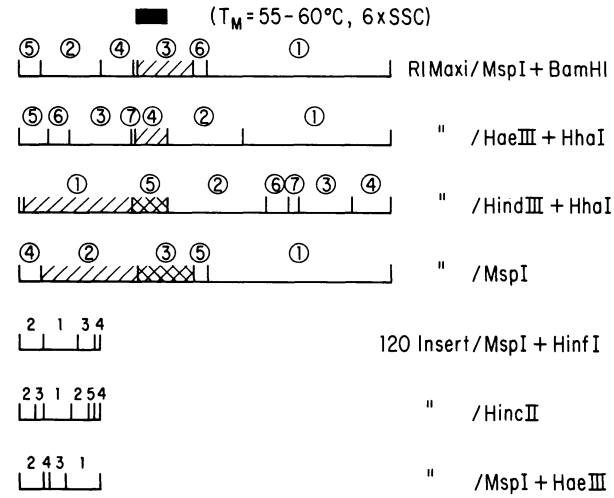


Fig. 3. Diagram of blot hybridization results using the COXII (DS302) probe.

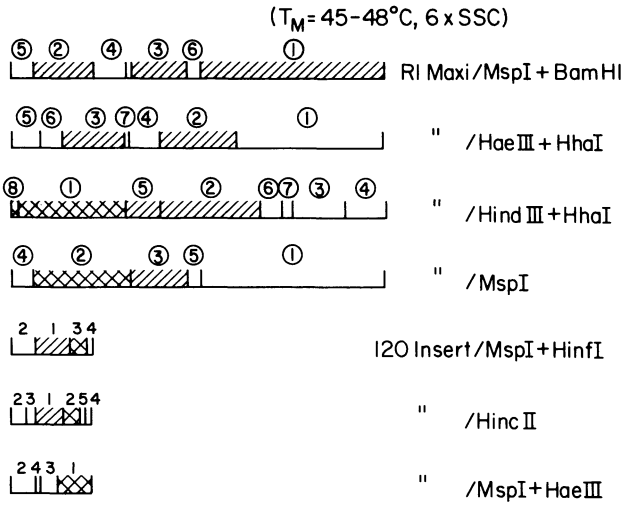


Fig. 4. Diagram of blot hybridization results using the COXIII (DS40) probe.

animal cell mitochondria and in view of the maxicircle transcriptional analysis we have presented previously [9]. A candidate for the transcript of the COXI gene is a 1.4 kb RNA localized in the same HhaI/MspI fragment which shows the major homology to the yeast COXI probe (Fig. 7). Several RNAs of the appropriate size for the COXII transcript are localized in the MspI/HhaI fragment that shows homology to the yeast COXII gene probe. These include a 0.7, a 0.95 and a 1.8 kb RNA species (Fig. 7). A 1.2 kb RNA localized in the 120 region is a candidate for the CYb transcript (Fig. 7). The ATPase 6 results are puzzling in that the hybridization of the yeast probe occurred over several maxicircle fragments representing approximately 9 kb and the hybrids appeared to be relatively thermally stable. The ATPase 6 genes in yeast and humans are relatively small

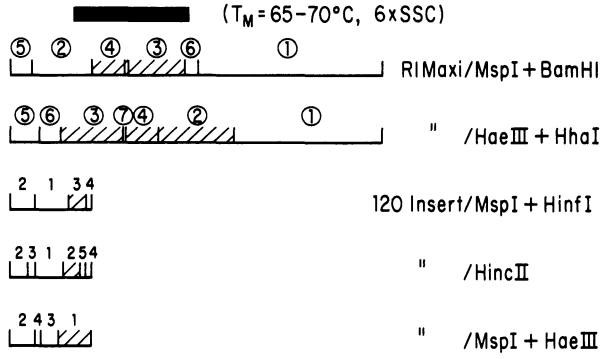


Fig. 5. Diagram of blot hybridization results using the ATPase 6 (DS14) probe.

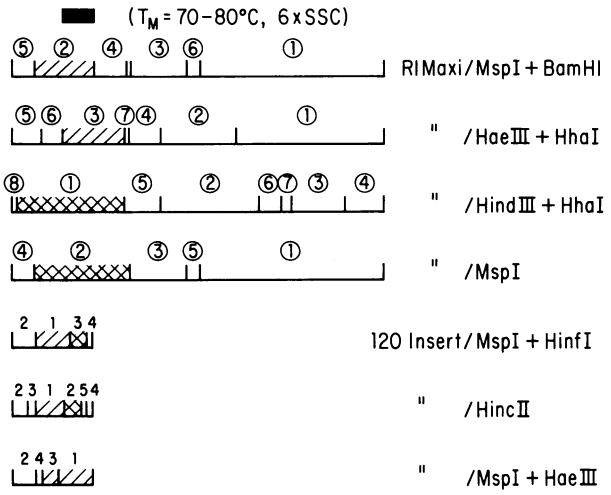


Fig. 6. Diagram of blot hybridization results using the CYb (ExonB1) (DS400/M8) probe.

(0.78, 0.68 kb) and it is unlikely that even the presence of large intervening sequences in the maxicircle gene would give rise to these hybridization results. There are however several maxicircle RNAs in the region of heterologous hybridization that would be appropriate for the ATPase 6 transcript [9]. In the case of COXIII, the lack of specific localization of stable heterologous hybrids with the yeast probe does not allow the identification of any particular presumptive COXIII transcripts, but again there are

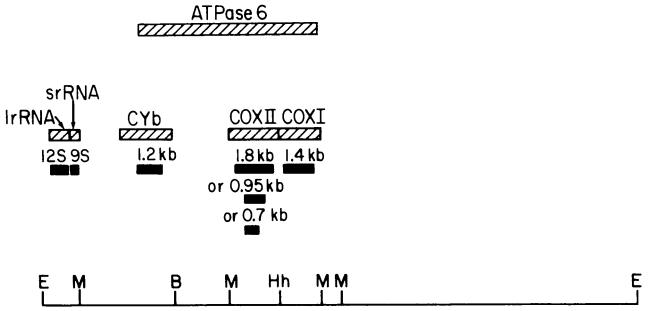


Fig. 7. Summary of localizations of structural genes and tentative RNA transcripts of the structural genes on the maxicircle DNA restriction map. The maxicircle is linearized at the EcoRI site (E), and the MspI (M) and BamHI (B) sites and one HhaI (Hh) are shown [9]. The cross-hatched bars indicate the structural gene localizations determined from the yeast hybridizations and the solid bars indicate the localization of tentative RNA transcripts for these genes. No tentative transcripts of the ATPase 6 and COXIII genes are presented due to the excessively broad localizations from the yeast hybridizations. Also indicated are the putative large and small ribosomal RNA genes and the 9 and 12 S transcripts of these genes.

sufficient RNAs of appropriate size in the general hybridization region to represent such a transcript.

The relative organization of the presumptive structural genes on the maxicircle has some resemblance to the relative organization of genes in the human mitochondrial DNA in which the sequence is COXI, COXII, ATPase 6, COXIII, ..., CYb (5′ to 3′ on the L strand). This however must be substantiated by more precise mapping of the maxicircle genes. Another similarity is the apparent absence of the ATPase 9 gene in the maxicircle.

Melting of the heterologous hybrids off the filter allowed the determination of the relative extent of sequence mismatch, which gives an idea of the evolutionary conservation of the various structural genes between yeast and *Leishmania* species. The CYb hybrid showed the highest thermal stability, followed by the ATPase 6 hybrid, the COXI and COXII hybrids and finally by the presumptive COXIII hybrid. It should be noted that the CYb and COXI probes only represented single exons of the complex yeast genes and that these data therefore only apply to those portions of the genes.

The hybridization of well defined heterologous mitochondrial gene probes under low stringency conditions seems to be a powerful method to obtain a preliminary localization of mitochondrial structural genes in new systems.

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