STUDIES ON MITOCHONDRIAL GENE PURIFICATION USING PETITE MUTANTS OF YEAST: CHARACTERIZATION OF MUTANTS ENRICHED IN RIBOSOMAL RNA CISTRONS.

Phillip Nagley, P.L. Molloy, H.B. Lukins and Anthony W. Linnane

Department of Biochemistry, Monash University, Clayton, Victoria 3168, Australia.

Received January 16,1974

SUMMARY: Normal yeast mtDNA contains one cistron each for the rRNA in the 37S and 50S subunits of mitochondrial ribosomes. Genetic information for 50S-rRNA is retained in mtDNA of petites carrying the loci for erythromycin or chloramphenicol resistance. In one strain retaining the erythromycin locus alone, 50S-rRNA hybridized to 14% of the mtDNA, representing a tenfold enrichment of these sequences compared with normal mtDNA. Where oligomycin or mikamycin loci are retained but chloramphenicol and erythromycin loci are deleted, little or no hybridization with 50S-rRNA is observed. Genes for 37S-rRNA are not retained in any of the petites investigated in this work. We conclude that the 50S-rRNA genes are located close to, or encompass, the chloramphenicol and erythromycin loci in mtDNA.

INTRODUCTION

Cytoplasmic petite mutants of yeast are mtDNA deletion mutants (for review see ref. 1) and we have proposed that they provide the potential for an *in vivo* purification of mitochondrial genes (2-4). It was deduced from genetic studies using ethidium bromide target analysis that amplification of erythromycin resistance loci can occur in some petite mutants (3,4). Further support for our interpretation was the observation of the enrichment in a petite clone of the region of mtDNA coding for leucyl-tRNA (5).

This communication presents direct evidence for the retention and amplification of rRNA genes in the mtDNA of petite mutants of defined genotype. Petite strains have been prepared from multiply marked grande strains, using resistance loci for erythromycin [*eryl*], chloramphenicol [*capl*], oligomycin [*olil*], and mikamycin [*mikl*] to monitor the retention or loss of loci. Results are presented for the hybridization of [^{125}I]-labelled

rRNA from the smaller (37S) and larger (50S) subunits of the mitochondrial ribosome with grande mtDNA, and with mtDNA isolated from petite mutants retaining only one of the four antibiotic resistance loci as well as one strain carrying both the [capl] and [eryl] loci. The results show that two petite strains carrying the [eryl] locus are highly enriched in sequences complementary to 50S-rRNA compared to the normal grande mtDNA.

MATERIALS AND METHODS

Strains: The petite clones used in this work are listed with their genotypes in Table 1 (first three columns). Petite clones were derived from three different haploid grande strains as follows: Petite K1E1 from strain L411 [rho$^+$ eryl-r]; petites EP1 and P2 from strain 761-7A [rho$^+$ capl-r eryl-r oli1-r]; petites S1 and S2 from strain 432-31 [rho$^+$ eryl-r oli1-r mik1-r]. Strain L410 [rho$^+$ cap-s ery-s oli-s mik-s] was used as the source of grande mtDNA, and also of mitochondrial rRNA.

Isolation of Mitochondria and Preparation of mtDNA: Mitochondria were prepared (6), treated with DNase to remove nuclear DNA, and mtDNA extracted as described (7) and purified in a CsCl gradient.

Preparation and [^{125}I] labelling of mitochondrial rRNA: Mitochondria from strain L410 were washed by centrifugation three times; the G+C content of bulk mtRNA was less than 28%, indicative of minimal cytoplasmic ribosomal contamination (8,9). Mitochondria were lysed at 6 mg protein/ml in a solution containing 1% Triton X-100, 600 mM KCl, 10 mM Tris pH 7.4, 10 mM $MgCl_2$. Undissolved membranes were pelleted at 27,000 g for 20 min. The supernatant was layered onto linear 15-30% sucrose gradients (34 ml) containing lysis buffer minus Triton, and centrifuged at 22,000 r.p.m. for 18 hr at 2° in a Spinco SW 27 rotor. The UV absorption peaks corresponding to the 37S and 50S mitoribosome subunits were pooled, and ribosomes collected by ethanol precipitation. RNA was extracted and purified as described(9). In order to obtain RNA of high enough specific activity for use in RNA-DNA hybridization studies, RNA was iodinated *in vitro* with [^{125}I] (10).

RESULTS AND DISCUSSION

Hybridization of mitochondrial 37S-rRNA and 50S-rRNA to strain L410 (rho$^+$) mtDNA is shown in Figure 1. In each case the input RNA is held constant whilst input DNA varies. At low DNA inputs, RNA is saturating on DNA; RNA is bound in

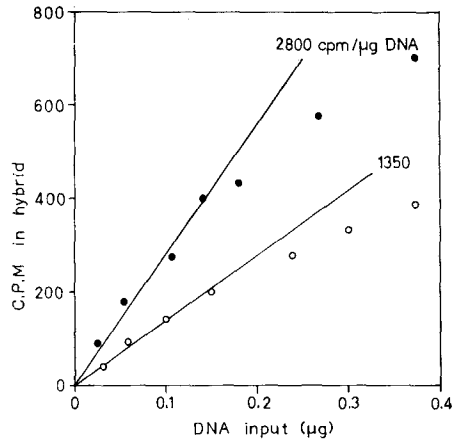

Figure 1: Hybridization of mitochondrial rRNA with mtDNA from strain L410 (rho$^+$).

Hybridization mixtures (0.21 ml) contained 2 x SSC, 30% formamide, 0.1 µg RNA (either 37S-rRNA, 1.92 x 10^5 cpm/µg; or 50S-rRNA, 1.85 x 10^5 cpm/µg) and the indicated amounts of denatured DNA (heated for 15 min at 98° in 0.1 x SSC, then rapidly chilled). Annealing was for 2 hr at 37°; then 2 ml 2 x SSC containing pancreatic RNase (10 µg/ml) and RNase T1 (25 units/ml) was added and left for 1 hr at room temperature. 8 ml of chilled 6 x SSC was added, and the mixture kept at 0° before filtration with mild suction through presoaked (6 x SSC) mitrocellulose filters (Millipore, 13 mm diameter, 0.45 µm pore size) to collect the RNA-DNA hybrids. Filters were washed with 10 ml 6 x SSC, blotted dry, and directly counted for radioactivity in a Philips Gamma Analyser. Backgrounds were subtracted from observed counts as follows (tubes not containing DNA): 37S-rRNA, 125 cpm; 50S-rRNA, 55 cpm; these figures include machine background of 15 cpm. With each RNA, denatured yeast nuclear DNA or *E. coli* DNA, or native mtDNA (all at 0.5 µg) did not bind more than 50 cpm above background.

Open circles, 37S-rRNA; closed circles, 50S-rRNA. The gradient of the linear portion of each curve (cpm/µg DNA) is shown on the Figure.

---

proportion to added DNA. At higher DNA inputs, renaturation of DNA interferes with RNA-DNA hybridization, which leads to deviation of the graph from linearity. From the initial slopes of the curves for 37S-rRNA and 50S-rRNA (indicated in Fig. 1), it is calculated that the rRNA's are complementary to 0.7% and 1.5% of L410 mtDNA, respectively. This measurement has been

made several times with three different rho$^+$ strains (including one chromosomal petite); the range of values obtained for 37S-rRNA is 0.65 to 0.75%, and for 50S-rRNA 1.45 to 1.55%. These values are very similar to those observed by Reijnders et al. (11), and are consistent with one cistron per mitochondrial genome (approx. 50 x 10^6 daltons) for each rRNA species.

In Figure 2 are shown the results of hybridization of

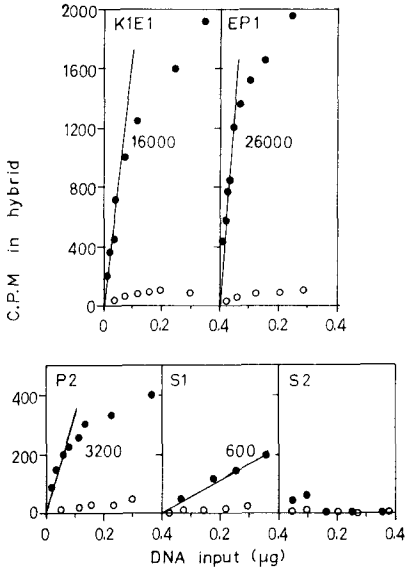

Figure 2: Hybridization of mitochondrial rRNA with mtDNA from petite clones.
The experimental conditions and the nomenclature in this figure are exactly as for Figure 1.

labelled 37S-rRNA and 50S-rRNA to mtDNA extracted from the five petite clones used in this work. In petites K1E1 (retaining [capl] and [eryl] loci) and EP1 (retaining the [eryl] locus alone) sequences complementary to the 50S-rRNA account for a far higher proportion of mtDNA than in rho$^+$ mtDNA (Table 1, fourth column). The enrichment of these 50S-rRNA sequences in the rho$^-$ mtDNA's is

TABLE 1. CHARACTERISTICS OF MITOCHONDRIAL GENOMES OF GRANDE AND PETITE STRAINS

Strain	Genotype	Loci retained[a]	Homology to[b] 50S-rRNA (% mtDNA)	Enrichment[c] over rho^+
L410	rho^+	[capl] [eryl] [olil] [mikl]	1.5	1
K1E1	rho^-	[capl] [eryl]	8.7	5.8
EP1	rho^-	[eryl]	14.0	9.3
P2	rho^-	[capl]	1.8	1.2
S1	rho^-	[mikl]	0.3	0.2
S2	rho^-	[olil]	not detectable	

a: This was tested as follows. Let [ant] be a generalised antibiotic locus. For detection of [ant-r] loci the haploid petites were crossed with a suitable [rho^+ ant-s] strain, and the appearance of [rho^+ ant-r] zygotic clones examined (for details see ref.3). The presence of [rho^+ ant-r] diploids indicates that the petite cells retain [ant-r] loci. Where the rho^+ diploids are exclusively sensitive, the deleted state [ant-o] is designated in the petite. It was also necessary to check for the [ant-s] locus in petites derived from [rho^+ ant-s] strains. This was done by crossing the petites with a suitable [rho^+ ant-r] strain and the resulting zygotes allowed to grow vegetatively for several divisions. The appearance of [rho^+ ant-s] diploids indicates that the [ant-s] loci are present in the petite clone. Conversely, the absence of [rho^+ ant-s] diploids indicates the deleted [ant-o] state in the petite cells.

b: Calculated from data in Figs 1 and 2.

c: i.e. (% mtDNA homologous to 50S-rRNA in rho^-) divided by (% mtDNA homologous to 50S-rRNA in rho^+).

shown in Table 1 (fifth column). For clone EP1, it is apparent that close to a tenfold enrichment of 50S-rRNA sequences has occurred. Clone P2 (retaining the [capl] locus alone) shows significant hybridization with 50S-rRNA, but as defined in Table 1 these sequences are not significantly enriched with

respect to rho$^+$ mtDNA. Clone S1 (retaining the [mikl] locus alone) shows a significant but greatly reduced hybridization with 50S-rRNA (0.3% of mtDNA), whilst clone S2 (retaining the [olil] locus alone) shows no detectable hybridization with 50S-rRNA.

In contrast to 50S-rRNA, the 37S-rRNA hybridizes very poorly with all the petite mtDNA's studied (Fig. 2). Indeed it could be shown by competition experiments that the observed hybridization of labelled 37S-rRNA with petite mtDNA results from minor contamination of this labelled RNA with sequences homologous to 50S-rRNA. The hybridization of 37S-rRNA with all these petite mtDNA's is totally competed by unlabelled 50S-rRNA and not at all competed by unlabelled 37S-rRNA (data not shown). By contrast, similar competition experiments using grande mtDNA showed that unlabelled 50S-rRNA competes far less efficiently with the hybridization of labelled 37S-rRNA than does unlabelled 37S-rRNA. It is concluded that 37S-rRNA cistrons are probably deleted from all of the five petite clones.

Analysis of the genetic behaviour in crosses of the four loci used in this work has led us and others to draw up maps of yeast mitochondrial genome (12,13) which place the [eryl] locus close to the [capl] locus which is at one end of a linear array; the [olil] and [mikl] loci are further away from this region. The retention of 50S-rRNA cistrons together with the [capl] and [eryl] loci, but their absence from clones carrying exclusively [olil] or [mikl] loci places the 50S-rRNA cistron in the region of the former two loci. Although in two clones (K1E1 and EP1) there is a considerable enrichment for DNA complementary to 50S-rRNA it is uncertain as to whether the whole cistron is retained or only a fragment. It is evident that the smaller the fraction of the cistron retained, the higher will be the

reiteration of these sequences within the petite genome. Nonetheless, for petite genomes which are of complexity smaller than the grande and which do not show enrichments of ribosomal genes with respect to the grande (such as clones P2 and S2) the 5OS-rRNA sequences retained can only be fragments of the whole cistron.

The very high level of hybridization of 5OS-rRNA with mtDNA of clone EP1 [rho$^-$ eryl-r] (14% of mtDNA) indicates that almost one third of the genetic information (in a single strand) in the mtDNA of this clone consists of rRNA sequences. The coincident retention of [eryl] loci and high enrichment for 5OS-rRNA sequences is consistent with the possibility that the [eryl] locus lies within the 5OS-rRNA cistron. Ribosomes of bacteria resistant to erythromycin may be changed either in a ribosomal protein or in rRNA itself (for review see ref.14), and in the case of mutant chloroplasts, ribosomal protein changes have been observed (15). A definitive demonstration of the altered component in yeast mitochondrial ribosomes in [eryl-r] cells is still to be established.

REFERENCES

1. Linnane, A.W., Haslam, J.M., Lukins, H.B. and Nagley, P. (1972) Ann. Rev. Microbiol. 26, 163-198.
2. Nagley, P. and Linnane, A.W. (1972) J.Mol.Biol.66,181-193.
3. Nagley, P., Gingold, E.B., Lukins, H.B. and Linnane,A.W. (1973) J. Mol. Biol. 78, 335-350.
4. Nagley, P., Gingold, E.B. and Linnane, A.W. (1974) in The Biogenesis of Mitochondria: Transcriptional. Translational and Genetic Aspects, eds. Kroon, A.M. and Saccone, C., Academic Press, New York (in press).
5. Cohen, M., Casey, J., Rabinowitz, M. and Getz, G.S.(1972) J. Mol. Biol. 63, 441-451.
6. Watson, K., Haslam, J.M. and Linnane, A.W. (1970)J. Cell Biol. 46, 88-96.
7. Nagley, P. and Linnane, A.W. (1970) Biochem. Biophys. Res. Commun. 39, 989-996.
8. Fauman, M., Rabinowitz, M. and Getz, G.S. (1969)Biochim. Biophys. Acta 182, 355-360.

9. Forrester, I.T., Nagley, P. and Linnane, A.W. (1970) FEBS Letters, 11, 59-61.
10. Getz, M.J., Altenberg, L.C. and Saunders, G.F.(1972) Biochim. Biophys. Acta 287, 485-494.
11. Reijnders, L., Kleisen, C.M. Grivell, L.A. and Borst,P. (1972) Biochim. Biophys. Acta 272, 396-407.
12. Linnane, A.W., Howell, N. and Lukins, H.B.(1974) in Biogenesis of Mitochondria: Transcriptional, Translational and Genetic Aspects, eds. Kroon, A.M. and Saccone, C., Academic Press, New York (in press)
13. Avner, P.R., Coen, D., Dujon, B. and Slonimski, P.P.(1973) Mol. Gen. Genet. 125, 9-152.
14. Benveniste, R. and Davis, J. (1973) Ann. Rev. Biochem. 42, 471-506.
15. Mets, L. and Bogorad, L. (1972) Proc. Nat. Acad. Sci. U.S. 69, 3779-3783.