Heterogeneity of mitochondrial DNA from *Saccharomyces cerevisiae* and genetic information for tRNA

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ABSTRACT

Mitochondrial DNA from wild-type *Saccharomyces cerevisiae* and from an "extreme" petite mutant were analyzed by hybridization of several tRNAs on DNA fragments of different buoyant density, obtained by sonication and fractionation on a CsCl gradient. The hybridization patterns show that the genes for tRNA_{ser}, tRNA_{phe}, tRNA_{his}, tRNA_{val}, tRNA_{ileu} are present on wild-type mitochondrial DNA, while only genes for tRNA_{ser} and tRNA_{his} are present on petite mitochondrial DNA; moreover the hybridization patterns indicate that these genes are not clustered and suggest that more than one gene might exist for tRNA_{ser} and tRNA_{his}.

INTRODUCTION

The existence on mtDNA of several genes coding for tRNAs has been demonstrated in rat liver (1), in HeLa cells (2), in *Xenopus laevis* (3) and in *Saccharomyces cerevisiae* (4-9). So far as *S. cerevisiae* is concerned, hybridization with w.t. mtDNA has been demonstrated by now for fourteen different aminoacyl-tRNAs, while several petite strains differ greatly in the capacity of their mtDNA to anneal with different tRNAs (5, 8).

In a previous work (7) we have demonstrated that mtDNA from an "extreme" petite mutant (3.6% G+C) still contains the information for tRNA_{ser}; this result indicates that guanine and cytosine residues should be concentrated in one or more informational regions of this DNA, thus confirming previous results on the intramolecular heterogeneity of w.t. and petite mtDNA (10,11). This heterogeneity has been now fully demonstrated by Prunell and Bernardi (12).

The possibility of fractionating fragments having different buoyant density provides an opportunity of localizing genes for tRNA in various (heavy or light) regions of mtDNA.

In this work we report some data on the tRNA genes present in heavy and light fractions of w.t. and petite mtDNA.

MATERIALS AND METHODS

Two diploid strains of Saccharomyces cerevisiae were used: strain 42 (DM) ρ^+ and strain 41 (DM₁) ρ^- , whose characteristics have been previously described (13). These strains were grown as previously reported (10).

Preparation of mitochondria: 200 gr of yeast cells collected in the stationary growth phase were suspended in 200 ml of buffer A (10^{-2} M Tris-HCl buffer pH 7.4, 0.4 M Sorbitol, 10^{-3} M β -Mercaptoethanol, 10^{-3} M EDTA) and disrupted with a mechanical disintegrator equipped with glass beads. Only in some cases, the spheroplast method described by Duell, Inoue and Utter (14) was used. All subsequent operations were performed at 3° C. The homogenate was centrifuged three times at 1000 x g in a Sorvall RC2-B centrifuge to eliminate cell debris. Supernatant was centrifuged for 15 min. at 30,000 x g and the pellet resuspended in TKM buffer (Tris-HCl 0.05 M pH 7.4, KCl 0.025 M, MgCl₂ 0.0025 M) and incubated for 30 min. at 30° C with 20 μ g/ml DNase I (Worthington). After centrifugation at 30,000 x g for 15 min., the mitochondrial fraction was resuspended in buffer A and subjected three times to the following cycle of differential centrifugation: the mitochondrial suspension was centrifuged at 1,000 x g for 15 min., the pellet was discarded and the supernatant centrifuged at 30,000 x g for 15 min.; the pellet of this centrifugation was resuspended in buffer A. Mitochondrial fractions, prepared following this procedure, when checked on a 1.1-1.9 M sucrose gradient (run for 4 h at 25,000 rpm in the SW 27 rotor of the Spinco mod. L2-65B preparative ultracentrifuge) showed a reddish mito-

chondrial band (exhibiting the characteristic absorption spectrum) and no relevant contaminating material in the other regions of the gradient.

Preparation, fragmentation and fractionation of mtDNA: w.t.

mtDNA was prepared by the method of Marmur (15) from DNase treated mitochondrial fractions. For the preparation of mtDNA from the petite mutant, DNase treatment was omitted and the mitochondrial preparation was lysed in SSC with 1% sodium dodecyl sulphate and DNA purified directly by preparative CsCl isopycnic density gradient. The solution was adjusted to a refractive index of 1.3990 by the addition of CsCl and run for 62 h at 15° C, 40,000 rpm in the 42.1 rotor of the Spinco mod. L2-65B preparative ultracentrifuge. Sonic degradation of DNA was carried out at 0° C by a MSE 100 watt ultrasonic disintegrator at 24 Kc/sec, 7 microns for 40 sec. in order to obtain 7-10 S DNA fragments from 23-25 S DNA. Only in some cases further fragmentation was obtained by lengthening the sonication period from 40 to 90 sec. DNA fragmented by sonication was fractionated with a preparative CsCl isopycnic density gradient prepared as reported above but run at 34,000 rpm. Fractions of 0.8 ml were collected from the tubes punctured at the bottom. Absorbances were measured at 260 mμ on a ZEISS PMQ II spectrophotometer. After dialysis overnight against 1/100 SSC, fractions were used for hybridization experiments.

Preparation of labelled aminoacyl-tRNA: mitochondrial tRNA was prepared by the method reported by Holley (16) and acylated by mitochondrial aminoacyl-tRNA synthetases. For the preparation of these enzymes, mitochondria were lysed with 0.3% Triton X 100 in 10⁻² M Tris-HCl buffer pH 7.4 containing 10⁻³ M β-Mercaptoethanol. The lysate was centrifuged for 90 min. at 100,000 x g in the Spinco mod. L2-65B preparative ultracentrifuge and the supernatant dialysed overnight against 10⁻² M Tris-HCl buffer pH 7.4. Synthetases were lyophilized and stored at -20° C for some months

without loss of activity. The acylation mixture contained in 1 ml: 50 μ moles Tris-HCl buffer pH 7.4, 0.2 mg tRNA, 250 μ Ci 3 H amino-acid (Radiochemical Center, Amersham, specific activity 10-50 Ci/mmmole), 10 μ moles ATP, 30 μ moles $MgCl_2$, 0.3 mg enzyme protein. The mixture was incubated for 30 min. at 28° C and the aminoacyl-tRNA was isolated by phenol extraction followed by precipitation with 2 volumes 95% ethanol. The precipitate was dissolved in 0.1 M Tris-HCl buffer pH 7.4, reprecipitated with ethanol and redissolved in 3 x SSC pH 4.2.

Hybridization procedure: the annealing procedure was essentially the same as reported by Casey et al. (4). DNA was denatured by heat treatment followed by quick cooling, the SSC concentration was adjusted to 6 x and DNA immobilized on membrane filters (Millipore GSWP, 0.22 μ , ϕ 25 mm). In the case of sonicated DNA each fraction of a gradient was dialysed, denatured, divided and fixed on two filters to compare the hybridization of different aminoacyl-tRNAs on the same DNA fractions. The hybridization was carried out at pH 5 in 2 x SSC containing 33% formamide for 4-5 h at 33° C. Filters were then washed by stirring in 1 l of 2 x SSC pH 6 for 15 min., treated for 30 min at 30° C in 2 x SSC pH 6 with 5 μ g/ml RNase T₁ (Worthington), washed again in 1 l of 2 x SSC pH 6 for 15 min., dried and counted in a Beckman liquid scintillation counter.

RESULTS

Results of the hybridization experiments between denatured unfractionated DNA from w.t. and petite mitochondria and seryl-, phenylalanyl-, histidyl-, valyl- and isoleucyl-tRNA are reported in fig. 1. As can be seen from the hybridization plateaux, DNA from the w.t. strain contains the information for the five tested tRNAs, while the information for only two of them (serine and histidine) is retained in the mutant. No competition was observed when an excess of cytoplasmic or bacterial tRNA was added to the

Fig. 1. Hybridization of ^3H labelled mitochondrial aminoacyl-tRNA with w.t. and petite mtDNA, yeast nuclear DNA and *E. coli* DNA. Filters containing 20 μg of w.t. mtDNA ($\bullet$) or 20 μg petite mtDNA ($\circ$) or 20 μg yeast nuclear DNA (Δ) or 20 μg *E. coli* DNA ($\blacktriangle$) were hybridized with mitochondrial a) seryl-tRNA (239 cpm/ μg); b) phenylalanyl-tRNA (660 cpm/ μg); c) valyl-tRNA (720 cpm/ μg); d) isoleucyl-tRNA (1,200 cpm/ μg); e) histidyl-tRNA (3,000 cpm/ μg). The final volume of the annealing mixture was 0.6 ml.

annealing mixture.

Hybridization between ^3H seryl- and ^3H phenylalanyl-tRNA and the same sonicated and fractionated w.t. mtDNA preparation is reported in fig. 2 (a, b) which shows that seryl-tRNA hybridizes preferentially on the heavy fragments, whereas phenylalanyl-tRNA hybridizes preferentially on the light ones. It is interesting to note that hybridization on the light and heavy fractions where the absorbance is very low, implies hybridization levels at least 100 times higher than those obtained in the region of the peak. Fig. 2 (c, d) shows the hybridization pattern of histidyl- and isoleucyl-tRNA on fractionated w.t. mt DNA; fig. 2 (e, f) shows the hybridization patterns of phenylalanyl- and valyl-tRNA

Fig. 2 Fractionation on CsCl density gradient of sonicated w.t. mtDNA and annealing of the fractions with aminoacyl-tRNAs. The annealing mixture contained 70 $\mu\text{g}/\text{ml}$ of: a) seryl-tRNA (2,000 cpm/ μg), b) phenylalanyl-tRNA (3,000 cpm/ μg), c) histidyl-tRNA (2,714 cpm/ μg), d) isoleucyl-tRNA (3,300 cpm/ μg), e) phenylalanyl-tRNA (5,000 cpm/ μg), f) valyl-tRNA (1,700 cpm/ μg). In a), b), c) and d) gradients contained DNA sonicated under standard conditions; in e) and f) DNA was sonicated for 90 sec. The average value (70-130 cpm) of five blanks with nuclear DNA (20 $\mu\text{g}/\text{filter}$) was subtracted from the data. Radioactivity retained on filters is indicated by the height of the bars.

on the same DNA preparation sonicated for 90 sec. In fig.3 hybridization of phenylalanyl-tRNA is tested on two mt DNA gradients obtained from the same DNA preparation sonicated for different periods of time: results show that the position of the hybridization peak (normally highly reproducible) shifts towards the heavier region of the gradient after further fragmentation of DNA.

Fig. 3. Fractionation on CsCl density gradient of w.t. mtDNA sonicated for 40 sec. (a) and for 90 sec. (b) and annealing of the fractions with phenylalanyl-tRNA (5,000 cpm/ μ g).

Fig. 4. Fractionation on CsCl density gradient of sonicated mtDNA from strain DM and annealing of the fractions with a) seryl-tRNA (800 cpm/ μ g), b) phenylalanyl-tRNA (700 cpm/ μ g), c) histidyl-tRNA (1,200 cpm/ μ g). Annealing conditions as in fig.1. An average of five blanks (35-100 cpm) was subtracted from the data.

The hybridization patterns of seryl-, phenylalanyl- and histidyl-tRNA on petite mtDNA, reported in fig. 4, show that in this case seryl- and histidyl-tRNA hybridize mainly on heavy fragments while no hybridization is found with phenylalanyl-tRNA.

DISCUSSION

The above results concerning the hybridization of five tRNAs on fractionated mtDNA exhibit two main characteristics which may be discussed in connection with the problem of the structure of

yeast mtDNA: a) the hybridization profiles are not superimposable to the absorbance profiles and b) the mtDNA from the mutant, which contains only 3.6% G+C, still retains the information for two tRNAs. This result strengthens the conclusion previously drawn of the presence on mtDNA from this petite mutant of informational regions having a high G and C content, while the remaining part of the molecule should contain mainly A and T. A similar structure should be present in w.t. mtDNA since hybridization peaks are obtained on fractions lighter or heavier than bulk DNA: the hybridization on fractions even lighter than the main peak (18% G+C) can actually be explained by the presence on these fragments of informational sections containing genes for tRNAs and having presumably a G+C content higher than 18% and regions containing mainly A and T. This is supported by the results reported in fig. 3 which show that further fragmentation of DNA shifts the hybridization peak of phenylalanyl-tRNA towards the heavier region of the gradient, presumably by detaching an A+T rich section from the heavier informational one. The picture of mtDNA resulting from these data, while confirming our previous results (7, 10), is consistent with the model proposed by Prunell and Bernardi for the structure of yeast mtDNA (12).

The differences in the position of the hybridization peaks which are localized for some tRNAs in the lighter and for some tRNAs in the heavier zone of the gradient, are not artifacts of the standard fragmentation procedure, since different hybridization profiles have been obtained with two aminoacyl-tRNAs on the fractions of the same DNA gradient, as can be seen from fig. 2 (a,b and e,f). Fig. 2 (a,b) shows that genes for tRNA_{ser} are localized on DNA fragments different from those containing genes for tRNA_{phe}; the same conclusion can be drawn from fig. 2 (e,f) as far as the genes for tRNA_{phe} and tRNA_{val} are concerned. In this second case we must stress the fact that these genes are both localized on the light fragments obtained by the standard

sonication procedure (results not shown), but after further fragmentation of DNA, the hybridization peak of tRNA_{phe} is found in the heavy region (fig. 2 e), while tRNA_{val} still hybridizes on lighter fractions (fig. 2 f). In conclusion genes for tRNA_{phe}, tRNA_{val} and tRNA_{ser} are found on different mtDNA fragments and should therefore be not clustered on the intact DNA molecule.

Comparison of the hybridization profiles of the five tested tRNAs shows another noteworthy difference: high hybridization levels are actually obtained on fractions of widely different density in the case of serine and histidine, while hybridization is displaced in the lighter region of the gradient in the case of isoleucine, valine and phenylalanine. It is worthwhile noticing that the genes for these three tRNAs are lacking in the mutant, as if an informational section containing these genes and immersed in a light region had been lost: the genes for tRNA_{ser} and tRNA_{his}, localized in the heavy fragments, are retained while no relevant hybridization is observed on the lighter fragments of petite DNA.

It would now be necessary to investigate the reason why tRNA_{ser} and tRNA_{his} hybridize both on light and on heavy fragments of w.t. mtDNA. The possible explanations are the following: a) peculiarities of the fragmentation procedure produce more than one class of fragments containing the same gene, b) more than one gene for these tRNAs are present on mtDNA. We are now investigating on these possibilities.

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ABBREVIATIONS

mtDNA, mitochondrial DNA; w.t., wild-type; SSC, standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate).

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