ANALYSIS OF GRANDE AND PETITE MITOCHONDRIAL DNA BY DNA–DNA HYBRIDIZATION

M. FAUMAN and M. RABINOWITZ

Departments of Medicine, Biology and Biochemistry, the University of Chicago, and the Argonne Cancer Research Hospital, Chicago, Illinois 60637, USA

Received 8 October 1972

1. Introduction

The cytoplasmic petite mutation in the yeast *Saccharomyces cerevisiae* results in a loss of the normal respiratory capacity found in the grande strain [1–3]. This loss of mitochondrial oxidative function may be associated with an alteration in buoyant density of petite mitochondrial DNA (mtDNA) which reflects varying degrees of base composition changes [4–6]. However, the mtDNA of many petite strains may have the same buoyant density as grande mtDNA. This has prompted us to develop a DNA–DNA microhybridization system which could be used as a more sensitive measure of changes or losses of mtDNA sequences in petites.

In this investigation we have examined a grande and a petite mtDNA by reciprocal DNA–DNA hybridization followed by thermal denaturation of the filter-bound hybrid to check the fidelity of base pairing [7–11]. We find that, under conditions which result in very specific hybridization, there is a reciprocal twofold difference in the hybridization of the grande and petite mtDNA's, even though their buoyant densities are not significantly different. Kinetic analysis of the rates of renaturation of the grande and petite mtDNA show that the major portion of the petite mtDNA renatures about twice as fast as the grande mtDNA. A small rapidly renaturing fraction may also be present in the petite mtDNA.

2. Materials and methods

2.1. *Yeast strains and growth*

The two chromosomal isogenic strains of yeast used are subclones from the original haploid strains D243-2B-R1 and D243-2B-R1-6 obtained from the laboratory of Dr. P.P. Slonimski (Centre de Génétique Moléculaire du C.N.R.S., 91 Gif-sur-Yvette, France). D243-2B-R1 is a grande and D243-2B-R1-6 is a spontaneously mutated cytoplasmic petite strain. The strains have previously been characterized [4,12] and the mitochondrial RNA and DNA studied [4,12, 13]. Cultures were grown according to the methods described earlier [14, 15]. Tritiated mtDNA was prepared by growth in [8-^{3}H]adenine (12–22 Ci/mmole, Schwarz Bioresearch, Orangeburg, New York).

2.2 *Mitochondrial isolation, DNA extraction and analytical ultracentrifugation*

These procedures have been described in previous publications from our laboratory [14, 15]. DNA was also prepared from *E. coli* strain K12-Hfr-H3,000 and from purified T4 phage obtained from Dr. Robert Haselkorn.

2.3. *Optical melting curves*

Mitochondrial DNA, dissolved in 1 × SSC (0.15 M NaCl, 0.015 M Na_3 citrate), was heated in quartz cuvettes in the sample chamber of the Gilford recording spectrophotometer while the optical density at 260 nm was monitored.

2.4. *Renaturation kinetics*

Grande and petite mtDNA and T4 DNA solutions were dialyzed against 1/10 X SSC, sonicated, adjusted to 1 X SSC, bubbled through with helium, and 0.4 ml denatured with 0.05 ml of 1.0 N NaOH for 20 min at room temp. in quartz cuvettes. The cuvette was cooled in ice, and 0.5 ml of cold water followed by 0.05 ml of cold 2.0 M NaH_2PO_4 added. The cuvette was then placed in water at 60° for 30 sec to warm it to the renaturation temperature, and rapidly placed in the pre-heated sample chamber of the Gilford recording spectrophotometer. The hypochromic shift of the DNA was recorded for 1 hr. The DNA was then heat-denatured to record the maximum hyperchromicity. The renaturation curves and kinetic constants were calculated according to the method of Wetmur and Davidson [17]. An aliquot of the DNA was sedimented through 3.0 M CsCl and 0.1 N NaOH at 42,040 rpm. Sedimentation values were calculated according to the method of Studier [16]. The sedimentation values of grande and petite mtDNA, and of T4 DNA, were 4.3 S, 4.3 S and 5.4 S, respectively.

2.5. *Hybridization of mitochondrial DNA*

Nitrocellulose filters were loaded with DNA according to the method of Gillespie and Spiegelman [18]. The DNA in 1 X SSC was denatured with 0.2 ml of 1.0 N NaOH/1.0 ml of DNA solution for 20 min at room temp. The solution was cooled to 4°, neutralized with cold 1.0 N HCl, adjusted to 4 X SSC, and loaded on filters. The filters were dried at room temp. for 1 hr and incubated in Denhardt's solution [7] for an additional hour. The filters were again dried at room temp. and then at 60° overnight.

The [^{3}H] mtDNA was dialyzed against 1 X SSC, sonicated, alkali denatured and neutralized as described above. The neutralized solution was adjusted to a pH of 6.5 to 7.0 with 0.01 M N-Tris (hydroxymethyl) methyl-2-aminoethane-sulfonic-acid.

A microhybridization system was developed for use with small amounts of labeled mtDNA. MtDNA filters and incubation solution containing labeled denatured mtDNA were sealed between two pieces of parafilm by pressing them together with a small hollow cylinder that had a diameter larger than the filters. The sealed microhybridization unit was immersed in a water bath at 35°. All hybridization units contained 3 filters, one charged with 5.0 μg of grande mtDNA, a second with 5 μg petite mtDNA, the third with 5 μg *E. coli* DNA, and 200 μl of solution. Hybridization solutions contained 2 X SSC and 36% formamide (Matheson, Coleman and Bell) to lower the normal melting temperature of mtDNA from 75° to about 50° [11]. Hybridization was carried out at 35°, or approx. 15° below the formamide lowered T_m.

2.6. *Hybrid melting curves*

The thermal stability of the DNA–DNA hybrids bound to filters was measured. After hybridization the filters were washed, dried, and incubated successively in vials with 1.0 ml of 1 X SSC for 5 min at temperature increments of 5°. Triton scintillation fluid was added to each vial and the [^{3}H] DNA released at each temperature counted in the Packard Tri-carb scintillation counter.

3. Results

3.1. *Buoyant density*

With SPO-1 DNA (density 1.742 gm/cm^3) as standard, the grande mtDNA had a buoyant density of 1.684 gm/cm^3 and the petite mtDNA a density of 1.683 gm/cm^3. Under the same circumstances yeast nuclear DNA had a buoyant density of 1.700 gm/cm^3.

3.2. *Hybridization*

Reciprocal hybridization was carried out with grande and petite mtDNA. Hybrids formed between grande [^{3}H] mtDNA in solution and grande mtDNA or petite mtDNA on filters are referred to as grande isohybrids and grande heterohybrids, respectively. Hybrids of petite [^{3}H] mtDNA in solution and petite mtDNA and grande mtDNA on filters are called petite isohybrids and petite heterohybrids.

Hybridization between grande [^{3}H] mtDNA in solution and grande, petite and *E. coli* DNA's on filters are shown in fig. 1a. Hybridization to both grande and petite mtDNA increases linearly as the amount of grande [^{3}H] mtDNA in solution is increased. The ratio of grande [^{3}H] mtDNA bound to the two different mtDNA filters is a measure of the difference between the grande and petite mtDNA. The ratio of radioactivity bound to the petite vs. the

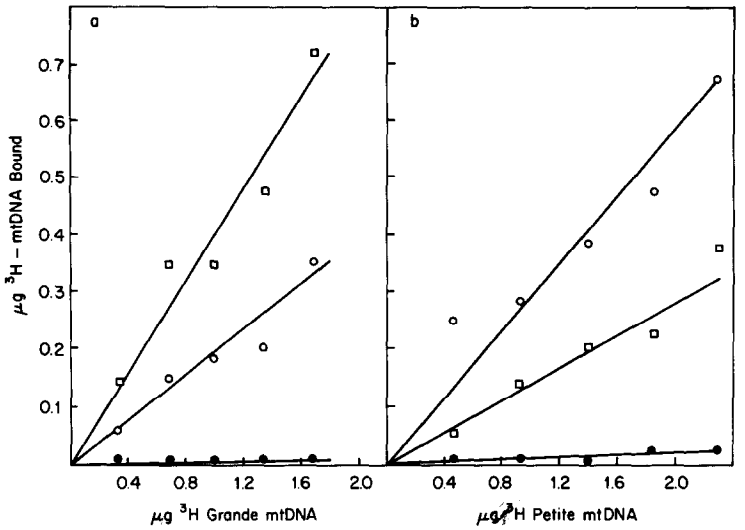

Fig. 1. Hybridization of grande [^{3}H] mtDNA (a) and petite [^{3}H] mtDNA (b). Filters contained 5.0 μg of grande mtDNA □—□—□, petite mtDNA ○—○—○, or *E. coli* DNA ●—●—●. Specific activity of [^{3}H] grande mtDNA was 1710 cpm/μg. Specific activity of petite [^{3}H] mtDNA was 670 cpm/μg.

grande mtDNA is 0.47 ± 0.04 S.E. The hybridization of petite [^{3}H] mtDNA in solution to grande and petite mtDNA on filters is shown in fig. 1b. Binding again increases linearly with increasing concentrations of petite [^{3}H] mtDNA in solution. The ratio of petite [^{3}H] mtDNA bound to the grande mtDNA filter to that bound to the petite mtDNA filter is 0.45 ± 0.08 S.E. Thus the isohybridizations were always about two times higher than the heterohybridizations. Binding to filters containing *E. coli* DNA, which were present in each hybridization set, was very low (1–4% of the isohybridization level). Nonspecific binding to blank filters was less than half that to the *E. coli* DNA.

3.3. *Optical and hybrid thermal denaturation curves*

The T_m of the grande mtDNA determined optically is 75.5° while that of petite mtDNA is 72.5°. The values are similar to those found for a number of yeast mtDNA's by Bernardi et al. [6]. Melting curves of the DNA–DNA hybrids were determined for each of the two iso- and heterohybridization combinations (fig. 2a, 2b). The T_m, *i* values (irreversible strand separation) [10] of the heterohybrids were very similar to that of the isohybrids. The T_m, *i* values of the isohybrids are several degrees higher than the T_m values derived from the optical melting curve, an effect that has been noted previously [19].

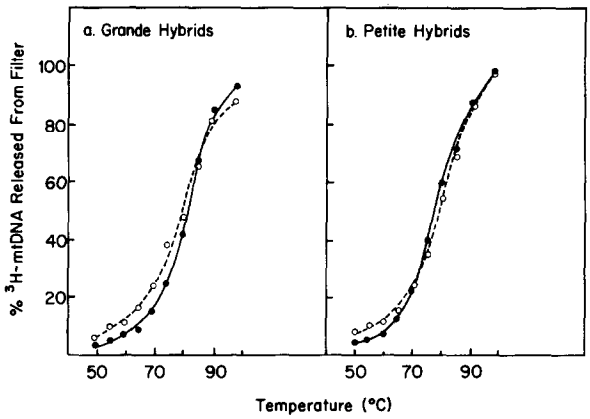

Fig. 2. Thermal denaturation curves of mitochondrial DNA–DNA hybrids. These DNA–DNA hybrids were melted as described in the text. a) Grande isohybrid ●—●—● (T_m, *i* 82°), grande heterohybrid ○- - -○- - -○ (T_m, *i* 81°). b) Petite isohybrid ●—●—● (T_m, *i* 78°), petite heterohybrid ○- - -○- - -○ (T_m, *i* 79°). Radioactivity initially bound to the hybrids: grande isohybrid 1241 cpm, grande heterohybrid 609 cpm, petite isohybrid 478 cmp, petite heterohybrid 272 cpm.

3.4. *Renaturation kinetics*

The relative rates of renaturation of grande and petite mtDNA were measured (fig. 3). The renaturation constants (K_2) of the grande and petite mtDNA's are 1.4 and 2.8 M^{-1} sec^{-1}, respectively; the renaturation constant of T4 DNA is 1.5 M^{-1} sec^{-1}. Thus the petite mtDNA shows a decrease in kinetic complexity. There is a downward skewing in the curves of all three of the DNA's at the earliest time periods (fig. 3), probably secondary to incomplete temperature equilibration. The effect was consistently observed to be considerably greater with petite mtDNA and may indicate the presence of a small, rapidly reassociating fraction of repeated sequences in petite mtDNA. This observation has been confirmed in this and in several other related spontaneously mutated petite strains [20].

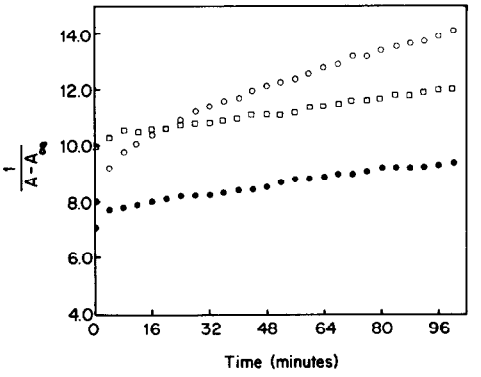

Fig. 3. Renaturation kinetic curves of grande mtDNA •, petite mtDNA ○, and T4 DNA □. DNA was denatured with NaOH, and renatured in 0.16 M Na ion at pH 6.8 to 7.0 at a DNA concentration of 17.5 to 18.0 μg/ml.

4. Discussion

A number of investigators have previously reported on the altered melting characteristics, base composition, buoyant density, and RNA and tRNA hybridization of cytoplasmic petite mtDNA [4–6, 12, 13]. The emphasis of this paper has been to adapt the technique of DNA–DNA hybridization to the investigation of possible subtle differences between grande and petite mtDNA's. Filter hybridization followed by thermal denaturation of the filter-bound hybrids has been used previously to evaluate the specificity and fidelity of hybridization [7–11]. Specific hybridization with little or no mismatching results in hybrids that have a thermal stability similar to that of native DNA determined optically. The similar hybrid thermal denaturation curves found for the iso- and heterohybrids and the low binding to *E. coli* DNA indicate that our hybridization results are quite specific.

Several possible changes in the petite mtDNA may account for our hybridization and renaturation data. There may be deletion, alteration of the nucleotide sequence, reiteration of part of the sequence, or a combination of these processes. The increase in renaturation rate of petite mtDNA indicates a loss of DNA sequences, i.e., deletion, but the apparent reciprocity of the hybridization results, with isohybridizations being greater than heterohybridizations suggests the presence of sequence changes. The possibility of reiteration is suggested by the rapidly renaturing fraction found in the petite mtDNA. However, the anomalous physical properties of yeast mtDNA [6] introduce uncertainties with regard to the relation between renaturation rates and genetic complexity [21], and the 2-filter DNA–DNA hybridization system used is difficult to interpret quantitatively. Therefore, we do not believe that the results presented can definitely establish the nature of the changes in the petite mtDNA. We can conclude from the hybridization and renaturation data, however, that there is a substantial qualitative difference between these grande and petite mtDNA's which is not detectable by CsCl buoyant density studies. Additional work elaborating upon the techniques and data in this paper are in progress, and may produce a more precise evaluation of the differences between the mtDNA's of the two strains.

Acknowledgements

This work was supported in part by Grants HL-04442, HL-09172, HD-174 and HL-05673 from the USPHS. The Argonne Cancer Research Hospital is operated by the University of Chicago for the United States Atomic Energy Commission.

References

[1] B. Ephrussi, Nucleo-cytoplasmic Relations in Microorganisms (Clarendon Press, Oxford, 1953).
[2] P.P. Slonimski, Ann. Inst. Pasteur 76 (1949) 510.
[3] P.P. Slonimski and B. Ephrussi, Ann. Inst. Pasteur 77 (1949) 47.
[4] J.C. Mounolou, H. Jakob and P.P. Slonimski, Biochem. Biophys. Res. Commun. 24 (1966) 218.
[5] F. Carnevali, G. Morpurgo and G. Tecco, Science 163 (1969) 1331.
[6] G. Bernardi, G. Faures, G. Piperno and P.P. Slonimski, J. Mol. Biol. 48 (1970) 23.
[7] D.T. Denhardt, Biochem. Biophys. Res. Commun. 23 (1966) 641.
[8] C.D. Laird and B.J. McCarthy, Genetics 60 (1968) 303.
[9] C.D. Laird and B.J. McCarthy, Genetics 60 (1968) 323.
[10] B.J. McCarthy and B.L. McConaughy, Biochem. Genet. 2 (1968) 37.
[11] B.L. McConaughy, C.D. Laird and B.J. McCarthy, Biochemistry 8 (1969) 3289.
[12] H. Fukuhara, M. Faures and C. Genin, Mol. Gen. Genet. 104 (1969) 264.
[13] M. Cohen, J. Casey, M. Rabinowitz and G.S. Getz, J. Mol. Biol. 63 (1972) 441.
[14] J. Casey, M. Cohen, M. Rabinowitz, H. Fukuhara and G.S. Getz, J. Mol. Biol. 63 (1972) 431.
[15] M. Rabinowitz, G.S. Getz, J. Casey and H. Swift, J. Mol. Biol. 41 (1969) 381.
[16] F. W. Studier, J. Mol. Biol. 11 (1965) 373.
[17] J.G. Wetmur and N. Davidson, J. Mol. Biol. 31 (1968) 349.
[18] D. Gillespie and S. Spiegelman, J. Mol. Biol. 12 (1965) 829.
[19] E.P. Geiduschek, J. Mol. Biol. 4 (1962) 467.
[20] J.W. Casey, P. Gordon and M. Rabinowitz, J. Cell Biol. (1972) in press (abstract).
[21] C. Christiansen, A.L. Bak, A. Stenderup and L. Christiansen, Nature New Biol. 231 (1971) 176.