EVIDENCE FOR AN EXTRA-CHROMOSOMAL ELEMENT INVOLVED IN MITOCHONDRIAL FUNCTION: A MITOCHONDRIAL EPISOME?

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1. Introduction

Biochemical genetic studies of cytoplasmic mutants of *Saccharomyces cerevisiae* resistant to inhibitors of aerobic (mitochondrial) metabolism such as oligomycin and venturicidin have shown that the resistance determinants are located on mitochondrial DNA [1,2]. Three separate loci, OL 1, OL II and OL III which probably specify two different subunits of the mitochondrial ATPase complex have been identified [2–4]. Other mutants resistant to inhibitors and uncouplers with a specific mode of action on aerobic energy conservation reactions are under investigation including triethyltin, '1799', bongkrekic acid, rhodamine 6G and valinomycin.

Detailed studies of cytoplasmic triethyltin mutants and another class of venturicidin resistant mutants [2,5] indicate that these resistance determinants are unlinked to other loci on mt-DNA suggesting that the $VEN^R TET^R$ determinants are located on a cytoplasmic DNA species different from mt-DNA [2]. However, there are several features of these mutants which suggest a close association with mitochondrial function, e.g. the loss of the $VEN^R TET^R$ phenotype on ethidium bromide treatment always leads to the ρ^- state and a significant proportion of spontaneous petites (ρ^-) have lost resistance determinants.

In this paper we present evidence that the VEN^R TET^R resistance determinants are located on a cytoplasmic DNA species other than mt-DNA. Our studies suggest that we are dealing with a DNA species which is necessary for mitochondrial function which has properties of a plasmid or possibly an episomal system associated with mt-DNA. The implications of these findings with respect to nucleo-cytoplasmic interactions involved in the biogenesis of mitochondrial inner membrane components is discussed.

Abbreviations: '1799' bis (hexafluoroacetonyl) acetone; R6G rhodamine -6G; TET, Triethyltin sulphate; Ven, Venturicidin; ρ^0 a specific 'petite' in which all mt-DNA has been deleted.

2. Materials and methods

2.1. *Yeast strains and genetic analysis*

The venturicidin resistant strain D22/69 of phenotype $VEN^R TET^R 1799^R R6G^R$ and its characterisation as a cytoplasmic (extrachromosomal) mutant and the properties of the sensitive strain D41 have been described previously [6]. This strain exhibits normal sensitivity to oligomycin, chloramphenicol and erythromycin [6].

Ethidium bromide induced petites were produced from strain D22/69 by subculturing twice in liquid glucose medium (1% yeast extract, 1% peptone, 2% glucose, sodium-potassium phosphate pH 6.25) containing 10 μg/ml ethidium bromide (E Br). Methods for determination of petite frequencies and identification of resistance markers remaining in petite strains by crossing with a ρ^+ sensitive tester to form a diploid were as described previously [5]. Diploid strains were assayed for resistance to inhibitors by dropping out cell suspensions onto solid glycerol medium (1% yeast extract, 1% peptone, 3% glycerol, 2% agar sodium-potassium phosphate pH 6.25) containing a suitable range of inhibitor concentrations.

Suppressiveness of petite strains was determined by forming synchronous zygotes between the petite and

the grande tester strain D41 followed by determination of the percentage petite zygotes formed using the tetrazolium overlay procedure [2]. This value, when corrected for the spontaneous production of petites in the control cross D22/69 x D41, is the value taken for suppressiveness.

2.2. *Growth of yeast cells and isolation of DNA*

Yeast strains were grown under forced aeration and rapid stirring at 30°C in 4 litres of culture medium and harvested in early stationary phase by centrifugation at 2000 *g* for 10 min and washed in distilled water before fractionation. Culture media contained 1% w/v yeast extract, 1% w/v peptone, 1% w/v glucose for grande strains, 2% w/v glucose for petite strains, Wickerhams salts and adenine sulphate (100 mg/litre).

Fractionation of yeast, preparation of a yeast mitochondrial fraction and ethidium bromide buoyant density centrifugation of DNA were as described by Clark-Walker [7] and O'Connor et al. [8]. The buoyant density analyses were performed in a Spinco Model E ultracentifuge under standard conditions.

3. Results

3.1. *Isolation of neutral (zero suppressive) petites*

Treatment of the venturicidin resistant strain D22/69 ethidium bromide under the conditions described leads to complete transformation to the cytoplasmic petite (ρ^-) but the venturicidin resistance determinant can either be lost or retained in these petites as described previously [2]. The majority of the petite strains analysed were found to be neutral petites (zero suppressive). Such petites provide a suitable system for establishing whether the V^R determinant is located on mt-DNA as previous mt-DNA analysis of neutral petites has shown that the mitochondrial genome has been completely deleted leading to the ρ^o form [9,10].

The properties of typical examples of petites isolated are listed in table 1. It is evident that loss or retention of a particular drug resistance is generally accompanied by the respective loss or retention of the other three drug resistance determinants.

3.2. *Analysis of mt-DNA and omicron-DNA*

The isolation of neutral (zero suppressive) petites which retain V^R determinant (i.e. ρ^oV^R) is further evidence in support of the genetic data indicating that the V^R determinant is not located on mt-DNA [2]. Further support comes from analyses of mt DNA in mitochondrial-enriched fractions by ethidium bromide buoyant density centrifugation of DNA (fig.1). Three bands corresponding to mt-DNA, n-DNA and omicron-DNA [7,8] can be seen in the grande strain, D22/69. No band corresponding to mt-DNA is seen in any of the ρ^o petites examined

Table 1
Ethidium bromide induced strains

Strain	Genotype	Phenotype of diploid strain (xD41)	% Suppressiveness of petite	No. of colonies analysed
D22/69 parental	ρ^+V^R	$VEN^RTET^RR6G^R1799^R$		
D22/69/D1	ρ^oV^R	$VEN^RTET^RR6G^R1799^R$	0	(198)
D22/69/D3	ρ^oV^o	$VEN^STET^SR6G^S1799^S$	0	(218)
D22/69/D4	ρ^oV^R	$VEN^RTET^RR6G^R1799^R$	0	(239)
D22/69/D8	ρ^oV^o	$VEN^STET^SR6G^S1799^S$	0	(89)

Induction of petites by ethidium bromide treatment of strains D22/69 and determination of suppressiveness of petite strains was as described in Materials and methods. The genetic markers for resistance determinants in petites were analysed by the marker rescue back crossing technique described previously [5]. Resistance to venturicidin, thriethyl tin, rhodamine-6G and 1799 was determined as the ability to grow on the following drug concentrations, 1.0 μg/ml, 40 μM, 50 μg/ml and 20 μg/ml, respectively.

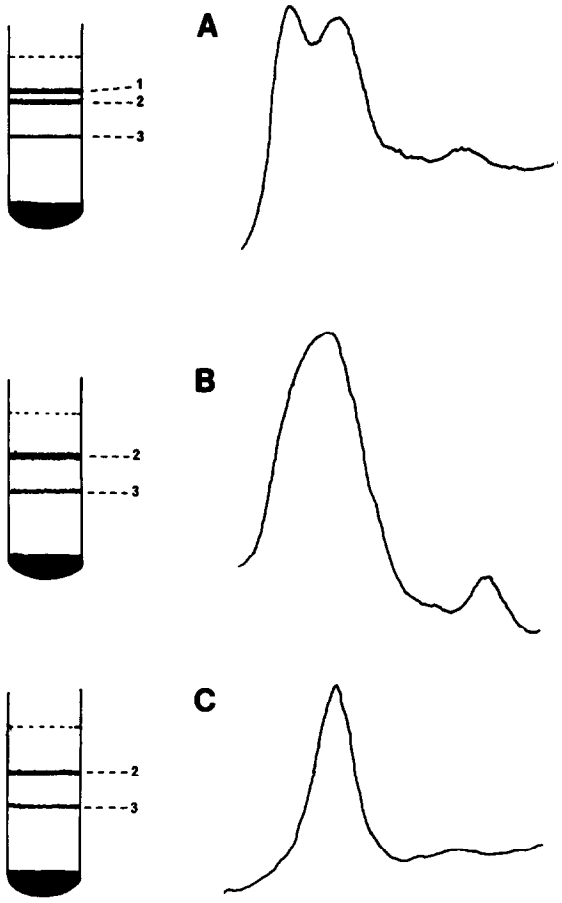

Fig.1. Ethidium bromide buoyant density centrifugation of mitochondrial membrane enriched fractions. The preparation of the mitochondrial membrane enriched fraction in the presence of ethidium bromide was as described by Clark Walker [7,8].

The mitochondrial-enriched pellet was resuspended in 2% (w/v) sarkosyl, added to a CsCl-E Br gradient and centrifuged to equilibrium in a Ti 50 rotor (48 k rev/min, 48 hr, 13°C). DNA bands were visualised and photographed under u.v. light at 366 nm and the relevant fractions withdrawn by side puncture with an 18 gauge neelde. EBr was removed by four successive extractions with isoamyl alcohol and the CsCl removed by dialysis against saline citrate buffer. Photographs were taken through an orange filter by illuminating with u.v. light at 366 nm. Negatives were scanned in a Joyce Loebl densitometer, heavy density is to the right of the trace.

The results of typical centrifugation runs are given. On the left are tracings of the approximate band positions observed in the density gradient. Band 1, mt-DNA; Band 2, n-DNA; Band 3, omicron-DNA.
A, strain D22/69 (ρ^+V^R); B, strain D22/69/D3 (ρ^oV^o); C, strain D22/69/D4 (ρ^oV^R).

(ρ^oV^R or ρ^oV^o). The band corresponding to n-DNA in the petite strain D22/69/D4(ρ^oV^R) and the clear area corresponding to the normal position of mt-DNA were removed and analysed in the analytical ultracentrifuge after removal of ethidium bromide. No evidence for a species of buoyant density corresponding to mt-DNA was obtained, thus confirming the results obtained by ethidium bromide buoyant density centrifugation.

Genetic analyses [2,5], isolation of ρ^oV^R petites and concomitant demonstration of retention of the V^R determination in ρ^o petites where mt-DNA has been deleted, provide strong evidence for the location of the V^R determinant on a cytoplasmic DNA species other than mt-DNA.

The location of the V^R determinant remains to be established and preliminary investigations of the relationship of the V^R determinant to the closed circular omicron-DNA species demonstrated to be present in mitochondrial [7,8] have been inconclusive. Analysis of the omicron-DNA content in ρ^+V^R grandes and ρ^oV^R and ρ^oV^o petites are of interest as retention of omicron-DNA in ρ^oV^R petites and loss of omicron-DNA in ρ^oV^o petites would provide a positive correlation of the V^R determinant with omicron-DNA. Fig.1 summarises preliminary investigations into this point. No positive correlation of the V^R determinant with omicron-DNA can be made on the basis of these experiments as both ρ^oV^R and ρ^oV^o petites retain the omicron-DNA band on ethidium bromide buoyant density centrifugation. However, quantitative analysis of the omicron-DNA species is difficult and no estimate of the composition of different sized circular DNA species in mitochondria can can be made in the absence of visualization of these species in the electron microscope and correlated size-distribution studies. Further investigations of the possible role of omicron-DNA are in progress.

4. Discussion

Mutants isolated as cytoplasmic mutants resistant to specific inhibitors of aerobic (mitochondrial) metabolism such as triethyl tin [5], venturicidin [6], the uncoupler '1799' [11] and rhodamine 6G [12] have now been shown to be closely related mutations of multiple resistance phenotype VEN^R

TET^R $R6G^R$ 1799^R with variable degrees of resistance to bongkrekic acid (BA). These are stable cytoplasmic mutations which satisfy all the accepted criteria for mitochondrial mutations, including loss on petite induction (association with *rho* factor), *except linkage with other mitochondrial loci.* A close association with mitochondrial functions is indicated by; (a) effects on transmission of known mitochondrial markers [13]; (b) phenotypic interaction between the VEN^RTET^R mutation and the mitochondrial oligomycin resistance mutation [5]; (c) loss of resistance determinants is observed in a significant number of spontaneous petites [12], indicating an association with *rho* factor; (d) changes in inhibitor affinities are observed at the mitochondrial membrane level [5,14], indicating modification of mitochondrial membrane components in resistant mutants; (e) all the ligands involved, VEN, TET, R6G and '1799' have a specific effect on aerobic growth of yeast [5,6,12] and have a specific mitochondrial mode of action relating to mitochondrial transport processes suggesting that a common structural or organisational feature of the mitochondrial inner membrane is involved.

The demonstration that the VEN^RTET^R resistance determinants are essentially unlinked to other known mitochondrial loci [2] poses the question as to whether the resistance determinants are located on a cytoplasmic DNA species other than mt-DNA and the intra-mitochondrial or extra-mitochondrial location of this cytoplasmic determinant. The isolation of ρ^oV^R petites (table 1) and the demonstration of the absence of mt-DNA in ρ^oV^R and ρ^oV^o petites (Figure 1) establishes clearly that the resistance determinants are not located on mt-DNA. A possible candidate for the location of the VEN^RTET^R determinants are a class of small circular DNA species found in yeast mitochondrial preparations by many workers and termed omicron-DNA by Clark-Walker [7]. Preliminary investigations have shown no correlation in complete loss of the omicron-DNA band concomitant with loss of the V^R determinant in the ρ^oV^o petites as compared with the ρ^oV^R petite (fig.1). Detailed investigations of the loss or retention of specific classes of small circular DNA [7] or changes in physical conformation of these molecules as well as studies of the intra-mitochondrial or extra-mitochondrial location of the resistance determinants are in progress.

The class of mutations described here have many properties typical of bacterial plasmids or episomes, e.g. (a) the mutations confer multiple resistances to drugs affecting a common target site, the mitochondrial membrane; (b) the resistance determinants can be lost by treatment with ethidium bromide; (c) the mutations are weakly linked to host genes (mitochondrial DNA). Genetic studies on a similar series of mutants resistant to thriethyl tin (TET^RVEN^R) reveal several interesting genetic interactions between these mutations and mutations located on mt-DNA. The presence or absence of these resistance mutations in a strain containing a mitochondrial resistance mutation will cause a strong increase or decrease in the per cent transmission of the mitochondrial gene to the zygotic progeny [13]. These results can be explained by a model involving incorporation of the drug resistance determinant into mt-DNA at specific stages during mitotic cell division and zygote formation, i.e. location of the resistance determinant on an episomal system associated with mt-DNA.

A series of stable cytoplasmic mutants specifically resistant to uncoupling agents showing many similarities to the VEN^RTET^R class of mutants are also under investigation in this laboratory [11,15,16]. However, in this case no close association with mt-DNA has been shown by genetic analysis but changes in mitochondrial uncoupler binding sites and marked effects on aerobic growth yield have been demonstrated, again indicating a modification of mitochondrial function. Evidence for an episomal system in yeast involving multiresistance determinants for oligomycin, venturicidin, triethyl tin, cycloheximide and chloramphenicol has recently been presented by Guerineau et al. [17]. In contrast to the stable cytoplasmic mutations described in this paper, these mutations appear to be nuclear mutations with episomal characteristics such as loss during vegetative multiplication and elimination by growth in ethidium. In addition, a correlation between the loss of small circular band of DNA (omicron-DNA) and loss of the oligomycin resistance determinant has been demonstrated. The presence of different episomal systems in yeast concerned with specification of membrane components, mitochondrial and cytoplasmic, must be considered.

These studies raise questions as to the definition

of the term 'mitochondrial DNA' and the informational role and cellular location (s) of small circular DNA (omicron-DNA). The cytoplasmic determinants described in this paper are not located on mt-DNA but the intra- or extra-mitochondrial location of the DNA species carrying these determinants and the relationship to omicron-DNA remain to be established. It is possible that this cytoplasmic DNA species has an intra-mitochondrial and extra-mitochondrial location and function at different phases of the growth cycle and this raises new questions in studies of nucleo-cytoplasmic interactions involved in mitochondrial biogenesis, e.g. it may be necessary to revise data on nuclear or cytoplasmic (mitochondrial) determination of mitochondrial membrane components which are based on the presence or absence of these components in petite mutants.

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References

[1] Avner, P. R. and Griffiths, D. E. (1973) Eur. J. Biochem. 32, 301–311.
[2] Lancashire, W. E. and Griffiths, D. E. (1975) Eur. J. Biochem. 51, 403–413.
[3] Avner, P. R. and Griffiths, D. E. (1973) Eur. J. Biochem. 32, 312–321.
[4] Avner, P. R., Coen, D., Dujon, B. and Slonimski, P. P. (1973) Mol. Gen. Genet. 125, 9–52.
[5] Lancashire, W. E. and Griffiths, D. E. (1975) Eur. J. Biochem. 51, 377–392.
[6] Griffiths, D. E., Houghton, R. L., Lancashire, W. E. and Meadows, P. M. (1975) Eur. J. Biochem. 51, 393–402.
[7] Clark Walker, G. D. (1973) Eur. J. Biochem. 32, 263–267.
[8] O'Connor, R. M., McArthur, C. R. and Clark-Walker, G. D. (1975) Eur. J. Biochem. (in press).
[9] Nagley, P. and Linnane, A. W. (1970) Biochem. Biophys. Res. Commun. 39, 989–996.
[10] Deutsch, J., Dujon, B., Netter, P., Petrochilo, E., Slonimski, P. P., Bolotin-Fukuhara, M., Coen, D. (1974) Genetics, 76, 195–219.
[11] Griffiths, D. E., Avner, P. R., Lancashire, W. E. and Turner, J. R. (1972) in: Biochemistry and Biophysics of Mitochondrial Membranes (Azzone, G. E., Carafoli, E., Lehninger, R. L., Quagliariello, E. and Siliprandi, N. eds.) pp. 605–621, Academic Press, New York.
[12] Carignani, G., Jones, A., Lancashire, W. E. and Griffiths, D. E. (unpublished results).
[13] Lancashire, W. E. (1974) 'A genetic approach to oxidative phosphorylation', Ph.D. thesis, University of Warwick.
[14] Cain, K., Lancashire, W. E. and Griffiths, D. E. (1974) Biochem. Soc. Trans. 2, 215–218.
[15] Griffiths, D. E. (1972) in: Mitochondria: Biogenesis and Bioenergetics (S.G. van den Bergh, P. Borst and E. C. Slater eds.) pp. 95–104. North Holland, Amsterdam.
[16] Skipton, M., Lancashire, W. E., Griffiths, D. E. and Hanstein, W. G. (unpublished results)
[17] Guerineau, M., Slonimski, P. P. and Avner, P. R. (1974) Biochem. Biophys. Res. Commun. 61, 462–469.