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Expression of petite mitochondrial DNA *in vivo*: Zygotic gene rescue

(mitochondrial protein synthesis/genes on mitochondrial DNA/yeast)

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ABSTRACT **A protocol is introduced for probing the organization and regulation of expression of the yeast mitochondrial genome, termed "zygotic gene rescue." The procedure is based on the notion that genes retained on mitochondrial DNA of petites can be expressed in zygotes of a cross between petite and wild type. To test the validity of this notion, we have taken advantage of our ability to discriminate, by mobility differences on sodium dodecyl sulfate/polyacrylamide gels, different forms of the product of alleles of the mitochondrial gene, *var1*. In petite strains that have retained the *var1* gene, its characteristic product appears in zygotes 4–5 hr after mating; no product is observed in petite strains deleted in the *var1* locus. Our studies indicate that (*i*) expression in the zygote of the *var1* gene in the petite genome is not exclusively the result of recombination with mitochondrial DNA of the wild-type tester, and (*ii*) the *var1* gene is probably reiterated in the petite mitochondrial genome. The strength of the technique of zygotic gene rescue in the analysis of the mitochondrial genome is discussed.**

Recent advances in genetic and physical mapping of the yeast mitochondrial genome have provided considerable information on the identification and localization of a number of mitochondrial genes. Among these are included drug resistance loci (1–3), genes specifying rRNAs (4–6) and tRNAs (7–9), and a variety of loci termed *mit* (10, 11). The latter, when mutated, lead to an inability of the strain to grow on nonfermentable carbon sources as a result of specific alterations in cytochrome oxidase, the cytochrome bc_1 complex, or the oligomycin-sensitive ATPase complex (12). These complexes are composed of nuclear encoded, cytoplasmically synthesized subunits and subunits that are translated on mitochondrial ribosomes and generally believed to be encoded by mitochondrial DNA (mtDNA). It is of obvious interest to know the precise location of sequences on the yeast mitochondrial genome corresponding to the structural genes for these and other, less well characterized gene products of mtDNA.

In this communication we introduce a procedure for probing the organization of the yeast mitochondrial genome. This procedure, termed "zygotic gene rescue," is based on the premise that the mtDNA of yeast cells carrying the vegetative petite mutation (ρ^-) is senseful and could, in principle, express structural gene sequences *in vivo* given a functional transcriptional and translational apparatus.

It is now well documented that petites are the result of extensive deletions of wild-type mtDNA sequences (13–15) and, moreover, that individual petite isolates can retain any part of the mitochondrial genome (16, 17), most probably in a gene-amplified configuration (18–20). Indeed, petites exist that carry, to various extents, virtually all known loci on mtDNA (17). This important result has been established by taking advantage of the fact that petite mtDNA is capable of recombining with wild-type mtDNA, resulting in the appearance of wild-type recombinant progeny (16, 21). Thus, one can readily determine the presence of specific loci on petite mtDNA even though the petite is unable to express that DNA as a protein product. The detection and localization of structural gene sequences on mtDNA of both wild type and petites could be achieved by the application of procedures used to map drug resistance (22) and mit^- loci (11). What is intrinsically uncertain in such analyses, however, is whether or not the particular allele localized by the phenotype measured represents the structural gene sequence for a specified polypeptide product. We reasoned that it might be possible to detect directly structural gene sequences on mtDNA of genetically defined petites in an analysis of zygotes of a cross between petite and wild type, assuming that, in zygotes, mitochondria undergo fusion. In principle, mitochondrial fusion in zygotes would allow petite mtDNA to be expressed by the wild-type transcription and translation apparatus. Importantly, zygotic gene rescue could be readily demonstrated if the petite mtDNA encoded a protein that could be easily distinguished from its allelic form present in the wild-type tester strain. This approach became feasible with the report by Douglas and Butow (25) of electrophoretic variants of mitochondrial translation products. One particular variant, termed *var1*, was shown to exist in several allelic forms based on strain-dependent mobility differences on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels (25). The strains exhibiting these various allelic forms all carry out mitochondrial protein synthesis and are respiratory sufficient. Recently, we have determined that *var1* maps between the *ery* and *oli1* region of the yeast mitochondrial genome.* In this report, we demonstrate that the *var1* allele present on genetically defined petite mtDNA can be expressed in zygotes of a cross between the petite and a wild-type tester. The system we describe should make it possible to probe aspects of the organization and regulation of expression of the yeast mitochondrial genome not possible with the methods currently available.

MATERIALS AND METHODS

Strains. The nuclear and mitochondrial genotypes of yeast strains used in this study are provided in Table 1. The strains were generously provided by Philip Perlman, Ohio State University.

Mating of Yeast Strains. An inoculum of each strain was grown overnight at 30° in liquid YP medium containing (wt/vol) 1% Bacto yeast extract, 1% Bacto peptone, 0.1% KH_2PO_4, and 0.12% $(NH_4)_2SO_4$. The carbon source for ρ^- and ρ^+ strains was 0.5% glucose and 2% galactose (wt/vol), respectively. In the morning, the strains were mixed to give a cellular ratio of $3\rho^-:1\rho^+$. Mating efficiency of the ρ^+ strain is improved by using this ratio. Mating was allowed to proceed in liquid YP 2% galactose, containing 0.5% glucose, for 5 hr at 30° with shaking.

Abbreviations: mtDNA, mitochondrial DNA; YP medium, 1% Bacto yeast extract/1% Bacto peptone/0.1% KH_2PO_4/0.12% $(NH_4)_2SO_4$; YNBD, 0.67% Bacto yeast nitrogen base/1% glucose; $NaDodSO_4$, sodium dodecyl sulfate.

* P. S. Perlman, M. G. Douglas, R. L. Strausberg, and R. A. Butow, unpublished data.

Table 1. Strains

Strain	Nuclear genotype	Mitochondrial genotype						
55R5-3C/221	a *ura*	$\rho^+ \omega^-$	chl^s	ery^r-221	$oli1^s$	$oli12^s$	par^s	$var1^s$
41-6/19	α *ad lys*	$\rho^+ \omega^-$	chl^r	ery^s	$oli1^r$	$oli12^s$	par^r	$var1^f$
O^R29	α *ad lys*	ρ^-	chl^0	ery^0	$oli1^r$	$oli12^0$	par^0	$var1^0$
O^R45	α *ad lys*	ρ^-	chl^0	ery^0	$oli1^r$	$oli12^0$	par^0	$var1^0$
O^R120	α *ad lys*	ρ^-	chl^0	ery^0	$oli1^r$	$oli12^0$	par^0	$var1^f$
O^R154	α *ad lys*	ρ^-	chl^0	ery^0	$oli1^r$	$oli12^0$	par^0	$var1^f$
P^R5	α *ad lys*	ρ^-	chl^0	ery^0	$oli1^0$	$oli12^0$	par^r	$var1^0$
1L16-06	α *his*	ρ^0	chl^0	ery^0	$oli1^0$	$oli12^0$	par^0	$var1^0$

All petites were generated from strain 41-6/19 by ethidium bromide mutagenesis with the exception of 1L16-06 ρ^0 which was generated from 1L16-10B ρ^+. Mitochondrial markers were determined by the replica-cross procedure of Deutsch *et al.* (30).

Determination of Mating Percentage. Aliquots of the mating mixture were plated on YP 1% glucose to give approximately 100 cells per plate. After 2 days' growth at 30°, the resulting clones were replica-plated onto YNBD (0.67% Bacto yeast nitrogen base/1% glucose) and YNBD + uracil (20 mg/liter). The strains used have nutritional requirements such that only diploids will grow on YNBD, and diploids and ρ^+ haploids can grow on YNBD + uracil; therefore, a determination of the percentage of each cell type in the mating mixture can be made.

Labeling, Cell Fractionation, and Gel Electrophoresis. The protocols for labeling yeast cells with $^{35}SO_4^{2-}$ in the presence of cycloheximide, preparation of mitochondria, and the separation of proteins on $NaDodSO_4$/polyacrylamide slab gels have been described (25).

RESULTS

The general principle underlying zygotic gene rescue is shown in Fig. 1. A mating mixture of a wild-type tester strain and a petite are labeled with $^{35}SO_4^{2-}$ in the presence of cycloheximide so as to label selectively products of mitochondrial protein synthesis. After aliquots are labeled at different times after the initiation of mating, mitochondria are isolated from cells of the bulk mating mixture and analyzed on $NaDodSO_4$/polyacrylamide gels. Since petites by themselves are incapable of carrying out mitochondrial protein synthesis, any mitochondrial translation products *characteristic of the wild-type strain from which the petite was derived* must have arisen in zygotes of the mating mixture from: (*i*) transcription and translation of wild-type sequences retained on petite mtDNA, (*ii*) transcription and translation of those sequences on petite mtDNA subsequent to recombination into wild-type mtDNA, or (*iii*) by a combination of both processes (see below).

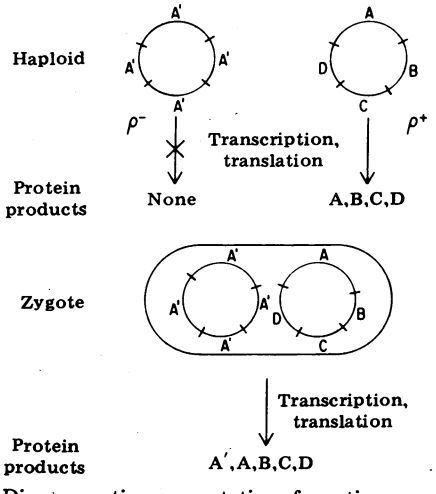

FIG. 1. Diagrammatic representation of zygotic gene rescue. The ρ^+ strain is shown with a complement of structural genes arbitrarily designated *A*, *B*, *C*, and *D*. The petite is shown to have retained an allelic form of gene *A*, designated *A'*, in a gene amplified configuration. In the zygote, the mtDNAs are indicated to be bound within the same mitochondrial structure.

Kinetics of Zygote Formation and Gene Rescue. In a typical experiment shown in Fig. 2, we followed the time course of zygote formation and analyzed products of mitochondrial protein synthesis on aliquots of the mating mixture labeled in the presence of cycloheximide with $^{35}SO_4^{2-}$. As we have shown previously, the ρ^+ tester strain (55R5-3C/221) carries the slow form of variant 1 polypeptide ($var1^s$), and the ρ^+ parental strain (41-6/19), from which most of the petites used here were derived, carries the fast form of variant 1 ($var1^f$) (25). The petite strain O^R120 was chosen for the experiment illustrated in Fig. 2 because we know from recombination analysis that it retains the $var1^f$ allele.* Fig. 2*A* demonstrates that the mating reaction is essentially complete after about 4 hr and that approximately 20% of the ρ^+ haploids participated in the formation of zygotes.

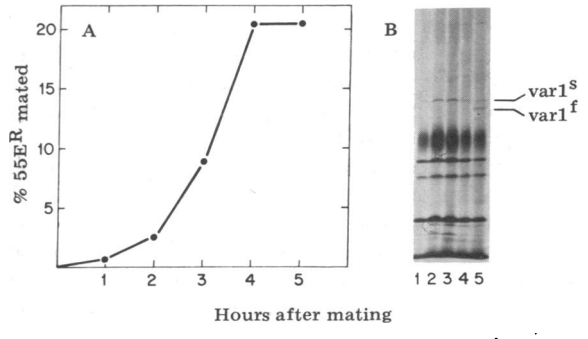

FIG. 2. Time course for zygotic gene rescue of var^f. Cells from logarithmic phase cultures of strains O^R120 and 55R5-3C/221 were mixed at a 3:1 cellular ratio, respectively, in 50 ml of YP 2% galactose medium containing 0.5% glucose. The total cellular concentration was approximately 10^7 cells per ml. Aliquots (5 ml) of the mating reaction were removed for labeling of mitochondrial translation products *in vivo* as described (25). The labeled cells were resuspended in 0.3 ml of 0.25 M mannitol/0.02 M Tris-SO_4, pH 7.1/0.001 M EDTA and mitochondria were isolated and prepared for $NaDodSO_4$/polyacrylamide gel electrophoresis as described (25). A second aliquot of the mating mixture was removed and plated at the appropriate dilutions to determine percentage mating as described in *Materials and Methods*. (*A*) Percentage of total 55R5-3C/221 cells (indicated as $55E^R$) mated; (*B*) $NaDodSO_4$/polyacrylamide gel profile of $^{35}SO_4^{2-}$-labeled mitochondrial translation products in mitochondria isolated from cells of the bulk mating mixture.

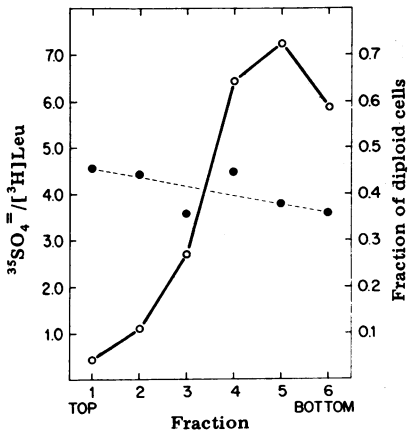

FIG. 3. Mitochondrial protein synthesis in unmated cells and zygotes *in vivo*. Separation by sorbitol gradient centrifugation. A logarithmic phase culture of strain 55R5-3C/221 was grown for 2 hr at 30° in 5 ml of YNB 2% galactose containing 100 μg of uracil and 200 μCi of [4,5-^{3}H]leucine (59 Ci/mmol). Ten milliliters of a 1% Casamino acid solution was added and the cells were incubated an additional 10 min. After cells were harvested and washed three times with 1% Casamino acids, they were mixed with 3 times the number of logarithmic phase cells of strain O^{R}120 in a final volume of 25 ml of YP 2% galactose medium containing 0.5% glucose. The mating mixture was incubated for 5 hr at 30°, after which time mitochondrial translation products were labeled *in vivo* with $^{35}SO_4^{2-}$ as described (25). After cells of the mating mixture were harvested and washed twice with a solution containing 1% Casamino acids and 0.3% Na_2SO_4, they were suspended in 1 ml of 0.25 M mannitol/0.02 M Tris-SO_4, pH 7.1/0.001 M EDTA, sonicated for 5 sec at full output in a Bronson Sonifier to disrupt cellular aggregates, and layered onto a sterile 15-ml gradient of 15–40% sorbitol. The gradient was centrifuged at room temperature at one-third maximum speed in an Adams Dynac bench top centrifuge for 10 min and then fractionated by pumping from the bottom of the tube. One aliquot from each fraction was removed to determine the percentage diploids (see *Materials and Methods*). The remaining cells in each fraction were pelleted and resuspended in 0.3 ml of mannitol/Tris-SO_4/EDTA. Mitochondria were isolated as described (25) and analyzed for hot 10% trichloroacetic acid-precipitable ^{35}S and ^3H radioactivity. Isotope ratios were obtained after correcting for channel spillover. (○ — ○) Fraction of diploid cells; (● - - - ●) $^{35}S/^3H$.

An autoradiogram of an NaDodSO_4/polyacrylamide gel of mitochondrial translation products synthesized between 0 and 5 hr in cells of the mating mixture is shown in Fig. 2*B*. Note that the var1^f band is just barely detectable 4 hr after mating and its intensity on the gel after 5 hr of mating is roughly equal to that of var1^s.

Specific Labeling of Haploids and Zygotes. The relative intensity of the var1^f band after 5 hr of mating is particularly interesting in view of the fact that only 20% of the ρ^+ strains participated in the mating reaction. In other words, since the entire mating mixture was labeled with $^{35}SO_4^{2-}$, the gel profile should reflect not only mitochondrial translation products in the zygotes, but also the translation products from the 80% of unmated ρ^+ cells in the population. One possibility to account for the extent of the var1^f band seen on the gel is that under the particular conditions of mating there is *selective* synthesis of mitochondrial translation products in zygotes compared to the unmated ρ^+ haploid cells. To test this possibility, we carried out a double label experiment shown in Fig. 3, taking advantage of the fact that it is possible to separate zygotes from unmated cells by centrifugation in a sorbitol gradient (26). A culture of the ρ^+ haploid parent was labeled in the absence of antibiotics with [^{3}H]leucine to label protein uniformly. The labeled cells were then mated for 5 hr to the ρ^- strain, O^{R}120; the entire

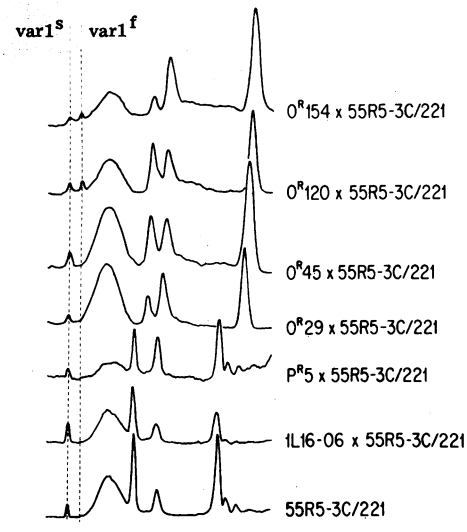

FIG. 4. Zygotic gene rescue of *var1^f* in various petite strains. The protocol for zygotic gene rescue between the petites indicated in the figure (see Table 1 for their mitochondrial genotypes) and the ρ^+ tester, 55R5-3C/221, is the same as indicated in Fig. 2, except that all the analyses were carried out after 5 hr of mating. Autoradiograms of the gel profiles were scanned with a Quick Scan (Helena Laboratories) and presented as the densitometer tracings. Although the analyses were not all done on the same gel, the scans have been aligned with respect to the var1^s and var1^f bands; consequently, the other bands do not necessarily align because of differences in the electrophoresis time.

mating mixture was labeled in the presence of cycloheximide with $^{35}SO_4^{2-}$. Aliquots of the doubly labeled cell population were layered on a 15–40% sorbitol gradient, fractions were collected, and the ratio of $^{35}S/^3H$ was measured in mitochondria isolated from cells in each of the gradient fractions. In separate aliquots, the fraction of mated cells was determined as described in *Materials and Methods*. The data in Fig. 3 show that the percentage of diploids in the fractions ranged from less than 5% at the top of the gradient to as high as 70% in the bottom fractions; thus, a marked separation between mated and unmated cells has been achieved by this technique. Importantly, however, the ratio of $^{35}S/^3H$ changes very little across the gradient. This result demonstrates that there is no appreciable difference in the extent of mitochondrial protein synthesis between unmated ρ^+ cells and zygotes. If anything, the extent of mitochondrial protein synthesis in zygotes appears to be slightly *less* than in unmated ρ^+ cells. Thus it is unlikely that the observed extent of rescue of var1^f after 5 hr of mating is the result of a marked inability of the ρ^+ haploid cells in the mating mixture to incorporate label into mitochondrial translation products. We favor the view that the extent of rescue of var1^f in zygotes reflects amplification of the *var1* gene in the petite.

Gene Rescue with Other Petites. To verify that the appearance of the var1^f band in zygotes is, in fact, the result of the expression of this allele on mtDNA from the petite strain, we have repeated the basic zygotic gene rescue experiment with another independently isolated petite (O^{R}154) carrying the *var1^f* allele and with a variety of other petites as controls. One petite lacks any detectable mtDNA (ρ^0); another, by genetic criteria, does not carry the *var1* allele and contains a segment of the mitochondrial genome encompassing a marker (par^r) completely unlinked to and quite distant from the *var1* locus; and two petites, also by genetic tests, do not contain the *var1* locus but, nevertheless, retain the *oli1^r* marker. Fig. 4 shows the results obtained with these strains. In all cases, the mating time

was 5 hr and the mating efficiency with the ρ^+ tester was approximately the same for all of the strains. Data for the ρ^+ tester alone and a repeat of the experiment described above with petite O^R120 are presented for comparison. The data clearly show that the $var1^f$ band appears in the mating population only in those petites that we deduced from independent genetic tests to retain the *var1* segment of the mitochondrial genome (O^R120 and O^R154). Thus we may conclude that the appearance of $var1^f$ is not the result of some nonspecific conversion of $var1^s$ to $var1^f$ in zygotes.

DISCUSSION

The procedure of zygotic gene rescue we have introduced here takes advantage of our ability to discriminate the gene products of allelic forms of the *var1* gene on mtDNA. As determined by recombinational and deletion mapping procedures,* we have available petite strains carrying the structural gene for the fast migrating form of the *var1* gene product ($var1^f$). Using these strains we have been able to show that the petite allele can be expressed as a protein product in zygotes. What is of particular interest is the observation that the $var1^f$ band appears with a delay of 1 hr after the time of maximum zygote formation at roughly equal intensity with $var1^s$, the gene product of the allelic form of the *var1* gene in the ρ^+ haploid tester strain. This delay, reproducibly seen for these particular crosses, can readily be explained by, and is consistent with, what we know about cytoplasmic mixing in zygotes. We have noticed in crosses with the ρ^+ tester 55R5-3C/221 and petites derived from strain 41-6/19, that more than 80% of the first buds issued from the zygote are end buds (R. L. Strausberg, unpublished). The significance of this observation is that by pedigree analysis it has been demonstrated that first end buds retain largely *parental* mitochondrial genotypes (27), implying a relatively slow rate of cytoplasmic mixing. Consequently, the delay in appearance of $var1^f$ subsequent to maximum zygote formation could be due to the time required for cytoplasmic mixing and subsequent mitochondrial fusion.

Our ability to observe directly the rescue of a structural gene on petite mtDNA in zygotes of a cross between petite and wild type, as well as the extent of rescue obtained, allows us to make some tentative conclusions about the organization of structural genes on petite and wild-type mtDNA and suggests new possibilities for future directions:

(*i*) In terms of mitochondrial translation products, our experiments demonstrate that it is possible to express sequences on petite mtDNA *in vivo*. The simplest interpretation of this finding is that the $var1^f$ allele present on mtDNA of the petites O^R120 and O^R154 is expressed by the mitochondrial transcription and translation system of the ρ^+ tester strain.†

(*ii*) It is unlikely that expression of the *var1* allele on petite mtDNA in zygotes requires prior recombination of that allele into ρ^+ mtDNA. If we make the assumption that the $var1^s$ allele present on all ρ^+ mtDNA molecules in zygotes was replaced through recombination with the $var1^f$ allele from the petites, generating ρ^+ $var1^f$ mtDNAs, and further, that none of the $var1^f$ sequences on ρ^- mtDNA was expressed, then at most 20% of the *var1* product in mitochondria isolated from the total mating mixture should be $var1^f$. The fact that after 5 hr of mating both the $var1^f$ and $var1^s$ bands appear at about the same intensity suggests that recombination into wild-type mtDNA is not an obligatory pathway for expression of the $var1^f$ allele present on petite mtDNA.

A corollary of this argument is that the $var1^f$ allele probably exists in a gene amplified configuration in the petite. It is now apparent that the conversion of cells from ρ^+ to ρ^- involves retention and subsequent amplification of wild-type mtDNA sequences (18–20). For example, some petite strains have been shown to have reiterated mitochondrial rRNA and tRNA genes (4–9), and it is reasonable to expect that retained structural gene sequences would be reiterated as well. Another consideration is that the extent of rescue of the $var1^f$ product is the result of a more efficient transcription of the *var1* gene from petite mtDNA, possibly because of a stronger promoter. However, we have shown previously that the amount of var1 product relative to the other mitochondrial translation products is roughly the same in strains 41-6/19 and 55R5-3C/221 (25). Unless, for some reason, the efficiency of transcription of the $var1^f$ allele in petite mtDNA is different from that of the wild-type parent, the simplest interpretation of our results consistent with what is known about mtDNA in petites is that duplication of the $var1^f$ gene has occurred. In this case, it should then be possible to estimate the extent of amplification of the *var1* locus if we knew the fraction of ρ^+ cells that have mated and the amount of product associated with the petite mtDNA relative to the amount of product from the ρ^+ tester. Final proof of the extent of amplification would, of course, require more direct analysis of petite mtDNA.

(*iii*) With the zygotic gene rescue protocol we have described, a number of interesting possibilities are available to explore regulatory aspects of mtDNA in yeast which up to now have not been possible with the methodology currently used. For example, interactions between mtDNA molecules, as might occur with *trans* acting regulatory elements, are best explored in a heterozygous (or in the case of mitochondrial genomes, a heteroplasmic) configuration. Generally, mitochondrial genotypes are deduced from analyses on clones representing many cell generations removed from the initial mating event (22). Because mtDNA molecules appear to segregate rapidly in diploids once issued from zygotes, resulting in clones of essentially pure mitochondrial genotypes (1, 23, 24), analysis of possible interactions between different allelic forms of mitochondrial genes is not possible. On the other hand, the feasibility of analyzing events associated with the expression of mtDNA in zygotes, as we have shown here, where transient heteroplasms do exist, offers excellent possibilities to now probe important aspects of the organization and regulation of expression of genes on mtDNA. Such possibilities include the mapping of additional genes on mtDNA, *in vivo* complementation studies, and the analysis of positive and negative regulatory elements like activators and repressors.

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† We do not rule out the possibility that the unmated petite cells are capable of accurately transcribing sequences on their mtDNA and that subsequent to zygote formation the transcripts are translated by mitochondrial ribosomes from the wild-type tester.

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