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Biogenesis of mitochondria: a mutation in the 5'-untranslated region of yeast mitochondrial *oli1* mRNA leading to impairment in translation of subunit 9 of the mitochondrial ATPase complex

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ABSTRACT

A temperature-conditional *mit*$^-$ mutant of *Saccharomyces cerevisiae* has been characterized; the mutant strain h45 cannot grow at 36°C on non-fermentable substrates yet appears to be normal at 28°C. The mutation in strain h45 maps genetically to the *oli1* region of the mitochondrial DNA (mtDNA) genome, and prevents the synthesis at 36°C of the *oli1* gene product, subunit 9 of the mitochondrial ATPase complex. Since the level of *oli1* mRNA in mutant h45 is close to normal at 36°C, it is concluded that there is a specific block in translation of this mRNA at the non-permissive temperature. DNA sequence analysis of mtDNA from strain h45 reveals an additional T residue inserted 88 bp upstream of the *oli1* coding region, in the A,T-rich sequence that is transcribed into the 5'-untranslated region of the *oli1* mRNA. Sequence data on two revertants show that one returns to wild-type parental (J69-1B) mtDNA sequence, whilst the other contains an inserted A residue adjacent to the T inserted in the original h45 mutant. The results are discussed in terms of the stability of folds in RNA upstream of putative ribosome-binding sites in mitochondrial mRNA, and the potential action of nuclear-coded proteins that might be activators of the translation of specific mitochondrial mRNAs in yeast mitochondria.

INTRODUCTION

The *oli1* gene in mitochondrial DNA (mtDNA) of *Saccharomyces cerevisiae* codes for the proteolipid subunit 9 component of the proton-translocating mitochondrial ATPase (mtATPase) complex (1-3). We have determined (4) the sequence of this gene and its flanking sequence in the respiratory competent (*rho*$^+$) strain J69-1B. These particular flanking sequences encompass the region coding for the putative mature mRNA transcript of this region, that is about 0.9 kb in length (5,6).

Recent studies in our laboratory (7) have concerned the molecular definition of mutants with altered subunit 9 polypeptides. Amongst the *oli1* mutants analyzed through nucleotide sequence analysis were oligomycin resistant strains (8-10), and *mit*$^-$ mutants (10,11) unable to derive energy from respiratory activity. A key step in the initial genetic characterization of *mit*$^-$ mutants carrying mutations in the *oli1* gene is a

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marker rescue test involving crosses with the *rho*$^-$ petite 23-3. Detailed DNA sequence analysis of mtDNA from strain 23-3 (4) showed this petite to carry a continuous 1.35 kb segment of the *rho*$^+$ mitochondrial genome of J69-1B encompassing the *oli1* gene. Thus, *mit*$^-$ mutants bearing lesions in the *oli1* gene, when mated to petite 23-3, give rise to *rho*$^+$ diploids and are so defined as being rescued by petite 23-3.

Amongst the collection of mitochondrial mutants derived from strain J69-1B we have identified a temperature conditional *mit*$^-$ mutant h45, whose defect is rescued by petite 23-3. Strain h45 is unable to grow on non-fermentable substrates at 36°C, but shows normal oxidative growth at 28°C. A preliminary analysis of the DNA sequence of the *oli1* gene coding region as such (11) did not reveal any mutations . This observation suggests that the expression of the *oli1* gene, rather than an abberation in subunit 9 itself, is responsible for the defective growth phenotype. The molecular characterization of strain h45 reported in this paper demonstrates that this defective growth results from the failure to translate the subunit 9 polypeptide at the non-permissive temperature. DNA sequence analysis of the primary mutant h45 and of two revertants shows the critical mutation to be a base-insertion in the DNA corresponding to the 5'-untranslated region of the *oli1* mRNA.

MATERIALS AND METHODS

Yeast strains and growth conditions

The temperature conditional mutant h45 was derived from *Saccharomyces cerevisiae* strain J69-1B *MAT*α *ade1 his5* [*rho*$^+$], following mutagenesis with manganese chloride (12) and enrichment with nystatin at 36°C (13). The spontaneous *rho*$^-$ petite strain 23-3 was derived from strain 70M *MATa ade1 lys2 trp1* [*rho*$^+$] (4,14). Genetic analysis and growth of strains were carried out as described (15).

RNA analysis

RNA was extracted from mitochondria as described (16). The phenol-extracted RNA was then subjected to ethanol precipitation. The RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde (8%), then stained with ethidium bromide or transferred to nitrocellulose sheets, essentially as described (17). RNA standards were prepared by *in vitro* transcription of fragments of defined length from the yeast phosphoglycerokinase gene; these fragments were radiolabelled by carrying

out RNA synthesis in the presence of [α-^{32}P]UTP using SP6 RNA polymerase (18).

Northern blot hybridization was carried out essentially as described (16) using a nick-translated ^{32}P-labelled DNA probe from mtDNA of petite 23-3, incubated at 42°C for 16-20 h in the presence of 40% formamide, 10 x Denhardt's solution, 2 x SSC, 50 mM phosphate pH 6.5, 0.1% SDS, 10% dextran sulphate and denatured calf thymus DNA (400 μg/ml). After washing, the nitrocellulose sheets were subjected to autoradiography on X-ray film using intensifying screens.

Analysis of mitochondrial translation products

Yeast cells were grown at 28°C overnight and the proteins labelled *in vivo* (14) for 60 min at 28°C or 36°C in a low-sulphate medium containing [^{35}S]-sulphate, in the presence of cycloheximide which specifically inhibits cytosolic protein synthesis. After labelling, the cells were washed and resuspended in a buffer containing 0.3 M mannitol, 0.26 M sorbitol, 0.6 mM EDTA, 12 mM Tris-HCl pH 7.2. Mitochondria were isolated from mechanically ruptured cells (19). The products of mitochondrial protein synthesis were analysed by SDS-polyacrylamide gel electrophoresis (14); the gel was dried and bands were visualized by direct autoradiography on X-ray film.

M13 cloning and DNA sequencing

Methods and strategies for sequencing the *oli1* gene and flanking regions have been described in detail (4). The general approach involves preparation of appropriate single-stranded mtDNA templates cloned in M13 vectors. Recombinants containing the 1.1 kb and 0.9 kb *Hpa*II fragments of *Saccharomyces cerevisiae* mtDNA which contain the sequences directly flanking the *oli1* gene were selected using 23-3 mtDNA as probe. The sequence of the relevant portions of these fragments was determined by the dideoxy chain termination method using an extended set of specially constructed oligonucleotide primers (4).

RESULTS

Preliminary genetic characterization of mutant h45

Amongst the products of $MnCl_2$-mutagenesis of strain J69-1B was found a mutant strain that grew normally at 28°C but which showed little or no growth on non-fermentable substrates at 36°C. The lesion in this heat-sensitive mutant, denoted h45, was found to exhibit characteristics of extranuclear inheritance (mitotic segregation and elimination by ethidium

bromide; data not shown). The mutation was localized to the *oli1* region of mtDNA based on marker rescue crosses to a panel of petite mutants, each of which carries a different segment of the wild-type mtDNA genome (20).

When the mutant h45 was mated with the respiratory deficient petite 23-3 the progeny included diploids that were respiratory competent at both 28°C and 36°C, indicating that the temperature-sensitivity mutation of strain h45 lies in the region of mtDNA defined by the petite 23-3. Since this petite contains the entire *oli1* gene (4), our initial suspicion was that h45 carries a lesion within the *oli1* gene itself. Therefore, a routine DNA sequence analysis of the *oli1* gene (11) in strain h45 was carried out. The *oli1* gene sequence of strain h45 was found to be identical to the J69-1B (wild-type) sequence. This finding necessitated our extending the DNA sequence analysis to cover all sequences flanking the *oli1* coding region that were covered by petite 23-3 mtDNA. Before reporting on these sequence data it is useful to consider the nature of the manifestation of the defect in strain h45 at the levels of transcription and translation of the *oli1* gene.

The effect of growth at 36°C on the synthesis of subunit 9

Cells of strain h45 were grown overnight at 28°C and their mitochondrial translation products (cycloheximide-insensitive) were labelled with [^{35}S]-sulphate either at the permissive (28°C) or restrictive (36°C) temperature. These mitochondrial translation products were then analyzed by SDS-polyacrylamide gel electrophoresis (Figure 1). Analysis of the mitochondrial translation products of the mutant reveals a normal labelling of all mitochondrial translation products at 28°C (lane 3) relative to wild-type (lanes 1 and 2). However, at 36°C (lane 4) strain h45 is capable of synthesizing only very small amounts of ^{35}S-labelled subunit 9. Moreover, reduced labelling of bands corresponding to subunits 8 and 6 of the mtATPase complex occurred in the mutant at 36°C, but the other products of mitochondrial protein synthesis were labelled to near normal extents. These data indicate a selective loss of some, but not all products of mitochondrial synthesis brought about by the mutation in strain h45. The defect in subunit 9 expression is the primary effect of the h45 lesion. The genes for subunit 6 and 8 (*oli2* and *aap1*, respectively) are quite distant from the *oli1* gene on mtDNA (21). The pleiotropic effect on the synthesis of other subunits of the F_0-sector of the mtATPase complex, particularly subunit 6, is a characteristic feature of *mit*$^-$ mutants in which subunit 9 cannot be synthesized (22). The failure of mutant h45 to grow on non-

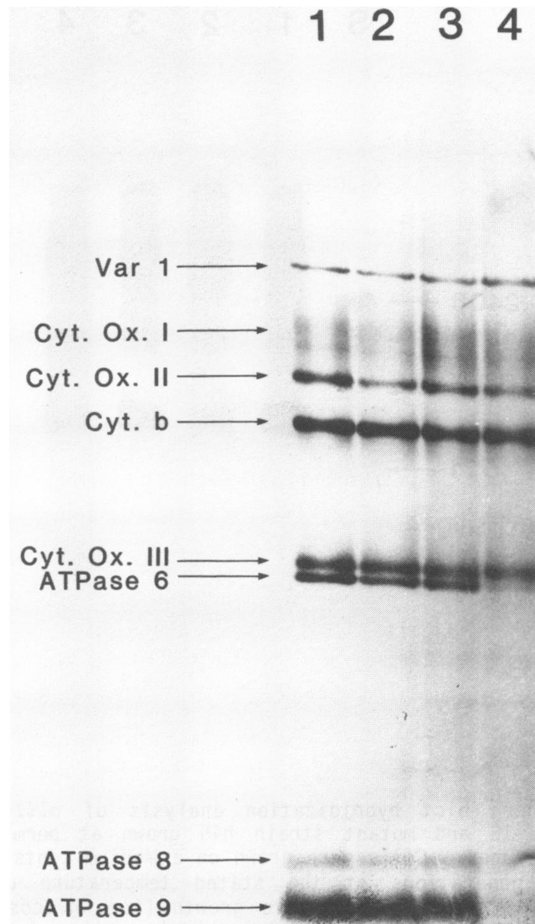

Figure 1. Mitochondrial translation products in parent strain J69-1B and mutant strain h45 labelled at 28°C and 36°C. Mitochondrial translation products resolved by SDS-polyacrylamide (12.5%) gel electrophoresis, were obtained from cells incubated with [^{35}S]-sulphate in the presence of cycloheximide, as follows: lane 1, J69-1B at 28°C; lane 2, J69-1B at 36°C; lane 3, h45 at 28°C; lane 4, h45 at 36°C. The various mitochondrial products are identified at the left of the Figure.

fermentable substrates at 36°C can thus be correlated with the inability of this mutant to synthesize subunit 9 (and other mitochondrially encoded mtATPase subunits) efficiently at this restrictive temperature; on the other hand, a normal subunit 9 is made at 28°C.

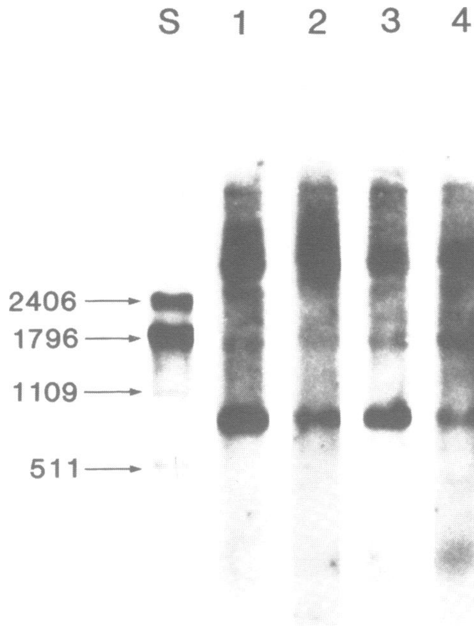

Figure 2. Northern blot hybridization analysis of *oli1* transcripts in parent strain J69-1B and mutant strain h45 grown at permissive and non-permissive temperatures. Cells were grown on a medium containing 1% glucose through about 6 generations at the stated temperature until the early stationary phase following fermentative growth (i.e. glucose depleted from media). RNA prepared from isolated mitochondria was analysed by agarose-formaldehyde gel electrophoresis, blotted to nitrocellulose and hybridized to a probe of ^{32}P-labelled mtDNA from petite 23-3. Lane 1, J69-1B at 28°C; lane 2, J69-1B at 36°C; lane 3, h45 at 28°C; lane 4, h45 at 36°C; lane S, RNA size standards (see Materials and Methods): sizes indicated at left in nucleotides.

Transcription of *oli1* gene in strain h45

To determine whether the failure of strain h45 to synthesize subunit 9 at 36°C results from a defect in transcription or mRNA processing, mitochondrial RNA extracted from the mutant cells cultured at least 16 hours at the permissive (28°C) or non-permissive temperature (36°C) was analyzed. The Northern blot analysis of mitochondrial RNA samples probed with labelled mtDNA from petite 23-3 (Figure 2) shows that the mutant at both temperatures (lanes 3 and and 4) contains the major transcripts of the

oli1 region. These transcripts are not significantly different in size or relative intensity from those of the wild-type parent J69-1B (lanes 1 and 2).

From the DNA sequence of the *oli1* region in J69-1B (4) it is predicted that the major mature *oli1* mRNA transcript is 931 nucleotides in length, measuring from the more active of the two possible promoters (5) upstream of the *oli1* coding region to the downstream consensus dodecamer sequence at which precursor cleavage occurs (23). The apparent size of this transcript seen in all mitochondrial RNA samples in Figure 2 is 860 nucleotides, with reference to the size standards used (lane S). The observation that this transcript is present in abundant quantities in h45 cells cultured at 36°C (lane 4) provides evidence that the mutant phenotype of strain h45 results from a temperature-sensitive block in the translation of the *oli1* mRNA and is not a result of the defective transcription of the *oli1* gene or processing of the mRNA transcripts.

Definition of the mutation in strain h45 by nucleotide sequencing

The full nucleotide sequence of the regions of mtDNA flanking the *oli1* gene in strain h45, defined by the segment of the mtDNA genome retained in petite 23-3, was determined using the approach previously adopted to determine the sequence of the corresponding region of mtDNA in the parent strain J69-1B (4). The sequences surrounding the *oli1* gene in strains J69-1B and h45 were identical, apart from an extra T residue inserted adjacent to position -87 in the mutant h45 sequence (Figure 3, compare panels 1 and 2). It should be mentioned that a stretch of 6 nucleotides in the G,C-rich cluster region containing three clustered *Hpa*II sites (at positions -349, -362 and -368) (see ref. 4) of the h45 sequence was unavailable for comparison. The missing nucleotides lie at positions -367 and -362 inclusive, and were not cloned into M13mp10 because *Hpa*II-digested fragments were used for cloning. Fortuitously, a partially *Hpa*II-digested 1.1 kb fragment of h45 mtDNA was cloned into M13 yielding the additional sequence between nucleotides -361 and -349. Studies on two revertants of mutant h45, considered in detail in the following section, further support the view that the T insertion in the mutant sequence at position -88 is the critical change in h45 mtDNA.

Isolation and sequence analysis of revertants

Two spontaneous revertants were isolated from the temperature-conditional mutant strain h45. Revertant h45.R.S-1 grew normally on YEPE-agar (ethanol carbon source) at 36°C, while h45.R.S-2 showed a slightly

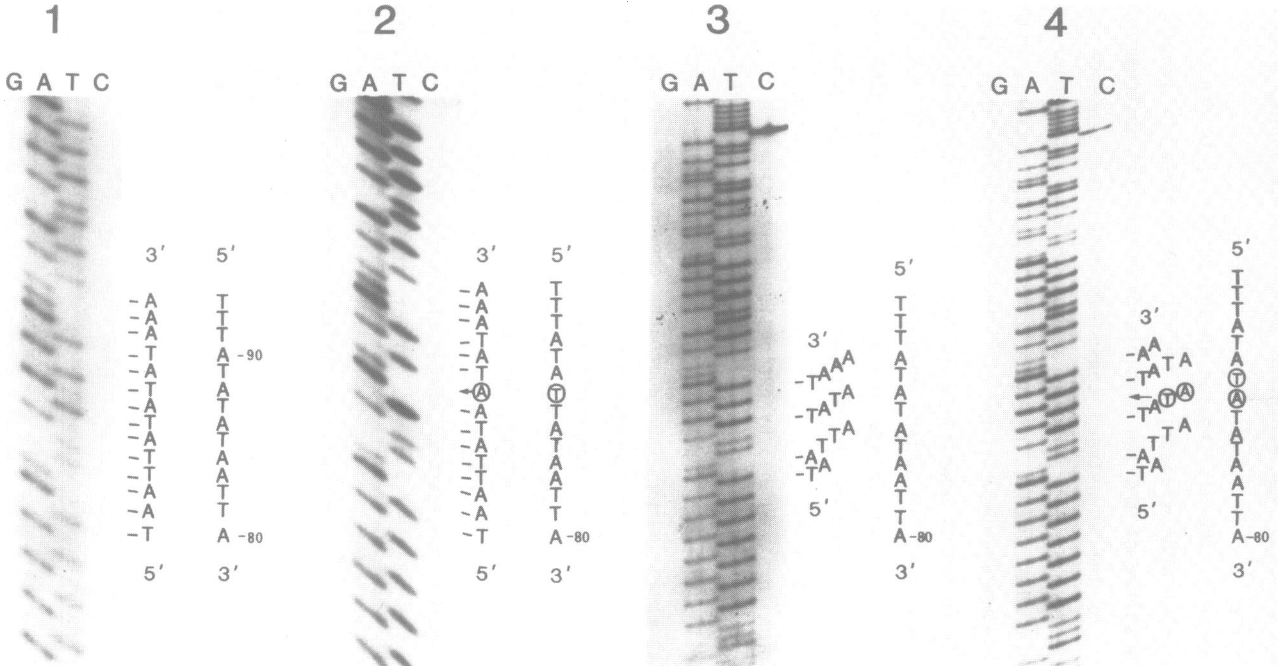

Figure 3. DNA sequencing gels showing the mutations in temperature-sensitive mutant h45 and its revertants. DNA sequence analysis (dideoxy procedure) was carried out in the 5'-untranslated region upstream of the *oli1* gene. The portions of sequence shown in the vicinity of nucleotides -80 to -93 are numbered according to Figure 3 of ref. 4 in which nucleotide +1 is the A of the ATG initiation codon of the *oli1* coding sequence. Panel 1, J69-1B; panel 2, h45; panel 3, h45.R.S-1; panel 4, h45.R.S-2. The sequences actually read, indicated adjacent to each autoradiogram, are in the non-mRNA-like sense. Complementary mRNA-like sequences are marked with nucleotide numbers. Nucleotide differences (referred to the parent J69-1B) of mutant h45 and its revertants are circled.

impaired growth rate but was clearly able to form colonies at 36°C on YEPE-agar. The nucleotide sequence in these two revertants of both the *oli1* coding region and the A,T-rich 5'-flanking region from nucleotides -1 to -348 (i.e. as far as the 5' end of the 1.1 kb *Hpa*II fragment of the mtDNA of each revertant cloned into M13 mp10) was determined. Revertant h45.R.S-1 had restored the wild-type sequence in this region, as shown in Figure 3 (panel 3). This result confirms that the T (at -88) observed in the h45 strain is in fact the mutation responsible for the heat-sensitive phenotype.

The other revertant, h45.R.S-2 (Figure 3, panel 4), still retains the inserted T residue at position -88 (characteristic of the mutant h45), and in addition, now has a further A residue inserted immediately adjacent. The substantial restoration of growth at 36°C in strain h45.R.S-2 suggests that some critical aspect of the structure of the *oli1* mRNA is modulated by this additional inserted base, now permitting the synthesis of subunit 9.

DISCUSSION

Evidence presented here shows that a mutation in the 5'-untranslated region of the *oli1* mRNA in strain h45 markedly alters the ability of this strain to produce subunit 9 of the mtATPase complex at elevated temperatures. At the restrictive temperature of 36°C a normal level of *oli1* mRNA is found in mitochondria of strain h45, yet very little subunit 9 is synthesized. It is likely that some aspect of the 5'-untranslated region of the mRNA important for efficient translation is destabilized when a single base is inserted in the A,T-rich region 88 nucleotides upstream of the *oli1* coding region. Presumably, the normal RNA structure can be partially maintained at 36°C in revertant h45.R.S-2 which carries an additional inserted A residue adjacent to the original base insertion. Little is known about the molecular interactions that must occur between a yeast mitochondrial mRNA and the organelle's translational apparatus to ensure the accurate and efficient initiation of translation.

It has been suggested a short sequence exists in each mitochondrial mRNA complementary to the 3'-end of the 15S mitochondrial rRNA (21,24). The sequence might be a ribosome-binding site analogous to the Shine-Dalgarno sequence (25) that plays a key role in the initiation of translation in bacteria (26). In the case of the *oli1* mRNA of strain J69-1B (from which mutant h45 was derived), this putative ribosome-binding site is a 10-base sequence located 26 nucleotides before the ATG start codon (4). Yet the mutation in strain h45 occurs some 60 bases upstream of this putative

ribosome-binding site. Recent studies on sequence alterations in the 5'-untranslated region of bacterial mRNA molecules (27,28) have shown that the efficiency of translation can be considerably modulated by changes outside the Shine-Dalgarno sequence itself. These changes apparently influence the secondary structure of RNA in the vicinity of this defined ribosome-binding site. Nevertheless, it is clear that the presentation of the Shine-Dalgarno sequence and the ATG start codon in single-stranded regions of mRNA does not in itself guarantee efficient initiation of translation in *E. coli* (27,28) suggesting that further subtle features of RNA structure and interactions remain to be elucidated.

In order to assess the possibility that gross secondary structure of the *oli1* mRNA plays a role in determining the efficiency of translation of subunit 9, we have undertaken a computer-assisted analysis (29) of the *oli1* mRNA, particularly the 5'-untranslated region encompassing both the putative ribosome-binding site and the site of mutation in strain h45. The series of predictive analyses of secondary structure we carried out collectively covered the upstream region from nucleotide -349 as far as the 3'-end of the *oli1* coding sequence at position +231. We were unable (data not shown) to assign predominant structural features to the *oli1* mRNA that were characteristic of both the wild-type strain and the revertant h45.R.S-2, but were disturbed in the mutant h45. Bearing in mind that these secondary structure analyses are carried out on nucleotide sequences of an extraordinarily A,T-rich character, and that the mutations are manifested as a temperature-sensitivity (implying that subtle changes in stability of secondary structures may be involved), we are therefore unable to resolve the question as to whether secondary structure of RNA alone is responsible for the observed phenotype.

A further aspect to be considered in the case of yeast mitochondrial protein synthesis is the possible role of nuclear-coded proteins proposed to be required for translation of certain mitochondrial mRNA species (30). Thus, nuclear *pet* mutants have been identified which block translation of individual mitochondrially encoded polypeptides, such as cytochrome *c* oxidase subunit III (31,32) and subunit II (30) and cytochrome *b* apoprotein (33,34). Suppression of the mutant phenotype can occur via rearrangements of mtDNA, whereby the 5'-untranslated regions of unaffected genes are fused to the coding region of the affected protein and so restore its translation (summarized in ref. 30). The rearranged mtDNA molecules comprise supernumerary self-replicating petite mtDNA segments. Interestingly, the

5'-untranslated region of the *oli1* gene is often found to provide sequences in such rearrangements which allow the translation of a downstream mitochondrial coding region (30,34).

It is thus possible that the mutation in h45 affects a recognition- or binding-site in the *oli1* mRNA for such a nuclear-coded specific activator of translation of subunit 9. This hypothesis may be tested genetically either by the identification and characterization of nuclear suppressors of the h45 mutant phenotype or by the isolation of nuclear *pet* mutants in which subunit 9 translation is specifically blocked. The *pet* mutants could then be screened for suppressors in which other 5'-untranslated regions are substituted upstream of the *oli1* coding region. In a more general sense, further molecular characterization of the function of such putative nuclear-coded translation factors specific for mitochondrial mRNA species awaits the future establishment of an *in vitro* mitochondrial protein synthesis system that can be reconstituted from yeast mitochondrial ribosomes, protein cofactors and natural mitochondrial mRNA species.

Many nuclear genes have been identified which affect the expression of particular mitochondrial genes at the level of mRNA processing and stability (35). On the other hand, most of the relevant mitochondrial mutations affect splicing and maturation of mosaic genes such as those coding for cytochrome *b* apoprotein or cytochrome *c* oxidase subunit I. These mitochondrial mutations (36) are either *cis*-acting and directly affect the RNA substrates subjected to intron splicing (see ref. 37) or they are *trans*-acting and affect the expression of intron-encoded proteins (maturases) that act to facilitate the processing of specific introns (see ref. 35). To our knowledge only two other types of mutation in mtDNA affect individual mRNA species. First is the mutation in a temperature-sensitive strain h56 which affects the post-transcriptional maturation (endonucleolytic cleavage) of the mRNA coding for the var1 protein (38); this mutation appears to be similar to PZ200L (39). Second is the mutation in strain h45 described here which affects the translation of mtATPase subunit 9. Thus, strain h45 appears to represent a novel class of mutants.

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