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PET111, a *Saccharomyces cerevisiae* Nuclear Gene Required for Translation of the Mitochondrial mRNA Encoding Cytochrome *c* Oxidase Subunit II

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ABSTRACT

Mutations in the nuclear gene *PET111* are recessive and specifically block accumulation of cytochrome *c* oxidase subunit II (coxII), the product of a mitochondrial gene. However, the coxII mRNA is present in *pet111* mutants at a level approximately one-third that of wild type. The simplest explanation for this phenotype is that *PET111* is required for translation of the coxII mRNA. The reduced steady-state level of this mRNA is probably a secondary effect, caused by increased degradation of the untranslated transcript. Mitochondrial suppressors of *pet111*, carried on *rho*$^-$ mtDNAs, bypass the requirement for *PET111* in coxII translation. Three suppressors are fusions between the coxII structural gene and other mitochondrial genes, that encode chimeric proteins consisting of the N-terminal portions of other mitochondrially coded proteins fused to the coxII precursor protein. When present together with *rho*$^+$ mtDNA in a heteroplasmic state, these suppressors allow coxII synthesis in *pet111* mutants. Thus in wild type, the PET111 product, or something under its control, probably acts at a site coded in the proximal portion of the gene for coxII to promote translation of the mRNA. *PET111* was isolated by molecular cloning and genetically mapped to a position approximately midway between *rna1* and *SUP8* on chromosome XIII.

YEAST mitochondrial genes are expressed by an organellar genetic system whose protein components are virtually all coded by nuclear genes (DUJON 1981). Interestingly, a number of recessive nuclear mutations that affect mitochondrial gene expression do not generally inactivate the mitochondrial genetic system, but rather block expression of specific mitochondrial genes. These positive activators of specific mitochondrial gene expression have thus far all been found to act post-transcriptionally (FOX 1986). The products of two yeast nuclear genes, *PET494* and *PET54*, have been shown to activate translation of the mitochondrial mRNA encoding cytochrome *c* oxidase subunit III (coxIII) (MÜLLER *et al.* 1984; COSTANZO and FOX 1986; FOX 1986; COSTANZO, SEAVER and FOX 1986) while two other nuclear genes, *CBP6* (DIECKMANN and TZAGOLOFF 1985) and *CBS1* (formerly *MK2*: RÖDEL, KÖRTE and KAUDEWITZ 1985; RÖDEL and FOX 1987) are required for translation of the mRNA encoding apocytochrome *b*. Thus there appears to be a group of nuclear genes in yeast that control the translation of specific mitochondrial mRNAs.

In this study we have investigated the nuclear gene defined by *pet111* mutations, originally characterized as being deficient in cytochrome *c* oxidase [formerly termed *petE11*: EBNER, MASON and SCHATZ (1973)]. Subsequent work indicated that a *pet111-1* mutant specifically lacked cytochrome *c* oxidase subunit II (coxII) (CABRAL and SCHATZ 1978), the product of the intronless mitochondrial gene *oxi1* (CABRAL *et al.* 1978; FOX 1979b; CORUZZI and TZAGOLOFF 1979). The pathway of expression of *oxi1* to yield coxII is of particular interest since coxII is the only known yeast mitochondrial gene product to be translated as a precursor-protein that is proteolytically processed to mature form (PRATJE *et al.* 1983).

Our examination of the phenotype of *pet111* mutants and the nature of mitochondrial mutations that suppress *pet111*, strongly suggests that the wild-type *PET111* gene product is required specifically to activate translation of the mRNA encoding coxII. Thus the *PET111* gene product appears to belong to the group of nuclearly coded regulators of mitochondrial translation. To facilitate further studies on *PET111* we have isolated the gene on a 2.7 kilobase (kb) restriction fragment of yeast DNA and mapped its chromosomal location to a position midway between *rna1* and *SUP8* on chromosome *XIII*.

MATERIALS AND METHODS

Media, genetic methods and yeast strains: YPEG medium contained 1% (w/v) yeast extract, 2% (w/v) peptone, 3% (v/v) ethanol and 3% (v/v) glycerol. YPGal was the same as YPEG except that it contained 2% (w/v) galactose instead of ethanol and glycerol. Glucose-containing medium (YPD), minimal medium (SD) and genetic techniques were as described (SHERMAN, FINK and LAWRENCE 1974).

The wild-type D273-10B (ATCC 25657) and DA1rho^0

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TABLE 1

Strains used in this study

Strain	Genotype
D273-10B	*MATα*, [*rho*$^+$]
Da1rho^0	*MATα, ade2*, [*rho*^{0}]
PTE12	*MATα, pet111-1, leu2-112, leu2-3, his3-11, his3-15, can1*, [*rho*$^+$]
PTE12R	*MATα, leu2-112, leu2-3, his3-11, his3-15, can1*, [*rho*$^+$]
LH114AC	*MATα, ade2, ino1-13, ino4-8*, [*rho*$^+$]
ECS108	*MATα, pet111-2, ade2, ino1-13, ino4-8*, [*rho*$^+$]
PTE14A	*MAT***a**, *pet111-1, leu2, ura3-52*, [*rho*$^+$]
14ANrF	*MAT***a**, *leu2, ura3-52*, [*rho*$^+$]
14AMr1	*MAT***a**, *pet111-1, leu2, ura3-52*, [*rho*$^+$, *MSU11-1*]
14AMr2	*MAT***a**, *pet111-1, leu2, ura3-52*, [*rho*$^+$, *MSU111-2*]
14AMr3	*MAT***a**, *pet111-1, leu2, ura3-52*, [*rho*$^+$, *MSU111-3*]
14AMr4	*MAT***a**, *pet111-1, leu2, ura3-52*, [*rho*$^+$, *MSU111-4*]
14AMr5	*MAT***a**, *pet111-1, leu2, ura3-52*, [*rho*$^+$, *MSU111-5*]
14AMr6	*MAT***a**, *pet111-1, leu2, ura3-52*, [*rho*$^+$, *MSU111-6*]
PTE17	*MAT***a**, *pet111-1, rna1, gal1, his4, ura3*, [*rho*$^+$]
GRF88	*MATα, his4-38, can1*, [*rho*$^+$]
X2318-26C	*MATα, SUP8, his5-2, lys1-1, can1-100, trp5-48, ade2-1, met1-1, ura3-1*, [*rho*$^+$]

Mitochondrial genotypes are indicated in brackets. Strain origins are described in MATERIALS AND METHODS.

TABLE 2

Tetrad analysis of the cross PTE17 × X2318-26C

Interval	Ascus type (no.) PD	NPD	TT	Distance (cM)
pet111–rna1	37	0	27	21
pet111–SUP8[a]	24	1	19	28
rna1–SUP8[a]	8	0	36	41

[a] Scored as described in MATERIALS AND METHODS.

have been previously described (Table 1) (MÜLLER *et al.* 1984). PTE12 was a segregant from a cross between strain petE11-1 (CABRAL and SCHATZ 1978) obtained from G. SCHATZ and GRF18 (DONAHUE *et al.* 1983). PTE12R was a spontaneous (nuclear) revertant of PTE12 selected on YPEG medium. LH114AC was constructed by first converting strain MC6A (HENRY, DONAHUE and CULBERTSON 1975; obtained from M. SOLIOZ) to a *rho*^{0} (GOLDRING *et al.* 1970) and then crossing the *rho*^{0} to D273-10B. A segregant from this cross was then crossed to DA1 (MÜLLER *et al.* 1984) yielding LH114AC. Strain LH114AC allows efficient enrichment of nonrespiring mutants by inositol starvation (L. HEDIN and E. C. SEAVER, unpublished data). ECS108 was a UV induced mutant of LH114AC. PTE14A was constructed by first crossing PTE12 to TD28 (DONAHUE *et al.* 1983) and isolating a strain that was *MAT***a**, *pet111-1, leu2-3, leu2-112, ura3-52*. This strain was then converted to a *rho*^{0} and crossed to D273-10B yielding PTE14A as a segregant. 14ANrF, 14AMr1, 14AMr2, 14AMr3, 14AMr-4, 14AMr5 and 14AMr6 were respiring revertants of PTE14A isolated after mutagenesis with $MnCl_2$ (PUTRAMENT, BARANOWSKI and PRAZMO 1973; MÜLLER *et al.* 1984). PTE17 was a segregant from a cross between strain STX77-6C (Yeast Genetics Stock Center, Berkeley, CA) and PTE14A. GRF88 was obtained from G. FINK and X2318-26C from the Yeast Genetics Stock Center.

The markers *lys1-1, trp5-48* and *ade2-1* in strain X2318-26C are suppressible by *SUP8* and not located on chromosome *XIII*. In the cross between PTE17 and X2318-26C (Table 2), *SUP8* segregation was followed by replica-plating to medium selective for Lys$^+$, Trp$^+$ and Ade$^+$. The 2:2 segregation for growth on this medium allowed identification of the *SUP8* carrying spores. Tetrads not showing 2:2 segregation on this medium were not scored for *SUP8*.

Labeling of mitochondrial translation products, sodium dodecyl sulfate (SDS) gel electrophoresis and immunological methods: Yeast cells were labeled with ^{35}S-methionine in the presence of cycloheximide in medium containing galactose as described (DOUGLAS and BUTOW 1976) except for strains 14AMr1, 14AMr2 and 14AMr6, which were labeled in medium containing ethanol and glycerol. Mitochondria were isolated (NEEDLEMAN and TZAGOLOFF 1975) and subjected to electrophoresis as described (MÜLLER *et al.* 1984).

Unlabeled total yeast protein was isolated from cells grown on YPGal as described (YAFFE and SCHATZ 1984). Aliquots of 50 μg each [except for diluted samples (Figure 2)] were subjected to electrophoresis on SDS gels containing 15% polyacrylamide, blotted to nitrocellulose and prepared for immune decoration as described (TOWBIN, STAEHELIN and GORDON 1979). The filter was incubated with a monoclonal antibody, CC06, that was directed against coxII and obtained from T. MASON. Immune complexes were detected as described (MÜLLER *et al.* 1984).

Plasmids and transformation procedures: DNA manipulations and transformation of *E. coli* strain HB101 were performed as described (MANIATIS, FRITSCH and SAMBROOK 1982). The *oxi1* gene of strain D273-10B was isolated as a 2.5-kb *Hae*III fragment of mitochondrial DNA (mtDNA), virtually identical to the 2.4-kb *Hpa*II fragment previously described (FOX 1979a). Plasmid pMT36 was constructed by tailing this *Hae*III fragment with poly-dC and inserting it into the plasmid pBR322 (BOLIVAR *et al.* 1977) which had been cleaved at the *Pst*I site and tailed with poly-dG.

Yeast transformations were as described (HINNEN, HICKS and FINK 1978), except that zymolyase was used to make spheroplasts.

Isolation, electrophoresis and hybridization of DNA and RNA: Total yeast DNA was isolated as described (SHERMAN, FINK and LAWRENCE 1974). DNA fragments were subjected to electrophoresis in a 1% agarose gel and blotted to nitrocellulose (SOUTHERN 1975). The *oxi1* specific probe for the experiment of Figure 4 was pMT36 that had been radioactively labeled by nick-translation (RIGBY *et al.* 1977).

Total yeast RNA was isolated as described (SPRAGUE, JENSEN and HERSKOWITZ 1983). RNA was subjected to electrophoresis in a 1% agarose gel containing formaldehyde, blotted to nitrocellulose and hybridized as described (MÜLLER *et al.* 1984). The probe for the experiment of Figure 3 was a 105 base pair (bp) *Hin*fI fragment of *oxi1* (FOX 1979a) that was isolated from pMT36 and radioactively labeled by nick-translation.

DNA sequence analysis of *MSU111* mutations: Restriction fragments spanning the breakpoints of *MSU111* deletions were isolated as follows: mtDNA was isolated (FOX 1979a) from cells of strains 14AMr1, 14AMr2 and 14AMr6 that had been grown on YPEG medium. For *MSU111-1* a 690-bp fragment of 14AMr1 mtDNA, extending from the most proximal *Pvu*II (*Alu*I) site in *oxi1* (FOX 1979a, b; CORUZZI and TZAGOLOFF 1979) to an *Mbo*I site in upstream DNA, was first cloned in pBR322 that had been cleaved with *Bam*HI and *Pvu*II. A 680-bp fragment extending from the proximal *Rsa*I site at position +49 in *oxi1* (CORUZZI and TZAGOLOFF 1979) to an *Msp*I site in pBR322 was taken from this plasmid and inserted into the M13 phage vector

mp19 (NORRANDER, KEMPE and MESSING 1983) that had been cleaved with *Acc*I and *Sma*I. For *MSU111-2* a 560-bp fragment of 14AMr2 mtDNA, extending from the proximal *Pvu*II (*Alu*I) site in *oxi1* to an *Msp*I site in upstream DNA, was first cloned in pBR322 that had been cleaved with *Cla*I and *Pvu*II. A 180-bp *Alu*I fragment extending upstream from the proximal *Pvu*II (*Alu*I) site in *oxi1* to the *Alu*I site at position +120 of *oxi3* (BONITZ *et al.* 1980) was taken from the plasmid and inserted into mp19 that had been cleaved with *Sma*I. For *MSU111-6* a 580-bp fragment of 14AMr6 mtDNA extending from the proximal *Rsa*I site in *oxi1* to an *Msp*I site upstream was cloned directly into mp19 that had been cleaved with *Acc*I and *Sma*I. Fragments cloned in mp19 were sequenced by the method of SANGER, NICKLEN and COULSON (1977).

RESULTS

Phenotype of *pet111* mutants

***pet111* mutants specifically fail to accumulate cytochrome *c* oxidase subunit II (coxII):** The *pet111-1* mutation causes a deficiency in the enzymatic activity of cytochrome *c* oxidase, but not other respiratory enzyme complexes (EBNER, MENNUCCI and SCHATZ 1973). An analysis of mitochondrial translation products by *in vivo* labeling in the presence of cycloheximide indicated that a *pet111-1* mutant specifically lacked coxII and contained lower than normal levels of cytochrome oxidase subunit I (coxI) (CABRAL and SCHATZ 1978). We have reexamined coxII accumulation in strains carrying both the original *pet111-1* mutation, and a newly isolated allele, *pet111-2*, found in a collection of Pet⁻ mutants generated in this laboratory (E. C. SEAVER and T. D. FOX, unpublished data).

First, mitochondrial translation products, selectively radioactively labeled in cells incubated in the presence of cycloheximide, were isolated and separated by SDS gel electrophoresis (MATERIALS AND METHODS). Autoradiography of the dried gels (Figure 1) confirmed that the *pet111-1* mutant strain PTE12 (lane C) specifically lacked coxII and was relatively deficient in coxI. (The diffuse band immediately above the band labeled "coxII" in Figure 1, corresponds to coxI.) An otherwise isogenic spontaneous revertant, PTE12R (lane B), contained coxII, and was similar in phenotype to a (nonisogenic) wild-type strain, D273-10B (lane A). In a separate experiment, the pattern of mitochondrial translation products in the *pet111-2* mutant, ECS108 (Figure 1, lane E), was very similar to that of the isogenic wild-type strain LH114AC (lane D), except for the absence of coxII and a slight reduction of coxI. Thus, while coxII was not detected in either *pet111* strain, the apparent effect on coxI accumulation varied with the allele and/or strain background.

Next, the steady-state levels of coxII in exponentially growing cells were examined. We used the immunoblotting method of TOWBIN, STAEHELIN and

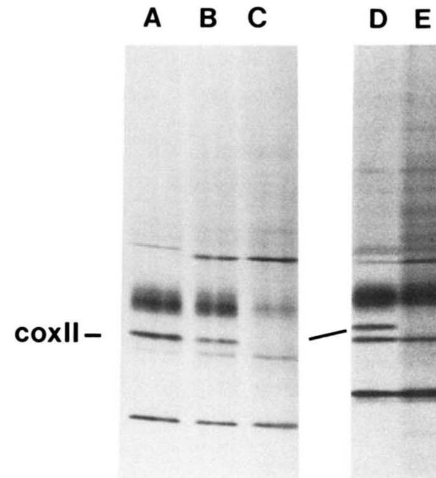

FIGURE 1.—Mitochondrial translation products of wild type, *pet111* mutants and a revertant strain. Mitochondrial proteins from yeast cells that had been radioactively labeled in the presence of cycloheximide were electrophoresed on SDS gels and autoradiographed in two separate experiments (MATERIALS AND METHODS). The band corresponding to coxII is indicated. The diffuse band immediately above it corresponds to coxI. The strains were: A, wild-type D273-10B; B, a *pet111-1* revertant, PTE12R; C, a *pet111-1* mutant, PTE12; D, wild-type LH114AC; E, a *pet111-2* mutant, ECS108. (D273-10B is not isogenic to PTE12R and PTE12. It carries a different allele of the mitochondrial gene *var1*: hence the altered mobility of the largest strongly labeled protein.)

GORDON (1979) with an anti-coxII mouse monoclonal antibody obtained from T. L. MASON (unpublished data) to detect coxII in total protein extracts. As a negative control we prepared total cell protein from the *oxi1* mutant M13-249, which contains a frameshift mutation in the mitochondrial structural gene for coxII, and therefore lacks the protein (CABRAL *et al.* 1978; FOX 1979a,b). Total proteins from cells of M13-249, the *pet111-1* mutant strain PTE14A, an otherwise isogenic revertant 14ANrF and the wild-type strain D273-10B were separated on an SDS gel, blotted to nitrocellulose and incubated with the anti-coxII antibody. Following incubation with radioactively labeled sheep anti-mouse immunoglobulin antibody, the filter was subjected to autoradiography (Figure 2). CoxII could not be detected in total protein from either the *pet111-1* mutant PTE14A (lane A) or the *oxi1* mutant M13-249 (lane B) while a strong coxII signal was observed in protein from the revertant 14ANrF (lane C) and the wild-type (lane D). Several cross-reacting proteins, not derived from coxII, were detected in all samples.

To establish the lower level of coxII detectable by this immunoblot assay, 50-fold and 100-fold dilutions of wild-type protein were also run on the gel of Figure 2 in lanes E and F, respectively. CoxII was easily detectable in a 100-fold diluted extract of wild type. Since no coxII could be detected in the *pet111-1* mutant, even when the autoradiograph was grossly overexposed (not shown), the nuclear mutation caused a reduction in the level of the mitochondrially coded

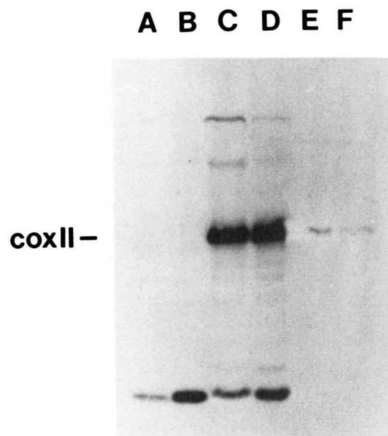

FIGURE 2.—Detection of steady-state levels of coxII by immunoblotting. Total yeast proteins were separated on an SDS gel, blotted to nitrocellulose and reacted with a monoclonal antibody directed against coxII (MATERIALS AND METHODS). The band corresponding to coxII is indicated. Proteins were isolated from the following strains: A, a *pet111-1* mutant, PTE141A; B, an *oxi1* mutant, M13-249; C, a *pet111-1* revertant, 14ANrF; D, wild-type D273-10B. Lane E contained a 50-fold diluted sample of D273-10B protein and lane F contained a 100-fold diluted sample of D273-10B protein. The identity of other cross-reacting proteins is unknown.

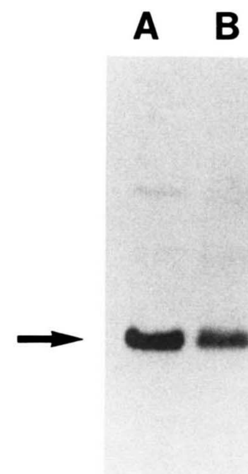

FIGURE 3.—CoxII mRNA in wild type and an isogenic *pet111* mutant. A sample of 25 μg RNA, isolated from the wild-type LH114AC (lane A) and the *pet111-2* mutant ECS108 (lane B), was electrophoresed in an agarose gel, blotted to nitrocellulose and hybridized with an *oxi1* specific probe (MATERIALS AND METHODS). The position of the 880-bp coxII mRNA is indicated by the arrow.

protein of well over 100-fold in logarithmically growing cells. Similar experiments on cells grown to stationary phase yielded the same result: absence of coxII in the *pet111-1* mutant strain. In addition, coxII could not be detected in immunoblots of isolated mitochondrial proteins (not shown).

***pet111-1* mutants contain the *oxi1* mRNA at reduced levels:** To determine whether the absence of coxII in *pet111* mutants could be due to a defect in transcription of the *oxi1* mitochondrial gene, total RNA was isolated from logarithmically grown cells of the wild-type LH114AC and the otherwise isogenic *pet111-2* mutant ECS108. Following electrophoresis, the RNA was blotted to nitrocellulose and hybridized with a radioactively labeled restriction fragment containing *oxi1* coding sequences (MATERIALS AND METHODS). Autoradiography (Figure 3) revealed that both wild type and mutant contained the 880-bp *oxi1* transcript (FOX and BOERNER 1980; CORUZZI *et al.* 1981), although the level in the mutant (lane B) was noticeably lower than in wild type (lane A). Densitometric scanning of several autoradiograms from the experiment of Figure 3 indicated that the *oxi1* mRNA was approximately 2.7-fold reduced relative to wild type, when normalized to the levels of 15 S mitochondrial rRNA, determined by a separate hybridization (not shown). Similar comparisons were also made of the levels of *oxi1* mRNA between the *pet111-1* mutants PTE12 and PTE14A, and their isogenic revertants PTE12R and 14ANrF, respectively. Both RNA gel-blot hybridization and "dot-blot" hybridization experiments confirmed that *oxi1* mRNA was present in logarithmically grown *pet111* mutant cells, and that its level was three- to tenfold lower than that in the revertants (POUTRE 1986). In cells grown to stationary phase, *pet111* caused an approximately tenfold greater reduction in the level of *oxi1* transcript than in logarithmic cells (POUTRE 1986).

The results of *in vivo* labeling in the presence of cycloheximide (Figure 1) indicated that different *pet111* mutants contained levels of coxI reduced to variable extents. However, in contrast to the effect of *pet111* on the level of the *oxi1* mRNA, the level of the *oxi3* mRNA, which encodes coxI, was not significantly affected by *pet111* (POUTRE 1986).

The fact that the coxII protein is undetectable in *pet111* mutants, while its mRNA is easily detectable at a level approximately one third that of wild type strongly suggests that *PET111* function is required to carry out some *post-transcriptional* step in *oxi1* expression. As discussed more fully below, the only simple hypothesis to explain the *pet111* phenotype is that the mutants are unable to translate the *oxi1* mRNA. The reduced steady-state level of the *oxi1* mRNA might be a secondary effect due to *in vivo* degradation of the untranslated transcript. The apparent reduction in the level of coxI by *pet111* is not understood, although as shown below, this too is probably a secondary effect of the mutation.

Suppression of *pet111* by rearrangements of *oxi1*

Isolation of mitochondrial suppressors of *pet111*: The biochemical analysis presented above suggested that the primary defect of *pet111* mutants was in expression of *oxi1*. To test this notion genetically, and to attempt to identify the mitochondrial site of action of the *PET111* product, we selected mitochondrial suppressors of the *pet111-1* mutation. This approach has been very valuable in the study of two other

nuclear yeast genes, *PET494* and *CBS1*, which are specifically required for translation of the mitochondrial mRNAs encoding cytochrome oxidase subunit III and apocytochrome *b*, respectively (MÜLLER *et al.* 1984; RÖDEL, KÖRTE and KAUDEWITZ 1985; FOX 1986; COSTANZO and FOX 1986). The mitochondrial mutations that suppress *pet494* and *cbs1* nuclear mutations are *rho*⁻ deletions of mtDNA with one breakpoint at or near the 5′-end of the affected mitochondrial structural genes. The deletions generate chimeric genes that encode novel transcripts and in some cases novel protein products. These chimeric genes are expressed despite the nuclear mutations when the *rho*⁻ chromosome is present heteroplasmically with wild-type *rho*⁺ mtDNA.

To obtain mitochondrial suppressors of *pet111*, 25 cultures of the *pet111-1* mutant, PTE14A, were mutagenized with manganese chloride (PUTRAMENT, BARANOWSKI and PRAZMO 1973) and plated on medium containing the nonfermentable carbon sources ethanol and glycerol (YPEG). Fourteen of these cultures gave rise to colonies with Pet⁺ phenotype. To distinguish the revertants arising as a result of mitochondrial mutations from those due to nuclear mutations, *rho*⁰ derivatives of each revertant, totally lacking mtDNA, were prepared by treatment with ethidium bromide (GOLDRING *et al.* 1970). Each revertant, and its *rho*⁰ derivative, was individually mated to the *pet111-1* strain PTE12 and the ability of the resulting diploids to grow on YPEG medium was scored. Revertants from eight of the 25 cultures retained the ability to confer the Pet⁺ phenotype on the diploids despite the loss of their mtDNA and were thus probably due to nuclear mutations. Revertants from six of the 25 cultures lost the ability to confer a Pet⁺ phenotype on the diploids when they lost their mtDNA and were thus due to mitochondrial mutations. One "mitochondrial revertant" from each of the six mutagenized cultures was analyzed further. These strains, called 14AMr1 through 14AMr6, carried mitochondrial suppressor mutations termed *MSU111-1* through *MSU111-6*.

The *MSU111* mutations are rearrangements of *oxi1* carried on *rho*⁻ mtDNAs: The genetic behavior of mitochondrially suppressed *pet111* strains was very similar to that of the previously studied "mitochondrial revertants" of *pet494* mutants (MÜLLER *et al.* 1984; COSTANZO and FOX 1986). Respiring cells rapidly gave rise to nonrespiring cells by mitotic segregation, and the nonrespiring mitotic segregants fell into two classes. Members of the first class contained wild-type mtDNA lacking the suppressor, as shown by their ability to yield respiring diploids when mated to DA1rho⁰, a *PET111* strain lacking mtDNA (*rho*⁰), but not when mated to the *pet111*, [*rho*⁺] strain PTE12. Members of the second class contained *rho*⁻ mitochondrial genomes that carried suppressor activity as shown by their inability to complement the *rho*⁰ tester, and their ability to yield mitotically unstable respiring diploids when mated to PTE12. Thus the ability to respire in the *pet111* nuclear background depended on the presence of both a *rho*⁻ mitochondrial genome carrying an *MSU111* suppressor, and *rho*⁺ mtDNA in the same cell.

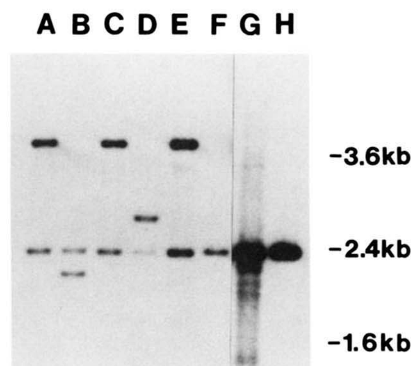

FIGURE 4.—Mitochondrially suppressed *pet111* strains contain both wild type and rearranged copies of the gene coding coxII. DNAs were digested with *Msp*I, subjected to electrophoresis, blotted and hybridized with the *oxi1* probe, pMT36 (MATERIALS AND METHODS). Yeast DNA was isolated from the following strains: A, 14AMr1; B, 14AMr2; C, 14AMr3; D, 14AMr4; E, 14AMr5; F, 14AMr6; G, wild-type D273-10B. MspI-digested DNA of pMT36 was run in lane H. The wild-type *oxi1* carrying fragment is 2.4 kb.

To determine whether the *MSU111* mutations involved rearrangement of the gene coding coxII, *oxi1*, total DNA was isolated from the revertant strains 14AMr1 through 14AMr6, cleaved with *Msp*I, subjected to agarose gel electrophoresis, blotted to nitrocellulose and hybridized with a radioactively labeled *oxi1*-specific probe (Figure 4). This analysis revealed that all of the "mitochondrial revertants" contained the wild-type *oxi1* gene, known to reside on a 2.4-kb *Msp*I (*Hpa*II) fragment (FOX 1979a,b) (Figure 4, lane G). In addition, 14AMr1 through 14AMr5 contained novel *oxi1*-carrying *Msp*I fragments (Figure 4, lanes A through E). The novel *oxi1* restriction fragments were carried on *pet111* suppressing *rho*⁻ mtDNAs, as shown directly by an examination of a *rho*⁻ mitotic segregant carrying *MSU111-5* (POUTRE 1986). 14AMr6 also contained a novel *oxi1* fragment, as shown below by DNA sequence analysis, that fortuitously co-migrated with the wild-type fragment in the experiment of Figure 4 (lane F). Thus, the *MSU111* mitochondrial suppressors of *pet111* appeared to correspond to rearranged copies of *oxi1*. Furthermore, the results of this analysis were consistent with the genetic data suggesting that the *MSU111* mutations were carried on *rho*⁻ mtDNA, present heteroplasmically with wild-type mtDNA in the revertant strains.

***MSU111* suppressors are chimeric genes with N-terminal coding sequences of other mitochondrial genes fused to *oxi1*:** Preliminary restriction-site map-

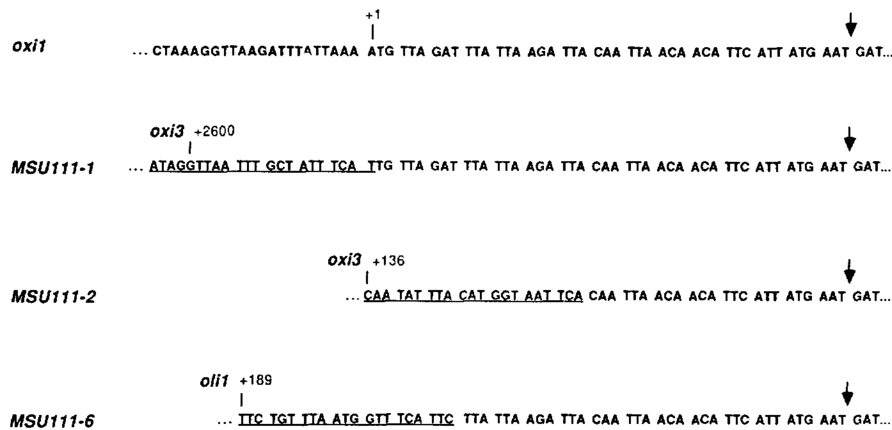

FIGURE 5.—DNA sequences of the breakpoints of *MSU111* deletions. DNA sequences were determined as described in MATERIALS AND METHODS. Protein coding regions are written as triplets, non-coding regions as continuous sequence. The first line shows the wild-type *oxi1* sequence encoding the N-terminal 15 amino acids of the coxII-precursor and the first residue of the mature protein. The arrows indicate the position of cleavage of the coxII-precursor protein (PRATJE *et al.* 1983). The underlined sequences indicate regions of *oxi3* and *oli1* that were fused to the coxII coding sequence by the deletions generating*MSU111-1*, *MSU111-2* and *MSU111-6*. The *oxi3* sequence shown for *MSU111-1* corresponds to the 3'-end of the first *oxi3* intron (see text). The indicated sequence positions were taken from CORUZZI and TZAGOLOFF (1979), BONITZ *et al.* (1980) and HENSGENS *et al.* 1979.

ping of the rearranged *oxi1* sequences indicated that in each case the breakpoint of the rearrangement was near the 5'-end of the coxII structural gene (POUTRE 1986). To examine precisely the nature of the *MSU111* suppressors, novel restriction fragments spanning the breakpoints of three *MSU111* mutations were isolated by molecular cloning and subjected to DNA sequence analysis as described in MATERIALS AND METHODS. The resulting sequences were then compared with published sequences of yeast mitochondrial genes.

The DNA sequence at the breakpoint of *MSU111-2* demonstrated that it was a simple chimeric gene consisting of the first 52 codons of the coxI structural gene, *oxi3*, fused to the eighth codon of *oxi1* (Figure 5). Similarly, *MSU111-6* was a chimeric gene with the first 70 codons of the ATPase subunit 9 structural gene, *oli1*, fused to the fourth codon of *oxi1* (Figure 5). *MSU111-1* appeared to be a more complex chimeric gene, consisting of the first exon and first intron of *oxi3* fused to the second two bases of the *oxi1* initiator ATG codon. [Restriction-site mapping suggested that *MSU111-3* and *MSU111-5* were similar or identical to *MSU111-1* (POUTRE 1986).] Thus in *MSU111-1*, *oxi1* was effectively substituted for the second exon of *oxi3*. Since the *oxi1* initiator codon was destroyed by the *MSU111-1* deletion, the coxII coding sequence was presumably co-translated with the 56 codons of the first exon of *oxi3* after splicing of the intron in the mRNA.

The size of transcripts copied from the *MSU111* suppressor genes could be predicted by assuming that their 5'-ends were determined by the upstream part of the chimeric gene and that they all had *oxi1* 3'-ends. The sizes of the novel *oxi1* transcripts present in RNA isolated from strains 14AMr6, 14AMr1 and 14AMr2 were examined by RNA-gel-blot hybridization experiments (POUTRE 1986). The predicted size for the *oli1-oxi1* fusion transcript from *MSU111-6* of 1600 bases was in good agreement with the observed size of approximately 1550 bases. In the case of *MSU111-1* the predicted size was either 1350 bases if the *oxi3* first intron sequences were spliced from the transcript, or 3860 if they were not. The observed size of the *MSU111-1* transcript was approximately 1350 bases, indicating that the intron was indeed spliced. In the case of *MSU111-2*, the major transcript was approximately 1100 bases, clearly different from the predicted 1350 bases. This discrepancy is probably accounted for by a second rearrangement in the *oxi3* 5'-leader coding region of *MSU111-2* (POUTRE 1986).

***MSU111* suppressors lead to accumulation of apparently wild-type coxII in *pet111* mutants:** CoxII is unique among known mitochondrially coded proteins of yeast in that it is synthesized as a precursor protein which is processed to the mature subunit. In wild type, the precursor is processed by removal of its 15 N-terminal residues (PRATJE *et al.* 1983). The DNA sequence analysis of *MSU111* chimeric genes indicated that their primary translation products should retain the proteolytic cleavage site between residues 15 and 16 (Figure 5). Thus, if the novel *MSU111* coded coxII "precursors" were processed at this site, wild-type coxII should accumulate in the mitochondrially suppressed *pet111* strains, instead of the longer variants of coxII predicted by the *MSU111* DNA sequences. Electrophoretic analysis of mitochondrial translation products of strains 14AMr1, 14AMr2 and 14AMr6 revealed that each produced coxII with wild-type mobility (Figure 6), strongly suggesting that the novel

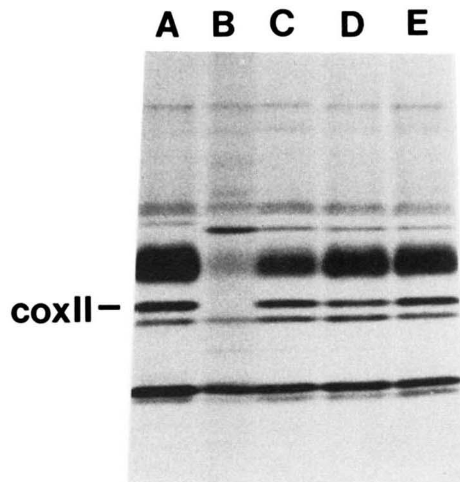

FIGURE 6.—Mitochondrial translation products of mitochondrially suppressed *pet111* strains. Mitochondrial translation products were analyzed as described in the legend to Figure 1. Strains were: A, wild-type D273-10B; B, *pet111* mutant PTE14A; C, D and E, the mitochondrially suppressed *pet111* strains 14AMr1, 14AMr2, and 14AMr6, respectively.

MSU111 coded coxII "precursors" were processed to yield wild-type coxII.

Taken together, the data presented here on the phenotype of *pet111* mutants and the nature of the *MSU111* suppressors argue strongly that the primary defect of *pet111* mutants is specifically in translation of the *oxi1* mRNA coding coxII. If, in a *pet111* mutant, coxII coding sequences are translated from chimeric *MSU111* mRNAs containing the translation initiation regions of other mitochondrial genes, coxII accumulates and the respiratory deficient growth phenotype is suppressed (as well as the apparent reduction in coxI levels).

Molecular cloning and genetic analysis of *PET111*

Isolation of *PET111*: To aid in the genetic characterization of *PET111* and the biochemical analysis of its product, the wild-type gene was isolated from a bank of yeast DNA that had been partially digested with *Bgl*II and inserted into the vector YEp13 (MÜLLER and FOX 1984; BROACH, STRATHERN and HICKS 1979). DNA from the bank was used to transform strain PTE12 to Leu⁺, after which Pet⁺ colonies were sought among the transformants. A *pet111*-complementing plasmid, YpA35, was isolated from a single Pet⁺ transformant. A restriction map of the 13.0-kb yeast DNA fragment in YpA35 (Figure 7) revealed that it contained five *Hin*dIII sites. To subclone the *PET111*-complementing sequence, YpA35 was digested with *Hin*dIII and the resulting fragments were ligated with *Hin*dIII digested YEp13. These recombinant plasmids were used to transform strain PTE12 and plasmid DNA was isolated from the resulting Pet⁺ transformants. One of these *PET111*-complementing plasmids, YpA2.7, contained only a 2.7-kb *Hin*dIII fragment (Figure 7). Genomic Southern analysis using

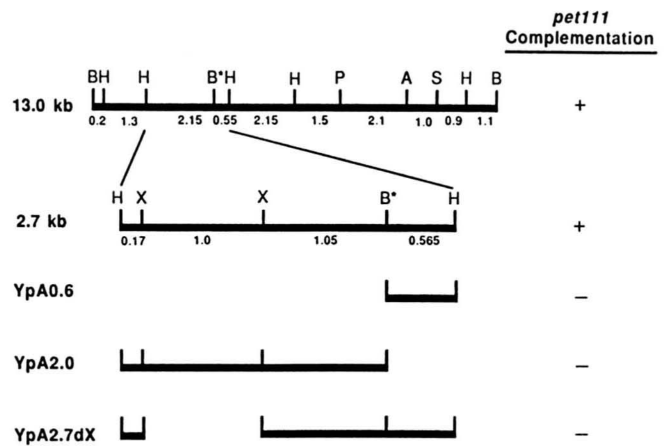

FIGURE 7.—Restriction maps of cloned DNA carrying *PET111* and deleted derivatives. The sites indicated are for the enzymes *Bam*HI (B), *Hin*dIII (H), *Pvu*II (P), *Ava*I (A), *Sal*I (S) and *Xba*I (X). Fragment sizes are indicated in kb. Sequences retained in the plasmids YpA0.6, YpA2.0 and YpA2.7dX, which carry deletions of the 2.7-kb fragment (see text), are indicated. The ability of each fragment to complement *pet111* in trans is indicated. The site labeled B* was used to disrupt *PET111* by insertion of *LEU2* (see text). Approximately 30 bp to the right of B* there is an *Eco*RI site, not indicated on the map.

this 2.7-kb *Hin*dIII fragment as a probe indicated that it was a unique sequence (POUTRE, 1986).

To confirm that the 2.7-kb *Hin*dIII fragment carried the *PET111* gene and not an unlinked suppressor, we examined the genetic linkage of this fragment to *PET111*. First, the 2.7-kb fragment was transferred to the integrating vector YIp5, which carries the selectable marker *URA3* (STRUHL *et al.* 1979). The resulting plasmid, YpB2.7, was used to transform strain PTE14A after cleavage at the unique *Bgl*II site in the 2.7-kb insert to promote integration by homologous recombination (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981). One of the Ura⁺, Pet⁺ transformants obtained was first crossed to the *pet111* mutant, PTE12. Pet⁺ phenotype segregated 2:2 in eight tetrads analyzed from this cross indicating that the *pet111* complementing fragment was integrated at a single site in the genome of the transformant. The same transformant was then crossed to a *PET111* strain, GRF88. Among 29 complete tetrads analyzed (116 spores) only two spores were Pet⁻ in phenotype, indicating that the site of integration of the *pet111*-complementing sequence was tightly linked to *PET111*.

Finally, as a direct demonstration that the *pet111-1* mutation resided on the 2.7-kb *Hin*dIII fragment, we cloned this fragment from DNA of the *pet111-1* strain PTE12 and demonstrated that it lacked the ability to complement the mutation. *Hin*dIII fragments of PTE12 nuclear DNA were size fractionated by electrophoresis. Fragments of approximately 2.7 kb were ligated with *Hin*dIII cleaved YEp13, transformed into *Escherichia coli*, and colonies containing the desired recombinant plasmids were identified by colony hy-

bridization using the wild-type 2.7-kb fragment as a probe. Four plasmids carrying the mutant fragment were obtained and each was used to transform strain PTE12. In each case, only Leu$^+$, Pet$^-$ transformants were obtained.

Localization of *PET111* on the cloned 2.7-kb fragment: As a first step in the detailed analysis of *PET111* we began to determine which regions of 2.7-kb *Hin*dIII fragment were necessary for function. The results of these experiments are summarized in Figure 7.

First, the wild-type fragment was cleaved with *Bgl*II and the resulting 2.15-kb and 0.55-kb fragments were inserted into YEp13, yielding plasmids YpA2.0 and YpA0.6, respectively. Both of these plasmids failed to complement *pet111-1* when transformed into strain PTE12, indicating that the *Bgl*II site lay in a functionally important region. The importance of this region was confirmed by inserting the *LEU2* gene, as a 3-kb *Bgl*II fragment of YEp13, into the *Bgl*II site of the *PET111* fragment, and using the disrupted linear fragment to transform a *leu2*, *PET111* strain to Leu$^+$ (Rothstein 1983). The resulting replacement of wild-type chromosomal DNA with the disrupted fragment produced a *pet111* mutation. Next, a 1-kb deletion in the 2.7-kb *Hin*dIII fragment was generated by *Xba*I cleavage and religation (Figure 7). This deleted fragment was inserted into YEp13 to generate plasmid YpA2.7dX, which failed to complement the *pet111-1* mutation. Thus *PET111* must at least extend from a point between the *Xba*I sites through the *Bgl*II site.

Interestingly, transformants of the *pet111-1* strain PTE12 carrying plasmid YpA2.0 gave rise to Pet$^+$ segregants (observed as papillae in patches of Pet$^-$ cells) at a rate far higher than the rate of reversion of *pet111-1*. We infer from this observation that YpA2.0 carries wild-type information corresponding to the *pet111-1* mutation and that a functional gene can be generated by mitotic recombination between the plasmid and the mutated chromosomal gene. Since transformants of PTE12 carrying either YpA0.6 or YpA2.7dX failed to give rise to Pet$^+$ segregants, it appears that the *pet111-1* mutation is located between the XbaI sites of the 2.7-kb *Hin*dIII fragment.

***PET111* is on chromosome *XIII* between *RNA1* and *SUP8*:** To determine the chromosomal location of *PET111*, the 2.7-kb *Hin*dIII fragment was hybridized to a filter, obtained from M. Kielland-Brandt, R. Gaber and G. Fink, carrying electrophoretically separated yeast chromosomes (Schwartz and Cantor 1984; Carle and Olsen 1984). The probe hybridized to a single band that appeared to correspond to chromosome XIII (Carle and Olsen 1985, Poutre 1986).

The segregation of *pet111* relative to known markers on chromosome *XIII* (Mortimer and Schild 1985) was studied by tetrad analysis. No linkage was detected to either *lys7* or *cdc5*. However, *pet111* was linked to both *rna1* and *SUP8*, and mapped to a point approximately equidistant between them (Table 2).

DISCUSSION

Mutations in the nuclear gene *PET111* are recessive and prevent accumulation of coxII, the product of the mitochondrial gene *oxi1*. Thus *PET111* is a positive activator of *oxi1* expression. However, *pet111* mutations do not appear to affect transcription of *oxi1* directly. Despite the fact that the level of coxII protein is reduced well over 100-fold relative to wild-type (Figure 2), the coxII mRNA (*oxi1* transcript) is present in *pet111* mutations at a level approximately one third that of wild-type (Figure 3). Clearly the mRNA level is not low enough to account for the absence of the protein.

The simplest explanation for our findings is that the *PET111* product is required for translation of the coxII mRNA. In the absence of translation, the stability of this mRNA would probably be reduced, accounting for the lower steady-state levels observed in *pet111* mutants. Destabilization of mRNAs in the absence of translation is a well known phenomenon in both *E. coli* and yeast (Morse and Yanofsky 1969; Schneider, Blundell and Kennell 1987; Losson and Lacroute 1979; Belasco *et al.* 1986). An alternative explanation for the mutant phenotype might be that the coxII protein is synthesized normally but that it is unstable in *pet111* mutants. However, this appears to be highly unlikely for two reasons. First, this hypothesis does not easily account for the lowered levels of coxII mRNA observed. Second, the mitochondrially suppressed *pet111* mutants described here accumulate coxII normally in the absence of functional *PET111* gene product (Figure 6). Thus, once synthesized, coxII is assembled into a stable functional complex, despite the absence of *PET111* function.

The mitochondrial suppressors of *pet111*, termed *MSU111*, bypass the requirement of coxII translation for *PET111* product. *MSU111* mutations correspond to deletions in mtDNA, carried on *rho*$^-$ chromosomes, that result in fusions between the coxII structural gene and other mitochondrial genes. When present together with *rho*$^+$ mtDNA in a heteroplasmic state, the *MSU111* suppressors allow coxII synthesis in *pet111* mutants. The *MSU111* gene fusions examined here by DNA sequence analysis encode chimeric proteins consisting of the N-terminal portions of either coxI or ATPase9 fused to coxII (Figure 5). However, despite the fact that the *MSU111* suppressors encode proteins 50 to 70 amino acids longer than wild-type coxII (which is 236 amino acids long), *MSU111* suppressed *pet111* mutants contain coxII that co-migrates in SDS gels with the wild-type protein. This apparent

paradox is probably resolved by the fact that coxII is synthesized as a precursor-protein. In wild type the coxII mRNA is translated to yield a precursor 15 amino acid residues longer at its N-terminus than the mature cytochrome oxidase subunit (PRATJE *et al.* 1983). Since the predicted novel coxII precursors encoded by the *MSU111* suppressors all contain the cleavage site between residues 15 and 16 (Figure 5) they are probably also processed to yield the wild-type mature subunit.

The nature of *MSU111* suppressors suggests that the *PET111* product, or something under its control, acts at a site coded in the proximal portion of the *oxi1* gene. By fusing the proximal portions of mitochondrial genes whose expression is independent of *PET111* to the coxII structural gene, the *MSU111* mutations allow translation of coxII and thus suppress the respiratory defect caused by *pet111*. Whether the site of action of the *PET111* function lies in the coxII mRNA or in the coxII precursor-protein itself cannot be determined from the *MSU111* suppressors described here, since they alter both. The nuclear genes *PET494* and *PET54* act in the 5′-untranslated leader of the mitochondrially coded coxIII mRNA to promote its translation (COSTANZO and FOX 1986; COSTANZO, SEAVER and FOX, 1986), and *CBS1* acts similarly to promote translation of apocytochrome *b* (RÖDEL, KÖRTE and KAUDEWITZ 1985; RÖDEL and FOX, 1987). One model for *PET111* function would be that it acts at a site in the 5′-untranslated leader of the coxII mRNA to activate translation of pre-coxII. However, another interesting possibility is suggested by drawing an analogy between the co-translational model of protein translocation across the endoplasmic reticulum (WALTER, GILMORE and BLOBEL 1984) and the pathway of coxII synthesis. One could imagine that the N-terminal amino acids of the nascent coxII-precursor are recognized by an SRP-like structure that represses polypeptide elongation until the entire complex interacts with a "receptor" that releases the elongation block. In this model *PET111* might code for the "receptor," and *pet111* mutants would be unable to overcome the blocked translation elongation, resulting in the observed phenotype. Either of these two models for *PET111* function is consistent with the *MSU111* suppressor mutations described here.

PET111 appears to be required *only* for expression of the mitochondrial gene *oxi1*. If *pet111* mutations also directly blocked the accumulation of other proteins required for respiration, for example coxI, we should not have obtained the *MSU111* suppressors described here. Furthermore, *PET111* does not appear to be required for any vital functions unrelated to respiration since strains carrying the presumed null allele caused by the disruption described here, as well as a complete deletion of *PET111* (C. A. STRICK, unpublished data), are viable. Interestingly, not all genes required for coxII synthesis are so specific. One of the two nuclear genes required for cleavage of the pre-coxII N-terminal extension (MANNHAUPT *et al.* 1983) is also required for processing of the precursor of cytochrome b_2, the product of a *nuclear* gene (PRATJE and GUIARD 1986).

The fact that expression of three yeast mitochondrial genes (those encoding coxII, coxIII and apocytochrome *b*) is known to be specifically controlled at the level of translation by nuclear genes, suggests that yeast mitochondrial gene expression in general may be translationally modulated. Previous studies of the levels of mitochondrially coded proteins (FALCONE, AGOSTINELLI and FRONTALI 1983) and mRNAs (ZENNARO *et al.* 1985) in yeast cells recovering from glucose repression are consistent with the notion that expression of mitochondrial genes is modulated individually at the level of translation. With respect to gene control in other organellar genetic systems it is interesting to note that expression of at least some chloroplast genes in several plant species is translationally regulated (MILLER *et al.* 1983; BERRY *et al.* 1985; FROMM *et al.* 1985).

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