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Translational regulation of mitochondrial gene expression by nuclear genes of *Saccharomyces cerevisiae*

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We describe several yeast nuclear mutations that specifically block expression of the mitochondrial genes encoding cytochrome *c* oxidase subunits II (COXII) and III (COXIII). These recessive mutations define positive regulators of mitochondrial gene expression that act at the level of translation. Mutations in the nuclear gene *PET111* completely block accumulation of COXII, but the COXII mRNA is present in mutant cells at a level approximately one-third of that of the wild type. Mitochondrial suppressors of *pet111* mutations correspond to deletions in mtDNA that result in fusions between the *coxII* structural gene and other mitochondrial genes. The chimeric mRNAs encoded by these fusions are translated in *pet111* mutants; this translation leads to accumulation of functional COXII. The *PET111* protein probably acts directly on *coxII* translation, because it is located in mitochondria. Translation of the mitochondrially coded mRNA for COXIII requires the action of at least three nuclear genes, *PET494*, *PET54* and a newly discovered gene, provisionally termed *PET55*. Both the *PET494* and *PET54* proteins are located in mitochondria and therefore probably act directly on the mitochondrial translation system. Mutations in all three genes are suppressed in strains that contain chimeric *coxIII* mRNAs with the 5′-untranslated leaders of other mitochondrial transcripts fused to the *coxIII* coding sequence. The products of all three nuclear genes may form a complex and carry out a single function. A direct demonstration that the wild-type nuclear gene products act in the *coxIII* 5′-leader has been obtained by showing that they are all required for translation of apocytochrome *b* from a novel mRNA consisting of the *coxIII* 5′-leader attached to the cytochrome *b* coding sequence. The site (or sites) of action maps at least 172 bases upstream from the *coxIII* initiation codon in the 600 base *coxIII* leader. Others have reported evidence which suggests that *coxIII* translation is repressed by glucose. Consistently with the possibility that the nuclear genes described here may play a role in modulating mitochondrial gene expression, we have found that *PET494* expression is glucose-repressed.

INTRODUCTION

In all eukaryotes, the abundance of mitochondrial gene products must be maintained at levels in balance with those of the nuclear gene products that are their partners in enzyme complexes. In addition, in facultative anaerobes like the yeast *Saccharomyces cerevisiae*, the levels of mitochondrial gene products must change in response to changes in environmental conditions, such as the level of glucose. The mechanisms by which mitochondrial gene expression is regulated are very poorly understood, but probably include controls at the levels of transcription, mRNA processing, translation and protein stability. By studying mutants of yeast that are defective in the expression of specific mitochondrial genes, we hope to obtain a better understanding of these molecular mechanisms.

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Among collections of yeast strains that are respiratory-deficient owing to nuclear mutations, a surprisingly large number of mutants have functional mitochondrial genetic systems but are defective in the expression of *specific* mitochondrial genes (Michaelis *et al.* 1982 ; Costanzo *et al.* 1986*a*; McEwen *et al.* 1986). Such mutations are of particular interest because it is likely that they define important regulatory genes that govern expression of the mitochondrial genome. We have chosen to concentrate our effort on the study of nuclear genes required for expression of the mitochondrial genes coding cytochrome *c* oxidase subunits II (COXII) and III (COXIII). (These genes are known formally as *oxi1* and *oxi2*, respectively, but will be referred to here as the *coxII* gene and the *coxIII* gene.)

All the nuclear mutations examined so far that affect expression of *coxII* and *coxIII* are recessive and are blocked in gene expression post-transcriptionally. In several cases, discussed here, we have shown that the wild-type nuclear genes are required to activate *translation* of the mitochondrially coded mRNAs. The requirements for specific translational activation can be surprisingly complex: in *coxIII* at least three nuclear genes must act at a site or sites in the 5'-untranslated leader of the mitochondrially coded mRNA. Here we summarize our findings on the control of mitochondrial translation by nuclear genes, and discuss them in light of studies, by others, on changes in mitochondrial gene expression in response to glucose levels.

COXII TRANSLATION REQUIRES THE PRODUCT OF NUCLEAR GENE *PET111*

The synthesis of yeast mitochondrial translation products can be easily studied *in vivo* by radioactively pulse-labelling cells in the presence of cycloheximide, separating the proteins by SDS-gel electrophoresis and radioautographing the gels. Strains with mutations in the nuclear gene *PET111* (formerly *PET11* (Ebner *et al.* 1973)) carried out mitochondrial gene expression normally, as judged by such labelling experiments, except that they specifically failed to accumulate COXII (Cabral & Schatz 1978; Poutre & Fox 1987) (figure 1). In addition, COXII was undetectable in *pet111* mutants by immunological assays of unlabelled cell extracts that easily detected COXII at one hundredth of the wild-type level (Poutre & Fox 1987). Despite the absence of the COXII protein in *pet111* mutants, the mitochondrially coded *coxII*

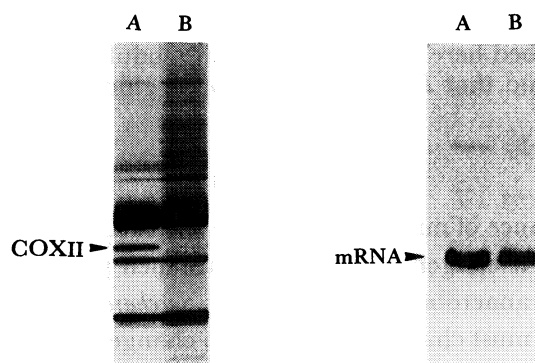


FIGURE 1. COXII does not accumulate in a *pet111* mutant, but the *coxII* mRNA is present. (a) Mitochondrial translation products were visualized by radioactive labelling, in the presence of cycloheximide, followed by SDS-gel electrophoresis and radioautography. The position of COXII is indicated. Lane A, wild type; lane B, an isogenic *pet111* mutant. (b) Total RNA was isolated from cells, subjected to electrophoresis, blotted and hybridized to a probe specific for the *coxII* mRNA. The filter was radioautographed; the position of the *coxII* mRNA is indicated. (Lanes A, B as in (a).)

mRNA was easily detectable in mutant cells, although its steady-state level was reduced by approximately three times relative to that of the wild type (Poutre & Fox 1987) (figure 1). Because the reduction in the level of *coxII* mRNA in *pet111* mutants is far too slight to account for the absence of the protein, we conclude that the nuclear mutation blocks a post-transcriptional step in *coxII* expression. The data are most easily explained by the hypothesis that *PET111* function is required for translation of the *coxII* mRNA and that, in the absence of translation (in a *pet111* mutant) the *coxII* mRNA is relatively unstable; this instability results in a lower mRNA steady-state level.

This hypothesis is strongly supported by the fact that it has been possible to isolate mitochondrial mutations that suppress *pet111* nuclear mutations, demonstrating that COXII can be stably incorporated into active cytochrome oxidase in the absence of *PET111* function, if the translational defect is bypassed. These suppressors, termed *MSU111*, correspond to deletions in mtDNA that result in fusions between the *coxII* structural gene and other mitochondrial genes (Poutre & Fox 1987). The *MSU111*-coded *coxII* mRNAs were thus substituted for the normal 5'-untranslated leader and translation initiation codon. The rearranged *coxII* genes were carried on ρ^- mtDNA molecules of the kind normally found in 'cytoplasmically petite' yeast strains. However, under selective conditions, these ρ^- mtDNAs were maintained heteroplasmically with wild-type mtDNA. Despite the fact that the *MSU111* suppressors encoded variants of COXII 50–70 amino acids longer than those of the wild type at the N-terminal end, *MSU111*-suppressed *pet111* mutants accumulated wild-type-sized COXII (Poutre & Fox 1987). Because COXII is normally made as a precursor with a 15 amino acid N-terminal extension (Pratje *et al.* 1983), and because the predicted chimeric proteins encoded by *MSU111* suppressors contained the processing site, the chimeric proteins must have been processed to yield wild-type COXII. These findings suggest that, in the wild type, the product of *PET111* (or something under its control) acts at a site coded in the proximal part of the *coxII* gene. This site of action may lie in the mRNA, as in nuclear gene products controlling *coxIII* translation (see below). However, other models (Poutre & Fox 1987) in which the product of *PET111* interacts with the COXII precursor protein itself cannot be excluded, because the *MSU111* suppressors that have been isolated so far alter both the *coxII* mRNA and the precursor protein.

We have isolated the *PET111* gene (Poutre & Fox 1987), determined its nucleotide sequence (Strick & Fox 1987) and generated antibodies to the polypeptide it encodes (unpublished results). As a first step towards understanding the mechanism by which the *PET111* gene product acts we have determined its intracellular location. The *PET111* protein was shown to be mitochondrially located by two lines of experimentation. First, expression in yeast of a *pet111::lacZ* fusion gene encoding a protein with the N-terminal 154 amino acids of *PET111* attached to β -galactosidase resulted in mitochondrial localization of β -galactosidase activity (Strick & Fox 1987). Second, direct immunological detection of the *PET111* protein in subcellular fractions from a yeast strain in which *PET111* was overproduced confirmed that it was located in mitochondria (unpublished results). We therefore conclude that the *PET111* protein acts directly to promote *coxII* translation rather than indirectly by, for example, activating another nuclear gene.

The poly(A) mRNA copied from *PET111* has an unusual structure (Strick & Fox 1987). The 5'-untranslated leader is 470 nucleotides long and contains four short open reading frames that exhibit an interesting pattern of overlap with each other and with the *PET111* structural

gene. We have not yet examined the regulation of *PET111*. However, another yeast regulatory gene, *GCN4*, which also has a long 5'-leader containing short open reading frames, is regulated at the level of translation (Hinnebusch 1984). It will be interesting to explore the possibility that *PET111*, a specific activator of *coxII* translation, may itself be translationally controlled.

We have continued to search among our collection of *pet* mutants for strains unable to express *coxII* owing to mutations in genes other than *PET111*. One strain, with a phenotype very similar to that of *pet111* mutants, carries a mutation in a new gene, termed *PET112*. The *pet112* mutation complements *pet111* and is unlinked to it (unpublished results). Like *pet111* mutants, the *pet112* mutant lacks COXII, as judged both by pulse-labelling experiments and immunoassay of steady-state protein levels. Moreover, the *pet112* mutant contains the *coxII* mRNA at a level reduced somewhat relative to that of the wild type. However, the *pet112* mutant is phenotypically distinguishable from *pet111* in that it accumulates a novel unidentified mitochondrial translation product that is immunologically unrelated to COXII. More importantly, the *pet112* mutation is *not* suppressed by the *MSU111* mitochondrial suppressors described above. Thus, either *PET112* is required for a step in *coxII* expression that occurs later than the *PET111* step, or *PET112* is required for other mitochondrial functions in addition to promoting translation of *coxII*.

ACTIVATION OF COXIII TRANSLATION BY THREE NUCLEAR GENE PRODUCTS

Mutations in the nuclear genes *PET494* (Ebner *et al.* 1973; Cabral & Schatz 1978; Mueller *et al.* 1984), *PET54* (Costanzo *et al.* 1986) and a newly identified gene, provisionally termed *PET55*, all block accumulation of the mitochondrial gene product COXIII (figure 2). *pet494*, *pet54* and *pet55* mutations complement each other and are genetically unlinked. Mutants in the same three complementation groups have also been isolated by others (McEwen *et al.* 1986; J. E. McEwen, personal communication; unpublished results).

Although mutations in any one of these three genes block COXIII accumulation, none of the mutations affects the steady-state level of *coxIII* mRNA (Mueller *et al.* 1984; Costanzo

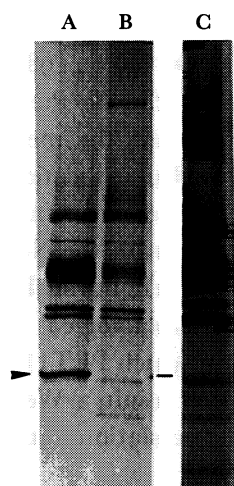


FIGURE 2. Mutations in *PET54* and *PET55* specifically block accumulation of COXIII. Mitochondrial translation products were visualized by radioactive labelling in the presence of cycloheximide followed by SDS-gel electrophoresis and radioautography. The arrowhead indicates the band corresponding to COXIII. Lane A, wild type; lane B, a *pet54* mutant; lane C, a *pet55* mutant.

et al. 1986 *b*) (figure 3). Unlike the mutants blocked in *coxII* translation described above, whose *coxII* mRNA levels were reduced relative to those of the wild type, *pet494*, *pet54* and *pet55* mutations have no measurable effect on the *coxIII* mRNA. Because the mutations are recessive, the wild-type genes must be positive activators of a post-transcriptional step in *coxIII* gene expression.

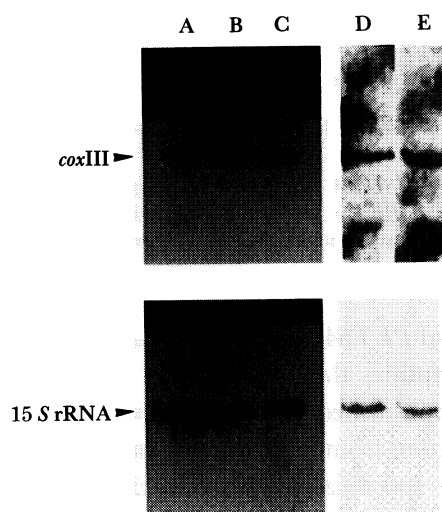


FIGURE 3. The *coxIII* mRNA is present at wild-type levels in *pet494*, *pet54* and *pet55* mutants. Total RNA was isolated from various strains, subjected to electrophoresis, blotted and hybridized to both a probe specific for the *coxIII* mRNA (*a*) and a probe specific for the mitochondrial 15S rRNA (*b*) as a control for quantitation. Lane A, wild-type; lane B, a *pet54* mutant; lane C, a *pet494* mutant; lane D, a *pet55* mutant; lane E, wild type. Lanes D and E are from a different hybridization experiment than lanes A, B and C.

The phenotypes of *pet494*, *pet54* and *pet55* mutations are consistent with two possible mechanisms of action. Either the wild-type gene products could be required to activate translation of the *coxIII* mRNA, or they could be required to stabilize the COXIII protein after synthesis to prevent its rapid degradation. That *pet494* mutations block *translation* of the *coxIII* mRNA was demonstrated by isolating and examining mitochondrial mutations that suppressed a *pet494* deletion mutation (Costanzo & Fox 1986). These mitochondrial suppressors, termed *MSU494*, were the result of deletions in mtDNA that fused the 5'-flanking sequences of the *coxIII* structural gene to the 5'-flanking regions of other mitochondrial genes without altering the *coxIII* coding sequence, as illustrated schematically in figure 4. Transcription of the chimeric genes generated novel *coxIII* mRNAs with the 5'-untranslated leaders of other mitochondrial transcripts attached to the wild-type *coxIII* structural gene (figure 3). These chimeric mRNAs, with 5'-leader substitutions, were translated (in cells heteroplasmic for the *MSU494* and wild-type mitochondrial genomes) to yield wild-type COXIII protein that accumulated and functioned in *pet494* mutant cells (Costanzo & Fox 1986). Interestingly, the defects in COXIII accumulation of both *pet54* (Costanzo *et al.* 1986 *b*) and *pet55* (unpublished results) mutations were also suppressed in cells containing the *MSU494*-encoded chimeric *coxIII* mRNAs. Thus, *PET494*, *PET54* and *PET55* are all required to activate translation of the mitochondrially coded *coxIII* mRNA. Furthermore, the observation of co-suppression suggests the possibility that the products of all three nuclear genes act together to carry out a single function.

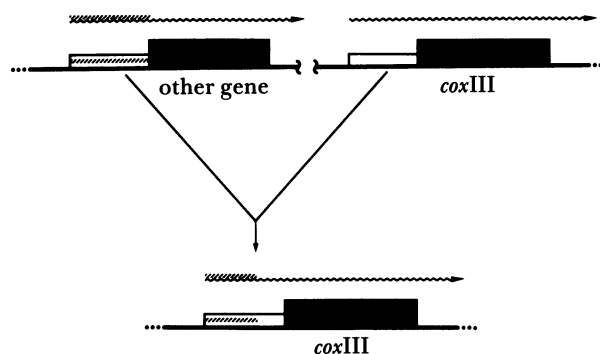


FIGURE 4. Schematic representation of the *MSU494* deletion mutations that lead to novel mRNAs for COXIII, translatable in the absence of *PET494*, *PET54* and *PET55* activity. The thin open bars represent regions coding for 5'-untranslated leaders; the wavy lines represent transcripts. (Reprinted from Fox (1986).)

We have isolated the *PET494* and *PET54* genes, determined their nucleotide sequences and generated antibodies to the polypeptides they encode (Mueller & Fox 1984; Costanzo *et al.* 1986*a*; Costanzo & Fox 1986; unpublished results). Direct immunological detection of the *PET494* protein in subcellular fractions from a yeast strain in which *PET494* was overproduced revealed that *PET494* co-purified specifically with mitochondria (Costanzo & Fox 1986). Similar experiments with an anti-*PET54* anti-serum that allowed detection of *PET54* in fractions from wild-type (non-overproducing) yeast cells demonstrated that the *PET54* protein was also specifically located in mitochondria (unpublished data). Thus both of these nuclear coded proteins must act directly in the organelles to promote *coxIII* translation. The subcellular location of the *PET55* gene product is currently unknown, leaving open the possibility that it acts indirectly. For example, one plausible explanation for the phenotype of *pet55* mutants would be that they are defective in expression of *PET494* and/or *PET54*. However, we have found that *pet55* mutants express both *PET494* and *PET54* normally (unpublished results), ruling out this particular hypothesis. We therefore consider it probable that, like the *PET494* and *PET54* proteins, the *PET55* gene product also acts directly in mitochondria to promote *coxIII* translation.

The analysis of mitochondrial mutations that suppressed *pet494*, *pet54* and *pet55* mutations suggested that the wild-type nuclear gene products act at a site (or sites) in the 5'-untranslated leader of the *coxIII* mRNA. We have recently obtained direct evidence that this is the case by showing that all three wild-type nuclear genes are required for translation of apocytochrome *b* from a novel chimeric mRNA consisting of part of the *coxIII* mRNA 5'-leader attached to the cytochrome *b* coding sequence. To obtain a chimeric mitochondrial gene encoding such an mRNA, we isolated mitochondrial suppressors of the nuclear mutation *cbs1*, which specifically blocks translation of the *cob* mRNA encoding cytochrome *b* (Rödel *et al.* 1985). One of 70 *cbs1* suppressor genes isolated carried the region encoding the 5' two-thirds of the *coxIII* mRNA leader fused to the *cob* structural gene (unpublished results).

To study translation of this chimeric mRNA, synthesis of cytochrome *b* was monitored in zygotes whose mitochondria contained both a wild-type genome and the chimeric gene. The zygotes were also homozygous for the *cbs1* mutation, and therefore could not translate the wild-type *cob* mRNA. Zygotes that were wild-type for *PET494*, *PET54* and *PET55* synthesized cytochrome *b*. However, homozygous *pet494*, *pet54* or *pet55* mutations specifically

blocked synthesis of cytochrome *b* in the zygotes (unpublished results). Thus, all three nuclear gene products were required to activate translation of this chimeric mRNA by acting at a site or sites in the *coxIII* 5'-untranslated leader. Furthermore, because the chimeric mRNA lacks the 3' 172 bases of the *coxIII* untranslated leader, we conclude that the target (or targets) for these nuclear gene products must lie at least 172 nucleotides upstream of the AUG initiation codon in the wild-type *coxIII* mRNA.

The nuclear genes required specifically to activate *coxIII* translation could play a role in modulating *coxIII* gene expression. If this were the case, one might expect to find that conditions known to lower COXIII levels, such as glucose repression (Falcone *et al.* 1983), also lead to lower levels of expression of the nuclear genes. We have studied the effect of glucose on *PET494* expression in a respiring diploid strain in which one chromosomal copy of *PET494* was replaced by a *pet494::lacZ* fusion. The specific activity of β -galactosidase was approximately fivefold higher in cells grown on medium containing ethanol as a carbon source than in cells grown on medium containing glucose (unpublished results). Addition of glucose to ethanol-glycerol medium repressed β -galactosidase activity. Thus, *PET494* expression is controlled in a manner consistent with its possible role in modulating expression of the mitochondrial gene for COXIII. Interestingly, expression of the *pet494::lacZ* fusion was unaffected by mutations controlling glucose repression of the enzyme invertase (Neigeborn & Carlson 1984; T. D. Fox *et al.*, unpublished results); this result indicates that multiple systems may control glucose repression in yeast.

DISCUSSION

Translation of the mitochondrially coded *coxIII* mRNA is dependent on the action of the nuclear genes *PET494*, *PET54* and *PET55*. Although the molecular mechanism by which *coxIII* translation is stimulated remains unknown, we have demonstrated that the *PET494* and *PET54* proteins are located in mitochondria and that a site of action for all three nuclear genes can be mapped genetically to a region of the 600 base 5'-untranslated leader of the *coxIII* mRNA, at least 172 bases upstream of the translation initiation codon. The results are thus consistent with a model (figure 5) in which the three nuclear genes encode subunits of a complex that functions in mitochondria as a *coxIII* mRNA-specific translation initiation factor. It remains to be determined whether the *PET55* gene product is a mitochondrial protein, as well as whether the products of all three genes directly interact with each other and with the *coxIII* mRNA.

As mentioned above, we are unable to distinguish whether the mitochondrially located *PET111* protein functions by interacting with the mitochondrially coded *coxII* mRNA or with the COXII precursor protein, owing to the fact that the *MSU111* suppressors alter both. However, because it is clear that *PET111* acts post-transcriptionally and that it is not required for the stability or function of the mature COXII protein, our working hypothesis is that *PET111* activates *coxII* translation. The mechanism of action of *PET112* in promoting COXII accumulation is even less well understood since the *pet112* mutation is not suppressed by the *MSU111* suppressors.

In addition to COXII and COXIII, the expression of the mitochondrial gene product apocytochrome *b* is known to be under translational control. Translation of the *cob* mRNA encoding cytochrome *b* requires the action of at least three nuclear genes (Dieckmann &

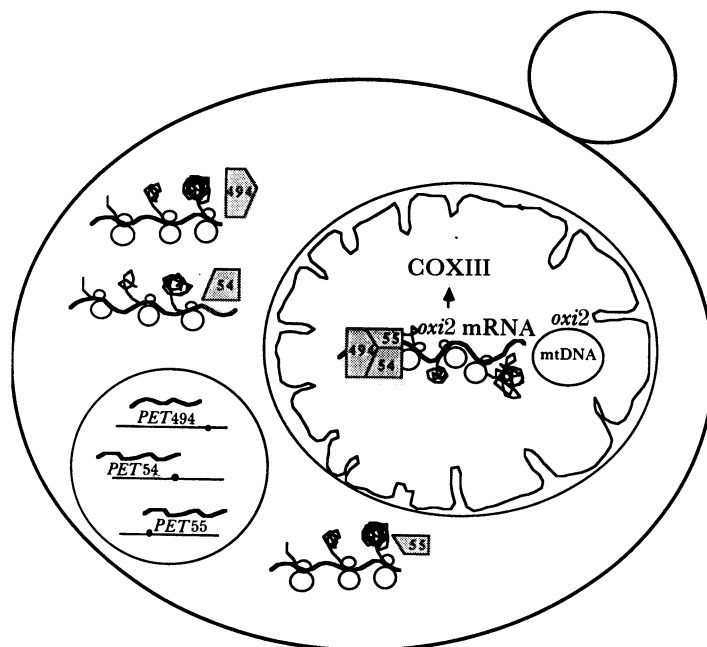


FIGURE 5. A hypothetical model for the action of *PET494*, *PET54* and *PET55* in promoting COXIII translation. See Discussion for a description. COXIII is encoded by the mitochondrial gene termed *oxi2*.

Tzagoloff 1985; Rödel *et al.* 1985; Rödel 1986). One of these genes, *CBS1*, has been shown to have a site of action in the 5'-untranslated leader of the *cob* mRNA (Rödel & Fox 1986) and may therefore act by a mechanism similar to that of the genes controlling COXIII.

Frontali and co-workers have reported that, although the synthesis of all mitochondrial translation products increased after the release of glucose repression (Falcone *et al.* 1983), the behaviour of their mitochondrially coded mRNAs varied (Zennaro *et al.* 1985). For example, the steady-state level of the *coxIII* mRNA did not change detectably, whereas the level of the *coxII* mRNA increased after release of glucose repression. One could interpret these data as indicating that some mitochondrial genes are controlled transcriptionally (*coxII*), whereas others are not (*coxIII*). However, these data are also consistent with a model in which transcription of both genes is constitutive and COXII and COXIII synthesis is controlled at the level of translation. One must simply assume that absence of translation decreases the stability of some mRNAs but not others. Indeed, our data on the levels of *coxII* and *coxIII* mRNAs in nuclear mutants specifically blocked in their translation strongly suggests that the *coxII* mRNA is less stable in the absence of translation and the stability of the *coxIII* mRNA is unaffected by translation. Thus, presently available data are consistent with the notion that modulation of mitochondrial gene expression in yeast occurs largely at the level of translation. It will be of great interest to determine whether any of the nuclear genes described here play a role in this modulation.

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Note added in proof (10 February 1988). The gene referred to above as *PET55* has since been renamed *PET122* to standardize nomenclature with that of other workers (McEwen *et al.* 1986).

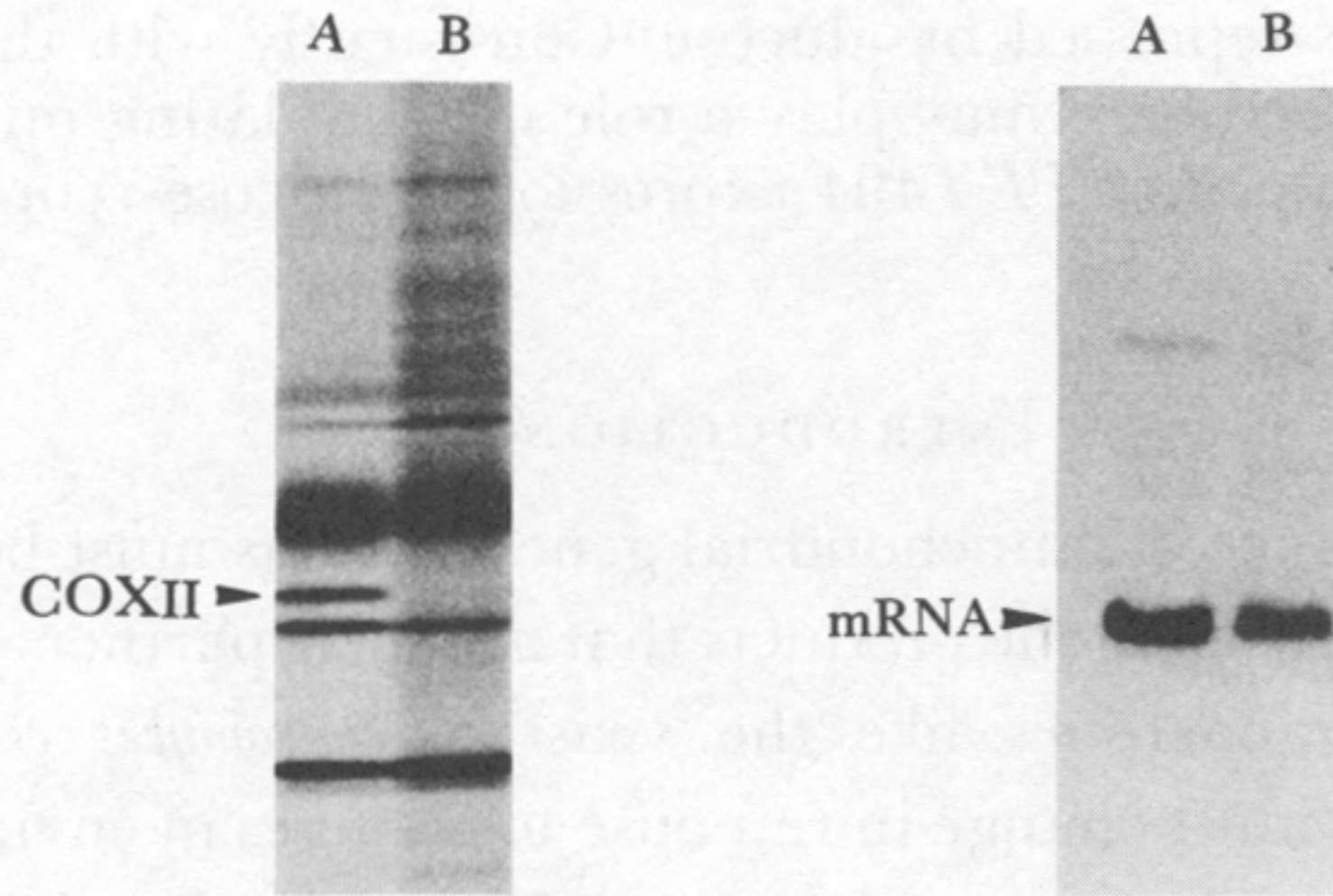


FIGURE 1. COXII does not accumulate in a *pet111* mutant, but the *coxII* mRNA is present. (a) Mitochondrial translation products were visualized by radioactive labelling, in the presence of cycloheximide, followed by SDS-gel electrophoresis and radioautography. The position of COXII is indicated. Lane A, wild type; lane B, an isogenic *pet111* mutant. (b) Total RNA was isolated from cells, subjected to electrophoresis, blotted and hybridized to a probe specific for the *coxII* mRNA. The filter was radioautographed; the position of the *coxII* mRNA is indicated. (Lanes A, B as in (a).)

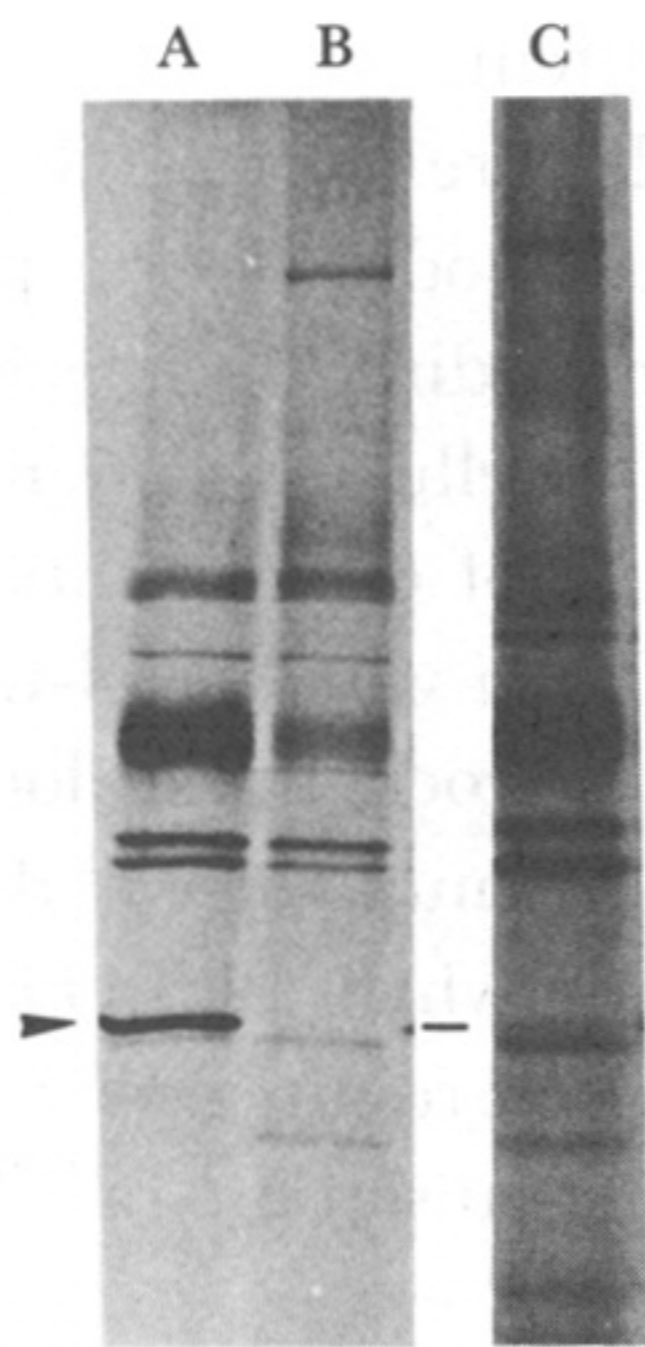


FIGURE 2. Mutations in *PET54* and *PET55* specifically block accumulation of COXIII. Mitochondrial translation products were visualized by radioactive labelling in the presence of cycloheximide followed by SDS-gel electrophoresis and radioautography. The arrowhead indicates the band corresponding to COXIII. Lane A, wild type; lane B, a *pet54* mutant; lane C, a *pet55* mutant.

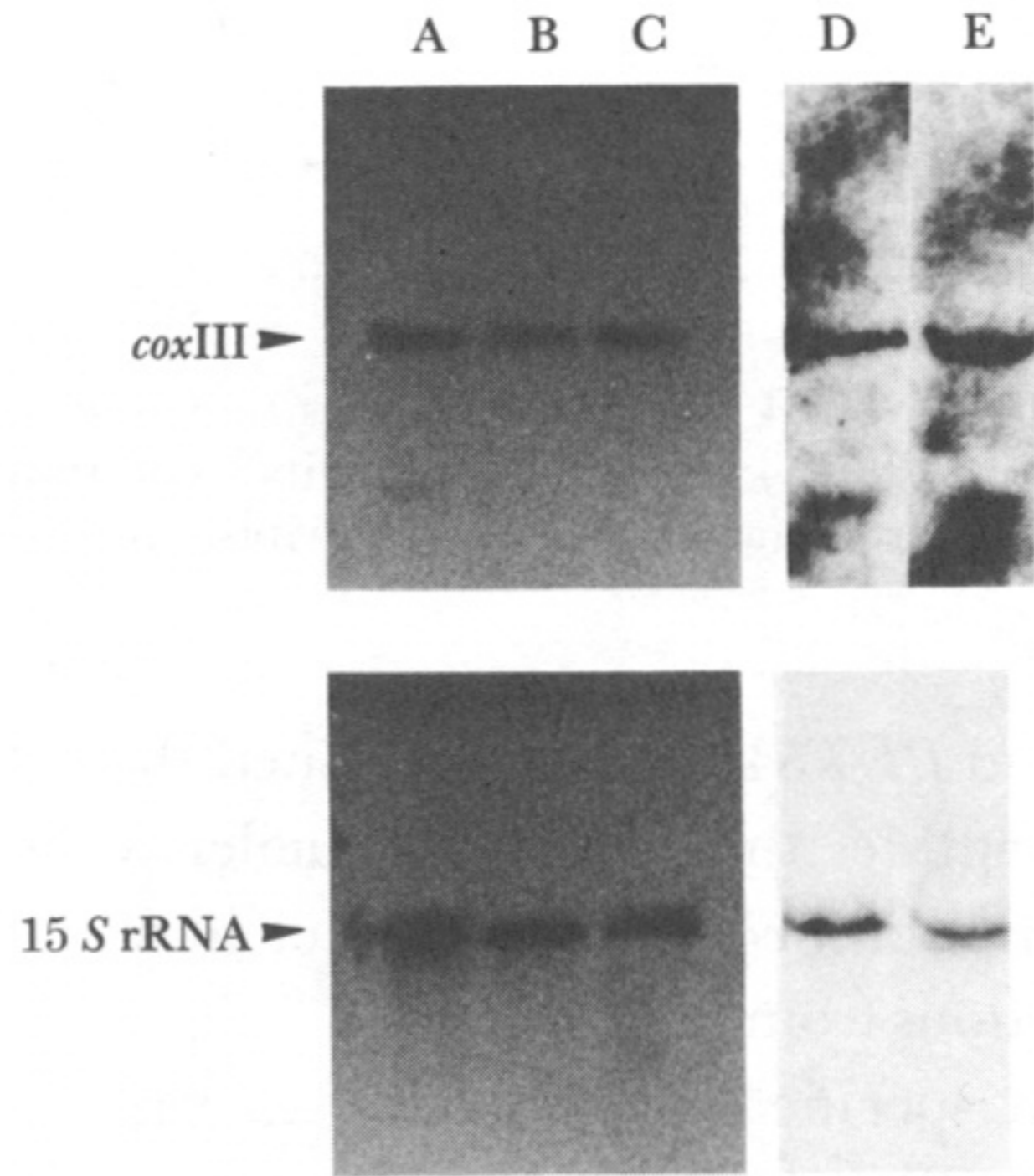


FIGURE 3. The *coxIII* mRNA is present at wild-type levels in *pet494*, *pet54* and *pet55* mutants. Total RNA was isolated from various strains, subjected to electrophoresis, blotted and hybridized to both a probe specific for the *coxIII* mRNA (*a*) and a probe specific for the mitochondrial 15S rRNA (*b*) as a control for quantitation. Lane A, wild-type; lane B, a *pet54* mutant; lane C, a *pet494* mutant; lane D, a *pet55* mutant; lane E, wild type. Lanes D and E are from a different hybridization experiment than lanes A, B and C.