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Identification of a Third Nuclear Protein-Coding Gene Required Specifically for Posttranscriptional Expression of the Mitochondrial *COX3* Gene in *Saccharomyces cerevisiae*

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A third nuclear protein-coding gene termed *PET122* has been shown to be required for a post-transcriptional step in expression of the mitochondrial *COX3* gene in *Saccharomyces cerevisiae*. *pet122* mutants fail to produce cytochrome *c* oxidase subunit III, which is the polypeptide product of the *COX3* gene, but produce normal amounts of mature *COX3* mRNA. A strain bearing the *pet122-1* allele is amber suppressible and correctly processes the 5′ end of *COX3* mRNA. Therefore, the *PET122* gene product is a protein required for the expression of *COX3* at some step after transcription and 5′-end processing of its transcript.

Mitochondrial gene expression in *Saccharomyces cerevisiae* is largely dependent upon nucleus-encoded factors that are synthesized in the cytoplasm and imported into mitochondria. Many of these nuclear factors (e.g., the DNA and RNA polymerases) are required for expression of all mitochondrial genes, whereas other factors are required in a gene-specific manner (6, 17). Recently, several nuclear mutants that are unable to translate specific mitochondrial mRNAs have been characterized in detail. The *cpb6* (3), *cbs1* (13), and *cbs2* (12) mutants are unable to synthesize apocytochrome *b*, the *pet111* mutant (11) lacks cytochrome *c* oxidase subunit II, and the *pet494* and *pet54* mutants (2, 9) are unable to synthesize cytochrome *c* oxidase subunit III.

We previously identified mutants in three nuclear complementation groups that are respiration deficient and defective in expression of the mitochondrial *COX3* gene, which encodes cytochrome *c* oxidase subunit III (6a). These mutants each carry a single recessive nuclear mutation. Their relative cytochrome *c* oxidase activities are listed in Table 1. To determine which, if any, of these mutants is allelic with mutants in two other genes, *PET494* and *PET54*, that are required for the expression of *COX3* (2, 9), complementation tests were carried out with strains 494 A1 (*pet494*) (9) and ECS54 (*pet54*) (2), respectively. The mutants in group 15 failed to complement strain 494 A1, whereas the mutant in group 17 failed to complement ECS54. All other combinations resulted in normal complementation. These results demonstrate that the mutants in groups 15 and 17 carry alleles of *PET494* and *PET54*, respectively, and suggest that mutants in complementation group 16 represent a third gene. To prove that this is the case, mutants from each complementation group were crossed to one another and subjected to meiotic segregation analysis. The segregation patterns of the cytochrome *c* oxidase-deficient phenotype are shown in Table 2. If mutations were tightly linked, the majority of tetrads should produce a parental ditype segregation pattern. In contrast, unlinked mutations should produce parental ditypes ($0^+:4^-$), nonparental ditypes ($2^+:2^-$), and tetratypes ($1^+:3^-$) in a ratio of about 1:1:4 (14). The data in Table 2 indicate that the mutations in strains B59 (*pet494*), B48 (complementation group 16), and B120 (*pet54*) are unlinked and therefore demonstrate that the three complementation groups represent three unlinked genes required for accumulation of cytochrome *c* oxidase subunit III. The gene represented by complementation group 16 is hereby named *PET122*.

The *pet494* mutation in strain 494 isolated by Ebner et al. (4) was shown previously to be an amber mutation (10), whereas the *pet54* mutation in strain ECS54 carries an ochre mutation (2). The *PET494* and *PET54* genes therefore encode proteins. To determine whether *PET122* encodes a protein, we isolated respiration-proficient spontaneous revertants of strain KG122-2A (*MATα pet122-1 his4-580 trp1-289 ura3-52 ade* Can^r), which is a derivative of strain B122 (Table 1), and screened for coreversion of the *pet* mutation with the known amber mutations *his4-580* and *trp1-289*. Out of 150 respiration-proficient revertants of KG122-2A tested, 72% were His^+ Trp^+. As shown for one of these revertants, reversion to respiration proficiency was accompanied by restoration of synthesis of cytochrome *c* oxidase subunit III (Fig. 1, lane 3). These data suggest that the *pet122-1* mutation is amber and that the Pet^+ His^+ Trp^+ revertants carry second-site amber suppressors that permit synthesis of a functional *PET122* gene product. To confirm this interpretation, meiotic segregation analysis was performed. Two representative revertants, KG122-R1 and KG122-R4, were crossed to strain STX20-1C (*PET122*$^+$ *lys2 gal2*; obtained from the Yeast Genetic Stock Center, Berkeley, Calif.). A high proportion of tetrads resulting from each cross exhibited $3^+:1^-$ (tetratype) or $2^+:2^-$ (nonparental ditype) segregation for cytochrome *c* oxidase activity (data not shown), indicating that the *pet122-1* mutation in these revertants is suppressed by an unlinked second-site mutation (14). Similar frequencies of tetratypes and nonparental ditypes were observed for the *his* and *trp* markers (data not shown), indicating the presence of an unlinked suppressor for these mutations as well. In crosses of the two revertants KG122-R1 and KG122-R4 to strain KG122-6B (*pet122-1 his4-580 trp1-289*), two spores of every tetrad exhibited cosegregation of the Pet^+, His^+, and Trp^+ phenotypes. These data demonstrate that, in each

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TABLE 1. Cytochrome *c* oxidase activities

Complementation group	Strain[a]	Cytochrome oxidase activity[b] (%)
Wild type	JM22	100
Group 15	N8-128	0
	B59	0.5
	BC124	ND
Group 16	B122	0
	B48	0
	BC129	7.3
	BC312	0
Group 17	B120	0

[a] All strains have been described previously (8).

[b] Mitochondria were isolated and assayed for cytochrome *c* oxidase activity according to published methods (7). The wild-type activity in strain JM22 was 135.4 μmol of cytochrome *c* oxidized per mg of mitochondrial protein per min. All activities are expressed as the percentage of wild-type activity. ND, Not determined.

TABLE 2. Linkage analysis of mutations in complementation groups 15, 16, and 17

Cross[a]	No. of tetrads analyzed	Segregation of cytochrome oxidase activity[b]			
		PD	NPD	T	Other
B59 × B48-19-2	19	5	0	12	2
B59 × B120-17-1	17	2	7	8	0
B120 × B48-19-2	22	4	2	16	0

[a] Standard yeast genetics methods were used in these crosses (14). Strain B59 represents complementation group 15; strain B48-19-2 represents complementation group 16; and strains B120 and B120-17-1 represent complementation group 17. Mutants in each complementation group were crossed to each other as indicated. Diploids were sporulated, tetrads were dissected, and spores were analyzed for cytochrome *c* oxidase activity by the TMPD colony stain (7).

[b] PD, Parental ditype ($0^+:4^-$); NPD, nonparental ditype ($2^+:2^-$); T, tetratype ($1^+:3^-$); and other, $3^+:1^-$).

case, a single nuclear mutation is responsible for suppression of all three markers. Together, these data demonstrate that the *pet122-1* mutation is amber and that the *PET122* gene encodes a protein.

As described elsewhere (6a) and shown for strain B122 (*pet122-1*) in Fig. 1 (lane 2), *pet122* mutants fail to accumulate cytochrome *c* oxidase subunit III, whereas other mitochondrial translation products are unaffected. To determine whether the block in subunit III accumulation occurs at the transcriptional or posttranscriptional level, blot hybridization analysis of *pet122-1* was carried out. Probes for the *COX1* and *COX2* genes, which encode cytochrome *c* oxidase subunits I and II, respectively, were used as controls in Fig. 2A and B since, as noted above, the expression of these genes is unaffected in *pet122* mutants. As expected, the *COX1* and *COX2* transcript patterns for the *pet122-1* mutant strain B122 (Fig. 2A and B, lanes 1) were identical to those seen in the wild-type strain JM22 (lane 2). The apparent size of the 3.6-kilobase (kb) *COX3* mRNA, which encodes cytochrome *c* oxidase subunit III, was also unchanged in the mutant (Fig. 2C, lane 1) relative to the *PET122*$^+$ strain (JM22). When normalized to the amount of *COX1* or *COX2* mRNA, the amount of *COX3* mRNA in strain B122 was essentially identical to that found in the wild-type strain JM22. These data demonstrate that the *PET122* gene is required for a posttranscriptional step in *COX3* gene expression.

In *pet122* mutants, the posttranscriptional block in expression of *COX3* could be the result of faulty RNA processing and/or modification that renders the mRNA untranslatable. It has been shown previously that *COX3* is cotranscribed with the upstream gene for tRNA^{Val} (15). The 5′ end of the 3.6-kb *COX3* transcript therefore results from processing of a longer primary transcript (15). In principle, the *PET122* gene could be required for the 5′ processing reaction. In this case, *pet122* mutations would prevent production of the correct 5′ end of *COX3* mRNA and in turn impair the translatability of *COX3* mRNA. Such a defect would be hard to detect in RNA blot analysis, such as that shown in Fig. 2C, because two similar-sized transcripts with different 5′ ends would not be resolved by these electrophoresis conditions (1% agarose; 2.2 M formaldehyde) (6a). To examine this possibility directly, the 5′ ends of *COX3* mRNAS in the wild type and the *pet122-1* mutant strains were analyzed by primer extension. The location of the primer (positions −271 to −293 in the *COX3* sequence) (16) was chosen based on the availability of G+C-rich regions between the proposed 5′ end of mature *COX3* mRNA in wild-type mitochondria (reported as nucleotide −488 or −489 [15] or −610 [9] in different studies) and the beginning of the subunit III coding region (position +1). In each reaction, the 5′ end-labeled primer was hybridized to total mitochondrial RNA, extended with reverse transcriptase, and analyzed by polyacrylamide gel electrophoresis. *COX3* mRNA from the wild-type strain (JM22) directed the synthesis of two extension

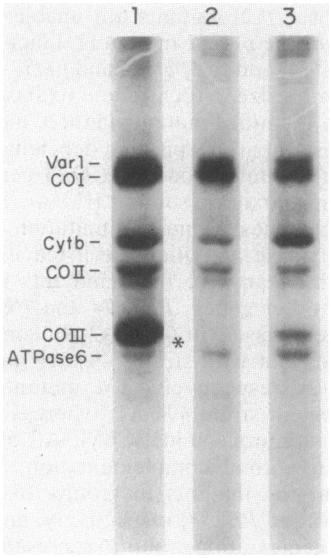

FIG. 1. Mitochondrial translation products. To selectively label mitochondrially synthesized proteins, the wild-type strain JM22 (lane 1), the *pet122* mutant strain B122 (lane 2), and a Pet$^+$ revertant KG122-R4 (lane 3) were incubated with [^{35}S]methionine in the presence of cycloheximide. Then mitochondria were isolated, and total mitochondrial proteins were separated on a 12.5% polyacrylamide gel in the presence of 27% glycerol and 3.5 M urea as described previously (8). Approximately 10^4 cpm was applied to each gel lane. The identities of each radiolabeled protein are indicated on the left of each panel. Var1, Polypeptide associated with the small mitochondrial ribosomal subunit; COI, COII, and COIII, subunits I, II, and III of cytochrome *c* oxidase, respectively; b, apocytochrome *b*; and ATPase 6, subunit 6 of the oligomycin sensitive ATPase. The asterisk indicates the position of a missing translation product.

products, which are labeled A and B in Fig. 3. We estimate the sizes of products A and B to be about 343 ± 10 and 201 ± 1 nucleotides, respectively, based on their migration relative to molecular weight markers. These sizes correspond to 5′ ends located at about positions −613 and −471 on the *COX3* DNA sequence, which may correspond to the 5′ ends mapped, by S1 nuclease protection analysis, to positions −610 and −488 or −489 by Mueller et al. (9) and Thalenfeld et al. (15), respectively. The primer extension products from the mutant B122 and the wild-type JM22 comigrated (Fig. 3). Hence, a defect in 5′ processing cannot account for the failure of *pet122-1* to accumulate cytochrome *c* oxidase subunit III.

Together, these results demonstrate that the *PET122* gene encodes a protein required for a posttranscriptional step in the expression of the mitochondrial gene *COX3*. Our data allow us to rule out an involvement of the *PET122* gene product in 5′-end processing of *COX3* mRNA but do not presently allow us to distinguish between a translational or a posttranslational mode of action. Future experiments will be aimed at resolving this question.

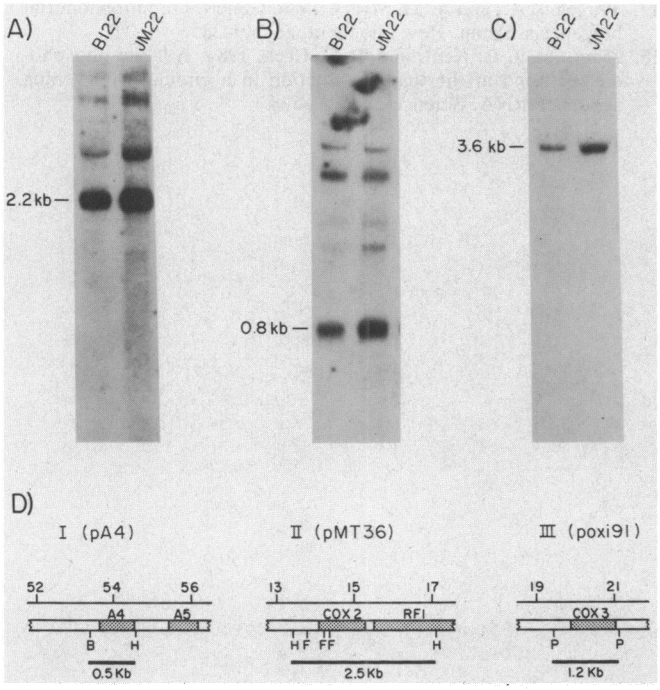

FIG. 2. Mitochondrial transcripts. Mitochondrial RNA was isolated from the wild-type strain JM22 (lanes 1) and the *pet122* mutant strain B122 (lanes 2), separated by electrophoresis in 1% agarose–2.2 M formaldehyde gels, and transferred to nitrocellulose as described previously (6a). The autoradiograms were obtained from a single nitrocellulose filter that was hybridized successively with each of the probes for *COX1* (A), *COX2* (B), and *COX3* (C). Before hybridization with the next probe, the old probe was washed off by incubation of the filter in 5 mM Tris hydrochloride (pH 8.0)–0.2 mM EDTA–0.1× Denhardt solution–0.05% sodium pyrophosphate for 60 min at 68°C. The effectiveness of probe removal was assessed by autoradiography. The mature mRNA sizes are given as 2.2 kb for *COX1* (5), 0.8 kb for *COX2* (1), and 3.6 kb for *COX3* (15). In panel D, the structure of the probes pA4, pMT36, and pOXI91 for *COX1*, *COX2* and *COX3*, respectively, are shown. The *COX* coding regions are represented as grey bars, and the numbers listed above the maps indicate the map units for the yeast mitochondrial genome. The restriction sites are abbreviated as B (*Bam*H1), H (*Hae*III), F (*Hin*F1), and P (*Hpa*II).

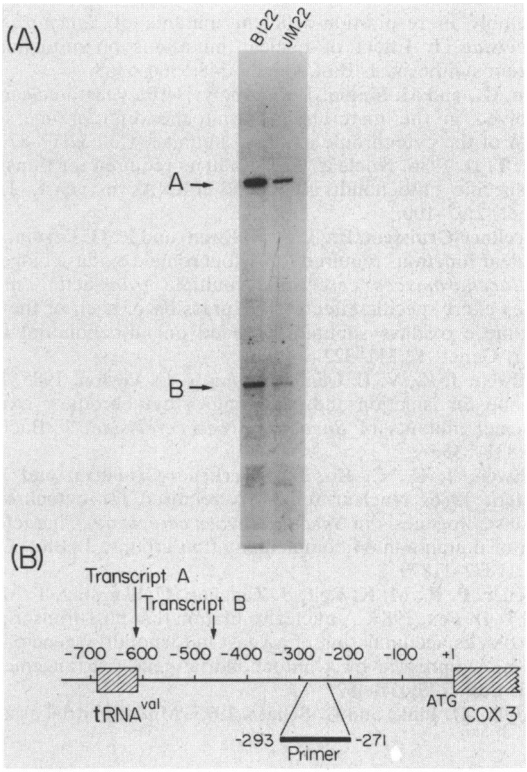

FIG. 3. Primer extension analysis of the 5′ ends of *COX3* mRNA. Mitochondria were prepared from the wild-type strain JM22 and the *pet122* mutant strain B122 as described previously (6a) and were lysed in 25 mM sodium acetate (pH 5.0) containing 4 M guanidinium thiocyanate as a RNase inhibitor. Mitochondrial RNA was then purified by ultracentrifugation through a 5.7 M CsCl cushion. Mitochondrial RNA was hybridized to a 5′ end-labeled synthetic deoxyoligonucleotide primer that is complementary to *COX3* mRNA at the indicated position (−271 to −293), and the labeled primer was extended with avian myeloblastosis virus reverse transcriptase as described by Zaug et al. (18). The extension products were analyzed on an 8% polyacrylamide sequencing gel. The sizes of the extended cDNAs were estimated by comigration with an M13 sequencing ladder that was run on the same gel.

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