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ATP13, a nuclear gene of *Saccharomyces cerevisiae* essential for the expression of subunit 9 of the mitochondrial *ATP*ase

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The respiratory deficient nuclear mutant of *Saccharomyces cerevisiae*, N9-168, assigned to complementation group G95 was previously shown to lack subunit 9, one of the three mitochondrially encoded subunits of the F_o component of the mitochondrial ATPase As a consequence of the structural defect in F_o, the ATPase activity of G95 mutants is not inhibited by rutamycin The absence of subunit 9 in N9-168 has been correlated with a lower steady-state level of its mRNA and an increase in higher molecular weight precursor transcripts These results suggest that the mutation is most likely to affect either translation of the *oli1* mRNA or processing of the primary transcript. We have isolated a nuclear gene, designated *ATP13*, which complements the respiratory defect and restores rutamycin-sensitive ATPase in G95 mutants Disruption of *ATP13* induces a respiratory deficiency which is not complemented by G95 mutants. The nucleotide sequence of *ATP13* indicates a primary translation product with an $M_{r_{pp}}$ of 42 897. The protein has a basic amino terminal signal sequence that is cleaved upon import into mitochondria No significant primary structure homology is detected with any protein in the most recent libraries

Yeast, Mitochondrion, *ATP13*, ATPase, Subunit 9, Translation

1. INTRODUCTION

The F_1-F_o complex of the mitochondrial inner membrane consists of a catalytic unit with the ATP hydrolase activity (F_1) and of a hydrophobic membrane sector (F_o) responsible for the proton translocating properties of the complex [1,2]. In *Saccharomyces cerevisiae*, F_o is composed of 4–5 different subunit polypeptides, three of which are encoded in mitochondrial DNA and translated on endogenous ribosomes [3–5]. The elaboration of this complex depends on a substantial number of nuclear gene products (6–10) whose functions are still poorly understood. One such gene, defined by the *pet* mutant N9-168, was reported to affect the expression of subunit 9 of the ATPase [7]. Even though the mutant was found to have catalytically active F_1, the enzyme was not associated with the normal F_o component of the membrane [7]. In addition, in vivo pulse-labeling of mitochondrial translation products indicated the absence of subunit 9 thereby accounting for the inability of F_o to bind F_1 in the mutant [7].

More recent screens for nuclear petite mutants of yeast, have yielded additional ATPase defective strains with mutations allelic to that of N9-168. These mutants have been assigned to complementation group G95 and the corresponding gene has been named *ATP13* in keeping with the convention used previously for the designation of genes involved in the synthesis of the ATPase [9–14]. In this communication we describe the cloning and sequence analysis of *ATP13*, and report the effect of mutations in the gene on the levels of two mitochondrial transcripts specifying subunits of F_o.

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Abbreviations. *pet* mutant, respiratory deficient mutant of yeast with a genetic lesion in a nuclear gene, ϱ^0 mutant, respiratory deficient mutant of yeast lacking mitochondrial DNA; DBM, diazobenyloxymethyl, kb, kilobase (pairs), bp, base pairs

2. MATERIALS AND METHODS

2.1 *Cloning of* ATP13

The *ATP13* gene was selected from a yeast genomic library by transformation of the mutant N230/U6 (*α,ura3-1,aip13*) by the procedure of Beggs [15]. The library, consisting of partial *Sau*3A fragments of yeast nuclear DNA averaging 5–15 kb ligated to the *Bam*H1 site of YEp24 [16], was obtained from Dr. Marian Carlson (Department of Human Genetics, Columbia University) Transformants complemented for the respiratory defect were selected on minimal glycerol medium containing 1 2 M sorbitol

2.2 *Miscellaneous methods*

Standard procedures were used for restriction endonuclease analysis of DNA, preparation and ligation of DNA fragments, transformation of *E coli* and small and large scale isolation of plasmid DNA [17] DNA was sequenced by chemical derivatization of 5'-end labeled single stranded restriction fragments [18]

Yeast were grown in media containing 2% galactose, 1% yeast extract, and 2% peptone The cells were harvested in early stationary phase and mitochondria were prepared by the procedure of Faye et al [19] except that Zymolyase 20000 (Miles Corp) was substituted for Glusulase during the conversion of cells to spheroplasts Mitochondrial RNA was extracted and subjected to Northern analysis as

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described previously [20]. The probe used to detect subunit 9 transcripts consisted of a 1.6 kb *Hae*III fragment containing the entire *oli1* gene sequence plus 5' and 3' flanking sequences [4]. Subunit 6 transcripts were detected with a 400 bp *Mbo*I-*Eco*RI fragment of mitochondrial DNA spanning nucleotides 295–696 of the *oli2* gene [3]. ATPase activity was measured as previously described [9].

Hybrid selected translation of *ATP13* mRNA was as previously published [21] Procedures for assaying mitochondrial import of proteins translated in programmed rabbit reticulocyte lysate have been published [22] Transport studies were performed with mitochondria prepared from *S. cerevisiae* strain D273-10B.

3. RESULTS AND DISCUSSION

3.1. *ATPase activity of mitochondria from G95 mutants*

Mitochondria from N230 (*α,met6,atp13*), and the derived *ura3* spore, N230/U6, when prepared by a procedure minimizing loss of matrix proteins, exhibit reduced ATPase activity which is not inhibited by rutamycin (Table I). This phenotype is also observed in ϱ_0 mutants lacking functional F_0. When mitochondria are prepared by a more damaging procedure which results in leakage of soluble matrix components, virtually all the ATPase activity of the mutant is recovered in the post-ribosomal supernatant [7]. This property distinguishes *atp13* mutants from other ATPase defective strains that have rutamycin-insensitive membrane-bound F_1 [9].

3.2. *Northern analysis of mitochondrial transcripts*

The absence of subunit 9 in N9-168 [7] could be a consequence either of defective transcription of *oli1* or of altered processing and/or stability of the transcript(s). To help distinguish among these possibilities, total mitochondrial RNA, prepared from N9-168 and from the respiratory competent parental strain D273-10B/A1 (*α,met6*), was analyzed by Northern blot hybridization with probes specific for *oli1* and *oli2* transcripts The results of these analyses indicate that the concentrations of *oli2* transcripts relative to the mitochondrial ribosomal RNAs are not affected in the mutant. The *oli2* probe detects two equally abundant transcripts commensurate in size with the two major RNAs reported to originate from the *aap1-oli2* region of the yeast mitochondrial genome [23] (Fig. 1). The 0.9 kb transcript corresponding to the *oli1* mRNA, however, is 2–3 times lower in the mutant than in the wild type (Fig. 1). In addition the mutant mitochondria appear to have higher levels of larger transcripts that are probably partially processed precursors of the *oli1* mRNA [24]. The detection of significant steady state levels of both mature size and partially processed *oli1* transcripts in N9-168 tends to argue against a role of the *ATP13* product in transcription of the gene. A block in processing is more difficult to exclude in view of the increased concentrations of precursor RNAs. However, the inability of the mutant to synthesize subunit 9 as evidenced by the results of in vivo pulse labeling of mitochondrial translation products [7] and the lack of measurable rutamycin-sensitive ATPase despite the presence of 20–30% processed *oli1* transcript (Table I) suggest that the principal effect of the mutation is on translation of the subunit 9 mRNA and that the higher concentrations of precursors may be secondary to the translational block. The product of *ATP13*, therefore, may have a function similar to protein factors that have been reported to promote translation of the specific mRNAs coding for subunits of cytochrome oxidase [25–27] and cytochrome *b* [28,29].

Table I

Mitochondrial ATPase activities

Strain	minus rutamycin	plus rutamycin
D273-10B/A1	5 7	0 8
D273-10B/A1ϱ^0	4.3	4.3
N230	1 2	0 9
N230/U6	2 6	2 6

Mitochondria were prepared from yeast spheroplasts and resuspended in 10 mM Tris-Cl, pH 7 5, to a final concentration of 8–15 mg/ml Rutamycin was used at a concentration of 10 μg/ml Specific activity is reported as unit/mg protein. A unit of ATPase activity is defined as that amount of enzyme that hydrolyzes 1 μmol of ATP per minute under the specified conditions

3.3. *Cloning and characterization of* ATP13

To clone the *ATP13* gene, the *atp13* mutant N230/U6 (*α,ura3-1,atp13*) was transformed with a yeast genomic library consisting of partial *Sau*3A fragment of yeast nuclear DNA ligated to the *Bam*H1 site of YEp24 [16]. The transformation yielded the respiratory competent and uracil independent clone N230/U6/T1. The two phenotypic traits cosegregated during vegetative growth of the transformant indicating that complementation is a function of the presence of an episomal plasmid. This plasmid (pG95/T1) was amplified in *E. coli* and its restriction map determined. Based on the sizes of different restriction fragments the nuclear DNA insert of pG95/T1 was estimated to be approximately 6 kb (Fig. 2). To map the complementing gene, different regions of the insert were transferred to the shuttle vector YEp352 [30] and the new constructs were tested for their ability to complement N230/U6. The results of the transformations suggested that the gene spans the *Sst*I and *Eco*RI sites located 2.2 and 2.5 kb, respectively, from the left hand edge of the insert as depicted in Fig. 2. This region as well as some 1 kb of DNA starting with a *Hin*dIII site upstream of the *Sst*I site and 250 nucleotides beyond the *Eco*RI site was sequenced. All the restriction sites used for 5'-end labeling were crossed from neighboring sites and most of the sequence was confirmed from the complementary strands.

The above region contains a single open reading frame starting with an ATG codon at nucleotide +1 and ending with an amber codon at nucleotide +1117

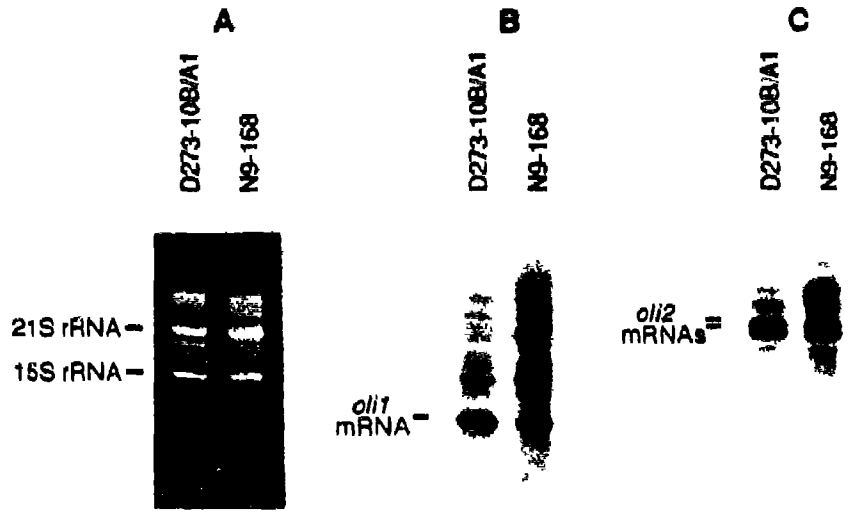

Fig 1 Northern blot analysis of *oli1* and *oli2* transcripts Mitochondrial RNA was prepared from the parental haploid strain D273-10B and from the *atp13* mutant N9-168 Approximately 4 μg of each RNA was separated by electrophoresis on a non-denaturing 1% agarose gel The RNA were stained with ethidium bromide (panel A), transferred by blotting to DBM paper, and hybridized to nick-translated fragments of DNA containing *oli1* (panel B) and *oli2* (panel C') sequences. The identical blot was used with both probes. The mitochondrial 15 S and 21 S ribosomal RNAs are identified in panel A The 0 9 kb subunit 9 mRNA and the two major *oli2* transcripts of 4 5 and 5 1 kb are marked in the margins of panels B and C, respectively

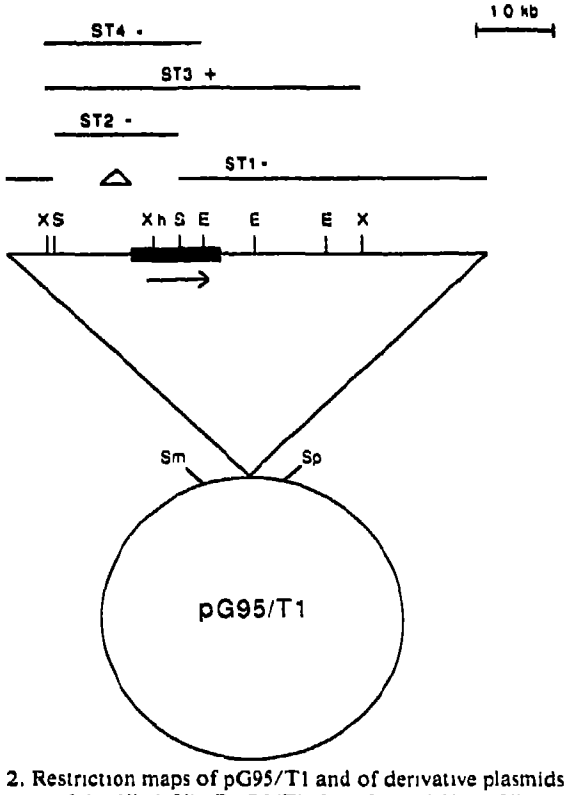

Fig 2. Restriction maps of pG95/T1 and of derivative plasmids The locations of the *Xba*I (X), *Eco*RI (E), *Sst*I (S), and *Xho*I (Xh) sites are marked on the insert of pG95/T1 The orientation of the insert in pG95/T1 is provided by the *Sma*I (Sm) and the *Sph*I (Sp) sites of YEp24 The region of DNA in pG95/ST1 removed by digestion of pG95/T1 with *Sst*I and religated is indicated by the deletion symbol (Δ). The fragments of DNA subcloned in YEp352 are denoted by the bars in the upper part of the figure. Complementation is indicated by the plus sign and lack thereof by the minus sign The location of the reading frame corresponding to *ATP13* is shown by the solid bar and the direction of transcription by the arrow

of the sequence reported in Fig. 3. The encoded protein consists of 372 residues with an M_{app} of 42 897. The results of in vitro import studies indicate the *ATP13* protein to be located in mitochondria. The 1.6 kb *Sst*I DNA fragment containing 65% of the reading frame was used to hybrid select *ATP13* mRNA. Translation of the enriched mRNA in a rabbit reticulocyte lysate yielded a labeled precursor protein of 42 kDa which was imported into mitochondria in a membrane potential-dependent manner (Fig 4). The imported protein was resistant to protease and had a lower molecular weight. The difference in the apparent sizes of the precursor and mature ATP13 protein indicated a transient presequence of 25-28 residues.

The overall composition of the protein is not very hydrophobic, although there are two non-polar regions of sufficient length to qualify as membrane spanning domains. At present we have no direct evidence bearing to indicate whether the protein is associated with the mitochondrial membrane.

Searches of the translated GenBank by the FASTA program [31] failed to reveal any homologous proteins.

3 4. *Disruption of* ATP13

To confirm that the gene cloned in pG95/T1 is *ATP13*, part of the sequence internal to the reading frame was deleted and substituted with the yeast *HIS3* gene. The substitution was effected by the one-step gene replacement method [32] in the wild-type haploid strain W303-1B(*α,ade2-1,his3-11,15,leu2-3,112,ura3-1, trp1-1*). Following transformation of W303-1B with a linear fragment of DNA containing the *atp13::HIS3* allele, clones were selected on minimal medium sup-

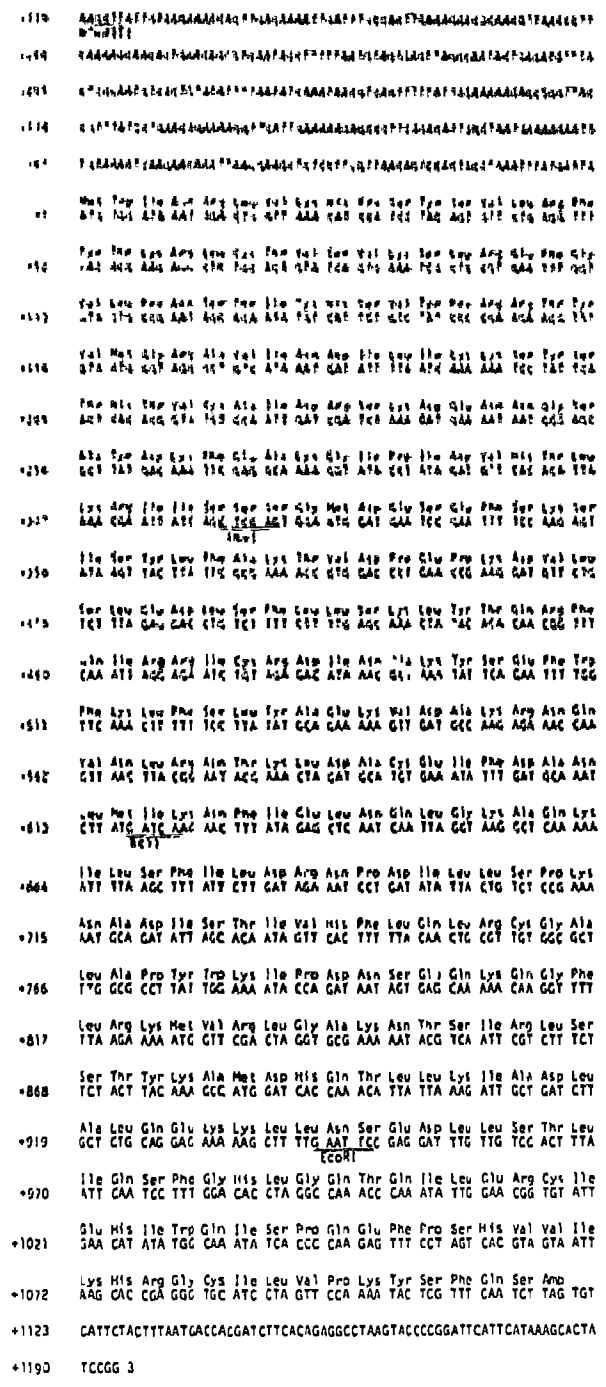

Fig 3 Nucleotide sequence of *ATP13* The sequence shown starts with the nearest *Hin*dIII site upstream of the gene Only the sequence of the sense strand is shown The *ATP13* reading frame starts with the ATG at nucleotide + 1 and ends with the TAG at nucleotide + 1117 The amino acid sequence of the protein encoded by the reading frame is shown above the sequence Some restriction sites have been marked for reference

plemented with all the auxotrophic requirements of W303-1B except histidine.

One of the respiratory deficient transformants (W303ΔATP13) was verified by genomic Southern

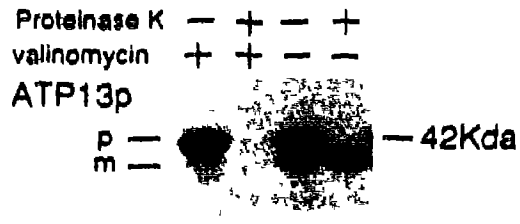

Fig. 4. Mitochondrial import of the *ATP13* translation product. *ATP13* mRNA was prepared by hybrid selection of total LiCl-precipitable yeast RNA. This RNA was prepared from a transformant harboring the complementary gene on a 2 μm plasmid (see Materials and Methods) Following translation of the selected messenger RNA, the labeled protein was incubated with isolated mitochondria in the presence or absence of 5 μM valinomycin. After incubation for 30 min at 30°C, one half of the mixture was treated with Proteinase K (200 μg/ml) on ice prior to polyacrylamide gel electrophoresis. The precursor (p) and mature (m) proteins are identified in the margin

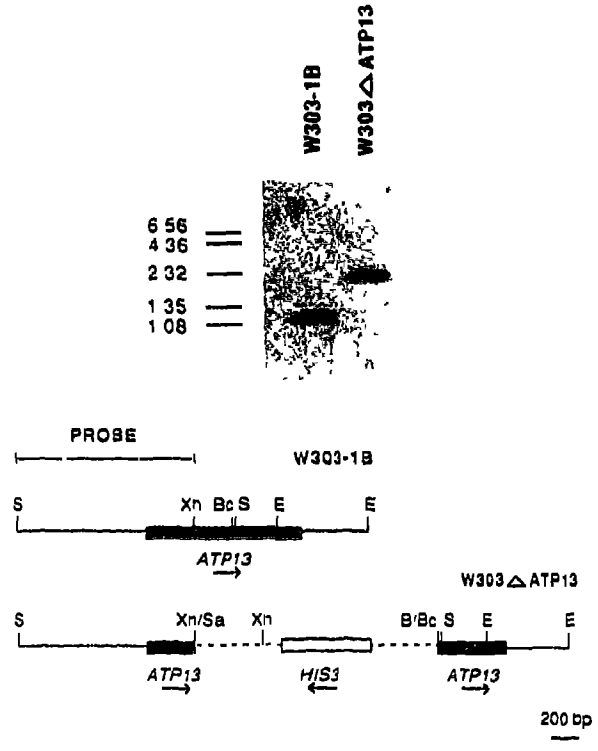

Fig 5 Disruption of *ATP13* The lower part of the figure shows the restriction maps of the wild-type and disrupted *ATP13* gene. The locations of the *Sst*I (S), *Xho*I (Xh), *Bcl*I (Bc) and *Eco*RI (E) sites are indicated on both maps The *ATP13* and *HIS3* genes are denoted by the solid and open bars, respectively The junctions between the *Xho*I and *Bcl*I sites of *ATP13* and the *Sal*I (Sa) and *Bam*HI (B) sites of *HIS3* are indicated with slashes The upper part of the figure shows the results of the Southern analysis Chromosomal DNA was prepared from the W303-1B parental and the W303ΔATP13 mutant strains The two DNAs were digested with *Sst*I and separated by electrophoresis on a 1% agarose gel Following transfer to nitrocellulose, the blot was hybridized to the nick-translated 1 3 kb *Sst*I-*Xho*I fragment (probe) The sizes of the DNA markers are indicated in the margin

analysis to have acquired the *atp13::HIS3* allele. In Fig. 5, the 1.6 kb *Sst*I fragment of wild-type DNA is seen to increase to 3 kb in the mutant consistent with the deletion of 313 bp between the *Bcl*I and *Xho*I sites and replacement of this sequence with the 1.7 kb fragment carrying the *HIS3* gene. Crosses of W303ΔATP13 to G95 mutants failed to produce respiratory competent diploid progeny indicating linkage of the *atp13::HIS3* allele with the mutant gene responsible for the phenotype of this group.

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