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A nuclear mutation prevents processing of a mitochondrially encoded membrane protein in *Saccharomyces cerevisiae*

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Subunit II of cytochrome oxidase is encoded by the mitochondrial *OXI1* gene in *Saccharomyces cerevisiae*. The temperature-sensitive nuclear *pet* mutant ts2858 has an apparent higher mol. wt. subunit II when analyzed on lithium dodecylsulfate (LiDS) polyacrylamide gels. However, on LiDS-6M urea gels the apparent mol. wt. of the wild-type protein exceeds that of the mutant. Partial revertants of mutant ts2858 that produce both the wild-type and mutant form of subunit II were isolated. The two forms of subunit II differ at the N-terminal part of the molecule as shown by constructing and analyzing nuclear ts2858 and mitochondrial chain termination double mutants. The presence of the primary translation product in the mutant and of the processed form in the wild-type lacking 15 amino-terminal residues was demonstrated by radiolabel protein sequencing. Comparison of the known DNA sequence with the partial protein sequence obtained reveals that six of the 15 residues are hydrophilic and, unlike most signal sequences, this transient sequence does not contain extended hydrophobic parts. The nuclear mutation ts2858 preventing post-translational processing of cytochrome oxidase subunit II lies either in the gene for a protease or an enzyme regulating a protease.

Key words: cytochrome oxidase subunit II/mitochondrial translation product/NH_2-terminal presequence/post-translational proteolysis/*Saccharomyces cerevisiae*

Introduction

Mitochondrial and nuclear genes cooperate in the biogenesis of functional mitochondria. The mitochondrial inner membrane contains at least three enzyme complexes consisting of subunits made on mitochondrial ribosomes, as well as subunits made on cytosolic ribosomes. Such oligomeric membrane proteins are mitochondrial oligomycin-sensitive ATPase, cytochrome bc_1 complex and cytochrome oxidase. For the latter enzyme the three largest subunits are of mitochondrial origin, whereas the four smaller subunits are encoded by nuclear genes. Therefore, a tight regulation between the two genetic systems must exist. In yeast, the mitochondrial DNA and its gene products are fairly well characterized, but little is known about their regulation and interaction with the nuclear genes. To study these nuclear-mitochondrial interactions, the mitochondrial translation products of 313 nuclear temperature-sensitive *pet* mutants were analyzed (Michaelis *et al.*, 1982). Here we describe a nuclear temperature-sensitive *pet* mutant leading to the accumulation of a precursor to subunit II of cytochrome oxidase. The precursor and mature cytochrome oxidase subunit II were characterized using genetic and protein chemical methods. A comparison of the N-terminal protein sequence of subunit II in wild-type and mutant allows us to establish the presequence.

Results

Mitochondrial translation products of mutant ts2858

Mutant ts2858 belongs to a large collection of nuclear temperature-sensitive *pet* mutants which can grow on lactate at 23°C but not at 36°C (Schweizer *et al.*, 1977). Analyzing the products of mitochondrial protein synthesis by lithium dodecylsulfate (LiDS)-polyacrylamide gel electrophoresis, cytochrome oxidase subunit II of mutant ts2858 shows a slightly decreased mobility compared with wild-type (Figure 1).

In a mixture of mutant and wild-type mitochondrial proteins the two forms of subunit II separated into two bands although the mobility difference is very small.

Because of the decreased mobility in a LiDS-polyacrylamide gel, mutant subunit II seems to have a slightly higher mol. wt. Surprisingly, the presence of 6 M urea in the LiDS-polyacrylamide gel changes the electrophoretic mobility of the mitochondrial proteins drastically. The order of the two forms of cytochrome oxidase subunit II is reversed. Whereas the mutant form of subunit II migrates more slowly than the wild-type form in a LiDS-polyacrylamide gel, it migrates faster than the wild-type form if the LiDS-polyacrylamide gel contains 6 M urea (Figure 2).

Genetic characterization of mutant ts2858

To ensure that the mutant phenotype is due to a single nuclear mutation and not to an additional change in the mitochondrial structural gene of cytochrome oxidase subunit II, a tetrad analysis was carried out. Mutant ts2858 was crossed with a wild-type strain and the resulting diploids were

Table I.

Strain	Nuclear genotype	Mitochondrial genotype	References or origin
Sc167	α ade1	ϱ^+	E.Schweizer
ts2858	α ade1 pet^{ts}_{2858}	ϱ^+	E.Schweizer; derived from Sc167
ts2858/R7	α ade1	ϱ^+	revertant of ts2858, this study
ts2858/R100	α ade1	ϱ^+	partial revertant of ts2858, this study
777-3A/P422	α ade1 op1	oxi1	this study
777-3A/P430	α ade1 op1	oxi1	this study
KL14-4A/2	a his1 trp2	ϱ°	Pratje *et al.*, 1979
KL14-4A/B1	a his1 trp2	ϱ^-	restores *oxi1* mutations, this study
JC8/AA1	a kar1 leu1	ϱ°	Lancashire and Mattoon, 1979
GM62	a his1 trp2 + + / α + + ade1 pet^{ts}_{2858}	ϱ^+	diploid from cross ts2858 x KL14-4A/2

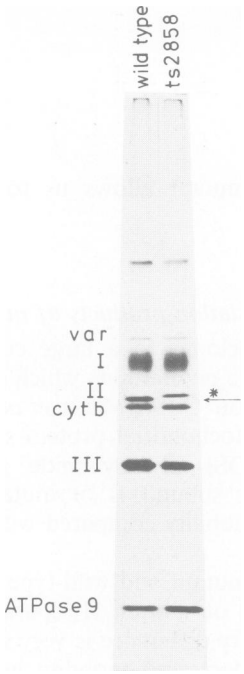

Fig. 1. Mitochondrial translation products of wild-type and mutant ts2858. After an incubation period of 14 h at 36°C, cells were labeled with $^{35}SO_4^{2-}$ in the presence of cycloheximide. Mitochondrial proteins were analyzed on a 10–15% LiDS-polyacrylamide gradient gel and visualized by fluorography as described in Materials and methods. Cytochrome oxidase subunit II from wild-type is indicated by an arrow, from mutant ts2858 by an asterisk.

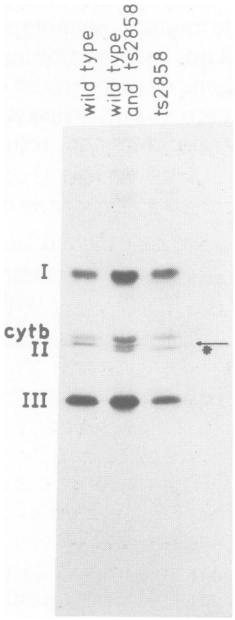

Fig. 2. Fluorogram of a 14% LiDS-polyacrylamide gel containing 6 M urea. In this gel system the mutant form (*) of subunit II of cytochrome oxidase migrates faster than the wild-type protein (←).

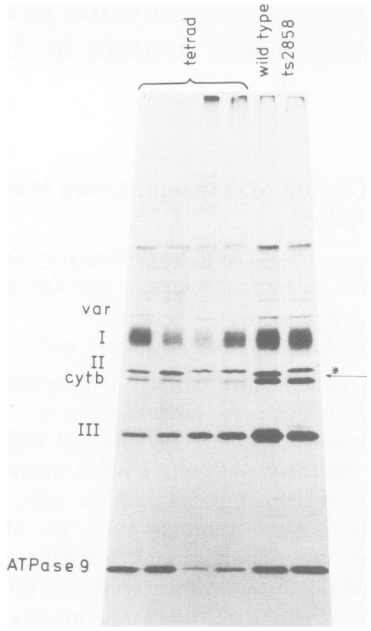

Fig. 3. Tetrad analysis of the nuclear ts2858 mutation. Diploids produced by mating of mutant and wild-type KL14-4A/2 cells were sporulated. The mitochondrially synthesized polypeptides of the four spores of one tetrad were analyzed on a 10–15% LiDS-polyacrylamide gradient gel. Only the two temperature-sensitive spores contained the mutant form of subunit II. An arrow indicates the wild-type form of subunit II, and an asterisk indicates the mutant form.

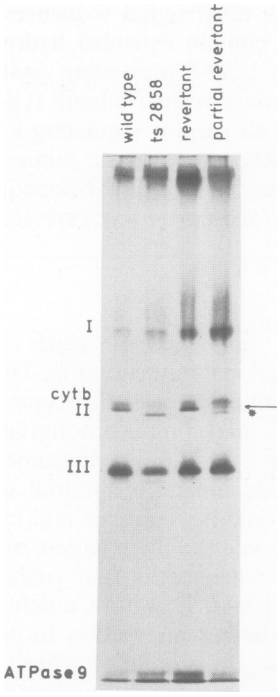

Fig. 4. Mitochondrially translated polypeptides in revertants of ts2858. The labeled proteins were analyzed on a 14% LiDS-polyacrylamide gel containing 6 M urea. An arrow indicates the wild-type form of cytochrome oxidase subunit II, and an asterisk the mutant form.

sporulated. The temperature-sensitive phenotype displayed a regular 2:2 segregation and only the temperature-sensitive spores transmitted the changed mobility of cytochrome oxidase subunit II (Figure 3). This establishes that one single nuclear mutation transfers temperature sensitivity as well as a modification of subunit II to the cell. Revertants could be isolated with a frequency of $\sim 5 \times 10^{-7}$. In two revertants analyzed, the mutant form of cytochrome oxidase was replaced by the wild-type polypeptide. In addition, partial rever-

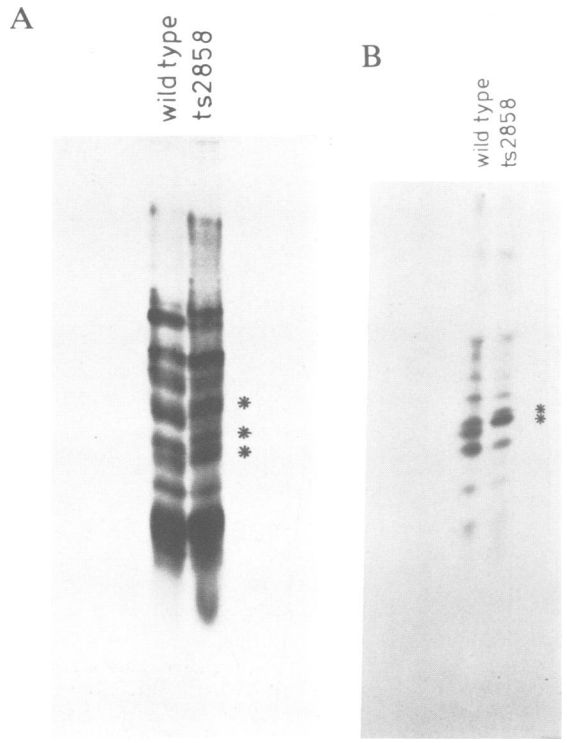

Fig. 5. Partial proteolysis of mutant and wild-type subunit II of cytochrome oxidase with 50 μg trypsin **(A)** or 50 μg *S. aureus* V8 protease **(B)**. Labeled cytochrome oxidase subunits II were excised from a polyacrylamide gel and subjected to partial proteolysis directly on the stacking gel of a 15–20% LiDS-polyacrylamide gradient gel as described in Materials and methods. Digestions were performed for ~4.5 h at 40 V without switching off the current. Asterisks indicate the proteolytic fragments of mutant subunit II shifted to a higher apparent mol. wt.

tants were found that produced small colonies at 36°C and contained both forms of subunit II (Figure 4).

Limited proteolysis of subunit II of cytochrome oxidase

Mutant and wild-type subunits II were subjected to partial proteolysis and the digests were analyzed on LiDS-polyacrylamide slabs. Both subunits II show a similar digestion pattern after limited proteolysis with trypsin, but three of the mutant polypeptides are shifted to an apparent higher mol. wt. (Figure 5A). After digestion with *Staphylococcus aureus* V8 protease, two fragments of the mutant subunit II migrate more slowly in the gel than those of the wild-type (Figure 5B). Thus, the difference between the two cytochrome oxidase subunits II could also be demonstrated with their proteolytic fragments.

The N-terminal part of subunit II of cytochrome oxidase carries the determinant for the altered migration

Which part of the mutant subunit II molecule is responsible for the decreased electrophoretic mobility in an LiDS-polyacrylamide gel? To answer this question we analyzed the effect of the nuclear ts2858 mutation on fragments of subunit II, created by nonsense mutations in the mitochondrial structural gene *OXI1*. *Oxi1* mutants were isolated in which subunit II was replaced by a shorter fragment, probably due to a nonsense mutation. Two of these *oxi1* mutants, P422 and P430, were crossed to mutant ts2858 and ts2858 *oxi1* double mutants were constructed. If the determinant for slower migration is localized at the C-terminal part of the polypeptide the electrophoretic behavior of the mitochondrial translation products should be identical in the *oxi1* and ts2858

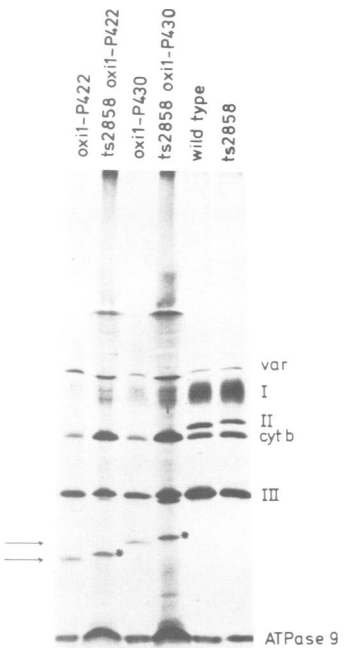

Fig. 6. Analysis of the mitochondrial translation products of *oxi1* and ts2858 *oxi1* double mutants. Construction of the double mutants and labeling conditions are described in Materials and methods. The proteins were analyzed on a 10–15% LiDS-polyacrylamide gradient gel. Arrows indicate the short fragments replacing subunit II in the *oxi1* mutants, and asterisks the fragments synthesized in the ts2858 *oxi1* double mutants.

oxi1 double mutants. On the other hand, if the N-terminal part of the polypeptide is involved, the fragments of subunit II of cytochrome oxidase might be shifted to a slightly higher apparent mol. wt. in the ts2858 *oxi1* double mutants. Figure 6 illustrates the results. The fragments of the two *oxi1* mutants display a faster electrophoretic mobility than the fragments of the respective double mutants.

These experiments demonstrate that the N-terminal part of subunit II of cytochrome oxidase is involved in the determinant for altered migration and that even fragments of subunit II are substrates for the processing activity.

Partial radiolabel sequence analysis of the N terminus of mutant and wild-type cytochrome oxidase subunit II

The two forms of subunit II might be the result of a protein modification, a second ribosomal initiation site or a processing of a higher mol. wt. precursor. To distinguish between these three alternatives, protein sequencing studies were performed. The two radioactive subunits II, extracted from a polyacrylamide gel, were subjected to automated sequence analysis in a liquid phase sequencer as described by Beyreuther *et al.* (1980). The positions of the ^{35}S-labeled methionines and cysteines could be determined by the radioactivity recovered at each sequencer cycle. From the published DNA sequence (Coruzzi and Tzagoloff, 1979; Fox, 1979), release of ^{35}S label is predicted at positions 1, 14 and 23, assuming the first ATG as the initiation codon. If the second ATG codon in position 14 of the *OXI1* open reading frame is considered as initiation site, radioactivity is expected in the 1st, 10th and 31st step of Edman degradation. The lack of a radioactivity peak at position 1 in wild-type subunit II of cytochrome oxidase would be expected if demethionylation occurs. The results of the sequence analysis are shown in Figure 7. In the mutant protein, radioactive signals were

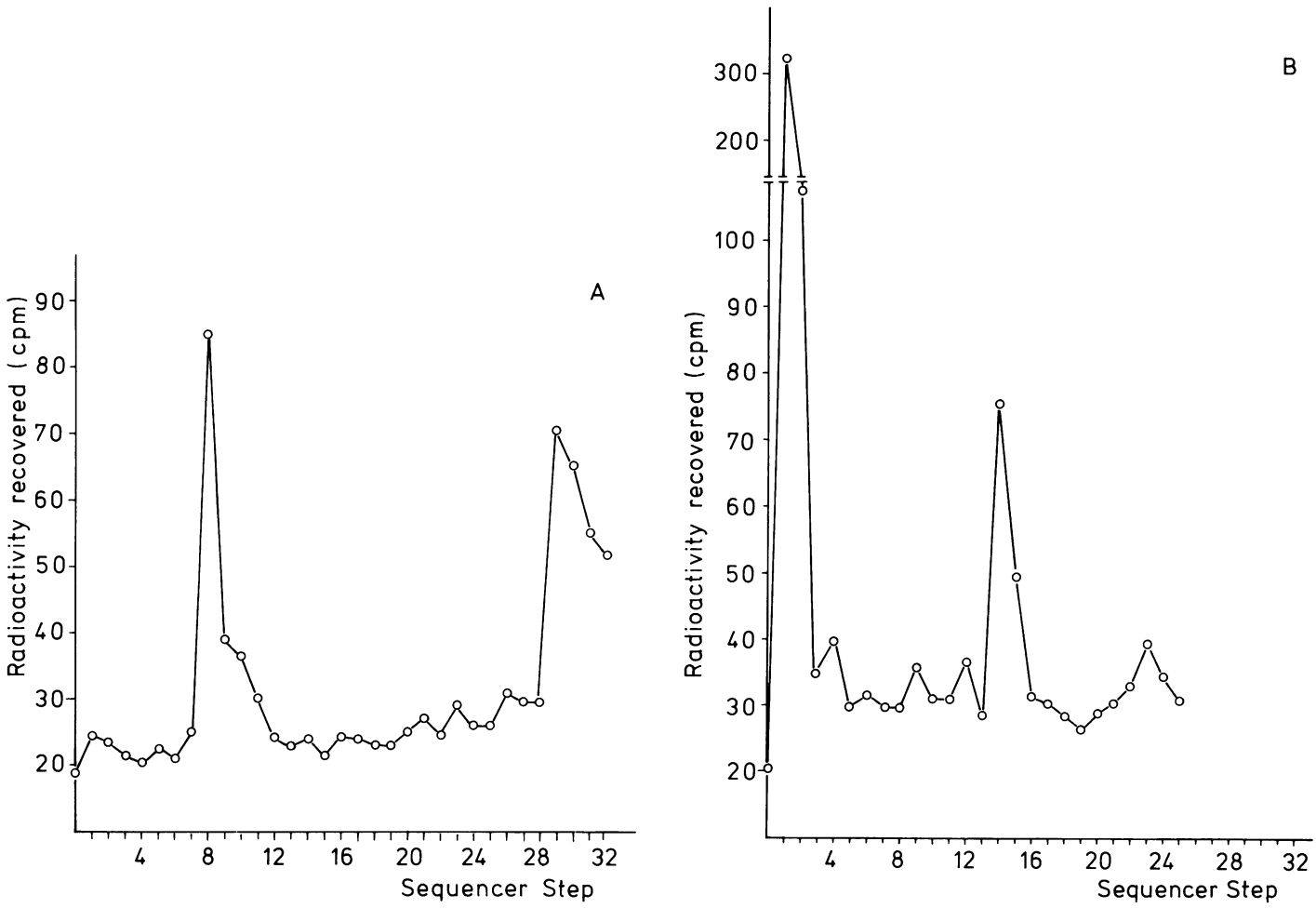

Fig. 7. Radiolabel sequence analysis of cytochrome oxidase subunit II from wild-type **(A)** or mutant ts2858 **(B)**. The ^{35}S-labeled subunits were subjected to automated Edman degradation in the presence of non-radioactive carrier proteins (Beyreuther *et al.*, 1980). Each degradation was started with a blank cycle in the sequencer. **A** shows the average of the radioactivity released at each degradation step of two experiments (20 000 c.p.m. and 148 000 c.p.m. in the two samples analyzed from wild-type). **B** shows the average of the radioactivity released at each degradation step of two experiments (20 000 c.p.m. and 36 000 c.p.m. in the two samples analyzed from mutant subunit II).

Table II. N-terminal amino acids of subunit 2 of cytochrome oxidase

beef heart protein																									fMet-	Ala-	Tyr-	Pro-	Met-	Gln-	Leu-	Gly-	Phe-	Gln-	Asp-
Neurospora protein																									Asp-	Ala-	Pro-	Ser-	Pro-	Trp-	Gly-	Ile-	Tyr-	Phe-	Gln-	Asp-
yeast DNA	Met-	Leu-	Asp-	Leu-	Leu-	Arg-	Leu-	Gln-	Leu-	Thr-	Thr-	Phe-	Ile-	Met-	Asn-	Asp-	Val-	Pro-	Thr-	Pro-	Tyr-	Ala-	Cys-	Tyr-	Phe-	Gln-	Asp-			
^{35}S peaks from ts2858	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–				
^{35}S peaks from wild-type																								–	–	–	–	–	–	–	+	–	–	–	–

The data are obtained from the amino acid sequence of beef heart (Steffens and Buse, 1979) and of *N. crassa* (Machleidt and Werner, 1979), from the DNA sequence of yeast (Coruzzi and Tzagoloff, 1979; Fox, 1979), and from the radiolabel protein sequence analysis of yeast wild-type and mutant ts2858 (this study).

found at positions 1, 14 and 23. These assignments are in full agreement with the amino acid sequence derived from the DNA sequence.

Wild-type subunit II of cytochrome oxidase does not start with methionine, because radioactivity is lacking at the first step corresponding to position 1. The first radioactive derivative was released at the 8th degradation step, and a second at the 29th degradation step. These data fit best with the DNA sequence, if the first amino acid of the wild-type protein corresponds to the 16th codon of the *OXI1* open reading frame (Table II). Therefore, the mutant subunit II seems to represent a precursor form of cytochrome oxidase subunit II and this precursor is processed in the wild-type by cleaving off the N-terminal 15 amino acids. The processing is prevented by the nuclear mutation ts2858. In conclusion, the presequence of cytochrome oxidase subunit II could be defined from the known DNA sequence and the starting point of the wild-type protein. Accordingly, the presequence consists of the 15 amino acids H_2N-Met-Leu-Asp-Leu-Leu-Arg-Leu-Gln-Leu-Thr-Thr-Phe-Ile-Met-Asn- and displays a mol. wt. of 2074.

Discussion

In all organisms studied so far, cytochrome oxidase subunit II is coded for by the mitochondrial genome. In the present

work we have shown that this subunit is synthesized as an N-terminal extended precursor in the yeast, *S. cerevisiae*. Radiolabel protein sequence analysis proved that subunit II accumulating in the nuclear mutant ts2858 contains a 15 amino acid presequence. The nuclear mutation prevents the processing of this precursor to the mature wild-type polypeptide. Indirect evidence for the existence of a precursor of yeast cytochrome oxidase subunit II was reported by Sevarino and Poyton (1980). They characterized a variant of cytochrome oxidase subunit II which accumulates while incubating isolated wild-type mitochondria with aurintricarboxylic acid. These authors interpreted the decreased electrophoretic mobility of the variant form in terms of an N-terminal extended precursor. However, changed electrophoretic mobilities of subunit II in SDS-gels have been found in revertants of *oxi1* mutants. These revertant proteins were characterized by amino acid substitutions and displayed mol. wts. more or less identical with the wild-type molecule (Cabral *et al.*, 1978; Fox, 1979; Weiss-Brummer *et al.*, 1979). If the mol. wts. of membrane proteins such as subunit II are calculated from the electrophoretic mobility in an SDS-gel precautions are necessary. Although the precursor form of subunit II displays a mol. wt. 2074 higher than that of the wild-type subunit, it migrates faster in a polyacrylamide gel in the presence of LiDS and 6 M urea. This effect may be due to increased LiDS binding of the completely unfolded chain or leader sequence.

Evidence for a N-terminal extended precursor for subunit II of cytochrome oxidase in *Neurospora crassa* was published by Machleidt and Werner (1979). They analyzed the N-terminal amino acid sequence of subunit II from *Neurospora* which begins with aspartic acid and not with N-formylmethionine, suggesting that a precursor might be processed to the mature subunit II. Recent DNA sequence studies by van den Boogaart *et al.* (1982) support this conclusion.

If the N-terminal amino acid sequence of the mature subunit II from *Neurospora* and yeast are compared, both proteins start with aspartic acid (Table II) and show a striking homology: 26 out of the first 39 amino acids are identical. A presequence of cytochrome oxidase subunit II is definitely lacking in the beef heart polypeptide because this protein starts with N-formyl-methionine (Steffens and Buse, 1979).

The occurrence of mitochondrially synthesized precursor proteins is not restricted to subunit II of cytochrome oxidase. In *N. crassa*, an elongated precursor of subunit I of cytochrome oxidase was analyzed by Werner and Bertrand (1979) and van't Sant *et al.* (1981). Presequences play an important role in the transport of proteins across membranes (Neupert and Schatz, 1981). What could be the biological function of a mitochondrially synthesized precursor protein which is not transported out of the organelle? It is possible that the extended sequence might be necessary for integrating and fixing the protein into the inner mitochondrial membrane and for the assembly of the enzyme complex. This idea is supported by the fact that the precursor of cytochrome oxidase subunit II can be integrated into a functional enzyme complex in yeast (Michaelis *et al.*, 1982). On the other hand, the lack of a precursor form of cytochrome oxidase subunit II from beef heart demonstrates that an enzyme assembly is possible without a transient presequence in bovines.

This paper describes the first complete presequence of a mitochondrially synthesized polypeptide. It is composed of 15 amino acids which are not typical in composition for a signal sequence. Six of them are polar, resulting in a polarity of 40% for the presequence. Therefore, the precursor and the mature subunit II display similar polarities of 37.5% and 37.3%, respectively (calculated according to Capaldi and Vanderkooi, 1972). It would be interesting to discover whether the presequence is cleaved by a protease located in the matrix, the inner membrane or the intermembrane space. Discrepancies exist between the amino acid composition of the mature subunit II of cytochrome oxidase from yeast (Poyton and Schatz, 1975) and the amino acid composition predicted from the DNA sequence (Coruzzi and Tzagoloff, 1979; Fox, 1979). These differences cannot be explained by our finding of a presequence. Mutant ts2858 was very useful for characterizing the precursor to subunit II. This mutation seems to inactivate a nuclear gene, coding either for a protease or an enzyme regulating a protease, which is now under study.

Materials and methods

Yeast strains

Strains of *S. cerevisiae* used in this study are listed in Table I.

Media

Strains were grown in the following media: YPGA = 1% Bacto-yeast extract, 1% Bacto-peptone, 2% glucose, 0.002% adenine sulfate, solidified with 2.3% Bacto-agar for plates; YPGalA = 2% glucose of the YPGA medium was replaced by 2% galactose; YPlac = 1% Bacto-yeast extract, 1% Bacto-peptone, 1% ethanol, 2% lactic acid pH 6.0, solidified with 2.3% Bacto-agar for plates; WO = 0.67% Bacto-nitrogen base free of amino acids, 2% glucose, 50 mM sodium-potassium phosphate buffer pH 6.25, solidified with 2.3% Bacto-agar.

Construction of ts2858 oxi1 double mutants

ts2858 *oxi1* double mutants were constructed by cytoduction using the *kar1* mutation (Lancashire and Mattoon, 1979). Mutants 777-3A/*oxi1* (αade1 oxi1) were mated with strain JC8/AA1 (a leu1 kar1 ϱ°). JC8 *oxi1* colonies (a leu1 kar1 oxi1) of intermediate size were selected on WO plates supplemented with 30 μg/ml leucine. These colonies were mated with ts2858 ϱ° (αade1 pet^{ts} ϱ°). The genotype of haploid ts2858 *oxi1* colonies (αade1 pet^{ts} oxi1) was verified by restoration tests with petite KL14-4A/B1 retaining the wild-type *OXI1* gene.

Sporulation

Published methods were used for tetrad analysis (Pratje *et al.*, 1979).

Biochemical analysis of mitochondrial translation products

Mitochondrial translation products were specifically labeled with $^{35}SO_4^{2-}$ in the presence of cycloheximide after a 14 h incubation period at 36°C. Isolation of mitochondria and the analysis by 10–15% exponential gradient gels of LiDS-polyacrylamide were carried out by the procedure of Douglas and Butow (1976) with the modifications described previously (Pratje *et al.*, 1979). Some gels contained 6 M urea, in addition to LiDS.

Proteolytic fingerprints

Partial digestions with 50 μg *S. aureus* V8 protease (Miles) or 50 μg trypsin (Boehringer) were performed directly on a 15–20% LiDS-polyacrylamide gradient gel using the methods of Cleveland *et al.* (1977), with the modifications described by Claisse *et al.* (1980).

Protein sequence analysis

After electrophoresis and autoradiography, labeled cytochrome oxidase subunit II was excised from dried, unstained gels. The proteins were eluted electrophoretically from the gel slices into a dialysis tube (Walker *et al.*, 1982). After the addition of 50 μg cytochrome c, the proteins were precipitated with 9 volumes of acetone and dried. Sequencing of the proteins was performed by the method of Beyreuther *et al.* (1980).

Acknowledgements

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