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The C-terminal domain of yeast cytochrome *b* is essential for a correct assembly of the mitochondrial cytochrome bc_1 complex

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Yeast mutants modifying the C-terminal region of mitochondrial cytochrome *b* were isolated and characterized. A nonsense mutation of the leucine codon 335 (TTA → TAA), 50 residues before the normal C-terminus, blocks incorporation of heme into the apocytochrome *b* and prevents growth on non-fermentable substrates. The same defects were observed in a frameshift mutant (after codon 348, TAT → TATT) in which the last 37 C-terminal residues are predicted to be replaced by a novel sequence of 33 amino acids. Function was regained in the nonsense mutant only by true back mutations restoring a protein of the wild-type sequence. The respiratory capacity was restored to wild-type levels in the frameshift mutant by a variety of single base subtractions located within a window of 24 bases before or after the original +T addition, these pseudo-reversions resulted in single or multiple (up to five) consecutive amino acid replacements between positions 346 and 354 and restored the wild-type sequence from position 355 to 385. These data, combined with hydropathy calculations and sequence comparisons, suggest that the C-terminal domain of cytochrome *b* forms a transmembrane segment essential for the correct assembly of the cytochrome bc_1 complex

Cytochrome bc_1 complex assembly; Yeast; Mutation; Mitochondrion; Genetics

1. INTRODUCTION

Hydropathy and amphipathy calculations indicate that the cytochrome *b* subunit of the cytochrome bc_1 complex form eight transmembrane helices [1–3]. The membrane location of the first seven helices has been supported by *pho*A fusion experiments [4] and extensive mutational studies [5–10] and di Rago et al., (in preparation) but no such supporting evidence has been provided until now for helix 8. The C-terminal tail of cytochrome *b* has been very poorly conserved during evolution of mitochondria and has no equivalent in chloroplasts where the shorter homologous protein of the cytochrome b_6f complex is predicted to form seven transmembrane segments only. It may therefore be asked whether the C-terminal tail of mitochondrial cytochrome *b* is essential for the functioun of the bc_1 complex and whether it really forms a transmembrane segment.

This question has been addressed in the present work by the search of yeast mutants modifying the structure of the apocytochrome *b*. First, we have asked whether respiratory-deficient mutants located in the C-terminal region could be found. Two such mutants were isolated. The first one resulted from a nonsense mutation (TTA → TAA in codon 335) 50 residues before the normal C-terminus; the second one is a frameshift mutant (+T after codon 348) in which helix 8 would be replaced by a non-hydrophobic amino acid sequence. We then asked whether the respiratory capacity could be recovered by these mutants by a second mutation not restoring the wild-type sequence of the protein. Only true back mutants were found amongst revertants issued from the nonsense mutant, whereas the function was fully regained by the frameshift mutant upon a variety of pseudo wild-type reversions leading to single or multiple (up to five) consecutive amino acid replacements in the vicinity of the original mutation. These data, combined with hydropathy calculations and sequence comparisons, provide evidence that the C-terminal tail of cytochrome *b* forms a transmembrane segment essential for the correct assembly of the cytochrome bc_1 complex.

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2. MATERIALS AND METHODS

2.1. *Construction of strains YL10/1-1B and YL11/1-8*

By a series of constructions beginning with the strain devoid of mitochondrial introns [11] in which we have isolated mitochondrial mutations conferring erythromycin-resistance (E^R, in the 23 rRNA gene) and oligomycin-resistance (O^R, in the ATPase subunit 9 gene, [12]) we have introduced, by crosses, the nuclear *op1* mutation (in the *AAC2* gene [13,23]) and the mitochondrial introns of the COXI gene. Strain YL10/1-1B has the nuclear genotype *MATα*, *ade1*, *op1*, and the mitochondrial genotype E^R, O^R, ail^+, while strain YL11/1-8 has the nuclear genotype *MATα*, *lys2*, *op1* and the mitochondrial genotype

E^R, O^R, ai2$^+$. Both strains are devoid of all remaining mitochondrial introns, including those of the cytochrome *b* gene.

2.2. *Media*

The media used in this study were described in [16].

2.3 *Cytochrome spectral analysis*

This was performed with whole cells grown for 2 days at 28°C on YPGAL plates. Spectra were recorded at liquid nitrogen temperature, with a Cary 128 spectrophotometer after reduction of the cytochromes by dithionite as described in [17].

2.4. *Isolation of the* mit$^-$ *cytochrome* b *mutants*

Mit$^-$ respiratory growth-deficient mutants were derived by Mn^{2+} mutagenesis from the haploid strains YL10/1-1B and YL11/1-8 using procedures described in [18,24]. Mutations belonging to the cytochrome *b* gene were identified by deletion mapping with the use of a battery of well-defined *rho*$^-$ tester mutants retaining different regions of the gene (see [10,14,15] for their characteristics). The respiration of the two *mit*$^-$ mutants described in this study (YL11/1-8/F16 and YL11/1-8/F17) was restored by crosses with a *rho*$^-$ clone (SD12/r96/1) covering the whole cytochrome *b* gene and a *rho*$^-$ (KL14-4A/P11) retaining the 3′ region only (intervals 24–39 of the genetic map of the gene), and was not restored by a series of *rho*$^-$ clones covering the beginning and middle portions of the gene. Therefore the mutations are allocated to the interval 24–39 of the genetic map of cytochrome *b*.

2.5. *Isolation of revertants*

The *mit*$^-$ mutants YL11/1-8/F16 and YL11/1-8/F17 were crossed to a strain devoid of mitochondrial DNA (KL14-4A/51 *Mata OPI trp2 his1* [*rho*°]) and the resulting diploids selected. Respiratory competent revertants were isolated from the diploid mutants using procedures described in [16].

2.6 *RNA sequencing*

RNA was sequenced by primer extension with avian myeloblastosis virus reverse transcriptase (Amersham Corp.) from total mitochondrial RNA as described in [19].

2.7 *Western analysis of total mitochondrial proteins*

Total mitochondrial proteins were prepared as described in [19]. They were resolved by SDS-PAGE (12% gel) and then electro-transferred onto nitrocellulose. The blots were pre-incubated with 5% non-fat milk powder in PBS, and immunserum was used at a dilution of 1 1,000 The protein bands were detected using anti-rabbit coupled to horseradish peroxidase in a 1·2,000 dilution (Amersham), then revealed with the ECL-KIT system from Amersham and exposed with Amersham hyperfilm for 1 min. The polyclonal antibodies directed against yeast cytochrome *b* were kindly provided by Dr. G. Getz, Chicago.

3. RESULTS

3.1. *Isolation of yeast respiratory-deficient mutants located in the C-terminal domain of cytochrome* b

Respiratory growth-deficient *mit*$^-$ cytochrome *b* mutants were isolated from a strain carrying the nuclear *op1* mutation and an intronless mitochondrial genome. *Rho*$^-$ mtDNA deletion mutations which would normally appear at very high frequency after Mn^{2+} mutagenesis are lethal in the presence of the *op1* mutation and are thereby counter-selected [13,18]. The absence of introns in the gene coding for cytochrome *b* avoids the selection of mutations leading to mRNA maturation deficiency.

Individual clones (8,200) resulting from Mn^{2+} mutagenesis of the parental strains, YL10/1-1B and YL11/1-8, were screened for respiratory growth-deficient mutations located in the mitochondrial DNA. To this end, each clone was crossed with a tester strain of opposite mating type carrying the *OPI*$^+$ wild-type allele and devoid of mitochondrial genome (KL14-4A/51: *MATa*,

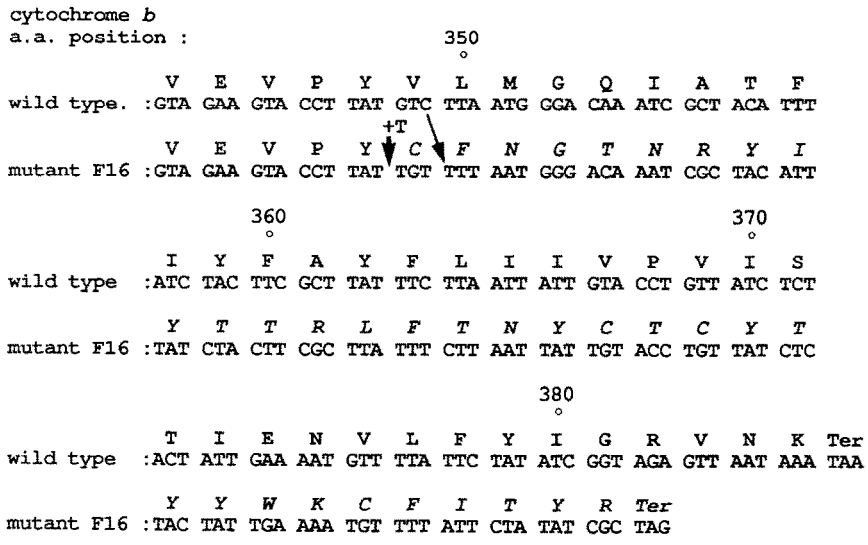

Fig. 1. Sequence of the 3′ region of the cytochrome *b* gene from the wild-type, YL11/1-8, and the respiratory-deficient mutant, YL11/1-8/F16. The nucleotide sequences were determined by reverse transcription of the cytochrome *b* mRNA. The corresponding amino acids (in the one letter code) and their positions are indicated. The mutant, YL11/1-8/F16, carries two nucleotide changes (indicated by arrows), a single +T addition between codons 348 and 349, and a C → T transition in codon 349 Consequently, the last 37 C-terminal amino acids of the wild-type sequence are predicted to be replaced in the mutant by a novel sequence of 33 residues (in *italics*). *G*, guanine; *A*, adenine; *U*, uracil; *C*, cytosine

his1, *trp2* [*rho*°]). The resulting prototrophic diploids were tested for respiratory growth; those which failed to grow were further tested by test-crosses with specific *rho*⁻ clones in order to localize the mitochondrial mutation (see section 2). Of 230 of such clones (about 3%), eight mapped to the cytochrome *b* gene. Two of these (YL11/1-8/F17 and YL11/1-8/F16) were located within the 3′ region of the cytochrome *b* gene, between codons 253 and 385 (interval 24–39 of the cytochrome *b* genetic map).

The mutant, YL11/1-8/F17, exhibited a single T → A transversion leading to replacement of Leu-335 by a stop codon (TTA → TAA). The mutant, YL11/1-8/F16, exhibited two nucleotide changes, a single +T addition between codons 348 and 349 and a C → T transition in codon 349 (Fig. 1). A stop codon (TAG) is found in-frame 33 residues downstream from the initial frameshift, and thus only four residues before the normal C-terminus. Consequently, the C-terminal tail of cytochrome *b* is predicted to be replaced in this mutant by a completely different amino acid sequence rich in polar residues when compared to the corresponding wild-type sequence (Fig. 2).

Both mutants had a stringent respiratory-deficient phenotype: they did not exhibit any growth on non-fermentable substrates even after prolonged incubation periods (up to 3 weeks). Cytochrome spectral analysis of whole cells revealed the almost complete absence in both mutants of dithionite-reducible cytochrome *b*, whereas cytochromes *c* and aa_3 were clearly detectable (Fig. 3). Western blot analysis of total mitochondrial proteins failed to detect apocytochrome *b* in the frameshift mutant: we believe (see section 4) that the absence of immunological signal is due to increased protease sentivity of the modified protein.

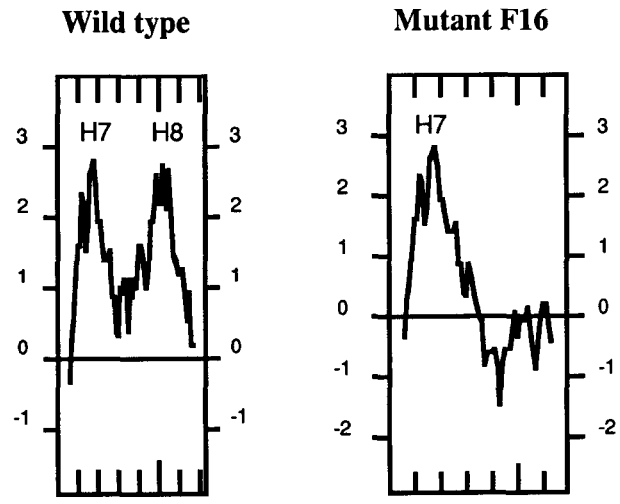

Fig. 2. Hydropathy plots of the C-terminal domains of cytochrome *b* from the wild-type strain, YL11/1-8, and the frameshift respiratory-deficient mutant, YL11/1-8/F16. The hydropathy plots of the C-terminal regions of cytochrome *b* from the wild-type strain, YL11/1-8, and the mutant, YL11/1-8/F16, were calculated according to Kyte and Doolittle [22] (see Fig. 1 for amino acid sequences).

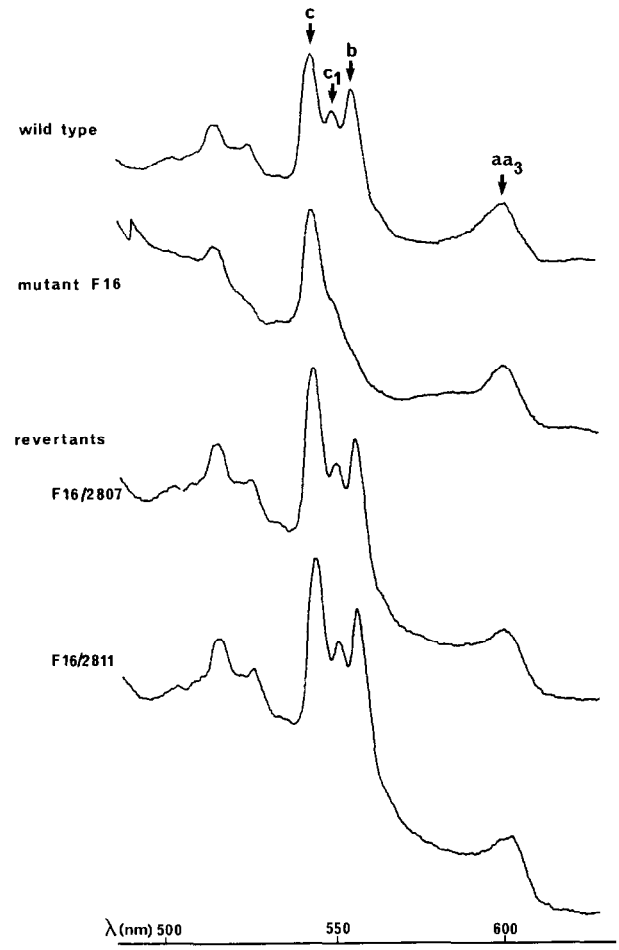

Fig. 3. Cytochrome absorption spectra of whole cells from the wild-type strain, YL11/1-8, the respiratory-deficient mutant, YL11/1-8/F16, and two respiratory-competent revertants of the mutant, YL11/1-8/F16. The spectra were recorded from whole cells at liquid nitrogen temperature after reduction of the cytochromes by dithionite. Arrows indicate the positions of the α band absorption maxima of cytochrome *c* (c), cytochrome c_1 (c_1), cytochrome *b* (b) and cytochromes *a* and a_3 (aa_3).

3.2. *Isolation and characterization of respiratory-competent revertants from the respiratory-deficient mutants, YL11/1-8/F16 and YL11/1-8/F17*

Revertants of the mutant, YL11/1-8/F16, appeared at a frequency of 0.3×10^{-8} (58 selection plates each inoculated with 5×10^8 cells gave in total 104; 31 plates did not give any revertants). Thirty five revertants representing 27 independent isolate groups were retained for analysis. The 3′ region of the cytochrome *b* mRNA was sequenced for each revertant from nucleotide 378 to at least 50 nucleotides beyond the initial frameshift.

Thirteen revertants proved to be the result of a single base pair subtraction within a window of 24 bases before or after the original +T addition (Fig. 4). Each reversion takes place within a base repetition: −T within TTTT, between codons 349 and 350 (in four revertants), −T within TT, between codons 347 and 348 (in two

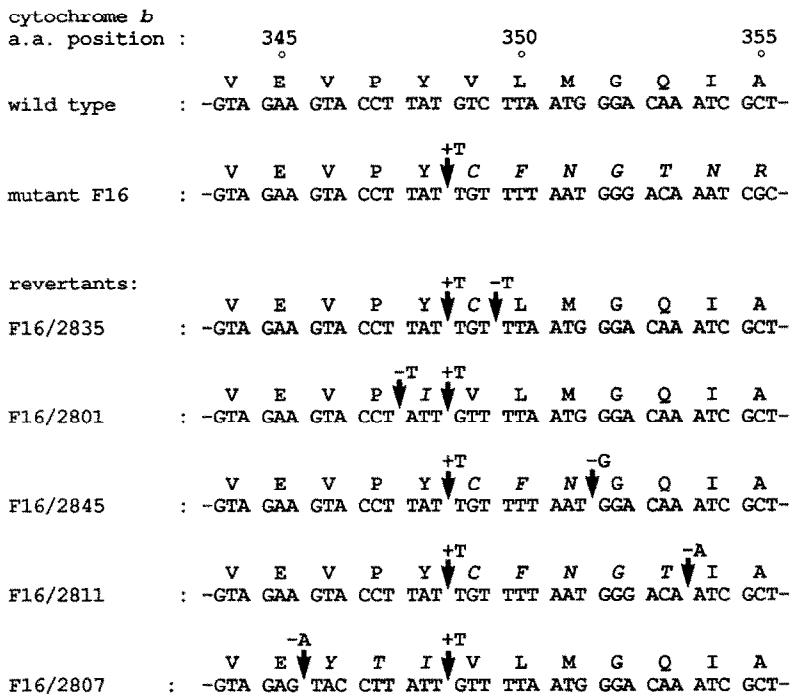

Fig. 4. Sequence of the cytochrome *b* gene region from codon position 344 to 355 in the wild-type, YL11/1-8, the respiratory-deficient mutant, YL11/1-8/F16, and five different respiratory-competent revertants of the mutant, YL11/1-8/F16. The nucleotide sequences were determined by reverse transcription of the cytochrome *b* mRNA. The corresponding amino acids (in the one letter code) and their positions are indicated. The frameshift mutant, YL11/1-8/F16, carries two nucleotide changes (indicated by arrows) leading to replacement of the last 37 C-terminal residues by a novel sequence of 33 residues (see Fig 1). Amino acids corresponding to the new reading frame are in italics. The revertants are the result of a single base subtraction (indicated by arrows) before or after the original +T addition, restoring the wild-type reading frame except between the two mutations.

revertants), −G within GGG, between codons 351 and 352 (in one revertant), −A within AAA, between codons 353 and 354 (in fiver revertants), and −A within AA, between codons 345 and 346 (in one revertant). Thus A/T base pair deletions (12 occurrences) were more frequent than G/C base pair deletions (1 occurrence). The suppressor mutations take place more frequently downstream (10 occurrences) than upstream (3 occurrences) from the initial mutation. These pseudo-reversions lead to single or multiple (up to five) consecutive amino acid replacements between positions 346 and 354 and to restoration of the wild-type sequence from position 355 to 385. Each reversion resulted in very efficient growth on respiratory substrates and gave spectrally, as well as immuno-detected cytochrome *b* contents similar to those of the wild-type (Figs. 3, 5 and 6). For each revertant, the cytochrome *b* hydropathy profile was not significantly different from that of the wild-type (not shown).

In the 22 other revertants no secondary mutation was found within the 3′ region of the cytochrome *b* gene, these maintained, therefore, in-frame, the novel reading frame resulting from the original frameshift mutation. All these revertants exhibited partial phenotype restoration with reduced respiratory growth rate and diminished cytochrome *b* contents. These revertants have not been characterized further.

Revertants of the mutant, YL11/1-8/F17, appeared at a frequency estimated to be 1×10^{-9} (55 selection plates were each inoculated with 5×10^{8} cells; only 7 gave revertants, 26 in total). Seven independent revertants were retained for analysis. They were all true back mutants (TAA → TTA) as revealed by sequence determination.

4. DISCUSSION

The present study shows that yeast lose the ability to grow on non-fermentable substrates when a translation termination codon is introduced 50 codons before the normal 3′ end of the apocytochrome *b* gene, and by the presence of a frameshift mutation which is predicted to lead to replacement of the last 37 C-terminal residues of apocytochrome *b* by a completely different sequence of 33 amino acids.

Both mutants were unable to incorporate heme into the apocytochrome *b*. In addition, no immunological response corresponding to the apocytochrome *b* could be detected by Western analysis of crude mitochondrial protein extracts. It may therefore be asked whether the

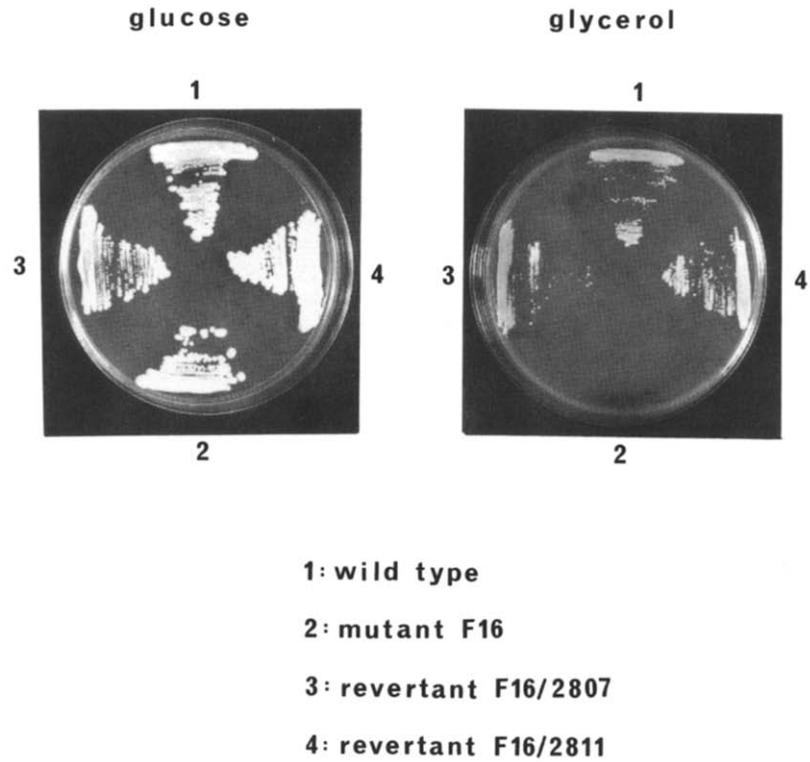

Fig. 5. Effects on the respiratory growth of yeast of the cytochrome *b* mutation, F16, and the pseudo-reversions, F16/2807 and F16/2811. Rich glucose and glycerol media were inoculated with cells from the wild-type strain, YL11/1-8, mutant F16, and revertants, F16/2807 and F16/2811. The plates were photographed after a 4 day incubation at 28°C (see Figs. 1 and 4 for nucleotide and amino acid sequence alterations).

modified apocytochrome *b* proteins are actually synthesized. At least, one can say that the mutants transcribe the cytochrome *b* gene and that the transcripts are stable since their nucleotide alterations were identified by the sequencing of their mRNAs. Rather than a defect in translation of mRNA we believe that proteolytic degradation is responsible for the absence of immunological response. In this respect, it should be remembered that mutations impairing the assembly of the cytochrome bc_1 complex lead to more or less severe reduction in the steady-state concentrations of the constituent subunits, presumably by increased protease sensitivity of the unassembled proteins [26]. Therefore, it seems reasonable to consider that the loss of the respiratory capacity by the two apocytochrome *b* mutants here described, results from a defect in the assembly of the cytochrome bc_1 complex.

The frameshift mutation would lead to replacement of helix 8 of the 8-helix cytochrome *b* model by a hydrophilic amino acid sequence. The respiratory capacity was restored to wild-type levels by a variety of pseudo-reversions leading to one or multiple (up to five) consecutive amino acid replacements in the vicinity of the original mutation. The absence of noticeable functional defects despite such radical structure modifications is consistent with the poor conservation during evolution of this region of the protein [25]. The amino acid replacements take place within the short connecting loop

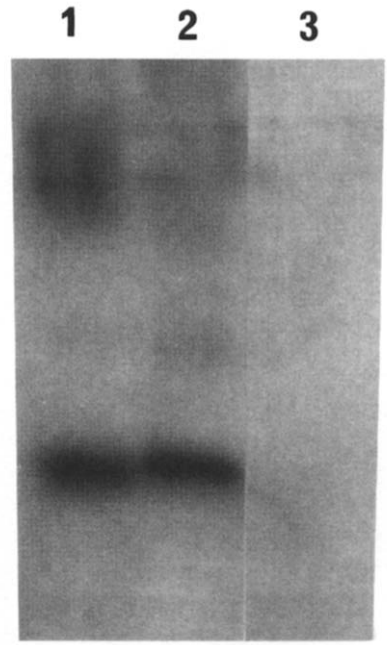

Fig. 6. Immunoblot analysis of the cytochrome *b* subunit of the cytochrome bc_1 complex in wild-type strain, YL11/1-8, mutant F16, and revertant, F16/2811. Total mitochondrial proteins were resolved by acrylamide gel electrophoresis. The proteins were transferred to nitrocellulose and probed with polyclonal antibodies raised against cytochrome *b*. Each lane contained 5 μg of total mitochondrial proteins. Lane 1, revertant F16/2811; lane 2, wild-type YL11/1-8; lane 3, mutant F16.

between helices 7 and 8 and at the beginning of helix 8. No significant change in comparison to the wild-type was observed in the hydropathy profiles of the revertants' apocytochrome *b*'s. Thus, efficient phenotype restoration was observed solely when the modified apocytochrome *b* recovered a normal hydropathy profile. The same holds for the nonsense mutant in which function was regained only by true mutations restoring a protein of wild-type sequence.

Taken together these data indicate that, although poorly conserved during evolution, the C-terminal tail of cytochrome *b* forms a membrane-spanning segment that is essential for the proper assembly and functioning of the cytochrome bc_1 complex.

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REFERENCES

[1] Rao, J K and Argos, P (1986) Biochim. Biophys. Acta 869, 197–205
[2] Crofts, A.R., Robinson, H., Andrews, K., Van Doren, S. and Berry, E. (1987) in: Cytochrome Systems (Papa, S., Chance, B. and Ernster, L., eds.) pp. 617–624, Plenum, New York.
[3] Brasseur, R. (1988) J. Biol. Chem. 263, 12571–12575.
[4] Yun, C.-H., Van Doren, S.R., Crofts, A.R and Gennis, R.B. (1991) J. Biol. Chem. 266, 10967–10973
[5] di Rago, J -P, Perea, X and Colson, A.-M. (1990) FEBS Lett. 263, 93–98.
[6] Crofts, A.R., Hacker, B., Barquera, B , Yun, C.-H. and Gennis, R.B (1992) Biochim. Biophys Acta 1101, 162–165.
[7] Atta-Asafo-Adjei, E. and Daldal, F. (1991) Proc. Natl. Acad. Sci USA 88, 492–496.
[8] di Rago, J.-P., Netter, P. and Slonimski, P.P. (1990) J. Biol. Chem 265, 15750–15757.
[9] Howell, N. and Gilbert, K. (1988) J. Mol. Biol. 203, 607–618.
[10] Lemesle-Meunier, D., Brivet-Chevillote, P, di Rago, J.-P., Slonimski, P.P., Bruel, C., Tron, T. and Forget, N (1993) J Biol. Chem. (in press).
[11] Séraphin, B., Boulet, A., Simon, M. and Faye, G. (1987) Proc. Natl. Acad Sci. USA 84, 6810–6814.
[12] Meunier, B., Tian, G.L., Macadre, C., Slonimski, P.P. and Lazowska, J. (1990) in: Structure, Function and Biogenesis of Energy Transfer Systems (Quagliariello, E., Papa, S., Palmieri, F. and Saccone, C., eds.) pp. 169–174, Elsevier, Amsterdam.
[13] Kovac, L., Lachowicz, T.M. and and Slonimski, P.P. (1967) Science 158, 1564–1567.
[14] Tian, G.-L., Michel, F , Macadre, C., Slonimski, P P. and Lazowska, J. (1991) J. Mol. Biol. 218, 747–760.
[15] Jacq, C., Lazowska, J. and Slonimski, P.P (1980) in: Organisation and Expression of the Mitochondrial Genome (Kroon, A.M. and Saccone, C., eds.) pp. 139–152, Elsevier, Amsterdam.
[16] di Rago, J.-P., Netter, P. and Slonimski, P.P. (1990) J. Biol. Chem 265, 3332–3339
[17] Claisse, M L. and Pajot, P.F (1974) Eur J. Biochem 49, 49–59.
[18] Putrament, A., Baranowska, H. and Prazmo, W. (1973) Mol. Gen. Genet 126, 357–366
[19] di Rago, J.-P. and Colson, A.-M. (1988) J. Biol. Chem 263, 12564–12570.
[20] Bonitz, S.G., Coruzzi, G., Thalenfeld, B E , Tzagoloff, A and Macino, G. (1980) J. Biol. Chem. 255, 11927–11941.
[21] Grivell, L A. (1989) Eur. J. Biochem 182, 477–493
[22] Kyte, J and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[23] Lawson, E.J. and Douglas, M G. (1988) J. Biol. Chem 263, 14812–14818.
[24] Kotylak, Z. and Slonimski, P.P (1977) in: Mitochondria 77: Genetics and Biogenesis of Mitochondria (Bandlow, W., Schweyen, R.J , Wolf, K. and Kaudewitz, F , eds.) pp 83–89, de Gruyter, Berlin.
[25] Hauska, G., Nitschke, W. and Hermann, R.G. (1988) J. Bioenerg. Biomembr. 20, 211–228.
[26] Trumpower, B L. (1990) Microbiol. Rev 54, 101–129.