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The cytoplasmically-made subunit IV is necessary for assembly of cytochrome *c* oxidase in yeast

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Yeast cytochrome *c* oxidase contains three large subunits made in mitochondria and at least six smaller subunits made in the cytoplasm. There is evidence that the catalytic centers (heme *a* and copper) are associated with the mitochondrially-made subunits, but the role of the cytoplasmically-made subunits has remained open. Using a gene interruption technique, we have now constructed a *Saccharomyces cerevisiae* mutant which lacks the largest of the cytoplasmically-made subunits (subunit IV). This mutant is devoid of cyanide-sensitive respiration, the absorption spectrum of cytochrome aa_3 and cytochrome *c* oxidase activity. It still contains the other cytochrome *c* oxidase subunits but these are not assembled into a stable complex. Active cytochrome *c* oxidase was restored to the mutant by introducing a plasmid-borne wild-type subunit IV gene; no restoration was seen with a gene carrying an internal deletion corresponding to amino acid residues 28–66 of the mature subunit. Subunit IV is thus necessary for proper assembly of cytochrome *c* oxidase.

Key words: cytochrome *c* oxidase/yeast/subunit IV/gene interruption

Introduction

Cytochrome *c* oxidase is one of three oligomeric enzymes located in the inner membrane of yeast mitochondria to which both the mitochondrial and nuclear genome contribute genetic information. It consists of at least nine non-identical subunits, two heme *a* groups and two copper atoms. The three large subunits (I, II, III) are coded by mitochondrial DNA and synthesized inside the mitochondria; the six small subunits (IV–VII, VIIa, VIII) are coded by nuclear DNA and synthesized in the cytosol (Schatz and Mason, 1974; Power *et al.*, 1984a).

What are the functions of these different subunits? Several observations indicate that subunits I and II carry the catalytic centers of cytochrome *c* oxidase which mediate the reduction of oxygen by ferrocytochrome *c*. First, antisera against subunit II strongly inhibit cytochrome *c* oxidase activity (Poyton and Schatz, 1975). Second, the amino acid sequence of subunit II shows significant homology with the two copper-binding proteins azurin and plastocyanin, suggesting that at least one of the two copper ions of cytochrome *c* oxidase is linked to subunit II (Steffens and Buse, 1979). Third, subunit I appears to carry a heme *a* group since mutations in subunit I can shift the absorption spectrum of cytochrome *c* oxidase (Cabral *et al.*, 1977) and since, upon mild dissociation of cytochrome *c* oxidase, some heme *a* remains associated with subunit I and II (Winter *et al.*, 1980). Fourth, cytochrome *c* oxidases from *Paracoccus* and *Thermus* (Ludwig and Schatz, 1980; Fee *et al.*, 1980) are catalytically and spectrally almost indistinguishable from mitochondrial cytochrome *c* oxidases, yet contain only two subunits. In the case of the *Paracoccus* enzyme, these two subunits are homologous to the mitochondrially-made subunits I and II of yeast cytochrome *c* oxidase (Steffens *et al.*, 1983).

Much less is known about the function of the other subunits. There is evidence to suggest that subunit III functions in proton pumping (Casey *et al.*, 1980; Wikström *et al.*, 1981). However, the function of the cytoplasmically-made small subunits is still a mystery. First, antisera directed against any one of these subunits inhibit cytochrome *c* oxidase activity only weakly, if at all (Poyton and Schatz, 1975). Second, the number of detectable small subunits appears to increase with the development of more highly resolving polypeptide separation methods (Kadenbach *et al.*, 1983; Power *et al.*, 1984a). Third, different organisms appear to have different numbers of small subunits (Wikström *et al.*, 1981). Finally, a given small subunit of cytochrome *c* oxidase may have a slightly different amino acid sequence in different tissues of the same animal (Kadenbach *et al.*, 1982).

Even though the small subunits consistently co-purify with eukaryotic cytochrome *c* oxidase activity, one may indeed question whether they are authentic subunits of the enzyme. This question might be answered by analyzing yeast mutants specifically lacking cytochrome *c* oxidase, but initial screening of such mutants only revealed defects in mitochondrially-made subunits (Ebner *et al.*, 1973). Recently, however, a chromosomally-inherited yeast mutant lacking cytochrome *c* oxidase activity (Tzagoloff *et al.*, 1975) was found to contain a subunit V of altered electrophoretic mobility (Cumsky *et al.*, 1983).

The advent of powerful gene cloning techniques in yeast has allowed the isolation and sequencing of the nuclear yeast genes for cytochrome *c* oxidase subunits IV, VI and VII (Maarse *et al.*, 1984; Wright *et al.*, 1985; Power *et al.*, 1984b). We have used the cloned gene for subunit IV to construct a yeast mutant specifically lacking this subunit. The properties of this mutant show that this subunit is necessary for cytochrome *c* oxidase assembly. Furthermore, we identified a stretch of 38 amino acids within the mature subunit IV protein which is indispensable for the subunit IV function.

Results

Construction of a yeast mutant lacking cytochrome c oxidase subunit IV (cox IV⁻ mutant)

In order to inactivate specifically the nuclear gene for cytochrome *c* oxidase subunit IV, a respiring yeast strain carrying a non-revertible *leu 2* auxotrophic marker (strain DL 1) was transformed with a linear piece of DNA containing the wild-type *LEU 2* gene flanked on either side by 5′- and 3′-terminal sequences of the *COX IV* gene. Six *leu*$^+$

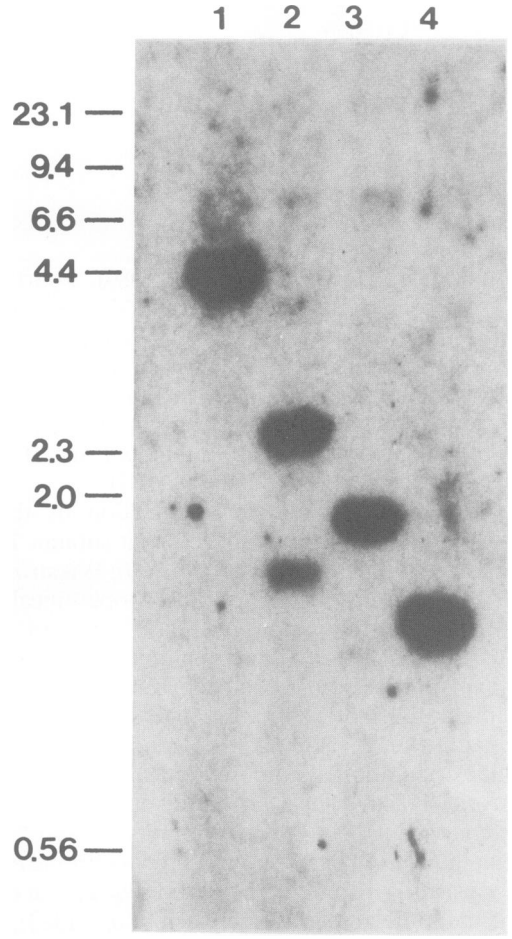

Fig. 1. Southern blot analysis showing the restriction pattern predicted for the interrupted *COX IV* gene. **Lane 1** WD 1 DNA digested with *Pvu*II and *Bgl*II. **Lane 2** WD 1 DNA digested with *Eco*RI and *Bgl*II. **Lane 3** DL 1 DNA digested with *Pvu*II and *Bgl*II. **Lane 4** DL 1 DNA digested with *Eco*RI and *Bgl*II. Hybridization was carried out with a ^{32}P-labeled probe covering the entire *COX IV* gene (an *Eco*RI-*Bgl*II fragment, cf. Figure 7). The numbers on the left indicate the position of the mol. wt. markers (λDNA fragments obtained by *Hind*III digestion; sizes given in kb).

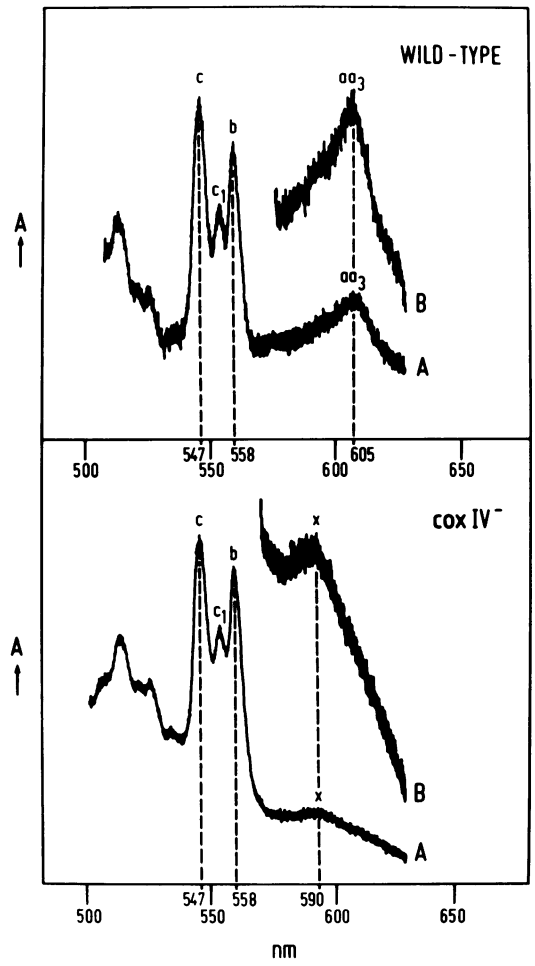

Fig. 2. The *cox IV*$^-$ mutant lacks cytochrome aa_3. Reduced-minus-oxidized difference absorption spectra were recorded at liquid nitrogen temperature for wild-type and *cox IV*$^-$ mitochondria (1.3 mg protein/ml; 0.2 M KP_i pH 7.4, 50% glycerol). Spectra were recorded at a light-path of 2 mm and a band-width of 0.8 nm at the following sensitivities (absorbance units/full scale): Wild-type (**trace A** = 0.05; **trace B** = 0.02); *cox IV*$^-$ (**trace A** = 0.10; **trace B** = 0.02).

Table I. Mitochondria of the *cox IV*$^-$ mutant lack cytochrome *c* oxidase activity but have wild-type levels of succinate-cytochrome *c* reductase activity

Source of mitochondria	Cytochrome *c* oxidase[a]	Succinate cytochrome *c* reductase
Wild-type	0.512	0.103
cox IV$^-$ mutant	0.000	0.104

[a]Units/mg mitochondrial protein at 23°C

transformants were obtained and five of these had lost the ability to grow on a non-fermentable carbon source such as glycerol. One of these five transformants (termed WD 1) was chosen for further study. The *leu*$^+$ phenotype was mitotically stable, suggesting that the transforming DNA had been stably integrated into the nuclear genome. If WD 1 was crossed to yeast strains lacking mitochondrial DNA (*rho*° mutants), the resulting diploids were respiration-competent; the respiratory defect in WD 1 is, thus, caused by the mutation of a nuclear gene. 'Southern blot' analysis of DNA from the parental wild-type and the *cox IV*$^-$ mutant with a ^{32}P-labeled probe containing the entire *COX IV* gene showed that the *cox IV*$^-$ mutant contained an altered chromosomal *COX IV* gene (Figure 1): a *Bgl*II-*Pvu*II fragment recognized by the ^{32}P-labeled probe has increased by ~3 kb compared with the wild-type; this is consistent with the integration of the linear 3-kb fragment containing the *LEU 2* gene. Similarly, double digestion with *Eco*RI and *Bgl*II reveals the additional *Eco*RI restriction site that the *cox IV*$^-$ mutant has acquired because of the *LEU 2* insert. The same result was obtained when plasmids pFL19-4 (harboring the wild-type *COX IV* gene) and pIV137-23 (harboring the interrupted gene) were analyzed in this manner; with ^{32}P-labeled *LEU 2* DNA as a probe, only plasmid pIV137-23 gave a hybridization signal (not shown). This indicates that the *cox IV*$^-$ mutant WD 1 has had its wild-type copy of the subunit IV gene replaced by an interrupted copy of this gene. The hybridization data also make it very unlikely that the *Saccharomyces cerevisiae* strain used here has a second, silent copy of the *COX IV* gene at a distant locus in the genome.

Phenotype of the cox IV$^-$ *mutant*

Mitochondria from the *cox IV*$^-$ mutant WD 1 are indistinguishable from wild-type mitochondria with respect to succinate-cytochrome *c* reductase activity and the presence of

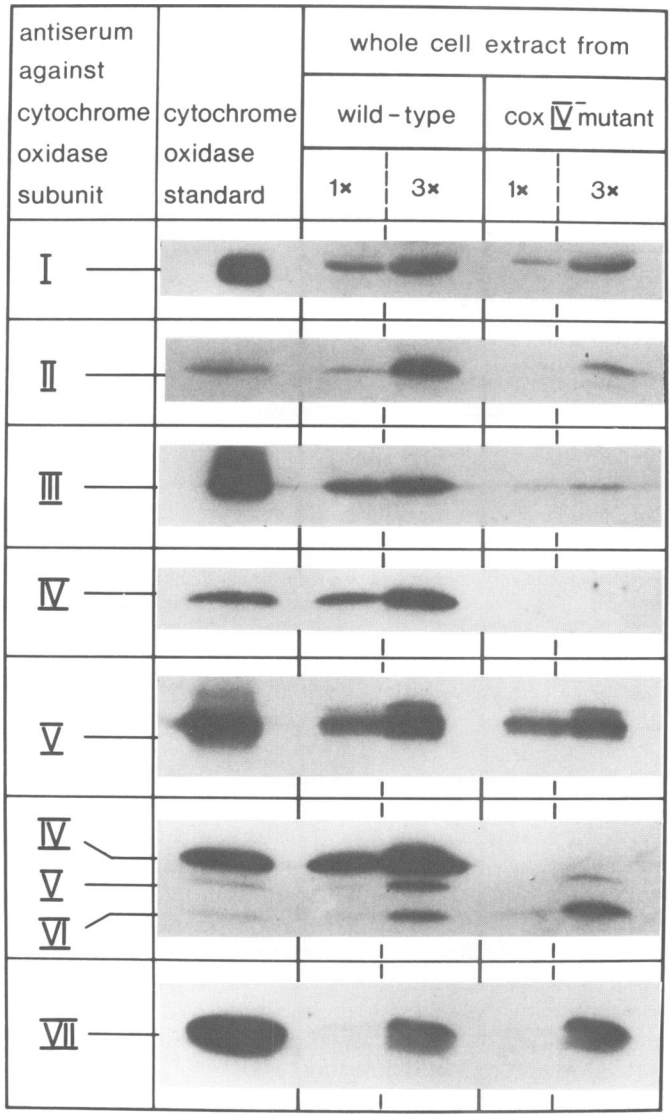

Fig. 3. The *cox IV*$^-$ mutant lacks subunit IV. Wild-type and *cox IV*$^-$ cells were grown on 1% yeast extract, 2% Bacto-peptone, 2% raffinose and harvested in mid-log growth phase. Protein extracts were analyzed by immune blotting with antisera against the various subunits. Two amounts of protein were analyzed (1x = 40–70 μg; 3x = 160–210 μg). Only the relevant portions of the autoradiograms are shown.

cytochromes *b*, c_1 and *c*; however, they lack spectroscopically-detectable cytochrome aa_3 (<5%) as well as cytochrome *c* oxidase activity (<0.1%; Table I and Figure 2). The respiratory deficiency of WD 1 can thus be fully explained by the lack of cytochrome *c* oxidase. In WD 1, the typical cytochrome aa_3 absorption band at 605 nm is replaced by a much weaker band at 590 nm which might reflect the presence of some incorrectly complexed heme *a*.

To test for the presence of individual cytochrome *c* oxidase subunits, extracts of wild-type and *cox IV*$^-$ cells were analyzed by immune blotting with antisera against these subunits (Figure 3). Subunit IV was undetectable in the *cox IV*$^-$ mutant; all other subunits were detectable, although usually at a lower level than in the corresponding wild-type strain. No attempt was made to work out conditions for obtaining a linear dose-response curve in these immune blots, but subunit IV was undetectable at every level of WD 1 extract tested. As expected, transformation of WD 1 with the plasmid-borne wild-

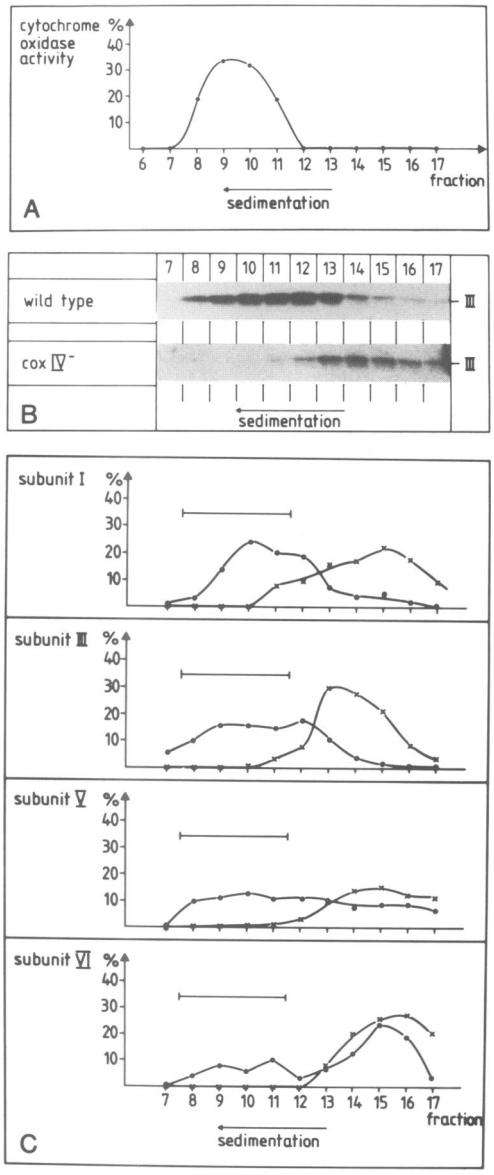

Fig. 4. Assembly of cytochrome *c* oxidase subunits is impaired in the *cox IV*$^-$ mutant. Mitochondria (5 mg/ml) of wild-type and *cox IV*$^-$ cells were extracted with 15 mM Zwittergent-14, 0.7 M NaCl, 10 mM NaP_i pH 7.0, 1 mM PMSF for 30 min at 0°C. Insoluble matter was removed by centrifugation (100 000 *g*, 30 min at 2°C) and 0.5 ml of the supernatant was layered on a 11 ml linear gradient of 20–50% glycerol, 2 mM Zwittergent-14, 0.7 M NaCl, 10 mM NaP_i pH 7.0. The gradient was centrifuged for 40 h at 200 000 *g* at 2°C. Seventeen 0.7 ml fractions were collected and assayed for cytochrome *c* oxidase activity and subunit content. The data shown in **panels A–C** were derived from a single experiment in which wild-type and *cox IV*$^-$ mitochondria were analyzed in parallel. **(A)** Sedimentation of cytochrome *c* oxidase activity in extracts of wild-type mitochondria. Fractions 1–6 were devoid of activity. Of the total cytochrome *c* oxidase activity loaded, 7% was recovered after the gradient centrifugation step; the abscissa denotes % of this recovered activity. The mitochondrial extracts of *cox IV*$^-$ cells contained no cytochrome *c* oxidase activity. **(B)** Sedimentation of cytochrome *c* oxidase subunit III in extracts from wild-type and *cox IV*$^-$ mitochondria (autoradiogram). Each gradient fraction (numerals on top) was subjected to SDS-polyacrylamide gel electrophoresis and immune blotting with a monoclonal antibody against subunit III. A photograph of the relevant portions of the autoradiograms is shown. The remainder of the autoradiograms was completely blank. **Panel C:** sedimentation of cytochrome *c* oxidase subunits (quantitation of immune blots): ●---●---● wild-type; x---x---x *cox IV*$^-$ mutant. The abscissa denotes % of subunit antigen recovered in all fractions. The horizontal bar indicates fractions containing cytochrome *c* oxidase activity.

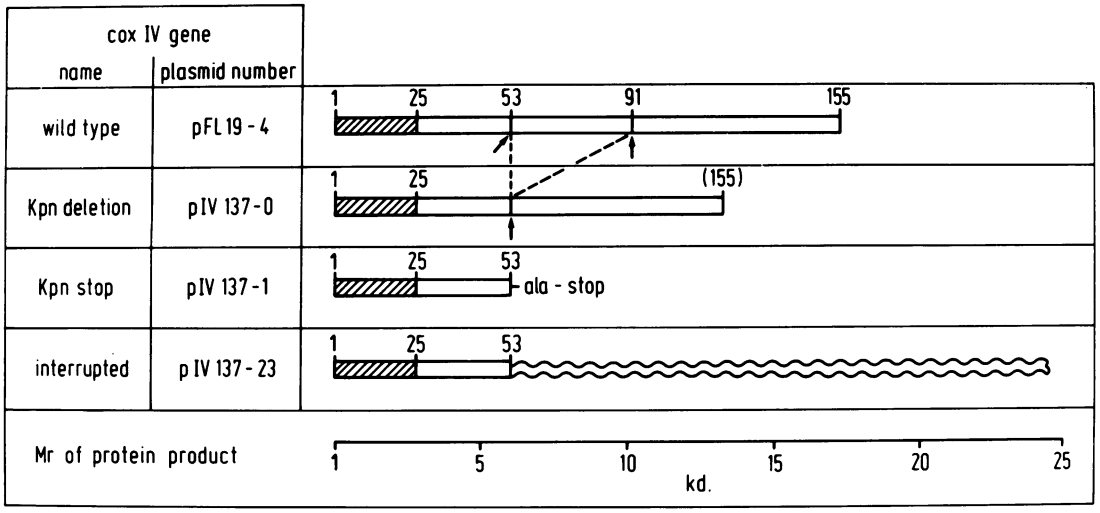

Fig. 5. Plasmids encoding the wild-type *COX IV* gene (pFL19-4) and three different altered versions of this gene (pIV137-0, pIV137-1, pIV137-23). The shaded bars represent the 25 amino acid long prepiece of the *COX IV* precursor, open bars the wild-type sequences of the mature protein, and the wavy bar the putative amino acid sequences coded by the inverted 3′ region of the yeast *LEU 2* gene. The numbers on top of the bars indicate amino acid position within the wild-type *COX IV* precursor protein. Arrows identify sites cleaved by the restriction endonuclease *Kpn*I. pIV137-0 was constructed by cleavage of pFL19-4 with *Kpn*I followed by in-frame sticky-end ligation. This results in the deletion of 38 amino acids between positions 53 and 91 of the precursor protein. pIV137-1 was constructed by cleavage of pFL19-4 with *Kpn*I, filling in the sticky ends, and ligation. The resulting mutant *cox IV* gene only codes for the first 53 amino acids of the precursor protein; in addition one C-terminal alanine is introduced by the gene manipulation. pIV137-23 was used for gene disruption. The mol. wt. of the fusion protein coded by this plasmid is tentative (cf. Discussion).

type *COX IV* gene (pFL19-4) restored both respiration and the presence of immuno-detectable subunit IV to the cells (not shown; cf. also Maarse *et al.*, 1984).

What is the state of the residual cytochrome *c* oxidase subunits in the *cox IV*$^-$ mutant? To answer this question, detergent extracts of mitochondria were centrifuged through a glycerol density gradient and the sedimentation profile of cytochrome *c* oxidase activity of wild-type cells was compared with the sedimentation profile of individual subunits in either the wild-type or the *cox IV*$^-$ extract (Figure 4). With an extract of wild-type mitochondria, cytochrome *c* oxidase subunits sedimented in two broad and partly overlapping peaks. The faster-sedimenting peak coincided with the peak of cytochrome *c* oxidase activity and probably represented subunits assembled within the holoenzyme. The more slowly sedimenting peak (which appeared more as a trailing edge with some subunits) was devoid of enzyme activity and probably reflected subunits that had dissociated from the enzyme during the prolonged analysis. Such a dissociation of subunits was not unexpected since >90% of the cytochrome *c* oxidase activity was lost during gradient centrifugation. In contrast, none of the cytochrome *c* oxidase subunits detectable in extracts from *cox IV*$^-$ mitochondria sedimented as rapidly as the holoenzyme. Taken by itself, this experiment does not exclude that the slowly sedimenting subunits represent an inactive, but assembled cytochrome *c* oxidase monomer (Wikström *et al.*, 1981) or that the holoenzyme is weakly assembled in the mutant *in vivo*. However, the lack of a cytochrome aa_3 spectrum in intact mitochondria provides independent evidence that the holoenzyme is not properly assembled *in vivo*.

A C-terminal and an internal section of subunit IV are both required for subunit IV function

Plasmid pIV137-1 codes for a truncated subunit IV which contains only the transient prepiece of 25 amino acids and the first 28 amino acids of mature subunit IV (Figure 5). Plasmid pIV137-0 encodes a subunit IV protein with an internal deletion of 38 amino acids (from residue 28 to 66 of the mature protein). When *cox IV*$^-$ cells were transformed with either one of these two plasmids, the transformants were unable to respire (not shown). Thus, the protein products of these mutated subunit IV genes are unable to fulfill the function(s) of subunit IV. Unpublished studies by E. C. Hurt in our laboratory have shown that the internally deleted subunit IV is still efficiently imported, and processed to a lower mol. wt. polypeptide by isolated mitochondria; the loss of subunit IV function is thus not merely a consequence of an impaired import of this protein into mitochondria.

Discussion

This study is a first attempt to exploit recombinant DNA technology for obtaining cytochrome *c* oxidase-deficient yeast mutants. The mutant described here lacks cytochrome *c* oxidase subunit IV as shown by 'Southern' and immune blotting analysis. The interrupted subunit IV gene should code for a fusion protein composed of the first 53 amino acids of the subunit IV precursor and whatever polypeptide is specified by the 3′-flanking sequences 900 bp downstream from the coding sequence of the *LEU 2* gene. (Note that these sequences would have to be read in the opposite orientation, cf. Figure 7). Sequence data for the *LEU 2* gene are only available up to ~600 bp downstream from the stop codon (Andreadis *et al.*, 1984; Andreadis and Schimmel, personal communication). We therefore do not know whether the 5′-proximal sequences of the subunit IV gene are fused in-frame to the sequences provided by the inverted 3′ region of the *LEU 2* gene. Attempts to detect a fusion protein in the *cox IV*$^-$ mutant WD 1 by immune blotting experiments with antiserum against holo-cytochrome *c* oxidase revealed a weak signal indicative of a 24-kd antigen in some, but not in all experiments. However, such an antigen was never detected with antiserum monospecific for subunit IV. Most likely, any fusion protein is either too unstable or too short to be reliably detected by our procedures. This, in turn, makes it unlikely

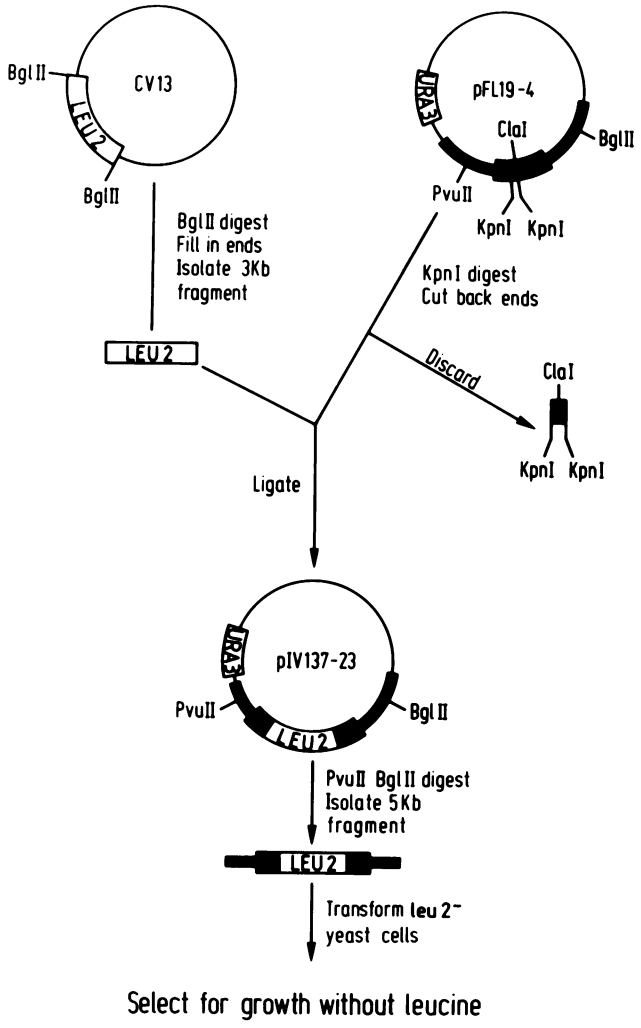

Fig. 6. Construction of the *cox IV*$^-$ mutant. See Results and Materials and methods for details.

that the defective assembly of cytochrome *c* oxidase in the *cox IV*$^-$ mutant is caused by interference from such a fusion protein rather than by the lack of subunit IV. Such a possibility is further weakened by the observations that the mutation is recessive and that introduction of multiple copies of the fused gene into wild-type yeast cells does not measurably affect the activity of cytochrome *c* oxidase.

The subunit IV-less mutant described here may open the way for tackling several questions relating to protein import into mitochondria or to cytochrome c oxidase assembly. For example, import of the subunit IV precursor could first be blocked by mutations within the presequence and second-site revertants (either within the subunit IV gene itself or within the host genome) could then be selected for by growth on glycerol. Alternatively, transformation of the mutant with differently altered versions of the subunit IV gene should reveal those parts of the subunit which are necessary for assembly as opposed to those that are necessary for any catalytic function. In this respect it will be interesting to learn whether subunit IV lacking 38 internal amino acids allows the formation of a stable (albeit inactive) cytochrome c oxidase complex.

The data reported here show that subunit IV of yeast cytochrome *c* oxidase has a function in assembling this oligomeric enzyme. This polypeptide is thus a *bona fide*

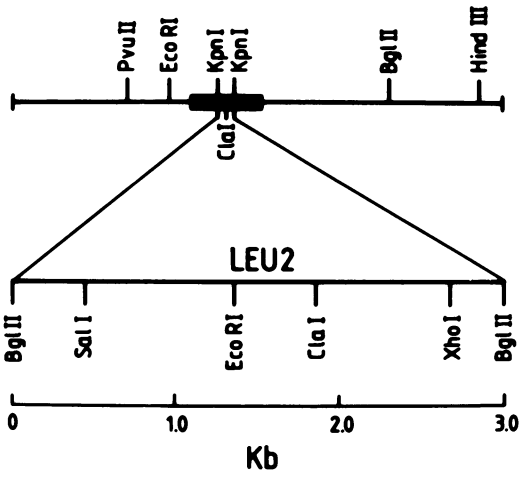

Fig. 7. Restriction map showing the orientation at which a *Bgl*II fragment carrying the *LEU 2* gene was inserted into the *COX IV* gene. The *COX IV* gene is indicated by the filled bar. The normal direction of transcription is from left to right for the *COX IV* gene and from right to left for the *LEU 2* gene.

subunit of cytochrome *c* oxidase. It has always been a puzzle why mitochondrial cytochrome *c* oxidases have so many subunits even though some corresponding bacterial enzymes have only two. In principle, this question could be approached by reconstitution of active cytochrome *c* oxidase from purified subunits, but efforts in this direction have so far been unsuccessful. The approach used here circumvents these problems and should lead to a better understanding of how cytochrome *c* oxidase is assembled and how it performs its catalytic function.

Materials and methods

Yeast strains

S. cerevisiae strains DL 1 (*α his 3*$^-$, *leu 2*$^-$, *ura 3*$^-$), E 69 (*a trp*$^-$, *ade*$^-$, *rho*^{0}) and AH 216-1A (*a his 3*$^-$, *leu 2*$^-$, *rho*^{0}) were used. WD 1 (*α his 3*$^-$, *ura 3*$^-$, *cox IV*$^-$) was constructed from DL 1 by integrative transformation (Orr-Weaver *et al.*, 1981) with a linear piece of DNA containing the *LEU 2* gene flanked by *COX IV* sequences corresponding to the N-terminal and C-terminal portions of the *COX IV* polypeptide (Figure 6). The plasmid pFL19-4 (Maarse *et al.*, 1984) was cut at both of its two *Kpn*I restriction sites. As these sites are located within the *COX IV* coding sequence, this step deletes an internal fragment of 38 amino acids. The deleted piece of DNA was replaced by a *Bgl*II fragment derived from plasmid CV13 (= YEp 13; Broach *et al.*, 1979). The resulting plasmid pIV137-23 was amplified in *Escherichia coli* HB101 and subsequently cut with *Pvu*II and *Bgl*II; the 5-kb fragment containing the *COX IV* gene interrupted by the *LEU 2* gene was used to transform a *leu 2*$^-$ yeast strain (DL 1) to growth without leucine. Restriction analysis (Figure 7) identified the orientation of the inserted *Bgl*II fragment with respect to the *COX IV* gene. Note that the two genes are transcribed in the opposite direction.

Media

For protein extractions and isolation of mitochondria, wild-type and *cox IV*$^-$ cells were grown on 1% yeast extract, 2% Bactopeptone and 2% raffinose (Lustig *et al.*, 1982).

Nucleic acid techniques

Published procedures were used for transforming yeast cells (Hinnen *et al.*, 1978) or *E. coli* HB101 (Mandel and Higa, 1970), for isolating yeast DNA (Riezman *et al.*, 1983a), for small-scale isolations of plasmids from *E. coli* (Birnboim and Doly, 1979), for isolation of DNA fragments from agarose gels using DEAE-paper (Dretzen *et al.*, 1981), for nick-translation (Rigby *et al.*, 1977) and for DNA blotting (Southern, 1975). Commercially-available restriction enzymes and other DNA modifying enzymes were used as advised by the suppliers.

Enzyme assays and low temperature spectra

Published procedures were used for the assay of cytochrome *c* oxidase (Mason *et al.*, 1973) and succinate-cytochrome *c* reductase (Tisdale, 1967) activities.

One enzyme unit is defined as the amount of enzyme converting 1 μmol cytochrome *c* per min under the specified assay conditions. Low temperature reduced-minus-oxidized difference spectra of mitochondria were obtained as described by Ebner *et al.* (1973) except that an Aminco DW 2 spectrophotometer was employed.

Miscellaneous

Published procedures were used for extraction of proteins from yeast cells (Riezman *et al.*, 1983a), for isolation of mitochondria (Daum *et al.*, 1982), and for extraction of proteins from mitochondria with Zwittergent-14 (Navarrete and Serrano, 1983). SDS-polyacrylamide slab gel electrophoresis was performed either with 15% gels (Douglas and Butow, 1976) or with 18.5% gels in the presence of glycerol and urea (Merle and Kadenbach, 1980). Immune blotting was performed as described by Haid and Suissa (1983) except that the nitrocellulose filters were saturated with 0.15 M NaCl-10 mM KP_i pH 7.4 containing 1% dried non-fat milk (Johnson *et al.*, 1984) instead of with bovine serum albumin. We used rabbit antisera directed against subunits I, II, IV, V or VII, a rabbit antiserum raised against holo-cytochrome *c* oxidase reacting mainly with subunit VI, and a monoclonal antibody against subunit III. Published procedures were used for protein estimation (Lowry *et al.*, 1951) as modified by Riezman *et al.* (1983b) and for spectrophotometric quantitation of silver grains on autoradiograms (Suissa, 1983).

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References

Andreadis,A., Hsu,Y.-P., Hermodson,M., Kohlhaw,G. and Schimmel,P. (1984) *J. Biol. Chem.*, **259**, 8059-8062.

Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513-1523.

Broach,J.R., Strathern,J.N. and Hicks,J.B. (1979) *Gene*, **8**, 121-133.

Cabral,F., Solioz,M., Deters,D., Rudin,Y., Schatz,G., Clavilier,L., Groudinsky,O. and Slonimski,P.P. (1977) in Bandlow,W., Schweyen,R.J., Wolf,K. and Kaudewitz,F. (eds.), *Mitochondria 1977*, de Gruyter, Berlin, pp. 401-413.

Casey,R.P., Thelen,M. and Azzi,A. (1980) *J. Biol. Chem.*, **255**, 3994-4000.

Cumsky,M.G., McEwen,J.E., Ko,C. and Poyton,R.O. (1983) *J. Biol. Chem.*, **258**, 13418-13421.

Daum,G., Böhni,P.C. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13028-13033.

Douglas,M.G. and Butow,R.A. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 1083-1086.

Dretzen,G., Bellard,P., Sassone-Corsi,P. and Chambon,P. (1981) *Anal. Biochem.*, **112**, 295-298.

Ebner,E., Mason,T.L. and Schatz,G. (1973) *J. Biol. Chem.*, **248**, 5369-5378.

Fee,J.A., Choc,M.E., Findling,K.L., Lorence,R. and Yoshida,T. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 147-151.

Haid,A. and Suissa,M. (1983) *Methods Enzymol.*, **96**, 192-205.

Hinnen,A., Hicks,J.B. and Fink,E.R. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1929-1933.

Johnson,D.A., Gautsch,J.H., Sprtsman,J.R. and Elder,J.H. (1984) *Gene Anal. Tech.*, **7**, 3-8.

Kadenbach,B., Hartmann,R., Glanville,R. and Buse,G. (1982) *FEBS Lett.*, **138**, 236-238.

Kadenbach,B., Jaransch,J., Hartmann,R. and Merle,P. (1983) *Anal. Biochem.*, **129**, 517-521.

Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall., R.J. (1951) *J. Biol. Chem.*, **193**, 265-275.

Ludwig,B. and Schatz,G. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 196-200.

Lustig,A., Padmanaban,G. and Rabinowitz,M. (1982) *Biochemistry (Wash.)*, **21**, 309-316.

Maarse,A.C., Van Loon,A.P.G.M., Riezman,H., Gregor,I., Schatz,G. and Grivell,L.A. (1984) *EMBO J.*, **3**, 2831-2837.

Mandel,M. and Higa,A. (1970) *J. Mol. Biol.*, **53**, 159-162.

Mason,T.L., Poyton,R.O., Wharton,D.C. and Schatz,G. (1973) *J. Biol. Chem.*, **248**, 1346-1354.

Merle,P. and Kadenbach,B. (1980) *Eur. J. Biochem.*, **105**, 499-507.

Navarrete,R. and Serrano,R. (1983) *Biochim. Biophys. Acta*, **728**, 403-408.

Orr-Weaver,T.L., Szostak,J.W. and Rothstein,R.J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6354-6358.

Power,S.D., Lochrie,M.A., Sevarino,K.A., Patterson,T.E. and Poyton,R.O. (1984a) *J. Biol. Chem.*, **259**, 6564-6570.

Power,S.D., Lochrie,M.A., Patterson,T.E. and Poyton,R.O. (1984b) *J. Biol. Chem.*, **259**, 6571-6574.

Poyton,R.O. and Schatz,G. (1975) *J. Biol. Chem.*, **250**, 762-766.

Riezman,H., Hase,T., Van Loon,A.P.G.M., Grivell,L.A., Suda,K. and Schatz,G. (1983a) *EMBO J.*, **2**, 2161-2168.

Riezman,H., Hay,R., Gasser,S., Daum,G., Schneider,G., Witte,C. and Schatz,G. (1983b) *EMBO J.*, **2**, 1105-1111.

Rigby,P.W.J., Dieckmann,M., Rhodes,L. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237-251.

Schatz,G. and Mason,T.L. (1974) *Annu. Rev. Biochem.*, **43**, 51-87.

Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503-517.

Steffens,G.C.M., Buse,G., Oppliger,W. and Ludwig,B. (1983) *Biochem. Biophys. Res. Commun.*, **116**, 335-340.

Steffens,G.J. and Buse,G. (1979) *Hoppe Seyler's Z. Physiol. Chem.*, **360**, 613-619.

Suissa,M. (1983) *Anal. Biochem.*, **133**, 511-514.

Tisdale,H.D. (1967) *Methods Enzymol.*, **10**, 213-215.

Tzagoloff,A., Akai,A. and Needleman,R.B. (1975) *J. Biol. Chem.*, **250**, 8228-8238.

Wikström,M., Krab,K. and Saraste,M. (1981) *Cytochrome Oxidase: A Synthesis*, published by Academic Press, Inc., London.

Winter,D.B., Bruyninckx,W.J., Foulke,F.G., Grinich,N.P. and Mason,H.S. (1980) *J. Biol. Chem.*, **255**, 11408-11414.

Wright,R.M., Ko,C., Cumsky,M.G. and Poyton,R.O. (1985) *J. Biol. Chem.*, in press.

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