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Mitochondrial Membrane Biogenesis: Characterization and Use of Pet Mutants to Clone the Nuclear Gene Coding for Subunit V of Yeast Cytochrome c Oxidase

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A nuclear pet mutant of Saccharomyces cerevisiae that is defective in the structural gene for subunit V of cytochrome *c* oxidase has been identified and used to clone the subunit V gene (*COX5*) by complementation. This mutant, E4-238 [24], and its revertant, JM110, produce variant forms of subunit V. In comparison to the wild-type polypeptide (M^r = 12,500), the polypeptides from E4-238 and JM110 have apparent molecular weights of 9,500 and 13,500, respectively.These mutations directly alter the subunit V structural gene rather than a gene required for posttranslational processing or modification of subunit V because they are cis-acting in diploid cells; that is, both parental forms of subunit V are produced in heteroallelic diploids formed from crosses between the mutant, revertant, and wild type. Several plasmids containing the *COX5* gene were isolated by transformation of JM28, a derivative of E4-238, with DNA from a yeast nuclear DNA library in the vector YEp13. One plasmid, YEp13-511, with a DNA insert of 4.8 kilobases, was characterized in detail. It restores respiratory competency and cytochrome oxidase activity in JM28, encodes a new form of subunit V that is functionally assembled into mitochondria, and is capable of selecting mRNA for subunit V. The availability of mutants altered in the structural gene for subunit V (*COX5*) and of the *COX5* gene on a plasmid, together with the demonstration that plasmid-encoded subunit V is able to assemble into a functional holocytochrome *c* oxidase, enables molecular genetic studies of subunit V assembly into mitochondria and holocytochrome *c* oxidase.

Key words: cytochrome oxidase, subunit V, nuclear genes, assembly, Saccharomyces cerevisiae, pet mutants, mitochondria, biogenesis

Mitochondrial biogenesis has been the focus of intense interest recently, both in terms of protein targeting and membrane assembly [for review, see 1–3]. The

Abbreviations used: SDS, sodium dodecyl sufate; leu^+, leucine positive; $glyc^+$, glycerol positive.

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majority of proteins in mitochondria are encoded by nuclear genes and synthesized on cytoplasmic ribosomes [4,5]. They are frequently made as larger precursors, which are proteolytically processed during assembly into mitochondria [for review see 6]. Their import into mitochondria occurs posttranslationally and, in some cases, requires an energized inner mitochondrial membrane [1,3]. In spite of the intensive investigation of several well-characterized mitochondrial proteins [6], precise information on the cellular mechanisms by which proteins are targeted to mitochondria and assembled into their correct location in one of four intramitochondrial compartments is so far unavailable. For example, the amino acid sequence and location of peptide extensions on higher molecular weight precursors are so far known for only two proteins (cytochrome *c* peroxidase and mitochondrial elongation factor TU from Saccharomyces cerevisiae [7,8] and subunit 9 of ATPase from Neurospora crassa [8]. Furthermore, experiments that directly test the functions of peptide extensions in mitochondrial protein targeting or transport have not yet been reported.

Genetics provides an extremely efficient way to identify specific regions of protein sequence or structure that are important for the targeting, or assembly, of proteins into membranes. Many elegant studies, in which fused gene products and site-directed mutagenesis were used to study protein targeting, have been done in procaryotes [10,11]. These approaches can be applied productively to mitochondrial biogenesis in S. cerevisiae also, as structural gene mutations and cloned genes for mitochondrial proteins become available.

Yeast cytochrome *c* oxidase, a hetero-oligomeric protein located in the inner mitochondrial membrane, is an excellent system for genetic analysis of mitochondrial membrane biogenesis [5] because the tools are now available for the molecular analysis of its biosynthesis and assembly. The genes for the mitochondrially-encoded subunits I, II, and III of this enzyme have been sequenced [12–15] and many mutants in these genes are available [16,17]. The nuclear-encoded subunits (IV, V, VI, VII, VIIa, and VIII) have been characterized biochemically [18–22], and partial or complete amino acid sequences are now known for each [21–23]. In addition, numerous nuclear mutants deficient in cytochrome *c* oxidase have been isolated and characterized recently in this laboratory [McEwen, Ko, and Poyton, in preparation]. This collection includes several that are likely to contain mutations in structural genes for nuclear-encoded cytochrome *c* oxidase subunits. We report here the characterization and use of one group of these mutants to clone the structural gene for subunit V. The availability of this gene, its DNA sequence, and the amino acid sequence of its protein product should enable us to elucidate: precursor-product relationships during the synthesis of this subunit, the pathway(s) involved in its targeting to the inner mitochondrial membrane, and those regions of the polypeptide chain that are essential for its assembly into a functional holoenzyme.

MATERIALS AND METHODS

Strains, Growth Media, and Plasmids

The following Saccharomyces cerevisiae strains were used: D273-10B (*Matα*, ATCC #24657); E4-238 (*Matα cox5-1*) [24], JM110 (*MATα cox5-1R lys*); JM33 (*Mata cox5-1 his4 ade2 opl*); JM23 (*Mata his4*); JM23/110 (diploid, JM23 × JM110); JM23/E4-238 (diploid, JM23 × E4-238); JM33/110 (diploid, JM33 × JM110); AB35-13D (*MATa leu2-3 leu2-112 ura3-52 trpl-289 his4-580 ade2*), a gift of Dr A.

Brake, University of California, Berkeley; AB320 (*HO ade2-1 lys2-1 trp5-2 leu2-1 can1-100 ura3-1* and/or *ural-17 met4-1*) [25]; and JM28 (*MATa leu2-3 leu2-112 his4-580 ura3-52 trpl-289 ade2 cox5-1*). JM28-511 and JM28-5210 are tranformants of JM28, as described in Results. All strain constructions were by standard yeast genetic techniques [26]. Yeast strains were grown at 28°C on one of the following media [26], as indicated in the text: YPD, YPGE, SD, SCD, SCG, (the same as SCD except containing 2% galactose instead of dextrose), MEG (0.67% yeast nitrogen base without amino acids, 3% [vol/vol] glycerol, 2% [vol/vol] ethanol), or semisynthetic galactose medium [27]. Amino acids and nucleotides were added or deleted as required. All solid media contained 2% bacto agar.

Escherichia coli strains RR1 (F^- *pro leu thi lacY* $Str^r r^-$ m^- $endoI^-$) or HB101 (F^- *pro leu thi lacY* Str^r *EndoI*$^-$ *recA*$^-$) were grown in LB medium [28]. Strains carrying plasmids were routinely grown in LB medium supplemented with 100 μg/ml ampicillin (LB-amp). When growing E coli strains for the large-scale preparation of plasmid DNA either the above LB-amp medium or M-9 casamino acids medium [28] containing ampicillin was used. All incubations were at 37°C.

The plasmid YEp13 [29] was used as a vector throughout this study. YEp13-511 and all other plasmids used in this study were obtained from a hybrid yeast library, which was constructed from a partial Sau3A digestion of total yeast DNA ligated into YEp13 [25].

Polyacrylamide Gel Electrophoresis and Immunoblotting

SDS polyacrylamide gel electrophoresis was performed in the discontinuous buffer system of either Laemmli [30] or Poyton and Schatz [18]. In order to achieve better resolution of small proteins, the resolving gel of the latter system was modified to contain 11.875% acrylamide, 0.625% bisacrylamide, 3.5 M urea and 27% glycerol [cf 31]. Protein blotting was performed as described [32].

Transformation of Yeast and E coli

All yeast transformations were performed using the alkali-cation technique of Ito et al [33], with minor modifications. Transformation of E coli was accomplished using the procedure of Lederberg and Cohen [34].

Preparation of Nucleic Acids

Large-scale preparation of plasmid DNA was performed following chloramphenicol amplification of E coli cultures [35] grown as described above. A slightly modified cleared lysate protocol [36] was used. The enriched plasmid DNA was then further purified by isopycnic centrifugation on ethidium bromide-CsCl gradients. Mini-preparations (< 50ml culture) of plasmid DNA were obtained as described [37].

All preparations of yeast DNA were obtained by the small-scale procedure of Nasmyth and Reed [38]. Total RNA from yeast cells was isolated according to Lustig et al [39].

Restriction Endonuclease Analysis

Restriction digests of purified plasmid DNA, agarose gel electrophoresis, and end-labeling of DNA fragments were performed according to standard procedures [40]. All enzymes were purchased from Bethesda Research Labs, New England Biolabs, or Boehringer Mannheim.

In Vitro Translation of Plasmid-Selected RNA

The plasmids YEp13-511 or YEp13 were either linearized with the restriction enzyme BamHI, or directly immobilized on nitrocellulose filters. DNA (10 μg) was immobilized on a 3mm-square filter, using published methods [40]. Hybridization reactions were performed for 6 hr at 45°C in 40% spectro-grade formamide, 25 mM Pipes (pH 6.8), 0.4% SDS, 0.5 M NaCl, using 200–300 μg total yeast RNA prepared from transformant JM28-511.The filters were washed and the RNA eluted as described [41]. In vitro translations were performed in nuclease-treated rabbit reticulocyte lysates purchased from Amersham-Searle (Arlington Heights, IL.). The manufacturer's directions were followed for all translations. After dilution into SDS gel sample buffer [18,30], in vitro translation products were either loaded onto 14% SDS-polyacrylamide gels [29] or immunoprecipitated [39] and then loaded onto 14% gels. Radiolabeled protein bands were localized by fluorography.

Miscellaneous Methods

Mitochondria were prepared after osmotic lysis of spheroplasted yeast, essentially as described elsewhere [42]. Holocytochrome *c* oxidase was purified from 500 g (wet weight) of cells from D273-10B or JM110 as described [20], except that the urea-wash step during octyl Sepharose chromatography was omitted. Cytochrome *c* oxidase activity was measured spectrophotometrically as the rate of oxidation of 32.5 μM reduced cytochrome c (Horse heart type VI from Sigma) [43]. It is expressed as the initial velocity (μmol cytochrome *c* per min) and normalized to either protein concentration or heme *a* content. Protein concentrations were determined by the method of Lowry [44]. Antisera were prepared in female New Zealand white rabbits as described [45]; purified subunit V or holocytochrome *c* oxidase were used as antigens.

RESULTS

Identification of Nuclear Pet Mutants That Synthesize Altered Forms of Subunit V of Cytochrome c Oxidase

In order to identify mutants altered in structural genes for nuclear-encoded cytochrome *c* oxidase subunits, we have screened a large number of nuclear cytochrome oxidase-deficient mutants, generated in this and other laboratories [McEwen, Ko, and Poyton, in preparation; 24], for their cytochrome oxidase subunit compositions. Thus far, we identified mutants in three complementation groups that lack, or have altered forms of, either subunit IV, subunit V, or subunit VII [McEwen, Ko, and Poyton, in preparation; McEwen, Power, and Poyton, in preparation]. The mutants that are defective in subunit V are the subject of this report. As shown in Figure 1 (lane 6), the mutant E4-238 appears to lack subunit V (M_r = 12,500) when antiholocytochrome *c* oxidase antibody is used in a Western immunoblot of mitochondrial proteins. However, when a subunit V-specific antiserum is used to probe these blots, an altered form of subunit V, which comigrates with subunit VI (M_r = 9,500), is detected (Fig. 1, lane 2). Another strain, JM110, also produces an altered subunit V, which migrates with an apparent M_r = 13,500 (Fig. 1, lanes 3 and 7). This strain, which will be described more completely elsewhere [McEwen, Power, and Poyton, in preparation], is a revertant of E4-238 that was isolated after mutagenesis with ethyl methane sulfonate. It has regained partial, rather than complete, respiratory compe-

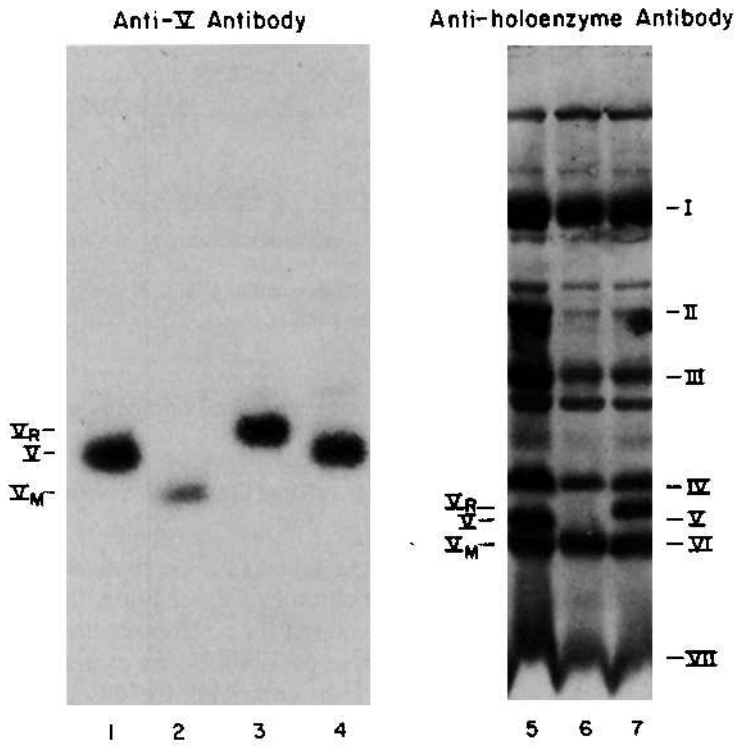

Fig. 1. Detection of altered forms of subunit V in the nuclear pet mutant E4-238 and its same-site revertant JM110. Strains were grown in semisynthetic galactose medium, harvested during the logarithmic phase of growth, and mitochondria prepared as described in Materials and Methods. 25 μg (lanes 1–3) or 50 μg (lanes 5–7) of mitochondrial protein were loaded per gel track; electrophoresed in a 12.5% polyacrylamide gel containing SDS, urea, and glycerol; and immunoblotted with antisera to subunit V (lanes 1–4) or holocytochrome *c* oxidase (lanes 5–7) (see Materials and Methods). The positions of cytochrome oxidase subunits are indicated by I-VII on the right of the figure; and the positions of wild-type, mutant, and revertant forms of subunit V are indicated by V, V_M, and V_R, respectively. The mitochondrial proteins loaded are lanes 1 and 5, strain D273-10B; lanes 2 and 6, strain E4-238, lanes 3 and 7, strain JM110; lane 4, 0.5 μg purified holocytochrome *c* oxidase.

tency, as assayed by its growth rate in nonfermentable medium (not shown) and by its level of cytochrome *c* oxidase activity in isolated mitochondria or purified holoenzyme (Table I). The new (revertant) lesion in JM110, which is responsible for these phenotypes, is located in the same gene as the original E4-238 mutation, as shown by tetrad analysis. For these analyses, JM110 was crossed to a wild-type strain, the diploid was isolated and sporulated, and the progeny from 18 tetrads were tested for growth on nonfermentable medium (YPGE). If the new lesion in JM110, which confers partial restoration of cytochrome oxidase activity in E4-238, was unlinked to the original E4-238 mutation, an average of 25% of the total haploid progeny should contain the E4-238 mutation alone and should fail to grow on YPGE. This was not observed. Instead, only five out of 90 progeny were respiratory-incompetent, and these proved to be rho− mutants, which arose spontaneously either during sporulation or upon germination of the spores. Therefore, the reversion event in JM110 either

TABLE I. Cytochrome Oxidase Activities of Wild-Type and Mutant Strains

Strain[a]	Specific activity	
	In mitochondria[b]	In holoenzyme[c]
D273-10B	3.8 (100%)	463
E4-238	0.12 (3%)	n.d.[d]
JM110	0.70 (18%)	251

[a]Cells were grown in semisynthetic galactose medium and harvested in the logarithmic phase of growth.
[b]μmol cytochrome *c* min^{-1} per mg mitochondrial protein $\times 10^{-1}$.
[c]μmol cytochrome c min^{-1} per μmol heme *a*.
[d]n.d. = not determined.

replaces the original E4-238 mutation, or creates a linked compensatory alteration in the mutated gene.

E4-238 and JM110 Are Altered in the Structural Gene for Subunit V of Cytochrome c Oxidase

How do different mutations in a single gene cause the production of different electrophoretic variants of subunit V of cytochrome oxidase? Either this gene encodes subunit V itself or it encodes an enzyme required for posttranslational processing or modification of subunit V. To distinguish these possibilities, we examined the form(s) of subunit V present in a diploid which is heterozygous for this gene. A protein processing, or modification, activity, should be trans-acting: ie, diploids containing both the dominant wild-type and the recessive mutant copies of the gene product should produce only the wild-type form of subunit V. In contrast, mutations in the subunit V structural gene should be cis-acting, so that both parental forms of subunit V are expressed in diploids heterozygous for the subunit V gene. As shown in Figure 2, the latter result was observed: both parental forms of subunit V are detected in mitochondria from diploid cells. Based on this evidence, it is likely that E4-238 and JM110 are altered in the subunit V structural gene. Additional proof is provided below, by the analysis of cloned DNA. We have given the subunit V gene the mnemonic *COX5*, which signifies *c*ytochrome *o*xidase subunit V.

Molecular Cloning of the *COX5* Gene

We used the yeast DNA library generated by Nasmyth and Tatchell [5], which contains 5–20 kilobase pair (kbp) pieces of yeast nuclear DNA inserted into the YEp13 vector, to clone the *COX5* gene by transformation of JM28 (*cox5-1, leu2-3, leu2-112*), a strain derived from E4-238 (see Materials and Methods). Putative $COX5^+$ transformants were selected by the following two-step protocol: Leu^+ transformants were first selected on SD(−leu) medium. Next, respiratory competent transformants, capable of growth on glycerol medium, were selected after replica-plating the leu^+ colonies onto either MEG (−leu), which simultaneously selects leu^{+1} and $glyc^+$, or YPGE medium, which selects only $glyc^+$. Eight stable leu^+/$glyc^+$ transformants were obtained. It should be noted that this two-step selection was required, since we were unable to obtain leu^+/$glyc^+$ transformants in a single step.

In order to characterize the cloned DNA in these strains, cellular DNA was isolated from 25 ml of cells grown in SD(−leu) and used to transform E coli strain

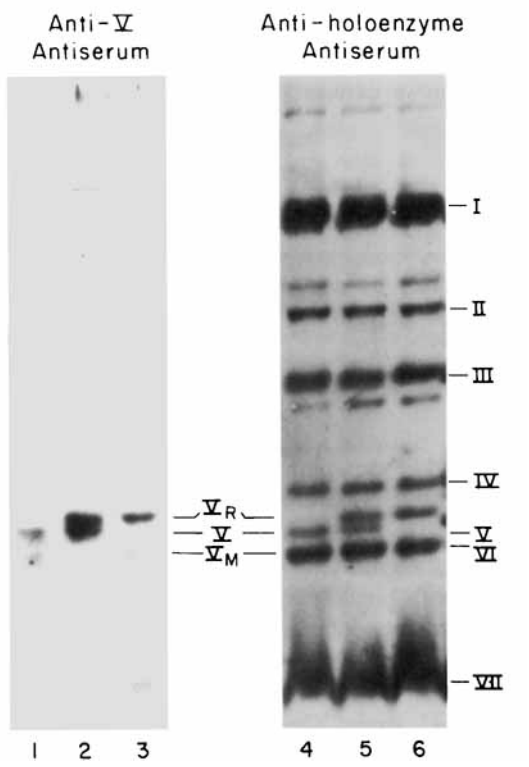

Fig. 2. Two forms of subunit V are produced in heteroallelic diploid cells. Mitochondrial proteins were prepared and immunoblotted as described for Figure 1. The mitochondrial proteins loaded are lanes 1 and 4, strain JM23/E4-238 (*COX5*$^+$/*cox5-1*); lanes 2 and 4, JM23/110 (*COX5*$^+$/*cox5-1R*); lanes 3 and 6, JM33/110 (*cox5-1*/*cox5-1R*).

HB101 to ampicillin-resistance. Plasmid DNA isolated from E coli transformants was then analyzed by agarose gel electrophoresis, and the size of each yeast DNA insert was determined. Inserts ranged in size from 4.8 kbp (from transformant JM28-5ll) to approximately 10 kbp (from transformant JM28-5212). Plasmid YEp13-511, which contained the smallest yeast DNA insert (4.8 kbp), was subjected to further analysis.

Two experiments were performed to demonstrate that transformant JM28-511, which harbors plasmid YEp13-511, is not a *COX5*$^+$ revertant but instead carries a plasmid-encoded gene that complements the chromosomal *cox5-1* mutation. In the first, plasmid DNA prepared from E coli clones carrying YEp13-511 was used to retransform JM28. All of the new transformants were capable of growth on glycerol medium, proving that the plasmid carries the gene that complements the *cox5-1* mutation. In the second, the segregation behavior of the leu$^+$ and glyc$^+$ phenotypes during growth of the original JM28-511 transformant under nonselective (YPD medium) or selective (SCD[−leu] or YPGE media) conditions was examined. As shown in Table II, the leu$^+$ and glyc$^+$ phenotypes were both lost during growth in YPD. In contrast, when only glyc$^+$ was selected, 100% of the cells remained leu$^+$. These two observations—loss of markers under nonselective growth conditions and retention of both markers when only one is selected—are evidence that the leu$^+$ and glyc$^+$ phenotypes are specified by the same plasmid.

TABLE II. Mitotic Co-Segregation of *COX5* and *LEU2* in Transformants*

	Liquid growth medium	No. of colonies on— YPD	YPGE	SCD(−leu)	%[a]
A. JM28-511	YPD	324	9	9	100
	YPGE	116	116	116	100
	SCD(−leu)	96	58	92	63
	SCD(−leu)shifted to YPGE	119	116	115	>99
B. JM28-5210	YPD	242	217	0	0

*Liquid cultures of either strain were grown in media as indicated. After at least 20 generations, aliquots of the various cultures were plated for single colonies on YPD agar plates. After 2 days of growth at 28° C, colonies were replica-plated onto selective agar (SCD[−leu] or YPGE).

[a]Percent is expressed as the number of leu^+ over the number of $glyc^+$ colonies or, for SCD(-leu)-grown cells, is the No. of $glyc^+$ over leu^+ colonies.

Surprisingly, when the segregation of $glyc^+$ was assessed after growth of JM28-511 under leu^+-selective conditions (SCD[−leu]), only 63% of the leu^+ colonies were $glyc^+$ (Table II). However, if the transformant was first grown in SCD(−leu) medium and then shifted to YPGE medium, greater than 99% of the plated colonies retained both phenotypes. We interpret this result to mean that the original JM28-511 transformant contained two plasmids: one, YEp13-511 carries both *COX5* and *LEU2*, while the other carries only *LEU2*. In support of this interpretation, we recovered two plasmids from E coli clones that had been transformed with DNA from the original JM28-511 strain. One is YEp13-511 and the other is the vector YEp13.

In Vivo and In Vitro Detection of Subunit V as a Plasmid-Encoded Gene Product

Transformants of JM28 that harbor the YEp13-511 plasmid (JM28-511) are capable of growth on a non-fermentable medium and should therefore produce a functional holocytochrome *c* oxidase containing the subunit V that is expressed from a plasmid-localized gene. As shown in Table III, mitochondria from JM28-511 contain more cytochrome *c* oxidase activity than untransformed JM28 mitochondria. When JM28-511 mitochondria were screened by Western immunoblots for their content of plasmid-encoded subunit V, two immunoreactive forms of subunit V were observed. As shown in Figure 3A, the antiserum to subunit V detected both the M_r = 9,500 polypeptide, which is present in untransformed JM28 and corresponds to the polypeptide observed in the original mutant E4-238 (cf Fig. 1), and a new polypeptide (M_r = 13,500), which represents the plasmid-encoded gene product. Antiholoenzyme antibody (Fig. 3B) detects only the latter because, as noted above, the M_r = 9,500 polypeptide comigrates with subunit VI. It is surprising that the plasmid-encoded subunit V has a higher apparent molecular weight than either the wild-type polypeptide (M_r = 12,500), or its mutant form (M_r = 9,500). We don't yet understand the reason for this, but several obvious explanations, such as strain polymorphisms, gene fusion, or a failure in precursor processing, can be ruled out (see Discussion).

Regardless of the explanation for the slow-migrating form of subunit V in transformants, it is important to note that the wild-type protein is produced after the plasmid and mutant genes have recombined. As shown in Figure 3A, (far left lane) transformant JM28-5210, as originally isolated, produced the slow-migrating form of subunit V. However, after maintenance in culture for several generations, the *COX5*

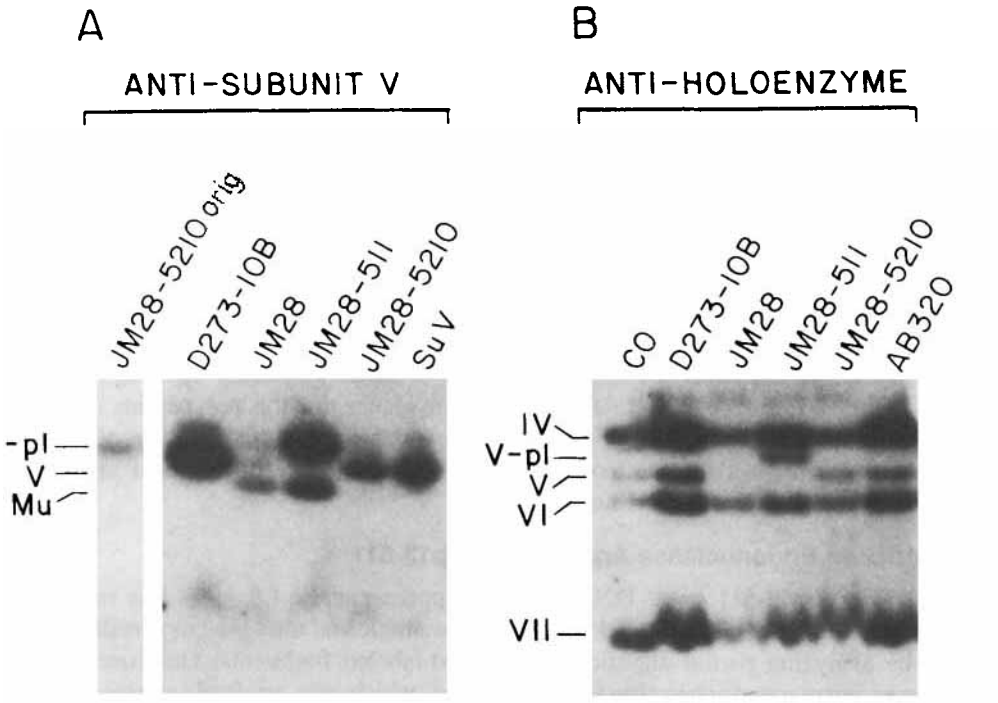

Fig. 3. Detection of plasmid-encoded subunit V in *COX5* transformants. Yeast strains were grown in SCD(-leu) medium (except JM28 and AB320, where 40 μg/ml leucine was added) and harvested in the logarithmic phase of growth. Mitochondria were prepared and proteins immunoblotted as described in Figure 1. In all cases, 50 μg of mitochondrial protein from the indicated strain, 0.5 μg holocytochrome *c* oxidase (CO), or 0.05 μg purified subunit V were loaded per gel track. The positions of the wild-type cytochrome oxidase subunits, the mutant subunit V (V-Mu), and the plasmid-expressed subunit V (V-p1) are as indicated. The gel track on the far left (JM28-5210 orig) shows that the V-p1 form of subunit V was originally present in transformant JM28-5210. Only the cytoplasmic subunit region of the gel is shown. A) Blot was incubated with anti-subunit V antiserum. B) Blot was incubated with antiholocytochrome *c* oxidase antiserum.

TABLE III. JM28 Transformants Regain Cytochrome *c* Oxidase Activity

Strain[a]	Growth rate[b]	Specific activity[c]	% Wild type
D273-10B	1.75	4.5	100
JM28	4	0.85	19
JM28-511	4.05	3.09	69
JM28-5210	4	3.52	78

[a]JM28 was grown in SCD medium and all other strains, in SCD(−leu) medium. Cells were harvested in the logarithmic phase of growth.
[b]Td, time (in hours) required for doubling of cell mass in SCD (JM28) or SCD(−leu) (other strains).
[c]μmol cytochrome c min^{-1} per mg mitochondrial protein × 10^{-1}.

allele from this plasmid appears to have recombined with the chromosomal *cox5-1* locus of JM28, thereby producing a recombinant gene that is expressed into wild-type protein (Fig. 3A, fifth lane). Additional evidence for recombination between the plasmid and chromosomal subunit V genes is shown in Table IIB. It is clear that, in contrast to transformants carrying *COX5* on a plasmid (see JM28-511, Table IIA) the

glyc$^+$ phenotype of JM28-5210 is mitotically stable, whereas the plasmid gene *LEU2* remains unstable. It should be noted that these observations can also be explained by a reversion of the chromosomal *cox5-1* locus in JM28-5210. However, we do not favor this explanation because the frequency of spontaneous revertants of *cox5-1* is very low ($< 10^{-7}$).

To identify subunit V as a plasmid-encoded gene product in vitro, the hybridization selection technique of Ricciardi et al [41] was used. When reticulocyte lysates were programmed with RNA isolated from JM28-511 and selected by hybridization to YEp13-511 DNA, an M_r = 15,000 product, which is the size expected for a subunit V precursor polypeptide [39], was seen both amongst total products (Fig. 4A, lane 3) and after immunoprecipitation with anti subunit V antiserum (Fig. 4B, lane 2). This polypeptide is not observed after translation of mRNA selected by YEp13 vector DNA (Fig. 4A, lane 4 and 4B, lane 5). Direct evidence that this polypeptide is related to subunit V was obtained in an experiment in which immunoprecipitation of the ^{35}S-labeled in vitro product was competitively inhibited in the presence of unlabelled subunit V (Fig. 4B, cf lanes 1 and 2).

Restriction Endonuclease Analysis of YEp13-511

The YEp13-511 yeast DNA insert is approximately 4.8 kbp. The restriction map of this region (Fig. 5) was determined after single and multiple enzyme digestions and by analyzing partial digestions of ^{32}P-end-labeled fragments. Only one BamHI site was regenerated when this DNA fragment, which was originally derived from a partial Sau 3A digest of total yeast DNA [25], was inserted into the BamHI site of the YEp13 vector. Restriction enzymes that did not cut within the YEp13-511 insert are Kpn1, Pvu11, Sal1, Sst1, Sst11 and Sma1.

DISCUSSION

In S cerevisiae, the most convenient way to clone a specific gene is by complementation of a well-characterized mutant altered in that gene. We therefore approached the cloning of the nuclear gene that encodes subunit V of cytochrome *c* oxidase by first identifying and characterizing mutants in the subunit V structural gene, which we call *COX5*. We have shown that the mutant E4-238 and its revertant JM110 produce altered forms of subunit V. In comparison to wild-type subunit V (M_r = 12,500), the polypeptides in E4-238 and JM110 exhibit apparent molecular weights of 9,500 and 13,500, respectively. Because the mutations in E4-238 and JM110 are cis-acting in diploid cells, we conclude that these mutants are altered in the subunit V structural gene rather than in a gene required for processing or modification of subunit V.

We cloned the gene for subunit V by transforming the *cox5-1* mutant to respiratory competency. One plasmid, YEp13-511, which carries the *COX5* gene on a 4.8-kbp fragment of yeast nuclear DNA ligated into the vector YEp13, has been characterized in detail. Several observations clearly establish that the structural gene for subunit V is localized on this insert. These include (1) the ability of the plasmid to restore respiratory competency and to increase enzyme activity in the *cox5-1* mutant after being shuttled from yeast to E coli and once again to yeast; (2) the cosegregation of a known plasmid-encoded gene, *LEU2*, and the *COX5* gene; (3) the appearance of a new form (M_r = 13,500) of subunit V in mitochondria from mutant

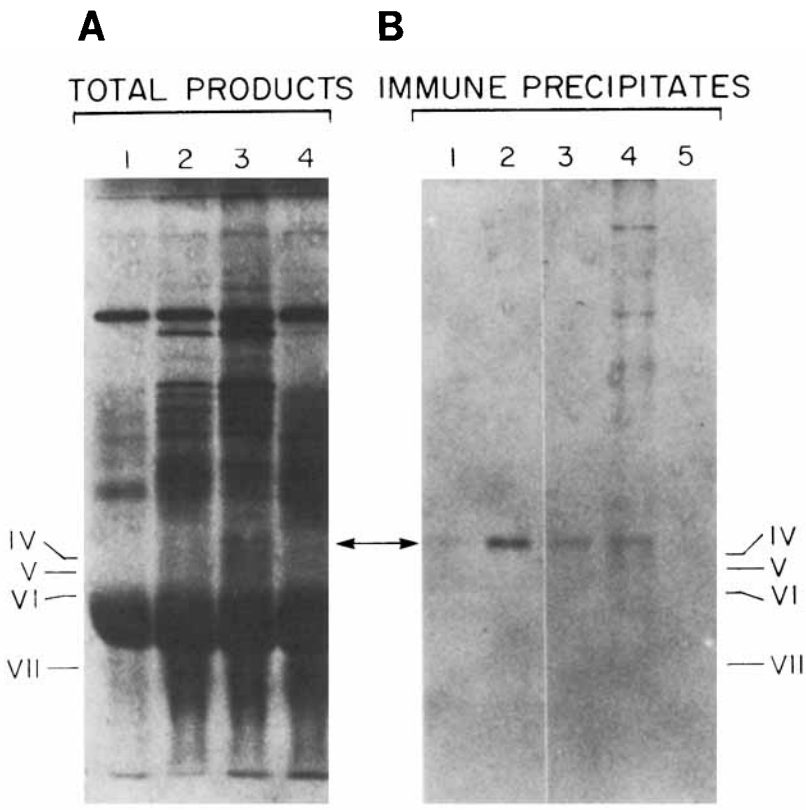

Fig. 4. In vitro translation of YEp13-511 selected RNA. Total RNA was prepared from strain JM28-511 after growth in YPGE medium. 200–300 μg of this RNA was hybridized to 10 μg filter-immobilized YEp13-511 or YEp13 DNA (see Materials and Methods). 2 μl of the selected RNA was translated in vitro using 10 μl of a nuclease-treated rabbit reticulocyte lysate. After 90-min incubation at 30°C, 3 μl of the translation mix was diluted into 10 μl of sample buffer and run directly on a 14% polyacrylamide gel (total products). The remainder was subjected to immunobinding using subunit V-specific antisera as described [39], and products were resolved on a 14% polyacrylamide gel (immune precipitates). The Laemmli buffer system was used in both gels [30]. Labeled proteins were detected after fluorography. The samples loaded under Total Products A) are lane 1, products from incubation minus RNA; lane 2, products from RNA selected by YEp13-511; lane 3, products from RNA selected by BamHI linearized YEp13-511; lane 4, products from RNA selected by YEp13. The samples loaded under Immune Precipitates B) are lane 1, products from RNA selected by BamHI linearized YEp13-511 and immunoprecipitated in the presence of competing cold subunit V (15 μg); lane 2, the same as lane 1, except that no cold subunit V was added; lane 3, the same as lane 2 except that the YEp13-511 DNA used to select mRNA was not cut with BamHI; lane 4, immunobound products from JM28-511 RNA that wasn't hybrid-selected; lane 5, the same as lane 3 except that the vector YEp13 was used to select RNA.

cells transformed with YEp13-511; (4) the restoration of a wild-type subunit V polypeptide after recombination of the plasmid-carried gene with the chromosomal *cox5-1* gene; and (5) the ability of the plasmid to select subunit V-specific mRNA.

Since the plasmid-encoded *COX5* gene results in the appearance of increased levels of cytochrome *c* oxidase activity in *cox5* mutant mitochondria, it is clear that the subunit V polypeptide expressed from the plasmid is capable of entering the

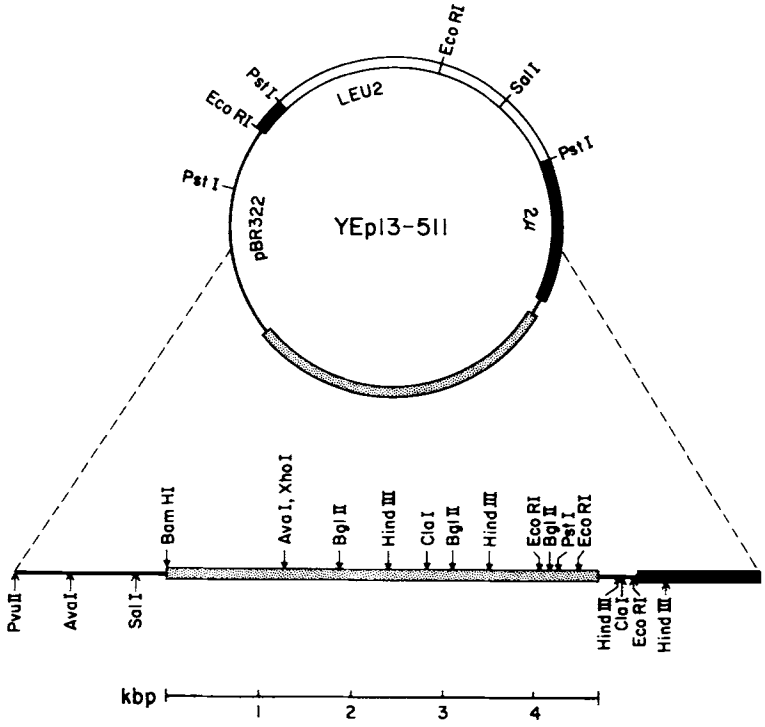

Fig. 5. Partial restriction map of the YEp13-511 insert. The position of the YEp13-511 insert relative to the vector YEp13 is schematically diagrammed. Restriction enzyme cleavage sites within the insert were determined as described in Materials and Methods. A few representative sites in the vector are also included.

mitochondrion and assembling, with the other eight polypeptide subunits of the enzyme, into a functional holoenzyme. Unexpectedly, the plasmid-derived gene product is slightly larger than its wild-type counterpart. At the moment, the reason for this is unclear. One possibility is that we have cloned a new allele of *COX5*, which produces an active protein of M_r = 13,500. However, the *COX5*$^+$ DNA on the plasmid YE13-511 was originally obtained from strain AB320 (25), and we have shown that AB320 produces the wild-type (M_r = 12,500) form of subunit V (Fig. 3B). Another possibility is that the *COX5* gene was fused to pBR322 sequences during construction of plasmid YEp13-511, resulting in improper translational expression of *COX5*. This is unlikely because we have observed the same slow-migrating form of subunit V in each of eight independently isolated *COX5* transformants whose plasmids contain yeast DNA inserts of different sizes, with different insert-vector junctions (data not shown). A third possibility is that the plasmid-expressed gene product is incompletely processed. This protein undergoes at least some posttranslational processing as it is targeted to, or enters, the inner mitochondrial membrane, because its molecular weight (13,500) is less than that of the subunit V precursor detected by in vitro translation of mRNA isolated from the transformant (M_r = 15,000; cf Fig. 4). It is possible that this processing is incomplete or improper when the *COX5* gene is located

on a plasmid. In this regard, it is interesting that the slow-migrating form in transformants has so far been observed only when the gene is expressed from the plasmid. In strain JM28-5210, in which the plasmid gene apparently recombined into chromosomal DNA, a protein of wild-type size is produced. One explanation for all of these observations is that it is the overexpression of the subunit V gene, in this case from the high copy number plasmid YEp13-511, which results in incomplete, or abnormal, processing of the subunit V precursor polypeptide. A precedent for this interpretation exists for the yeast mating pheromone alpha factor: overexpression of the alpha factor precursor from a high-copy-number plasmid results in incomplete processing of the protein product [46].

The successful cloning of the subunit V gene reported here represents the first step toward our goal of using molecular genetics to probe the assembly of a mitochondrial membrane protein, cytochrome *c* oxidase. The availability of *COX5* mutations, the cloned *COX5* gene, and the observation that the cloned gene complements *COX5* mutations enable us to use in vitro mutagenesis to dissect the pathway by which subunit V of cytochrome *c* oxidase is targeted to the inner mitochondrial membrane and assembled into a holoenzyme.

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