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Functional Analysis of Mitochondrial Protein Import in Yeast

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In order to facilitate studies on protein localization to and sorting within yeast mitochondria, we have designed an experimental system that utilizes a new vector and a functional assay. The vector, which we call an LPS plasmid (for leader peptide substitution), employs a yeast *COX5a* gene (the structural gene for subunit Va of the inner membrane protein complex cytochrome *c* oxidase) as a convenient reporter for correct mitochondrial localization. Using in vitro mutagenesis, we have modified *COX5a* so that the DNA sequences encoding the wild-type subunit Va leader peptide can be precisely deleted and replaced with a given test sequence. The substituted leader peptide can then be analyzed for its ability to direct subunit Va to the inner mitochondrial membrane (to target and sort) by complementation or other in vivo assays. In this study we have tested the ability of several heterologous sequences to function in this system. The results of these experiments indicate that a functional leader peptide is required to target subunit Va to mitochondria. In addition, leader peptides, or portions thereof, derived from proteins located in other mitochondrial compartments can also be used to properly localize this polypeptide. The results presented here also indicate that the information necessary to sort subunit Va to the inner mitochondrial membrane does not reside in the leader peptide but rather in the mature subunit Va sequence.

Key words: cytochrome oxidase, subunit Va, in vitro mutagenesis

Recent results from several laboratories have clearly established the critical role of the amino terminal leader peptide (or presequence) in the selective targeting of proteins to, and into the mitochondrion [for recent reviews see references 1 and 2]. Numerous studies, most of them involving chimeric gene fusions, have demonstrated that the leader peptide is necessary and often sufficient for mitochondrial targeting [3,4]. In some cases, it has been shown that the leader peptide contains sequences

Abbreviations used: kb, kilobase pair; dNTPs, deoxynucleotide triphosphates; SDS, sodium dodecyl sulfate; TRP^+, tryptophan positive; TMPD, tetramethyl-p-phenylenediamine.

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necessary for correct intramitochondrial localization (sorting) as well [5]. Recently, it has been proposed that many leader peptides on imported proteins have the ability to form amphipathic helices which might be a common structural feature important in their function [6,7].

While much has been learned about mitochondrial leader peptides and their role in protein import, several important questions remain answered. Are specific amino acid residues required for targeting or is secondary structure alone sufficient? Does all of the information required to target and sort an imported protein reside in its leader peptide or does the mature polypeptide contribute? How many ancillary factors (e.g., receptors, soluble factors) [8,9] are required for import, and do they interact with the leader peptide?

To address these and other questions, we have developed a new experimental system. Central to it is the yeast gene *COX5a*, which encodes subunit Va of the mitochondrial inner membrane protein complex cytochrome *c* oxidase. The *COX5a* gene, one of two nuclear genes specifying divergent forms of subunit V in yeast [10,11], is typical of the majority of yeast nuclear genes encoding mitochondrial proteins in that it is initially synthesized in precursor form. The preprotein contains a basic 20-residue leader peptide which is proteolytically removed during or after import into the organelle [12]. Gene disruption studies which have inactivated one or both *COX5* genes have established that a form of subunit V is required for cytochrome oxidase activity and hence for cellular respiration [11,13]. Thus, in yeast strains lacking these genes, there is no detectable respiration [11,13]. Such strains are unable to grow on nonfermentable substrates like glycerol or lactate, although wild-type respiration can be restored by the introduction of a single copy of *COX5a* or by multiple copies of *COX5b* [11,13].

In this study, we have constructed a plasmid in which the wild-type *COX5a* gene has been modified such that the sequences encoding the Va leader peptide can be removed and replaced with a heterologous sequence of choice. When a recombinant plasmid is transformed into a yeast host deleted for both subunit V genes, the ability of the new sequence to direct the attached protein to the inner mitochondrial membrane can be assayed by screening for the ability of transformants to respire. We have thus far tested the ability of several different sequences to target subunit Va in this system. We report here the results of these initial studies.

MATERIALS AND METHODS

Strains and Growth Media

The wild-type *Saccharomyces cerevisiae* strains used in this study were D273-10B (*mat*α, ATCC 24657) and JM43 (*mat*α *leu2-3 leu2-112 his4-580 ura3-52 trp1-289*), and both have been described previously [11]. The strain used for complementation studies was JM43-GD5ab (*mat*α *leu2-3 leu2-112 his4-580 ura3-52 trp1-289 cox5a*Δ*::URA3 cox5b::LEU2*). This strain is a derivative of JM43 in which the chromosomal copies of the *COX5a* and *COX5b* genes were sequentially disrupted with the *URA3* and *LEU2* genes, respectively [13]. When not harboring an extrachromosomal plasmid it has no detectable respiration and thus will not grow on any nonfermentable substrates [13]. Yeast strains were routinely grown at 30°C in selective or nonselective media as described [11]. Respiratory proficiency was tested on YPEG or YP lactate media [14].

E. coli strains used for plasmid propagation were HB101, RR1, or DH1 [11]. For phage cloning and propagation, JM101 or a *recA*$^-$ derivative of JM103 was used [11]. *E. coli* strains were grown at 37°C in LB or YT medium [11]. When necessary, transformants were selected in the above medium containing 100 μg/ml ampicillin.

Plasmid Construction

The plasmid YCpLPS-5a (Fig. 1) was constructed in several steps, as follows. A 3.4-kb EcoR1 fragment containing the *COX5a* structural gene and over a kilobase of both 5′ and 3′ flanking sequence, was isolated from the plasmid YEp13-552 [11] and cloned in the M13 phage vector mp19 [15]. Single-stranded template DNA prepared from JM103 cells carrying this phage (named mp19-5aEco) was then used as a template for in vitro mutagenesis. A synthetic oligonucleotide (5′-GCTCAA-ACGCATGCTCTT-3′) was used to create a unique SphI restriction site near the amino-terminal end of the mature subunit Va sequence (Fig. 2). This mutation changes codon 3 of the mature sequence from ACA to ACG but does not alter the amino acid specified at that position (thr). The mutagenesis procedure followed was essentially the two-primer method of Zoller and Smith [16] except that the ratio of primers to template was 1:1, the annealing was for 5 min at 65°C followed by 5 min at 37°C, the final concentration of dNTPs in the extension reaction was 50 μM, and the extension/ligation proceeded for 30–60 min at 37°C. The double-stranded mixture was transformed into JM103, and positive clones were identified by plaque hybridization [16]. Confirmation that the correct change had been introduced was by restriction digestion of the phage RF and by DNA sequence analysis using the upstream synthetic primer illustrated in Figure 2. Single-stranded template from one clone, mp19-5aEco-S^{2}13, was used in a second mutagenesis step designed to introduce a unique BamHI site just 5′ to the start of translation of the *COX5a* gene (Fig. 2). In this step, the synthetic oligonucleotide (5′-CAACTAAGAACGGATCCTACAATGTTACG-3′) introduced two changes to the DNA sequence, a C to G transversion at position −9, and the introduction of an additional C at position −5 (Fig. 2). The procedures used

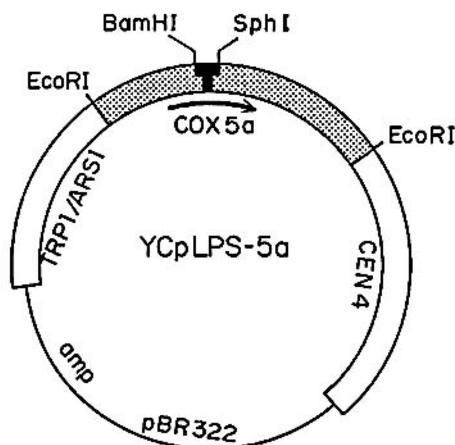


Fig. 1. The plasmid YCpLPS-5a. The leader peptide substitution (LPS) plasmid YCpLPS-5a, a yeast–*E. coli* shuttle vector useful for studies on mitochondrial protein import. Details on the construction of this plasmid are described in Materials and Methods. The BamHI and SphI restriction sites bracket the *COX5a* leader peptide.

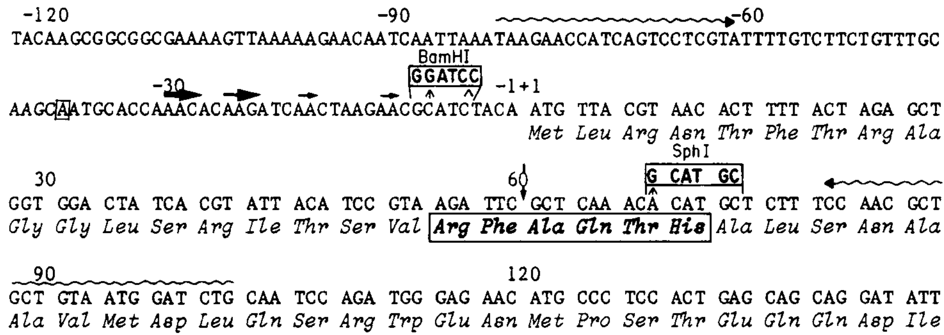


Fig. 2. DNA sequence of the *COX5a* gene in the region of the leader peptide. A partial DNA sequence of the yeast *COX5a* gene [12] from YCpLPS-5a, showing the changes introduced in order to create the two unique restriction sites. The boxed DNA sequences are the result of two separate in vitro mutagenic experiments, using oligonucleotides, as described in Materials and Methods. The boxed A at position −39 has also been changed to a T (see text). The bold arrows represent the 5′ ends of the *COX5a* transcripts; the wavy arrows designate the direction and sequence of two synthetic oligonucleotide primers used in sequencing YCpLPS constructs; and the vertical arrow at position 61 shows the site of processing of the subunit Va precursor [12]. The boxed amino acids from the *COX5a* protein sequence correspond to the boxed arrows from Figure 4. These residues are encoded on the oligonucleotides used to generate the heterologous presequences. Inclusion of the ArgPhe is optional (see text).

were as described above, and, as before, confirmation that the changes were introduced correctly was by restriction analysis and DNA sequencing of replicative form DNA. It should be mentioned that the clone selected for further use, mp19-5aEcoBS^2, which contained both new restriction sites, also carries an additional point mutation introduced by the mutagenesis procedure. However, several experiments, presented in Results, confirm that this mutation, an A to T transversion at position −39 of the *COX5a* 5′ flanking sequence (Fig. 2), has no effect on *COX5a* gene expression.

RF from the phage mp19-5aEcoBS^2 was prepared and cut with EcoRI, and the 3.4-kb fragment containing the *COX5a* region was purified by electroelution. This insert was then ligated into the EcoRI site of the yeast centromere plasmid YCp199, a derivative of YCp19 [17] deleted for the PvuII fragment containing the *URA3* gene (R.M. Wright, personal communication). In the final step, an endogeneous SphI site was deleted from the upstream region of the *COX5a* gene by partial digestion with SphI, purification of full-length linear molecules, treatment with T4 DNA polymerase to flush the ends, followed by religation and transformation into DH1. The final construct, YCpLPS-5a, is shown in Figure 1.

Synthetic Oligonucleotides

Synthetic oligonucleotides used for mutagenesis, as sequencing primers, or for cloning, were synthesized on an Applied Biosystems (University of Colorado, Boulder) or a Biosearch 8600 (University of California, Irvine) DNA synthesizer. In all cases, purification was accomplished by elution from polyacrylamide/urea preparative gels [11]. When the synthetic sequences were to be used to reconstruct leader peptide sequences in YCpLPS-5a, one of two cloning schemes was followed. For a short sequence, in this case LL5a, the oligonucleotide was cloned in single-stranded form by the "notch-cloning" procedure [18]; 50 ng of phosphorylated oligonucleotide was ligated directly into 100 ng of BamHI-SphI cut vector, and the mixture was used to

directly transform *E. coli* strain DH1 (Fig. 3). When oligonucleotides coding for longer sequences were to be cloned, a procedure similar to that described by Rossi et al. [19] was followed. Briefly, two strands containing an overlap of about 10 base pairs at their 3′ termini were synthesized separately, annealed, and extended with the Klenow enzyme or reverse transcriptase. After repeated extraction with phenol-chloroform and purification of the DNA by ethanol precipitation, the extended hybrids were cut with BamHI and SphI, ligated into YCpLPS-5a prepared as above, and transformed into DH1 (see Fig. 3). Screening for recombinants produced by either technique was by colony hybridization using an end-labelled oligonucleotide [16]. Before using any recombinant plasmid, it was subjected to DNA sequence analysis to confirm that the correct sequence had been cloned.

DNA Sequence Analysis

In all cases, sequencing was performed by using the dideoxy chain termination method [20]. In order to facilitate the sequence analysis of leader peptide recombinant clones in YCpLPS-5a, a double-stranded method was used. The procedure followed was a modification of that described by Zaug et al. [21]. Routinely, plasmid DNA was denatured in 0.2 N NaOH for 5 min at room temperature and then mixed with 7 ng of a 5′-end-labelled sequencing primer. The mixture was neutralized with NaOAc and ethanol precipitated. Following centrifugation, the pellet was washed, resuspended, and sequenced with AMV reverse transcriptase [11].

Miscellaneous Methods

Transformation of yeast was by the alkali cation [22] method, and transformation of *E. coli* was by the modified method of Hanahan [23]. Established procedures were used for all recombinant DNA and labelling work. Methods for SDS-polyacrylamide gel electrophoresis, immunoblotting, preparation of mitochondria, preparation of nucleic acids, and oxygen consumption assays have been described [11,13].

RESULTS

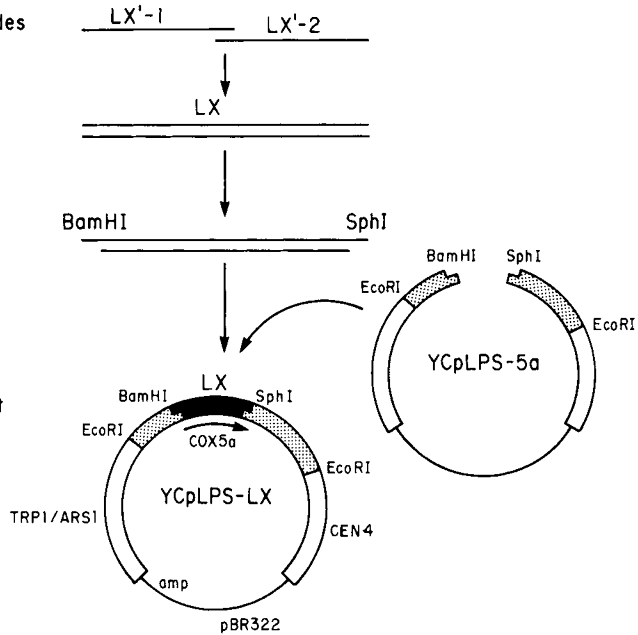
The Vector YCpLPS-5a

Using the procedures described in Materials and Methods we have constructed the LPS plasmid YCpLPS-5a (Fig. 1). This vector has several important features that make it useful for studies on the import of proteins into mitochondria. First, it is a yeast-*E. coli* shuttle plasmid, containing sequences that allow its propagation and selection in either organism. In addition, when propagated in yeast, the presence of a centromere (*CEN4*) maintains the copy number at approximately one per cell [17]. Also contained on the plasmid is a yeast *COX5a* gene that has been modified by in vitro mutagenesis, so that two unique restriction endonuclease cleavage sites bracket the sequences encoding the Va leader peptide (Fig. 2). When the plasmid is cleaved with these enzymes (BamHI and SphI), this region is removed, and in its place a given test sequence, or "leader peptide cassette," can be substituted. By using synthetic DNA, inserted by either of the two methods shown in Figure 3, virtually any sequence can be positioned in front of subunit Va and assayed for its ability to direct the import of the attached Va polypeptide.

There are several additional features of YCpLPS-5a that are noteworthy. First, during its construction we have not altered the subunit Va polypeptide sequence, nor

A.

1. Synthesize oligonucleotides
2. Anneal
3. Fill in (Klenow or RT)
4. Digest with BamHI and SphI
5. Ligate into Bam/Sph cut YCpLPS-5a



B.

1. Synthesize oligonucleotide
2. Anneal to Bam/Sph cut YCpLPS-5a
3. Ligate, transform E. coli

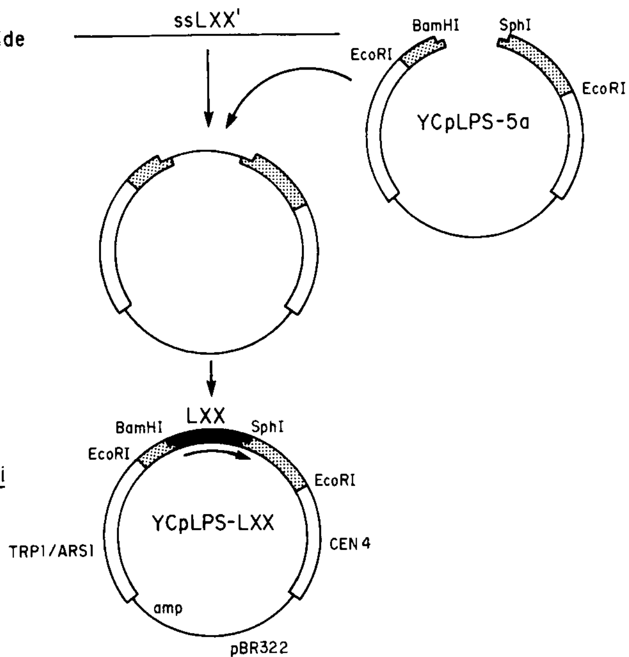


Fig. 3. Construction of YCpLPS derivatives containing heterologous leader peptides. **A:** Schematic representation of the two oligonucleotide method used to generate the hypothetical leader peptide LX (leader X). **B:** The "notch cloning" method used to generate the hypothetical leader LXX (leader XX) [18].

have we changed the translation initiation context (Fig. 2). Second, although creation of the BamHI site introduced three changes to the *COX5a* upstream DNA sequence (Fig. 2), none of the mutations has a detectable effect on the expression of the *COX5a* gene as determined by Northern blot analysis (not shown) and the immunoblot and respiration studies described below.

Another important feature of YCpLPS-5a is the location of the unique SphI site; it lies with the *COX5a* mature sequence at codons 3–5 (Fig. 2). Thus, when synthetic oligonucleotides are used to introduce a given presequence in front of subunit Va, one has the option of retaining or eliminating the amino acid residues immediately before and after the site of proteolytic processing. Therefore, we can begin to examine which residues are important for processing the precursor and whether proteolysis is important for the import or function of subunit Va.

Construction of YCpLPS Derivatives Carrying Heterologous Leader Peptides

In initial experiments, YCpLPS-5a was used to construct several heterologous leader peptide fusions schematically depicted in Figure 4. Using the "notch cloning" procedure first described by Childs et al. [18], and illustrated in Figure 3B for YCpLPS-5a, a *COX5a* gene completely lacking a leader peptide was constructed (YCpLPS-LL5a). It contains only the initiator methionine codon in front of the mature Va sequence. The second derivative shown in Figure 4, positioned sequences coding for the *COX5b* leader peptide in front of subunit Va. As mentioned, the yeast *COX5b* gene is similar to but not identical with *COX5a*. It encodes an isologous polypeptide (Vb) that is functionally equivalent to Va but is not usually observed in mitochondria prepared from aerobically grown, wild-type yeast cells [11–13]. The presequences of these two proteins share 45% homology, significantly less than the 68% homology

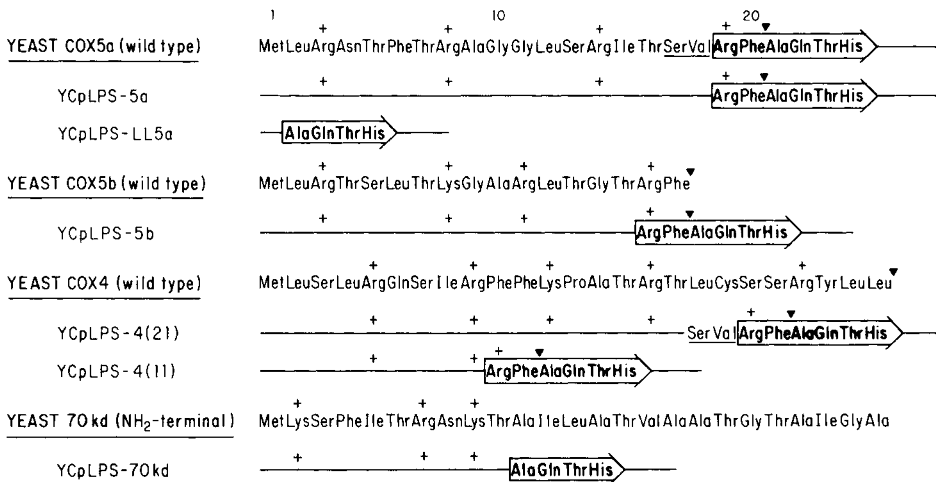


Fig. 4. Constructions in YCpLPS-5a. The polypeptide sequence of the heterologous leader peptides used in this study. The wild-type sequence of the different leader peptides is also shown (we show the amino-terminal sequence for the 70kd protein, since it is not proteolytically processed). Boxed or underlined residues are derived from *COX5a*. Basic residues in the presequence, as well as the *COX5a* proteolytic processing site, are also indicated (+ and arrowhead, respectively).

observed in their respective mature sequences. Substitution of the Vb leader for that of Va permits a comparison of the relative targeting efficiency of each presequence, as well as providing insight into whether the low levels of subunit Vb found in mitochondria prepared from aerobically grown yeast might result from a difference in the inability of this leader peptide to direct the import of its attached subunit [11,13]. The amino acid sequence of the construct, YCpLPS-5b, is shown in Figure 4. It was constructed by using the overlapping oligonucleotide method diagrammed in Figure 3A.

We next constructed two YCpLPS derivatives that carried portions of the yeast *COX4* leader peptide. We chose *COX4* for two reasons. First, it has been used extensively in gene fusion studies on mitochondrial import [1–3,24,25]; consequently, the minimal number of amino-terminal residues required to direct the import of several proteins has been defined [24]. Second, the *COX4* gene product (subunit IV of cytochrome *c* oxidase) appears to be a peripheral membrane protein, located on the inner face of the inner mitochondrial membrane, unlike subunit V, which is imbedded in the bilayer [26–28]. Thus, the use of the subunit IV presequence allows us to determine whether a leader peptide derived from a peripheral inner-membrane-associated protein can correctly localize a heterologous integral inner membrane protein (subunit Va). In the initial subunit IV construct, YCpLPS-4 (21), the first 17 amino acids of the subunit IV leader peptide (previously shown by Hurt et al. to direct mouse dihydrofolate reductase (DHFR) to the mitochondrial matrix) [24] were fused to the COOH-terminal four residues of the wild-type Va leader peptide (SerValArgPhe, Fig. 4). We therefore created a hybrid 21-amino acid leader peptide that contained the amino acids immediately adjacent to the Va processing site. In the second construct, YCpLPS-4 (11), the first nine amino acids of the subunit IV leader peptide (which the same authors also showed could not effectively direct the import of DHFR into mitochondria) [24] were joined to the COOH-terminal 2 (ArgPhe) of subunit Va. As above, the additional amino acids derived from Va were added in order to reconstruct the sequence(s) flanking the wild-type Va processing site. Although these residues increase the length of the leader peptide (by two amino acids) and contribute an extra positive charge as well, they were included because we were interested in determining whether the sequence ArgPheAlaGln constituted a site of proteolytic processing and whether processing was important for the import of subunit Va. This is an interesting question in light of the finding that the NH_2-terminal 12 residues of the *COX4* leader were not processed when used to direct DHFR into yeast mitochondria [24] but that the lack of cleavage did not affect the ability of the protein to be imported. It is also important to mention that the 11 amino acid presequence of YCpLPS-4 [11] differs from the wild-type *COX4* preseqnece at only one position. This is at residue 10, where Arg replaced Phe in the wild-type sequence.

In the last construction shown in Figure 4, YCpLPS-70kd, the subunit Va leader peptide was replaced by the NH_2-terminal 10 residues of the yeast 70-kd protein [29,30]. This construct was made in order to investigate whether a targeting sequence derived from a mitochondrial outer membrane protein (70-kd) can direct an inner membrane protein (subunit Va) to its proper location. While the 70-kd protein does not have a leader peptide that is proteolytically processed, its amino terminus has sequence features reminiscent of the majority of cleaved leader peptides and has been shown to function as a targeting sequence [29,30]. Specifically, it has been demonstrated that the NH_2-terminal 12 residues of this protein direct both subunit IV and

DHFR to the yeast mitochondrial matrix [30]. In YCpLPS-70kd, we have not attempted to reconstruct the processing site from *COX5a* by adding ArgPhe to the presequence.

Import of YCpLPS Derivatives Into Mitochondria

All YCpLPS-5a constructions were transformed into the yeast host JM43-GD5ab (referred to as GD5ab), a strain containing chromosomal disruptions of both endogenous subunit V genes, *COX5a* and *COX5b* [13]. Representative TRP^+ transformants were analyzed for their ability to grow on the nonfermentable substrates glycerol-ethanol and lactate and for staining with the cytochrome oxidase dye TMPD [31]. The results of these experiments are presented in Table I. YCpLPS-5a and its derivatives 5b, 4 (21), 4 (11), and 70 kd complement the subunit V defect in GD5ab and restore positive staining for cytochrome oxidase with TMPD. The control, JM43, also exhibits the predicted result. Table I also indicates that YCpLPS-LL5a, the plasmid encoding the leaderless subunit V gene, fails to complement the defect in GD5ab and stains only very faintly with the dye. To establish that LL5a was expressed in GD5ab, we performed Northern blot and pulse-chase analysis on representative transformants in order to visualize both the LL5a transcript and protein product. The results of these studies (not shown) indicated that the LL5a gene is expressed normally in GD5ab. Since no subunit Va is observed in mitochondria prepared from LL5a transformants (below), we conclude that a leader peptide is required for import of this polypeptide. From the results presented in Table I, we also conclude that targeting sequences derived from proteins located in other mitochondrial compartments can substitute for the Va leader peptide.

Since yeast strains exhibiting significantly less than wild-type levels of cytochrome oxidase activity can grow on nonfermentable substrates (unpublished observations), the results in Table I do not allow us to conclude that the hybrid proteins are targeted as efficiently as wild-type subunit Va. In order to analyze import in a more rigorous manner, we measured the cyanide-sensitive respiration and growth rates (in glycerol-ethanol) of all the strains listed in Table I. The results are shown in Table II. For all strains except those transformed with YCpLPS-LL5a and YCpLPS-70kd, growth and respiration rates are essentially equal to that of JM43. These results, together with the immunoblots shown below, indicate that the 5b, 4 (21), and 4 (11)

TABLE I. Complementation of Growth and Cytochrome *c* Oxidase Deficiency in YCpLPS Transformants

Strain	Complementation[a]		TMPD[b] colony assay
	Glycerol-ethanol	Lactate	
JM43 (no plasmid)	+	+	++++
GD5ab (no plasmid)	−	−	−
GD5ab-YCpLPS-5a	+	+	++++
GD5ab-YCpLPS-LL5a	−	−	±
GD5ab-YCpLPS-5b	+	+	++++
GD5ab-YCpLPS-4(21)	+	+	++++
GD5ab-YCpLPS-4(11)	+	+	++++
GD5ab-YCpLPS-70kd	+	+	++

[a]Complementation refers to growth of the GD5ab transformant on solid lactate media.
[b]The degree of staining in the TMPD colony assay [31] is indicated as ++++ for wild-type activity, ++ for a reduced staining reaction, ± for a faint reaction, and − for no staining reaction.

TABLE II. Growth and Respiration Rates of YCpLPS Transformants

Strain	Cyanide-sensitive respiration[a]	% Wild-type	Growth rate[b]	% Wild-type
JM43 (no plasmid)	33.9	100	2.8	100
GD5ab (no plasmid)	1.0	2.9	na[c]	na
GD5ab-YCpLPS-5a	34.3	101	2.8	100
GD5ab-YCpLPS-LL5a	1.9	5.6	na	na
GD5ab-YCpLPS-5b	38.8	114	2.9	96
GD5ab-YCpLPS-4(21)	43.4	128	2.8	100
GD5ab-YCpLPS-4(11)	nd[d]	nd	3.0	92
GD5ab-YCpLPS-70kd	17.1	50	4	57

[a]Cyanide-sensitive respiration is expressed as pmol O_2 consumed per min OD_{600}. Experiments were performed as described in reference 11 except that the assays were done in 50 mM phosphate buffer, pH 7, containing 0.66% dextrose. Typically, midlog-phase cells, suspended at 1.5 g per 3 ml buffer, were used. Percent wild-type refers to strain JM43 in all cases.
[b]Growth rates were determined in YPEC medium, and are expressed as t_d, the doubling time, in hours. Percent wild-type was calculated by subtracting the percent difference in growth rate of a given strain and JM43 from 100.
[c]na = not applicable.
[d]nd = not determined.

leader peptides function as efficiently as the wild-type subunit Va leader peptide in targeting this subunit to mitochondria.

Although transformants that carry YCpLPS-70kd grow on nonfermentable carbon sources, their growth and respiration rates are significantly lower than wild type (i.e., about half that of JM43 and the other positive transformants). These strains also differ from wild type in other respects. As indicated in Table I, the 70kd construct exhibits reduced staining with TMPD, indicating an intermediate amount of cytochrome oxidase activity. In addition, it takes much longer for YCpLPS-70kd transformant colonies to appear on lactate plates than for the other YCpLPS transformants. Taken together, these results support the conclusion that the 70kd targeting sequence functions less well than the others tested here.

It is interesting that while YCpLPS-LL5a does not restore the ability of GD5ab transformants to respire, it appears to provide a very low level of cytochrome oxidase activity, as determined by TMPD staining (Table I) and cyanide-sensitive respiration (approximately 2% above background, Table II). These results are interesting in that they may indicate an extremely small amount of subunit Va may be capable of entering the mitochondrion without a leader peptide, although this has not been demonstrated directly.

In order to examine the steady-state level of the polypeptide encoded by each YCpLPS construct and to determine if it was proteolytically processed, mitochondria from representative transformants were prepared and examined by immunoblotting, using an antibody to subunit Va. The results of that experiment are presented in Figure 5. It is clear that the steady-state levels of subunit Va observed in transformant mitochondria correspond well with the data from Table I. No subunit Va is observed in GD5ab or GD5ab-YCpLPS-LL5a. Wild-type levels of a mature-sized Va polypeptide can be observed in mitochondria prepared from all other strains, with the exception of GD5ab-YCpLPS-70kd. The 70kd product appears to be larger and less abundant (about 2–3-fold) than wild type. Since the presence of mature-sized subunit Va indicates proteolytic processing of a given preprotein, and since processing

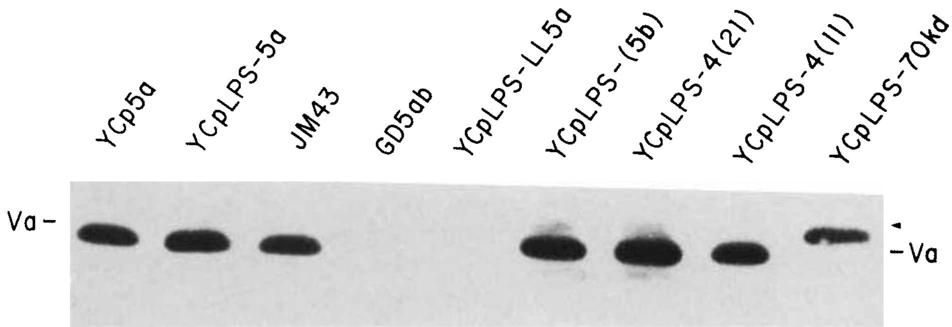


Fig. 5. Steady-state levels of subunit Va in mitochondria. Western immunoblot of mitochondria prepared from the wild-type strain JM43, the *COX5* double null mutant JM43-GD5ab (GD5ab), or GD5ab transformed with the indicated plasmids. In each case, 25 μg of mitochondrial protein was loaded on an 11.8% glycerol-urea polyacrylamide gel and blotted as described [10]. An antisubunit V antisera and ^{125}I-protein A were used to probe the blot. YCp5a is a wild-type *COX5a* centromere plasmid, used as a control [13].

correlates well with the presence of the ArgPheAlaGln sequence (which is absent from YCpLPS-70kd), we suggest that this sequence constitutes at least part of a cleavage site. The finding that the two subunit IV constructs appear to be processed to mature subunit Va is interesting in light of previous results which showed that 16- and 12-amino acid leader peptides derived from subunit IV were not processed when attached to mouse DHFR [24]. Furthermore, cleavage of the YCpLPS-4 (21) leader does not occur solely at an alternative site found in the subunit IV leader (24), since we would have been able to distinguish between the different length mature species in our gel system.

DISCUSSION

In this study we describe a new vector which can be used to conveniently position any sequence at the amino terminus of a well-characterized mitochondrial "passenger" protein. In turn, the ability of that sequence to direct the passenger (the *COX5a* gene product) to the inner mitochondrial membrane can be easily assayed in a phenotypic screen. When this screen (genetic complementation of a strain completely lacking subunit Va) is coupled with additional, more sensitive analyses, an estimate of the efficiency of import can be obtained.

Several of the experimental results presented here are in good agreement with those from other laboratories regarding the targeting of proteins to the mitochondrion. Included among these are that a functional leader peptide is essential for the import of subunit Va and that heterologous leader peptides or targeting sequences derived from proteins located in other mitochondrial compartments can be used to direct the import of subunit Va. However, not all of these heterologous sequences work with equal efficiency.

It is interesting that the 70kd construct functions less well than the others tested here. This result is in line with that of Hurt et al. [30], who found that the NH_2-terminal 12 residues of the 70kd protein also targeted subunit IV to mitochondria with markedly reduced efficiency. Moreover, like YCpLPS-70kd, the *COX4*-70kd construct was not proteolytically processed, yet it is clear that the protein product of

either is able to function as a subunit of the cytochrome oxidase holoenzyme. At present, it is not clear if the 70kd constructs function less efficiently because of the extra amino acids at their respective NH_2-termini or because this outer membrane targeting sequence functions less well as an import signal. We favor the latter explanation for two reasons. First, we observe lower steady-state levels of the 70kd-Va polypeptide in transformant mitochondria. Second, we have found that a mutant 32-amino acid leader peptide derived from the human ornithine transcarbamylase gene can effectively target subunit Va to yeast mitochondria. While this leader is not removed from the mature Va sequence during import, GD5ab transformants harboring the construct function normally, as judged by all the criteria used in this study (S.M. Glaser, B.R. Miller, J. Foreman, and M.G. Cumsky, manuscript in preparation).

Two additional conclusions drawn from the results presented here are significant. They are that the passenger protein is probably important for proper targeting, as others have suggested [32], and that the sequence(s) or signal(s) required for correct intramitochondrial sorting of the *COX5a* gene product are contained within the mature Va sequence and not within the leader peptide per se. Evidence in support of the latter conclusion stems from the fact that the NH_2-terminal 11 residues of the subunit IV leader peptide can be used to target subunit Va to the inner membrane with wild-type efficiency. In contrast, the NH_2-terminal 12 amino acids of the same leader direct DHFR to the mitochondrial matrix and subunit IV to the inner face of the inner mitochondrial membrane [24] (A.P.G.M. van Loon, personal communication). Therefore, essentially the same leader peptide targets three different proteins to three distinct intramitochondrial locations, implicating the mature sequence in intramitochondrial sorting. The nature of the subunit Va sorting signal is currently unclear but under investigation.

The experimental system described here should ultimately prove useful for several types of studies on mitochondrial protein import. Here we have used it to test the targeting efficiency of heterologous leader peptides; however, the system should prove useful for additional, broader studies on import, including random mutagenesis of the leader peptide region. Should studies of this nature prove successful in identifying mutants that fail to import the attached protein, revertants that restore import (identified easily by a return to respiratory competancy, i.e., complementation in GD5ab) can then be isolated. In turn, these revertants would prove useful in a genetic analysis of the import pathway. This type of analysis is currently in progress.

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