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Amino-Terminal Fragments of Δ^1-Pyrroline-5-Carboxylate Dehydrogenase Direct β-Galactosidase to the Mitochondrial Matrix in *Saccharomyces cerevisiae*

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Δ^1-Pyrroline-5-carboxylate (P5C) dehydrogenase, the second enzyme in the proline utilization (Put) pathway of *Saccharomyces cerevisiae* and the product of the *PUT2* gene, was localized to the matrix compartment by a mitochondrial fractionation procedure. This result was confirmed by demonstrating that the enzyme had limited activity toward an externally added substrate that could not penetrate the inner mitochondrial membrane (latency). To learn more about the nature of the import of this enzyme, three gene fusions were constructed that carried 5′-regulatory sequences through codons 14, 124, or 366 of the *PUT2* gene ligated to the *lacZ* gene of *Escherichia coli*. When these fusions were introduced into *S. cerevisiae* either on multicopy plasmids or stably integrated into the genome, proline-inducible β-galactosidase was made. The shortest gene fusion, *PUT2-lacZ14*, caused the production of a high level of β-galactosidase that was found exclusively in the cytoplasm. The *PUT2-lacZ124* and *PUT2-lacZ366* fusions made lower levels of β-galactosidases that were mitochondrially localized. Mitochondrial fractionation and protease-protection experiments showed that the *PUT2-lacZ124* hybrid protein was located exclusively in the matrix, while the *PUT2-lacZ366* hybrid was found in the matrix as well as the inner membrane. Thus, the amino-terminal 124 amino acids of P5C dehydrogenase carries sufficient information to target and deliver β-galactosidase to the matrix compartment. The expression of the longer hybrids had deleterious effects on cell growth; *PUT2-lacZ366*-containing strains failed to grow on proline as the sole source of nitrogen. In the presence of the longest hybrid β-galactosidase, the wild-type P5C dehydrogenase was still properly localized in the matrix compartment, but its activity was reduced. The nature of the effects of these hybrid proteins on cell growth is discussed.

The eucaryotic cell is divided into various compartments containing proteins involved in diverse metabolic pathways and required for specific functions. The proteins that reside in these compartments are members of a group that move from their cytoplasmic site of synthesis to their sites of function. Nuclear, vacuolar, mitochondrial, and chloroplast proteins carry information to direct them to their ultimate subcellular locations (7). The vast majority of the proteins that reside in mitochondria and chloroplasts is imported from the cytoplasm; only a small proportion is actually encoded by the organellar genomes (for reviews, see references 32, 46, and 56).

Much information has been acquired concerning mitochondrial protein import, primarily from biochemical studies in *Saccharomyces cerevisiae* and *Neurospora crassa*. The proteins to be imported are made as precursors, either physically larger than or in a different conformation from their mature forms (for reviews, see references 32 and 46). After completion of protein synthesis, precursors are believed to bind to receptor proteins on the outer mitochondrial membrane (50, 59, 60). Unlike proteins destined for the outer membrane, proteins in the intermembrane space, inner membrane, and matrix compartments require an electrochemical gradient for their import (45), and the majority of these proteins undergoes specific proteolytic cleavages that remove amino-terminal presequences (9, 32, 43).

Many of the proteins involved in electron transport and in the generation of ATP and reducing equivalents in the form of NADH and NADPH must be imported into mitochondria. In addition to these important functions, mitochondria play a significant role in amino acid metabolism. In *S. cerevisiae*, certain steps in the biosynthesis of arginine (37), lysine (5), leucine (30, 53), and valine and isoleucine (52) are carried out in the mitochondria. Less is known about amino acid catabolism associated with mitochondria, although enzymes involved with tryptophan and proline degradation have been so localized (3, 15).

The placement of these enzymes in mitochondria puts them in proximity to the electron transport chain and to intermediates in the tricarboxylic acid cycle, provides a physical barrier to separate them from competing pathways located in the cytoplasm, and permits the accumulation of metabolite pools in a confined space.

In this study we continued our characterization of the proline utilization (Put) pathway in *S. cerevisiae*. Proline serves as a source of nitrogen by its conversion to glutamate, a process requiring a functional electron transport chain (13). Like the pathways in mammalian liver (38), insect flight muscle (16), and plant cells (8), the yeast enzymes are associated with the mitochondria (15). We have cloned (12), sequenced (40), and characterized (12–14) the expression of the *PUT2* gene which encodes Δ^1-pyrroline-5-carboxylate (P5C) dehydrogenase, the second enzyme in the pathway. In an in vitro transcription, translation, and import system, P5C dehydrogenase is made as a precursor polypeptide that is processed to its mature form by the removal of 2 kilodaltons of sequence during mitochondrial import (J. Kaput and M. C. Brandriss, unpublished data).

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TABLE 1. Strains used in this study

Strain	Genotype	Reference or source
MB1000	*MAT* α wild type	Σ1278b; J.-M. Wiame; 12
MB1057	*MAT* **a** *his 4-42*	12
MB1433	*MAT* α *trp1 ura3-52 PUT2*	11
MB1452	*MAT* α *trp1 ura3-52 PUT2::PUT2-lacZ14 URA3*	This study
MB1455	*MAT* α *trp1 ura3-52 PUT2::PUT2-lacZ366 URA3*	This study
MB1457	*MAT* α *trp1 ura3-52 PUT2::PUT2-lacZ124 URA3*	This study
MB331-17A	*MAT* α *trp1 ura3-52 put2-57*	This study
MB634-1B	*MAT* **a** *arg4 thr1 cup1*	This study
MB643-3B	*MAT* **a** *trp1 his4-42 PUT2::PUT2-lacZ14 URA3*	This study
MB643-3C	*MAT* α *his4-42 ura3-52 PUT2::PUT2-lacZ14 URA3*	This study
MB650-1C	*MAT* α *trp1 his4-42 PUT2::PUT2-lacZ366 URA3*	This study
MB652-3A	*MAT* **a** *trp1 ura3-52 PUT2::PUT2-lacZ124 URA3*	This study
JW913-4D	*MAT* α *arg4 thr1 cup1*	27

We report here the matrix localization of P5C dehydrogenase. In addition, we used gene fusions to form three hybrid proteins carrying amino-terminal fragments of P5C dehydrogenase and the catalytically active carboxy-terminal portion of the bacterial enzyme β-galactosidase. Two of these hybrids were targeted and delivered to the mitochondrial matrix, suggesting that the amino-terminal region of P5C dehydrogenase carries all the necessary information for proper localization. These findings are in agreement with those from other studies (23, 24, 31, 34–36, 39, 57), in which it was concluded that the presequence contains all the necessary information to direct a protein to the mitochondrion and deliver it to its appropriate compartment.

MATERIALS AND METHODS

Strains. The strains of *S. cerevisiae* used in this work are described in Table 1. Strains MB1452, MB1455, and MB1457 are Ura$^+$ transformants that resulted from the integration of *PUT2-lacZ*-containing plasmids into strain MB1433. Their construction is described below. Strains MB643-3B, MB650-1C, and MB652-3A, respectively, are meiotic products derived from crosses of the original transformants with strain MB1057. While these strains are not strictly isogenic, they are closely related. Strains MB634-1B and JW913-4D (provided by J. Welch) were used for mapping the integration sites of the *PUT2-lacZ* fusions.

Genetic analysis. The methods used for genetic analysis have been described previously (13).

Media and chemicals. The media used for the growth of *S. cerevisiae* have been described previously (13). The nitrogen source used was ammonium sulfate (0.2%) or proline (0.1%); the carbon source used was glucose (2%) or galactose (2%). For large-scale preparation of mitochondria, the medium described by Daum et al. (21) was modified to include proline (0.1%). Media containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma Chemical Co., St. Louis, Mo.) were used as described previously (28), except that proline (0.1%) was added to, or replaced, the ammonium sulfate as the nitrogen source.

Escherichia coli was grown in LB broth or agar (41) supplemented with ampicillin (100 μg/ml; Bristol Laboratories, Syracuse, N.Y.) as required. M9 medium (44) supplemented with Casamino Acids (1 g/liter; Difco Laboratories, Detroit, Mich.) or LB was used for large-scale plasmid preparation.

DL-(+)-Allo-δ-hydroxylysine hydrochloride was purchased from Calbiochem-Behring (La Jolla, Calif.) and was used in the synthesis of DL-Δ^1-pyrroline-5-carboxylic acid by the method of Williams and Frank (58), as described previously (13).

DNA preparation. Large-scale plasmid DNA was prepared from *E. coli* by the method of Clewell and Helinski (18). Rapid isolation of plasmid DNA from either *E. coli* or spheroplasts of *S. cerevisiae* was performed essentially as described by Birnboim and Doly (6). *S. cerevisiae* genomic DNA was isolated by the method of Davis et al. (22). Procedures for ligation and restriction endonuclease digestion were as provided by the suppliers: New England Biolabs, Inc. (Beverly, Mass.); Boehringer Mannheim Biochemicals (Indianapolis, Ind.); or Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

Electrophoresis of DNA, transfer to nitrocellulose, and hybridization. Agarose gel electrophoresis of DNA was carried out in 50 mM Tris base–67 mM boric acid–10 mM EDTA (pH 8.0) or 40 mM Tris base–5 mM sodium acetate–1 mM EDTA (pH 7.9). The DNA fragments were transferred from the gels to nitrocellulose filters by the method of Southern (55). Hybridizations were carried out at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 1% Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.) overnight with probes that were nick translated by the method of Rigby et al. (51) or end-labeled with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) by the method of Bahl et al. (2).

The filters were exposed to Kodak XRP-5 film at −70°C with DuPont Cronex Lightning-Plus Screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

DNA transformation. Plasmid DNA transformation of *E. coli* was carried out by the method of Cohen et al. (20). DNA transformation of *S. cerevisiae* was done by the method of Hinnen et al. (33).

Plasmids were linearized by restriction endonuclease digestion prior to transformation to increase the frequency of integration at a particular region in the yeast genome, by methods described by Orr-Weaver et al. (47).

Construction of *PUT2-lacZ* gene fusions. (i) *PUT2-lacZ14*. The *Bgl*II sites at codons 14 and 124 of the *S. cerevisiae PUT2* gene (40) are inframe with the *Bam*HI site in the polylinker preceding codon 8 of the *E. coli lacZ* gene carried on plasmid pSKS107 (17; a gift from M. Casadaban). The *lacZYA* fragment from this plasmid was prepared by partial *Sal*I and complete *Bam*HI digestion. This fragment was ligated to plasmid pKB1 (12) cut with *Bgl*II and *Sal*I. The resulting plasmid pKB25 carried the *URA3* gene and 2μm sequences for selection and autonomous replication in *S. cerevisiae* as well as the gene fusion *PUT2-lacZ14* (Fig. 1A)

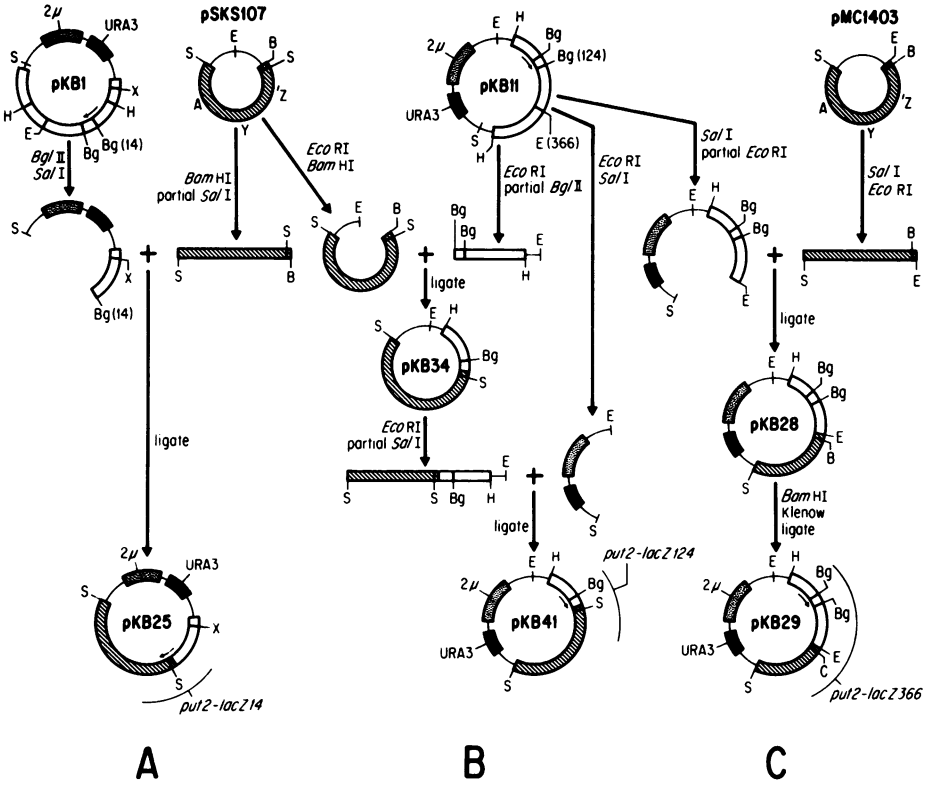

FIG. 1. Construction of autonomously replicating *PUT2-lacZ* fusion vectors. (A) *PUT2-lacZ14*; (B) *PUT2-lacZ124*; (C) *PUT2-lacZ366*. The details are given in the text. Symbols and abbreviations: stippled boxes, 2μm sequences; solid boxes, *URA3*; hatched boxes, *lacZYA*; open boxes, *PUT2*; thin line, pBR322; arrow, start of *PUT2* mRNA; X, *XhoI*; Bg, *Bgl*II; S, *Sal*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; C, *Cla*I.

with 1.9-kilobase (kb) sequences 5′ to the transcription starts and the initial 14 codons of *PUT2* attached to codon 8 of *lacZ*.

A plasmid capable of integration into the yeast genome, pKB27, was constructed from plasmid pKB25 by *Eco*RI digestion to remove the 2μm sequences.

(ii) *PUT2-lacZ124*. As mentioned above, the *Bgl*II site at codon 124 of the *PUT2* gene is in frame with the *Bam*HI site near codon 8 of *lacZ* carried on plasmid pSKS107. This plasmid was linearized by digestion with *Eco*RI and *Bam*HI and ligated to a 1.15-kb *Eco*RI-*Bgl*II fragment derived from the *PUT2* plasmid pKB11 (12). The latter fragment carried about 0.7 kb of sequences 5′ to the transcriptional start sites of the *PUT2* gene, allowing its proper regulation, as well as the structural portion of the gene through codon 124. The resulting plasmid pKB34 was digested partially with *Sal*I and completely with *Eco*RI to yield the entire *PUT2-lacZYA* gene fragment. When ligated to plasmid pKB11 that had been digested with *Eco*RI and *Sal*I, a new, autonomously replicating plasmid carrying the *URA3* gene, 2μm sequences and the *PUT2-lacZ124* gene (Fig. 1B) was formed.

An integrating plasmid pKB42 was constructed by moving the *Eco*RI-*Sal*I *PUT2-lacZ124* fragment into plasmid YIp5 (10) digested with the same enzymes.

(iii) *PUT2-lacZ366*. Plasmid pKB11 was partially digested with *Eco*RI and completely digested with *Sal*I, and the large fragment was ligated to the *Eco*RI-*Sal*I fragment carrying *lacZYA* from plasmid pMC1403 (17; a gift from M. Casadaban). The resulting plasmid pKB28 carried *URA3*, 2μm sequences and a predicted out-of-frame *PUT2-lacZ366* gene fusion (Fig. 1C). To put the *PUT2* and *lacZ* genes in frame with each other, the plasmid was cut at its *Bam*HI site that lies just 9 base pairs 3′ to the *Eco*RI site, the ends were filled with the Klenow fragment of polymerase I, and then the plasmid was religated. If the ends were properly filled, the new plasmid should have an additional *Cla*I site. This method was used successfully by Finkelstein and Strausberg (26; see their Fig. 4.). Plasmid pKB29 was isolated and shown to have a *Cla*I site at the appropriate position (data not shown).

An integrating plasmid carrying this gene fusion was constructed by ligating the *Hin*dIII-*Sal*I *PUT2-lacZ366* gene fragment to the YIp5 vector linearized by *Hin*dIII and *Sal*I digestions. An additional 1.2 kb of contiguous 5′ *PUT2* sequences was then introduced at that *Hin*dIII site to extend the upstream region so that it was almost as long as that found in plasmid pKB27 and carried the same unique *Xho*I site. This plasmid pKB35 was used to integrate the gene fusion into the *S. cerevisiae* genome.

Formation and analysis of stable *PUT2-lacZ* genomic integrants. Each of the three integrating plasmids pKB27, pKB42, and pKB35 was linearized at a unique site in the *PUT2* gene and used in transformations of *S. cerevisiae* MB1433 to direct integration of the plasmid to the *PUT2* locus on chromosome VIII (47). Plasmids pKB27 and pKB35 cut with endonuclease *Xho*I and plasmid pKB42 cut with *Bgl*II, respectively, were used to generate strains MB1452, MB1457, and MB1455. Each strain was crossed to MB1057, and meiotic products were used for mapping the location of the integration event. All three *PUT2-lacZ* genes showed tight linkage to the chromosome VIII marker, *thr1* (*PUT2-lacZ14*, 9 parental ditypes, 0 nonparental ditypes, 1 tetratype; *PUT2-lacZ124*, 16 parental ditypes, 0 nonparental ditypes, 0 tetratypes; *PUT2-lacZ366*, 13 parental ditypes, 0

nonparental ditypes 0 tetratypes and were therefore present in tandem with a wild-type copy of the *PUT2* gene.

Quantitative Southern analysis. Genomic DNA from strain MB643-3B was isolated and digested with enzymes *Sac*I and *Bgl*II. The DNA was separated by electrophoresis, transferred to nitrocellulose membranes, and probed with a ^{32}P-labeled 0.7-kb *Hin*dIII-*Pvu*II fragment from the 5′ end of the *PUT2* gene. The ratio of intensities of the 4.2-kb band (from the *PUT2-lacZ14* gene) to the 2.2-kb band (wild-type *PUT2* gene) was used to determine the *PUT2-lacZ14* copy number.

Genomic DNA from strain MB652-3A was isolated, digested with enzymes *Sac*I and *Pvu*II, and treated as described above. The same probe was used. The ratio of intensities of the 2.8-kb band (*PUT2-lacZ124* gene) to the 1.85-kb band (junction fragment) determined the number of copies inserted.

Genomic DNA from strain MB650-1C was isolated, digested with *Sac*I and *Pvu*II, and treated as described above. The probe was the ^{32}P-labeled, 2.3-kb *Pvu*II-*Hin*dIII fragment of plasmid pBR322. In this case if there were a single integration event, a 3.5-kb band was expected. With a multiple integration event, two bands of 4.2 and 3.5 kb would appear, with intensity ratios that could be used to determine the number of copies integrated.

The bands were traced with a Zeineh scanning densitometer (model SL-DNA; Biomed Instruments, Inc., Chicago, Ill.) with a tungsten lamp, and the relative peak heights were determined with an integrator (model 3392A; Hewlett-Packard Co., Palo Alto, Calif.).

Cellular fractionations. *S. cerevisiae* cells were fractionated into cytosolic and crude mitochondrial fractions by previously published methods (37). Total activities of glucose-6-phosphate dehydrogenase (cytoplasmic marker), fumarase (mitochondrial marker), and β-galactosidase were determined in lysed spheroplasts and cytoplasmic (30,000 × *g* supernatant) and particulate (30,000 × *g* pellet) fractions.

Mitochondrial fractionations. The mitochondrial fractionation procedure of Daum et al. (21) was followed, with the following modifications. In each experiment, 6 liters of cells was grown in a medium containing either galactose and proline (strain MB1000) or the medium described by Daum et al. (21; *PUT2-lacZ* strains) supplemented with proline (0.1%). Zymolyase 60000 (25 μg/ml; Miles Laboratories, Inc., Elkhart, Ind.) was used to make spheroplasts within 15 to 20 min at 30°C. Mitoplasts (mitochondria with ruptured outer membranes lacking the soluble contents of the intermembrane space) were formed as described previously (21) in the experiment for which the results are shown in Fig. 2. For the experiments for which the results are shown in Fig. 3, they were exposed to additional ultrasonic irradiation (four times for 15 s each at 0°C; model W-375; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). An increase in the sonication time appeared to decrease the activity of fumarase and P5C dehydrogenase in the membrane fraction (compare Fig. 2 with Fig. 3). The purity of the fractions was monitored by measuring the activity of marker enzymes: cytochrome b_2 (as lactate dehydrogenase, intermembrane space), cytochrome *c* oxidase (inner membrane), and fumarase (matrix space). No attempt was made to separate inner from outer mitochondrial membranes because these separations generally do not purify the fractions (see Fig. 2 in reference 21; also see reference 49).

The fumarase activity found in the intermembrane space is believed to be due to premature rupture of the inner membrane at a point in the procedure at which only the outer membrane should lyse. Nonetheless, the fumarase profile appears to be characteristic of a matrix marker and is clearly distinguishable from inner membrane or intermembrane space proteins.

The mitochondria from each strain were fractionated in three independent experiments to assess the inherent repeatability of the procedures and to be able to recognize significant changes in enzyme localization.

Assays of enzymes and protein. Previously published procedures were used to assay fumarase (48), using an extinction coefficient of 2.44 (4); glucose-6-phosphate dehydrogenase (19); cytochrome *c* oxidase (42); cytochrome b_2 (as lactate dehydrogenase [1]); P5C dehydrogenase (13); and β-galactosidase (44). Protein concentrations were determined by the method of Bradford (11), with crystalline bovine serum albumin used as the standard.

Growth of *S. cerevisiae* cells for enzyme assays. Amounts of 20 ml of the appropriately supplemented minimal medium (glucose-proline, glucose-ammonia-proline, or glucose-ammonia) in 125-ml sidearm flasks were inoculated to a density of 1 to 10 Klett units (blue filter) with a Klett-Summerson colorimeter. Cultures were shaken at 30°C until their densities reached 80 to 100 Klett units.

Preparation of cell extracts. The cells were harvested and washed with an equal volume (20 ml) of water and then with 5 ml of 50 mM potassium phosphate buffer (pH 7.7) or Z buffer (44), depending on the enzyme to be assayed. Glass beads (0.45 to 0.50 mm; B. Braun Melsungen AG, West Germany; roughly equal in volume to the cell pellet) were added, and the slurry was blended vigorously with a mixer (Genie; The Vortex Manufacturing Co., Cleveland, Ohio) for four 30-s intervals interspersed with periods of cooling on ice.

Cell extract was removed from the beads and transferred to a centrifuge tube. The glass bead slurry was rinsed twice with 1 to 1.5 ml of buffer. The rinses were combined with the extract and centrifuged at 12,100 × *g* for 20 min. The supernatant was used to assay P5C dehydrogenase or β-galactosidase activity and protein. The data presented in Table 3 are the average of two or more determinations.

Protease protection experiments. Mitochondria or mitoplasts were isolated as described above and diluted to a concentration of approximately 2 mg/ml. Proteinase K (Sigma) and Triton X-100 were added at a final concentration of 100 μg/ml and 0.5%, respectively. After incubation at 30°C for 30 min, phenylmethylsulfonyl fluoride, at a final concentration of 1 mM, was added to all reactions. Each reaction was measured for fumarase and β-galactosidase activities and compared with a control reaction that contained no additions.

Latency experiments. Mitochondrial enzymes are considered latent when limited activity is found toward an externally added substrate that cannot penetrate an intact inner mitochondrial membrane (25). These experiments take advantage of the fact that P5C dehydrogenase uses NAD as a substrate and that NAD can diffuse through the pores of the outer, but not the inner, membrane (25). If the enzyme is located on the inner face of the outer membrane or the outer face of the inner membrane, it should react with the NAD to form NADH. However, if the enzyme and its substrate are separated from each other by the inner membrane, no formation of NADH will occur unless the inner membrane is disrupted by the addition of a detergent. Mitochondria and mitoplasts were isolated as described above and maintained in a solution of 0.6 M mannitol–10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.5).

P5C dehydrogenase and fumarase activities were determined as described above, except that the reactions proceeded in 0.6 M sorbitol instead of water. The activity was determined in the presence or absence of Triton X-100 at a final concentration of 0.5%.

RESULTS

Submitochondrial localization of P5C dehydrogenase activity. Mitochondria were prepared as described above from the wild-type strain MB1000 under conditions that would allow maximum expression of P5C dehydrogenase while maintaining some respiratory capacity (galactose as carbon source, proline as nitrogen source). The results of one mitochondrial fractionation are displayed according to Daum et al. (21) (Fig. 2). The specific activity of each enzyme was determined in each fraction (intermembrane space, inner and outer membranes, matrix) as well as in unfractionated mitochondria. The ratio of specific activity in each fraction to specific activity in unfractionated mitochondria (relative specific activity) was determined and plotted as a function of the percentage of mitochondrial protein found in each fraction. Typically, the membrane fraction contained 50 to 60% of the protein, while the matrix had about 20% and the intermembrane space had the remainder. When plotted in this manner, the enrichment of a particular enzyme activity is discernible as it is purified away from other mitochondrial proteins. The area of each bar in the graphs (fraction of activity times fraction of total protein) is an indication of the total activity present in each fraction.

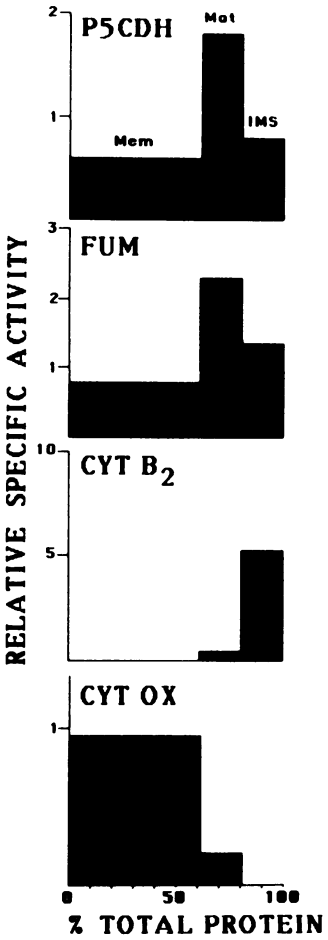

FIG. 2. Distribution of enzymes in fractions of *S. cerevisiae* mitochondria. *S. cerevisiae* mitochondria were fractionated as described in the text into membranes, intermembrane space, and matrix space; and each fraction was assayed for the indicated enzymes. Relative specific activity is the specific activity of an enzyme in a fraction divided by the specific activity of that enzyme in unfractionated mitochondria. Percent total protein denotes the percentage of mitochondrial protein recovered in each fraction. For each enzyme, the actual recovery value was taken as 100% for calculating the values shown in the figure. Abbreviations: P5CDH, P5C dehydrogenase; FUM, fumarase; CYT B_2, cytochrome b_2; CYT OX, cytochrome *c* oxidase; Mem, membranes; Mat, matrix; IMS, intermembrane space.

TABLE 2. P5C dehydrogenase is located inside the inner mitochondrial membrane

Sample	Triton X-100 treatment[a]	P5C dehydrogenase sp act[b]	Fold unmasking of activity by detergent
Mitochondria	−	64	
	+	1,377	22
Mitoplasts	−	92	
	+	1,205	13
Cell extract	−	299	
	+	272	0.9

[a] Added at a final concentration of 0.5%.
[b] Nanomoles of NADH formed per minute per milligram of protein. Strain MB1000 was grown on galactose and proline.

The marker enzymes displayed characteristic profiles in these fractionation procedures, and these profiles agree well with previously published results (21). Cytochrome *c* oxidase was found exclusively in the membrane fraction, while cytochrome b_2 was found in the intermembrane space. Fumarase was found predominantly in the matrix space with some cofractionation with both the membrane and intermembrane space markers. P5C dehydrogenase fractionated in a manner analogous to that of fumarase and therefore appears to be a matrix-localized protein.

Latency. Although the results displayed in Fig. 2 show that P5C dehydrogenase is not located in the soluble intermembrane space, they do not rule out the possibility that the activity could be associated with the inner face of the outer membrane or the outer face of the inner membrane. An enzyme in those locations would not be released from the mitoplasts on disruption of the outer membrane as was cytochrome b_2 but would appear, instead, to be contained in the matrix space after sonication of the mitoplasts. To determine on which side of the inner membrane the P5C dehydrogenase is located, latency experiments were performed as described above. In Table 2 it is shown that P5C dehydrogenase activity is latent in both mitochondria and mitoplasts because addition of detergent caused an increase in its activity by 22- and 13-fold, respectively. Triton X-100, itself, did not stimulate enzyme activity in cell extracts. From this result and the fractionation data, we conclude that P5C dehydrogenase is located in the soluble matrix space of the mitochondria.

Construction of *PUT2-lacZ* gene fusions. To determine the nature of molecular signals required to target and deliver P5C dehydrogenase to the mitochondrial matrix and to attempt import of a nonmitochondrial protein, three gene fusions of *PUT2* and *lacZ*, encoding the bacterial enzyme β-galactosidase, were constructed as described above. Because the *PUT2* gene sequence has been determined (40), it was possible to select *lacZ* genes from existing plasmids that had restriction sites that would match the *PUT2* reading

TABLE 3. Enzyme levels in the *PUT2-lacZ* strains

Strain (genotype)	Nitrogen source[a]	Doubling time (h)	Sp act[b]		No. of copies of *PUT2-lacZ*[c]
			β-Gal	P5CDH	
MB643-3B (*PUT2-lacZ14*)	Amm	2.4	73 (1)	15 (1)	7
	Amm + Pro	2.4	489 (7)	77 (5)	
	Pro	3.4	1,290 (18)	199 (13)	
MB652-3A (*PUT2-lacZ124*)	Amm	2.6	56 (1)	6 (1)	2
	Amm + Pro	2.5	413 (7)	36 (6)	
	Pro	5.0	1,237 (22)	170 (28)	
MB650-1C (*PUT2-lacZ366*)	Amm	2.4	36 (1)	25 (1)	1
	Amm + Pro	2.5	117 (3)	73 (3)	
	Pro	NG[d]			

[a] The cultures contained 2% glucose; 0.2% ammonium sulfate (Amm), 0.1% proline (Pro), or both; tryptophan (20 mg/liter); and histidine (20 mg/liter).
[b] The units of specific activity for β-galactosidase (β-Gal) and P5C dehydrogenase (P5CDH) are nanomoles of *o*-nitrophenol or NADH, respectively, formed per minute per milligram of protein. Relative values appear in parentheses. Each value represents the average of two or more determinations.
[c] Values were determined by quantitative Southern blotting, as described in the text.
[d] NG, the culture failed to maintain balanced growth.

frame. The three fusions, referred to as *PUT2-lacZ14*, *PUT2-lacZ124*, and *PUT2-lacZ366*, carry the initial 14, 124, and 366 codons, respectively, of the *PUT2* gene attached to *lacZ* at codon 8. All fusions have at least 0.7 kb of upstream DNA sequences containing the *PUT2* regulatory region.

Behavior of *S. cerevisiae* strains carrying *PUT2-lacZ* fusions on multicopy plasmids. When the gene fusions carried by the 2μm-*URA3* plasmids pKB25, pKB29, and pKB41 were introduced into strain MB331-17A or its isogenic *PUT2* derivative MB1433, each caused the production of proline-inducible β-galactosidase (data not shown). Only the shortest fusion-bearing plasmid pKB25 was maintained by the *S. cerevisiae* cells at a frequency (90%) seen for other 2μm-containing plasmids maintained under selective conditions (minus uracil) in the presence or absence of proline. Both plasmids pKB29 and pKB41 were considerably more unstable and were maintained in only about 50% of the cells grown under noninducing, selective conditions. When proline was added to the medium, even greater plasmid loss was seen.

These results strongly suggest that the presence of the *PUT2-lacZ124* and *PUT2-lacZ366* gene fusions have deleterious effects on the cells that appear to be related to the level of gene product made. The conditions of high gene dosage and induction by proline were associated with greater plasmid loss. Given the instability of the plasmids carrying the longer gene fusions, all further work was done with strains that carried stably integrated copies. The three gene fusions were introduced into the *S. cerevisiae* genome by integrative transformation of plasmids pKB27, pKB35, and pKB42 (described above) that were directed to integrate at the *PUT2* locus on chromosome VIII.

Behavior of strains with integrated *PUT2-lacZ* gene fusions. Three strains were chosen for analysis of strain behavior with integrated *PUT2-lacZ* gene fusions: MB643-3B (*PUT2-lacZ14*), MB652-3A (*PUT2-lacZ124*), and MB650-1C (*PUT2-lacZ366*). Mapping crosses, as described above, demonstrated that each gene fusion was tightly linked to the chromosome VIII marker *thr1* and was therefore located adjacent to the chromosomal wild-type *PUT2* gene.

The strains were grown under noninducing (ammonia), intermediately inducing (ammonia and proline), and fully inducing (proline) conditions; and the growth rate and levels of β-galactosidase and P5C dehydrogenase were determined. In Table 3 it is shown that the integrated *PUT2-lacZ* fusions caused the production of proline-inducible β-galactosidase, with expression that was roughly comparable to that seen for P5C dehydrogenase; this result is consistent with the fact that both the *PUT2* and *PUT2-lacZ* genes have at least 0.7 kb of identical upstream 5′ sequences. The generation times of strain MB643-3B on the various media were similar to those of the wild-type strain MB1000. In contrast, the strains bearing the *PUT2-lacZ124* and *PUT2-lacZ366* gene fusions showed increased doubling times on proline medium; the latter strain could not maintain balanced growth under these conditions. Comparable levels of β-galactosidase activity were found for the *PUT2-lacZ14* and *PUT2-lacZ124* fusions; in the *PUT2-lacZ366* fusion strain, β-galactosidase had lower activity. Fully induced levels of P5C dehydrogenase activity appeared to be slightly reduced in the *PUT2-lacZ124* strain compared with that in the *PUT2-lacZ14* strain.

The numbers of integrated copies of the plasmids were determined by quantitative Southern hybridization (data not shown), as described above. The copy numbers were found to be seven for the *PUT2-lacZ14* fusion, two for the *PUT2-lacZ124* fusion, and one for the *PUT2-lacZ366* fusion. The β-galactosidase levels do not reflect this difference in copy number. However, the number of integrated copies is found for each strain correlated well with the stability seen for the fusions on 2μm plasmids, as well as their effects on cell growth, and again strongly suggested that there is a toxic effect associated with high levels of expression of the two longer fusions.

Cellular location of *PUT2-lacZ* hybrid proteins. The cellular location of the β-galactosidases encoded by the three *PUT2-lacZ* gene fusions was determined by the cell fractionation procedure described above. In each fractionation the activities of a cytoplasmic enzyme marker (glucose-6-phosphate dehydrogenase) and a mitochondrial matrix marker (fumarase) were also followed to determine the purity of the fractions.

In Table 4 are shown the results of these fractionations. The *PUT2-lacZ14* fusion encoded a cytoplasmic β-galactosidase that behaved like glucose-6-phosphate dehydrogenase and like the β-galactosidase encoded by the *CYC1-lacZ* fusion carried on plasmid pLG669Z (29). The latter β-galactosidase is regulated like iso-1-cytochrome *c* in response to glucose and remains in the cytoplasm of *S. cerevisiae* cells (23, 29).

In contrast, the β-galactosidases encoded by the two longer gene fusions were found to be associated with the pellet fraction, behaving analogously to the mitochondrial matrix marker fumarase. In a previous study (15), we reported that P5C dehydrogenase cofractionated with another mitochondrial matrix marker, citrate synthase. These

results strongly suggest that the amino-terminal fragment of P5C dehydrogenase directs the β-galactosidase to the mitochondria. To confirm this finding and to determine in which submitochondrial compartment these proteins are located, additional fractionation experiments were carried out.

Submitochondrial location of *PUT2-lacZ* hybrid proteins. Mitochondria were isolated from strains MB643-3B (*PUT2-lacZ14*), MB652-3A (*PUT2-lacZ124*), and MB650-1C (*PUT2-lacZ366*), which were grown on lactate to derepress mitochondrial function and proline to induce the *PUT2* and *PUT2-lacZ* genes. The fractionation procedures of Daum et al. (21) were followed, as described above, and the results are presented in Fig. 3.

Although the β-galactosidase in strain MB643-3B was predominantly localized in the cytosol (Table 4), mitochondria were analyzed from this strain to serve as a control and to determine the location of the small amount of β-galactosidase that fractionated with the mitochondria. The pattern of enzyme localization in MB643-3B (Fig. 3A) was virtually identical to that seen for the wild-type strain MB1000 (Fig. 2). The marker enzymes behaved as expected, and the β-galactosidase was found predominantly in the intermembrane space. The P5C dehydrogenase reached its normal location in the matrix. Although the β-galactosidase contained the initial 14 amino acids of the signal of P5C dehydrogenase, this was apparently insufficient to target most of the protein to the mitochondria. The 1% of activity that did reach the mitochondria appeared to be localized in the intermembrane space.

The β-galactosidase encoded by the *PUT2-lacZ124* gene was found in the mitochondrial matrix compartment (Fig. 3B). The total activity of P5C dehydrogenase was reproducibly reduced by about 20%, but it was properly localized in the matrix. Of the marker enzymes, cytochrome *c* oxidase fractionated as in the control strains. There was some variation from experiment to experiment involving strain MB652-3A in the relative specific activity of cytochrome b_2 (although not in its location, which was always very definitive) and in the amount of fumarase in the intermembrane space, making it difficult to say with certainty whether their mitochondrial activities or locations were affected by the presence of the hybrid protein.

The behavior of the β-galactosidase encoded by the *PUT2-lacZ366* gene fusion is shown in Fig. 3C. Although it contained the same amino-terminal sequences as the *PUT2-lacZ124* hybrid protein as well as 242 additional amino acids, this hybrid protein was delivered to two compartments: the inner membrane (see below) and the matrix. In addition, the amount of P5C dehydrogenase activity was reduced still further, although it did reach its appropriate compartment. The activities of the marker enzymes appeared to be unaffected by the presence of the hybrid protein.

These results were confirmed by demonstrating that the β-galactosidase activity found in both strains is protected from protease digestion, as described below.

Protease protection of *PUT2-lacZ* hybrid proteins. Mitochondria and mitoplasts from strains MB652-3A (*PUT2-lacZ124*) and MB650-1C (*PUT2-lacZ366*) were treated with proteinase K in the presence or absence of Triton X-100, as described above. The results shown in Table 5 demonstrate that the β-galactosidase, like the marker enzyme fumarase, was resistant to digestion by proteinase K when the inner mitochondrial membrane was left intact. That mitoplasts behaved identically to whole mitochondria indicate that the β-galactosidase activities are either within the inner membrane or in the matrix and not in the outer membrane, in the intermembrane space, or on the outer face of the inner membrane. These results confirm the fractionation data; and we conclude, therefore, that the initial 124 amino acids of P5C dehydrogenase can target and deliver β-galactosidase to the appropriate compartment. Possible explanations for the dual localization of the longest fusion will be presented below.

TABLE 4. Cellular location of hybrid β-galactosidases

Gene fusion[a]	Enzyme[b]	Percent activity in:	
		Cytosol	Pellet
PUT2-lacZ14	G6PDH	99	1
	Fumarase	23	77
	β-Gal	99	1
PUT2-lacZ124	G6PDH	98	2
	Fumarase	41	59
	β-Gal	50	50
PUT2-lacZ366	G6PDH	97	3
	Fumarase	27	73
	β-Gal	16	84
CYC1-lacZ	G6PDH	99	1
	Fumarase	31	69
	β-Gal	99	1

[a] The *PUT2-lacZ14* fusion was contained in strain MB1452 stably integrated in seven copies on chromosome VIII. The strain was grown in a medium containing galactose, ammonia, proline, and tryptophan. The *PUT2-lacZ124* fusion was carried on plasmid pKB41 in strain MB1433. The growth medium contained galactose, ammonia, and tryptophan. The *PUT2-lacZ366* fusion was carried on plasmid pKB29 in strain MB1433. The growth medium contained galactose, ammonia, and tryptophan. The *CYC1-lacZ* fusion was contained on plasmid pLG669Z in strain MB1433. The growth medium contained galactose, ammonia, proline, and tryptophan.

[b] Abbreviations: G6PDH, Glucose-6-phosphate dehydrogenase; β-Gal, β-galactosidase.

DISCUSSION

Several methods are currently in use to determine the precise intramitochondrial location of a given mitochondrial protein. Mitochondrial fractionations physically separate the four compartments (inner and outer mitochondrial membranes, intermembrane space, and matrix) and use enzymatic or immunologic markers to determine the purity of the fractions. Protease protection and latency experiments can distinguish a protein that is either contained entirely within the inner membrane or in the matrix space from one that is associated with the outer face of the inner membrane or the inner face of the outer membrane. Individually, these tests provide incomplete evidence of localization of a protein. Taken together, however, these two types of data provide strong evidence for the position of a protein within the mitochondrion.

P5C dehydrogenase was determined to be located in the matrix space by both mitochondrial fractionation and latency experiments. Attempts to perform protease protection experiments were inconclusive due to the relative resistance of P5C dehydrogenase to digestion by proteinase K under the conditions used.

On the basis of evidence presented here, P5C dehydrogenase is a member of the class of mitochondrial proteins that is imported from the cytoplasm and resides in the matrix compartment. The information for its localization lies at least within the initial 124 amino acids. When the *PUT2* gene was transcribed and translated in vitro, P5C dehydrogenase was made as a precursor polypeptide that was imported into

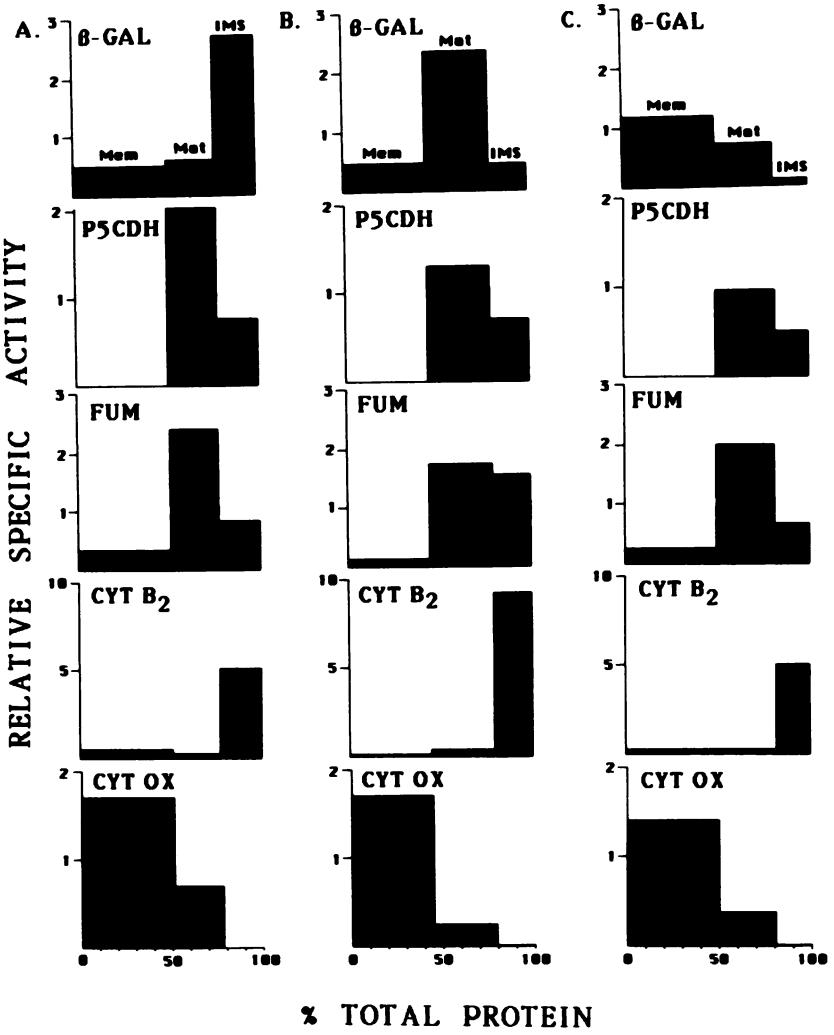

FIG. 3. Distribution of enzymes in fractions of *S. cerevisiae* mitochondria in the *PUT2-lacZ* fusion strains. (A) MB643-3B, *PUT2-lacZ14*; (B) MB652-3A, *PUT2-lacZ124*; (C) MB650-1C, *PUT2-lacZ366*. Axes and abbreviations are as described in the legend to Fig. 2. β-GAL, β-Galactosidase.

isolated mitochondria. Import was associated with removal of approximately 2 kilodaltons of protein sequence (Kaput and Brandriss, unpublished data). These observations, coupled with the behavior of the hybrid β-galactosidases presented here, indicate that P5C dehydrogenase is synthesized with an amino-terminal extension that is needed to deliver it to the mitochondrion. The amino-terminal 15 to 20 residues are removed at some point in the import process.

The amino acid sequence of the amino terminus of the protein, as deduced from its DNA sequence (37), is highly basic. There are 9 positively charged residues and 4 serines among the first 30 amino acids, with the first negative charge

TABLE 5. Protection of β-galactosidase activity from proteinase K digestion

Strain	Treatment[a]		Percent activity of the following remaining[b]:			
			β-Galactosidase		Fumarase	
	Triton X-100	Proteinase K	Mitochondria	Mitoplasts	Mitochondria	Mitoplasts
MB652-3A (*PUT2-lacZ124*)	−	−	100	100	100	100
	+	−	247	236	97	117
	−	+	137	74	88	81
	+	+	3	2	2	11
MB650-1C (*PUT2-lacZ366*)	−	−	100	100	100	100
	+	−	168	100	130	91
	−	+	119	71	90	75
	+	+	2	3	1.4	0.7

[a] The final concentrations of Triton X-100 and proteinase K were 0.5% and 100 μg/ml, respectively.
[b] One hundred percent represents the activity of each enzyme in the absence of detergent and protease.

at position 31, and there are no obvious hydrophobic stretches. This region of the protein resembles other mitochondrial matrix protein presequences in that it is rich in hydroxylated and basic amino acids and lacks acidic residues and extended stretches of uncharged amino acids (57).

Several laboratories have used gene fusions to deliver nonmitochondrial proteins to the mitochondria of *S. cerevisiae*. In the laboratories of Douglas and Emr and co-workers (23, 24), a series of *ATP2-lacZ* and *ATP2-SUC2* gene fusions have been constructed that resulted in the inner membrane localization of both β-galactosidase and invertase and also, in some cases, caused the cells to become respiratory deficient. Schatz and co-workers (31, 35, 36) have attached the presequence of an outer membrane protein to β-galactosidase and that of a matrix protein to murine dihydrofolate reductase and successfully delivered those proteins to the proper compartments (5). In one fusion, it was possible to get correct localization with only 12 amino acids of the presequence (normally 25 residues in length) (36). In addition, they have begun to dissect the various functions of the presequence of an intermembrane space protein (57). The import of nonmitochondrial proteins has also been accomplished with the presequences of ornithine transcarbamylase in mammalian mitochondria (34) and the small subunit of ribulose 1,5-bisphosphate carboxylase in chloroplasts (56).

On the basis of the results obtained with β-galactosidase in protein export studies in *E. coli* and in the initial studies reported by Douglas et al. (23), it was expected that β-galactosidase would not be able to pass through the inner mitochondrial membrane without getting stuck. The finding that it is possible for this rather large protein to be imported into the matrix of the mitochondrion suggests that a different mechanism is used for mitochondrial import from the one used to export proteins in *E. coli*. A recent report by Schleyer and Neupert (54) provides the first evidence for contact points between inner and outer mitochondrial membranes associated with protein import.

The small amount of β-galactosidase associated with mitochondria in the *PUT2-lacZ14* strain was found exclusively in the intermembrane space. It is tempting to speculate that the lack of the proper processing site or full signal prevented proper matrix localization. However, we have not ruled out the possibility that this level of β-galactosidase represents nonspecific association with the mitochondrial outer membrane that was released during the fractionation.

We found that the *PUT2-lacZ124* hybrid protein was localized entirely in the matrix, whereas the *PUT2-lacZ366* hybrid protein was found in the inner membrane as well as in the matrix. The nature of this difference is not understood. The longer protein contains the initial 124 residues of P5C dehydrogenase plus the next 242 residues, and these sequences do not cause the wild-type protein to be membrane associated. We cannot rule out the possibility that the *PUT2-lacZ366* hybrid assumes a conformation that partitions between the two compartments or slows down the import process such that we can actually "see" the protein in the membrane as it is imported. Alternatively, residues 125 to 366 of the dehydrogenase may be the region for dimerization or tetramerization among monomer units. Because both the wild-type dehydrogenase and the hybrid protein share this region, protein-protein interactions are possible that might interfere with complete passage through to the matrix. We are currently constructing a strain with a complete deletion of the *PUT2* locus that can be used to test this hypothesis.

It is clear from the instability of the longer *PUT2-lacZ*-bearing plasmids, as well as the increased doubling times of the strains carrying integrated copies of these two gene fusions, that, when expressed at elevated levels, the hybrid proteins are toxic. Under the conditions used, the strains were not respiratory deficient and, at least with the marker enzymes measured and to the level of accuracy possible by these fractionation procedures, other enzymes appeared to be unaffected by the presence of the hybrid β-galactosidase. The only enzyme that may have been somewhat affected was the wild-type P5C dehydrogenase, which shared the same signal sequence. Its activity was reduced compared with that of the wild-type strain. It is possible that the *PUT2-lacZ366*-bearing strain failed to grow on proline (the condition of maximum gene expression) because the hybrid β-galactosidase prevented the entry of P5C dehydrogenase into the matrix compartment, causing the strain to be Put^-. Alternatively, the strain could have become respiratory deficient because of exclusion of important respiratory chain proteins. The Put^- phenotype would then be caused by an indirect effect, because respiration is required for proline utilization (13). Whatever the cause, selection for *PUT2-lacZ366* strains that can grow in the presence of proline may lead to the isolation of mutations affecting mitochondrial protein import.

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This paper is dedicated to the memory of Marion K. Brandriss.

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