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The Nine Amino-Terminal Residues of δ-Aminolevulinate Synthase Direct β-Galactosidase into the Mitochondrial Matrix

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δ-Aminolevulinate synthase, the first enzyme in the heme biosynthetic pathway, is encoded by the nuclear gene *HEM1*. The enzyme is synthesized as a precursor in the cytoplasm and imported into the matrix of the mitochondria, where it is processed to its mature form. Fusions of β-galactosidase to various lengths of amino-terminal fragments of δ-aminolevulinate synthase were constructed and transformed into yeast cells. The subcelluar location of the fusion proteins was determined by organelle fractionation. Fusion proteins were found to be associated with the mitochondria. Protease protection experiments involving the use of intact mitochondria or mitoplasts localized the fusion proteins to the mitochondrial matrix. This observation was confirmed by fractionation of the mitochondrial compartments and specific activity measurements of β-galactosidase activity. The shortest fusion protein contains nine amino acid residues of δ-aminolevulinate synthase, indicating that nine amino-terminal residues are sufficient to localize β-galactosidase to the mitochondrial matrix. The amino acid sequence deduced from the DNA sequence of *HEM1* showed that the amino-terminal region of δ-aminolevulinate synthase was largely hydrophobic, with a few basic residues interspersed.

The vast majority of mitochondrial proteins are encoded by nuclear genes (12). Most of these proteins are made as larger precursors in the cytoplasm before they are transported to the mitochondria (21, 35, 43); the precursors are then cleaved into their mature forms by a mitochondrial protease (5). Mitochondrial precursor proteins can be transported posttranslationally into mitochondria both in vitro and in vivo (13, 14, 38). Although import into the mitochondrial matrix requires an electrochemical potential across the inner membrane of the mitochondrion (13, 45), an association of precursors with the outer surface of the mitochondrion is found in the absence of the potential in several cases (22, 39). The first step of any posttranslational import process is probably binding of some targeting sequence present in the precursor to a receptor protein on the outer surface of the mitochondrion; for cytochrome *c* in *Neurospora crassa*, kinetics of the protein-mitochondrion association are consistent with the receptor hypothesis (22, 53). For several imported proteins, amino-terminal peptide signals have proved sufficient to direct heterologous proteins to a mitochondrial location in vivo (11, 20, 25–28).

To begin a genetic analysis of mitochondrial protein import, we have initiated studies on the enzyme δ-aminolevulinate (δ-ALA) synthase in *Saccharomyces cerevisiae*. This enzyme catalyzes the first step in the heme biosynthetic pathway (48). It is found in the mitochondrial matrix and is encoded by a nuclear gene, *HEM1* (16). The enzyme is made as a precursor of 61,000 daltons and is processed to a mature form of 58,000 daltons (M. Douglas, personal communication). By complementation of the heme auxotrophy caused by a mutation in the *HEM1* gene, we have obtained a clone of this gene.

To study the δ-ALA synthase amino acid sequences that are needed to direct the protein to the inner matrix of the mitochondria, we have chosen the approach of fusion of the protein to β-galactosidase. β-Galactosidase is an *Escherichia coli* protein that can be expressed as a cytoplasmic protein in yeast cells (11, 20). By fusing various amino-terminal portions of δ-ALA synthase to a *lac* repressor–β-galactosidase moiety and examining the final location of each of these fusion proteins in the cell, sequences responsible for targeting δ-ALA synthase to the mitochondria can be identified. We report here that nine amino acid residues from the amino terminus of this protein are sufficient to direct a foreign protein, β-galactosidase, to the mitochondrial matrix.

MATERIALS AND METHODS

Strains. *S. cerevisiae* BWG1-7a (*MATa leu2-3 leu2-112 his4-519 ade1-100 ura3-52*) was used in this study. Strain 8D (*MATα ura3-52 met13 gal80* [oli^R]) was obtained from Brian Osborne, Massachusetts Institute of Technology. Strain TM2 (*ura3-52 hem1*) was obtained from Tom Mason, University of Massachusetts, Amherst. *E. coli* YMC9 (*ΔlacU169 hsd$^-$ hsm$^+$*) was used for plasmid isolation and propagation.

Chemicals and media. Synthetic selective and YEP media were as described by Sherman et al. (46). Galactose, lactate, and glucose, when added, were used at 2%. Buffered synthetic media with 5-bromo-4-chloro-3-indoyl-β-D-galactoside (XG) were as described previously (17). All nutritional supplements were added at 4 mg/ml. Heme was made up in 0.1 M NaOH and added to a final concentration of 50 μg/ml. δ-Aminolevulinic acid was added to 50 μg/ml.

Complementation of heme auxotrophy. *S. cerevisiae* TM2, a *hem1* mutant, was transformed by the spheroplast method (23), with 1 μg each of RB161, RB112, or RB113 DNA. The DNAs represent banks of partial *Sau*3A digests of yeast chromosomal DNA ligated into a YEp24 vector and were obtained from D. Botstein, Massachusetts Institute of Technology (8). The transformants were plated on synthetic glucose plates supplemented with heme and were selected for uracil prototrophy. Approximately 10^3 to 10^4 transformants were obtained per μg of DNA. Approximately 0.1 to 1% of transformants had become heme prototrophs. In each

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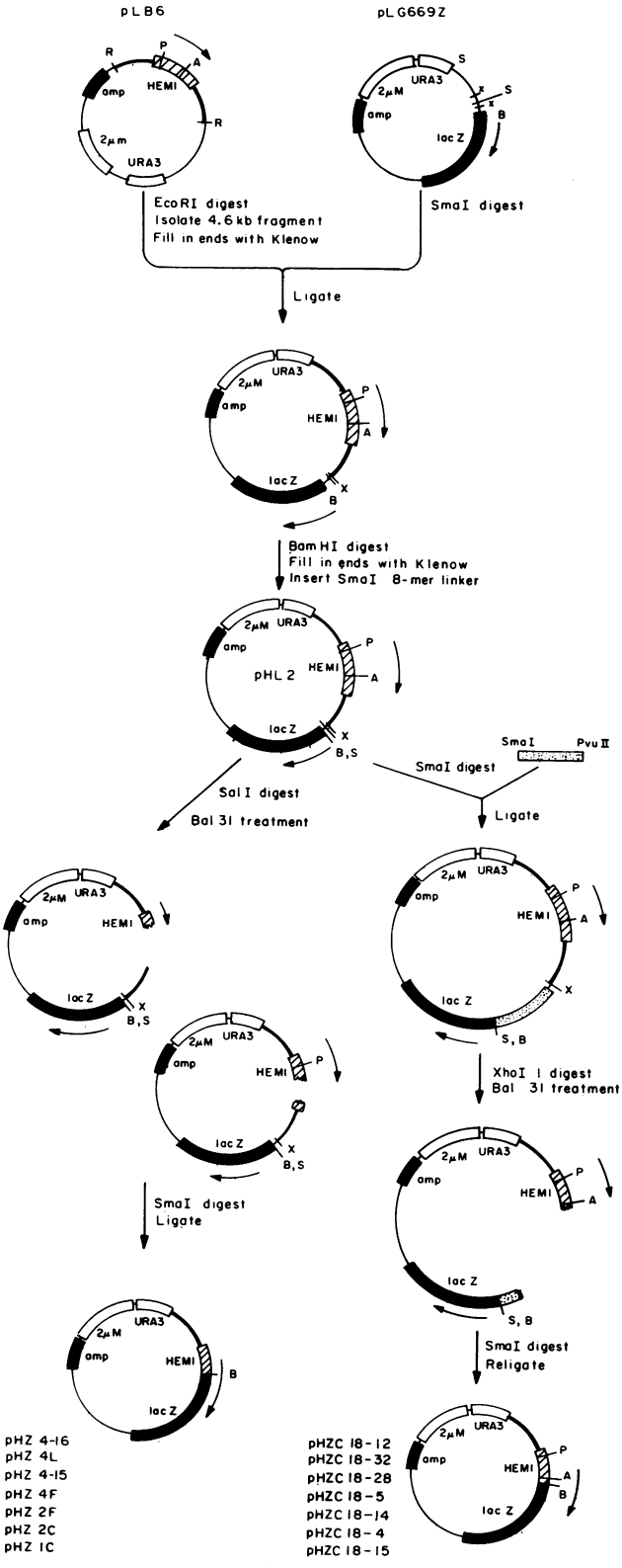

FIG. 1. Construction of *HEM1-lacZ* fusions. A 4.6-kb *Eco*RI fragment from pLB6 which contains *HEM1* was ligated to *Sma*I-digested pLG669Z in the proper orientation. A *Sma*I linker was inserted at the *Bam*HI site of the resulting plasmid to yield pHL2. To generate *HEM1-lacZ* fusions, pHL2 was cut at the unique *Sal*I site in *HEM1*, digested with BAL 31 nuclease, and restricted at the unique *Sma*I site before religation. Ligated plasmids were scored for β-galactosidase activity as described in Materials and Methods. This procedure yielded fusion plasmids pHZ328, pHZ329, pHZ233, pHZ4-16, pHZ4L, pHZ4-15, pHZ4F, pHZ2F, pHZ2C, and pHZ1C. To generate longer fusions, a *Sma*I-*Pvu*II fragment was inserted at the *Sma*I site of pHL2. The resultant plasmid was cut at the unique *Xho*I site, treated with BAL 31 nuclease, restricted at the *Sma*I site, and religated. This procedure resulted in plasmids pHZC18-12, pHZC18-32, pHZC18-28, pHZC18-5, pHZC18-15, pHZC18-4, and pHZC18-15. Restriction sites: R, *Eco*RI; P, *Pst*I; A, *Sal*I; X, *Xho*I; S, *Sma*I; and B, *Bam*HI.

case, loss of the plasmid, detected by loss of uracil prototrophy, was accompanied by loss of heme prototrophy. The plasmids which could complement the heme auxotrophy were used to transform other *hem1* mutants; each plasmid could complement more than one *hem1* mutant. Plasmids which were able to complement the *hem1* mutants were mapped and were found to contain a 4.6-kilobase (kb) *Eco*RI fragment in common and were later determined to represent the same locus as that isolated by Bard and Ingolia (2) and by M. Douglas. The 4.6-kb *Eco*RI fragment was subcloned into the unique *Eco*RI site of the vector β72 to give the plasmid pLB6. A restriction map of pLB6 was derived.

A noncomplementing 1.6-kb *Hin*dIII fragment from these plasmids, when introduced into the *hem1* mutant strain TM2 on an autonomously replicating plasmid, generated heme prototrophic recombinants at a high frequency. Thus, we conclude that we have cloned the *HEM1* gene.

DNA extractions and restriction site mapping. DNA was isolated from yeast cells as described previously (46) and used to transform bacterial strain YMC9 (33). Plasmid DNA was recovered from bacterial cells by the rapid boiling method (24). DNA used in plasmid constructions was isolated from cesium chloride gradients (15). All conditions for ligations and restriction endonuclease digests were as recommended by the suppliers (New England BioLabs, Boehringer Mannheim Biochemicals, or International Biotechnologies, Inc.).

Construction of *HEM1-lacZ* fusions. The first step toward constructing a *HEM1-lacZ* fusion was to construct a plasmid which contained both *HEM1* and *lacZ* sequences. The vector which provides the *lacZ* portion of the fusion is plasmid pLG669Z, which contains a fusion of upstream *CYC1* sequences from *S. cerevisiae*, including the initiation codon, to *lacZ* (18). The fusion junction is marked by a *Bam*HI site. The 4.6-kb *Eco*RI fragment containing *HEM1* was isolated, and the ends were made flush with Klenow fragment. This DNA fragment was ligated into the *Sma*I site of pLG669Z such that both *HEM1* and *lacZ* sequences were transcribed in the same direction. (The direction of *HEM1* transcription was kindly provided by E. Skekly and M. Douglas [personal communication].) A *Sma*I octanucleotide linker was then introduced into the *Bam*HI site of this vector. This intermediate plasmid, pHL2, was used to generate *HEM1-lacZ* fusions (Fig. 1).

pHL2 was cut at the unique *Sal*I site internal to the *HEM1* coding region, and BAL 31 was added to digest the DNA in both directions from the *Sal*I site. The DNA was then cut at the unique *Sma*I site adjacent to the *lacZ* coding region on the plasmid to remove *CYC1* DNA. The DNA was subsequently ligated. The addition of the *Sma*I linker at the *Bam*HI site next to *lacZ* obviates the need to add a linker after BAL 31 treatment and regenerates a *Bam*HI site at the fusion junction. The *Bam*HI junction faciliates DNA sequencing of the fusions. Plasmids pHZ328, pHZ329,

pHZ233, pHZ4-16, pHZ4L, pHZ4-15, pHZ4F, pHZ2F, pHZ2C, and pHZ1C were made in this manner.

To generate fusions of the C18 series, which fuses longer *HEM1* coding regions to *lacZ*, the plasmid pHL2 was cut at the *Sma*I site adjacent to the *lacZ* coding region. A 2-kb *Pvu*II-*Sma*I fragment of DNA from a ferrochelatase-encoding plasmid was inserted as a buffer region at this *Sma*I site of pHL2 such that a *Sma*I site is regenerated adjacent to *lacZ* sequences. This intermediate plasmid was cut at the unique *Xho*I site, treated with BAL 31, and recut at the *Sma*I site before ligation. This removed all non-*HEM1* coding sequences introduced. Plasmids pHZC18-12, pHZC18-32, pHZC18-28, pHZC18-5, pHZC18-4, pHZC18-14, and pHZC18-15 were constructed in this manner.

Construction of galactose-inducible *HEM1-lacZ* fusions. To make galactose-inducible *HEM1-lacZ* fusions, plasmid pLGSD5 was used. This plasmid contains the UAS_{GAL} segment between *GAL1* and *GAL10*. The segment is placed 5′ to a *CYC1-lacZ* region that is equivalent to that found in the plasmid pLG669Z (19). A *Bam*HI site also marks the fusion junction between *CYC1* and *lacZ*. pLGSD5 was cut at the *Xho*I site adjacent to UAS_{GAL}, and the ends were made flush with deoxynucleoside triphosphates and Klenow fragment. The plasmid DNA was then cut with *Bam*HI, removing all *CYC1* DNA. (Fig. 2). All the *HEM1-lacZ* fusions constructed in the original vector were cut at the *Kpn*I site upstream of the *HEM1* coding region, and the ends were made flush with T4 DNA polymerase. The plasmids were then cut again at the *Bam*HI site at the fusion junction, and the fragments coding for parts of *HEM1* were isolated and ligated into the prepared pLGSD5 vector.

Screening of *HEM1-lacZ* fusions. All the fusions constructed were put into *E. coli* YMC9. Because of the nature of the construction, two-thirds of the constructs would be expected to generate fusions in which the translational frames would be discontinuous across *HEM1* and *lacZ*. To obtain fusions in which the translational frames were uninterrupted, the transformed *E. coli* cells were plated onto LB plates with XG-ampicillin. Transformants that expressed β-galactosidase formed blue colonies, and their plasmid DNAs were isolated. Yeast transformants carrying these plasmids were selected as Ura^+ colonies on synthetic glucose plates. Transformants were picked and streaked on to XG plates, and after 2 days at 30°C, blue colonies were grown in synthetic medium to an optical density at 600 nm of 1.0 and assayed for the level of β-galactosidase activity after permeabilization with sodium dodecyl sulfate and chloroform (17, 18).

DNA sequencing. DNA sequencing was performed by the Sanger chain termination method (42) as modified by Biggin et al. (4); the M13 phages mp10 and mp11 were used to generate single-stranded templates. pHZ4-16 was cut at the *Bam*HI site and an upstream *Alu*I site, and the sequence of the *Alu*I-*Bam*HI fragment was determined on both strands.

Immunoblotting procedures. The immunoblotting procedure was based on a procedure described by Burnette (7). Crude protein extracts from exponentially growing yeast cells were prepared as described by Yaffe and Schatz (51). The protein concentration of the extracts were determined by the Bio-Rad protein assay (Bio-Rad Laboratories) (6). Approximately 4 μg of protein was run on each lane of a 5% sodium dodecyl sulfate-polyacrylamide gel (31). Proteins were electrophoretically transferred onto a nitrocellulose filter. The filter was washed and probed with anti-β-galactosidase antibodies (Cappel Laboratories) and with ^{125}I-labeled, affinity-purified protein A (Amersham Corp.).

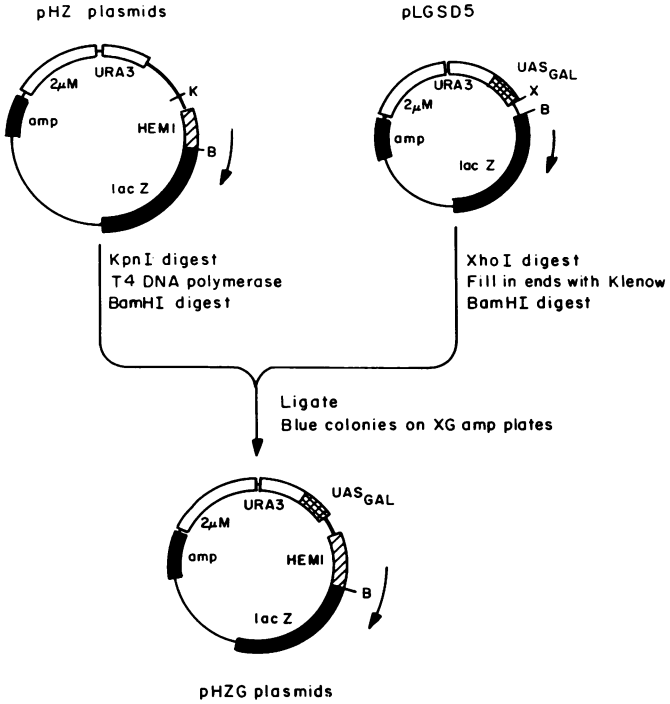

FIG. 2. Construction of galactose-inducible *HEM1-lacZ* fusions. *Kpn*I-*Bam*HI fragments containing different lengths of the *HEM1* coding region was inserted into pLGSD5 cut at *Xho*I and *Bam*HI. The resultant plasmids placed expression of *HEM1-lacZ* fusion proteins under control by UAS_{GAL}. Restriction sites: K, *Kpn*I; B, *Bam*HI; and X, *Xho*I.

The filters were then washed, and an autoradiogram of the filter was made at −80°C with Cronex Lightning Plus screen onto Kodak SB5 film. Protein standards transferred onto the nitrocellulose filter were visualized by using a 0.1% (vol/vol) India ink solution in 10 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–0.05% Nonidet P-40.

Subcellular fractionation. Transformants of yeast strain BWG1-7a, carrying various galactose-inducible *HEM1-lacZ* fusion plasmids, were grown in a rich lactate medium as described by Daum et al. (9). Spheroplasts were prepared from late-log-phase cells and homogenized in buffer A′ (0.6 M sorbitol, 20 mM Tricine-KOH [pH 7.4; Sigma Chemical Co.], 10 mM KCl, 1 mM dithiothreitol). The homogenate was fractionated into a mitochondrial pellet and a postmitochondrial supernatant by the method of Reizman et al. (40). The mitochondrial pellet was washed once with buffer A′ and repelleted by centrifugation at 17,000 × *g* for 10 min. The postmitochondrial fraction was centrifuged at 17,000 × *g* to remove residual mitochondria. To monitor mitochondria during the fractionation procedure, marker enzyme assays were performed. Density gradient analyses of washed mitochondria were performed on linear 20 to 70% sucrose gradients containing 50 mM Tris hydrochloride (pH 7.4) and 0.5 mM phenylmethylsulfonyl fluoride. The gradients were spun for 16 h at 20,000 rpm in a Beckman SW27.1 rotor (11).

Mitoplasts were prepared by selective rupture of the outer membranes of the mitochondria (20). Crude mitochondria were suspended in buffer A′ at a concentration of 3 to 5 mg/ml and diluted 10-fold with 20 mM Tricine-KOH (pH 7.4)–10 mM KCl–1 mM dithiothreitol and kept on ice for 30 min. The shocked mitochondria were collected by centrifu-

gation at 27,000 × *g* for 20 min. The protein concentration of the fractions was determined by the Bio-Rad dye binding method (6). To ensure that the mitoplasts were free of intact mitochondria, assays for cytochrome b_2, an intermembrane space enzyme, were performed (1). All mitoplast preparations were free of this enzyme activity, which was quantitatively released into the supernatant.

The compartments of the mitochondria were fractionated by the procedure of Daum et al. (9). To ensure that each submitochondrial compartment was not contaminated with other compartments, assays for cytochrome b_2, an intermembrane space enzyme, cytochrome *c* oxidase, an inner-membrane enzyme, and fumarase, a matrix enzyme, were performed on each fraction (1, 34, 37). Specific activity measurements of the enzymes indicated that the fractionated compartments were not significantly cross-contaminated.

Protease protection experiments. Mitochondria or mitoplasts were diluted to a concentration of 0.5 to 2 mg/ml in buffer A′ (21). Proteinase K and Triton X-100, when added, were used at concentrations of 100 to 400 μg/ml and 0.5%, respectively. After incubation at 30°C for 30 min, the protease was inactivated by addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM. The fractions were then assayed for fumarase and β-galactosidase activities.

Enzyme assays. Fumarase, a mitochondrial matrix enzyme, and L-lactate ferricyanide reductase (cytochrome b_2), an intermembrane space enzyme, were assayed as described by Racker (37) and Appleby and Morton (1). Cytochrome *c* oxidase, an inner-membrane enzyme, was assayed by the method of Mason et al. (34). The cytoplasmic enzyme glyceraldehyde 3-phosphate dehydrogenase was assayed as described by Krebs (30).

RESULTS

Construction of galactose-inducible *HEM1-lacZ* fusions. Our aim was to identify the sequences in *HEM1* which are needed to direct the protein to the mitochondria. This was accomplished by constructing plasmids containing various lengths of *HEM1* coding region fused to *lacI′-lacZ* and looking at the subcellular location of these hybrid proteins, as described in Materials and Methods (Fig. 1 and 2). The fragment of β-galactosidase used in these studies is normally found in the cytoplasm of yeast cells (11, 20).

The initial constructs contained the *HEM1* promoter and upstream sequences, such that expression of β-galactosidase activity depended on the *HEM1* regulatory sequences. These constructs were introduced into the yeast strain BWG1-7a, and the transformants were streaked onto XG plates and assayed for the level of β-galactosidase activity present. Transformants which gave white colonies on XG plates were assumed to be out-of-frame fusions and were not studied further. Blue colonies on XG plates represented cells with plasmids bearing in-frame fusions and gave rise to levels of β-galactosidase activity ranging from 2 to 25 U (Fig. 3).

The positions of the fusion junctions for all the fusion plasmids are shown in Fig. 3. The fusion endpoints of these plasmids were determined by DNA sequencing. The fusion plasmids pHZ328, pHZ329, pHZ233, pHZ4-16, pHZ2C, and pHZC18-28 were found to contain 9, 35, 47, 75, 232, and 404 codons of *HEM1*, respectively. None of these fusion plasmids could complement a *HEM1* mutation, indicating that none of them contains an intact *HEM1* gene and that none of the fusion proteins has δ-ALA synthase activity in addition to β-galactosidase activity.

To obtain levels of expression of the fusion proteins which could be more easily detected in subcellular fractions, fragments containing the *HEM1* portion of some of the plasmids were inserted into another vector, pLGSD5 (Fig. 2). This vector contains a *lacZ* portion that is identical to the one on the original fusion plasmids. In addition, this vector contains a UAS_{GAL} positioned upstream of the *lacZ* region (19). Insertion of the *HEM1* fragments into this vector rendered the *HEM1-lacZ* fusions galactose inducible. Since *HEM1* upstream DNA in the constructs extends out to position −448, we might also expect the *HEM1* promoter to remain functional. These plasmids were used to transform *S. cerevisiae* BWG1-7a. The transformants displayed β-galactosidase activity levels of 2 to 25 U in the absence of galactose, indicating the presence of a functional *HEM1* promoter, and 25 to 300 U in the presence of galactose (Fig. 3).

Partial DNA sequence of *HEM1* coding region. The DNA sequence of the *HEM1* coding region of pHZ4-16 is shown in Fig. 4. Because the *lacZ* translational frame is known, the *HEM1* coding sequence can be determined by counting back from the fusion junction to an AUG codon which is preceded by two in-frame UAA stop codons 9 and 11 codons upstream. pHZ4-16 was deduced to contain 75 amino acid residues of *HEM1*. Translation of the DNA sequence into the amino acid sequence revealed that, like other imported mitochondrial proteins, the amino-terminal 35 residues of this protein mostly consist of uncharged residues with 5 basic residues; there are no acidic residues (32, 41, 49, 50). One striking feature of this sequence is an alanine-rich region between residues 26 and 62, with 21 of 37 residues consisting of alanine, including a run of nine consecutive alanine residues between residues 54 and 62.

Characterization of levels of *HEM1-lacZ* fusion gene products. The fusions can be divided into two categories, based on the levels of β-galactosidase activity expressed in yeast cells. When expression is from the *HEM1* upstream region, fusions pHZ328, pHZ329, pHZ233, pHZ4-16, pHZ4L, pHZ4-15, pHZ4F, and pHZ2F, containing 175 codons or fewer of *HEM1*, all expressed approximately 20 to 30 U of β-galactosidase activity, whereas fusions pHZ2C, pHZ1C, pHZC18-12, pHZC18-32, pHZC18-28, pHZ1C8-5, pHZC18-4, pHZC18-14, and pHZC18-15, containing at least 232 codons of *HEM1*, all exhibited approximately 5 U of activity. The decreased level of expression of these longer fusion genes is not due to a shift in the translational frame, as the DNA sequences of junctions of these fusions have established their reading frame to be correct (T. Keng and L. Guarente, unpublished data). The lower levels of activity of the longer fusion gene products were also observed in the constructs, which placed them under UAS_{GAL} control.

The difference in expression of β-galactosidase activity could be due to a difference in the stability of the proteins such that the longer fusion proteins are more readily degraded, or to a difference in the structure or localization of the fusion proteins. In the latter case, levels of the long and short fusion proteins may be comparable, but the activity of the long fusion proteins may be reduced because of a failure to oligomerize their β-galactosidase moieties. To distinguish between these two possibilities, crude extracts of strains containing some of the plasmids with galactose-inducible *HEM1-lacZ* fusions were run on sodium dodecyl sulfate-polyacrylamide gels, and the fusion proteins were detected by immune blotting with anti-β-galactosidase antibodies (7). The immunoblot (Fig. 5) shows that the polypeptide levels of fusion proteins detected in the extracts approximately correlate with the relative levels of β-galactosidase activity

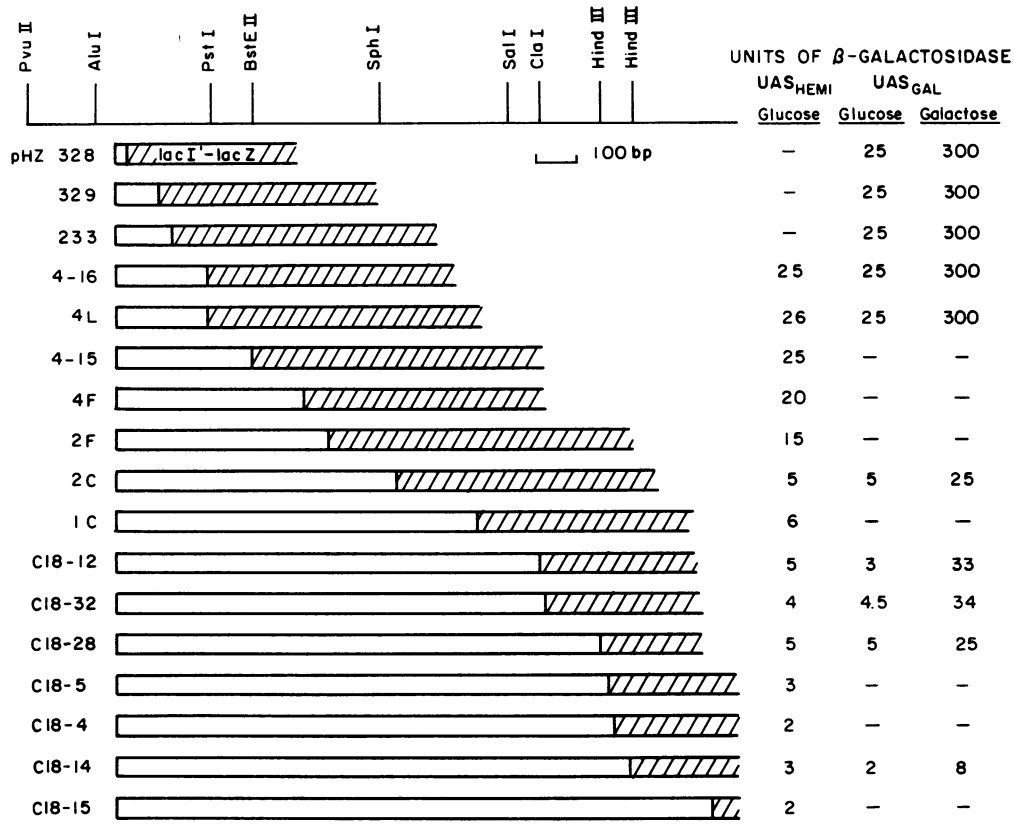

FIG. 3. Schematic representation of *HEM1-lacZ* fusions constructed and levels of β-galactosidase activity observed. A restriction map of the entire *HEM1* coding region is diagrammed above, with the position of the initiation codon as indicated. The *HEM1* coding region in each fusion is marked with an open bar. All fusion junctions were determined by DNA sequencing. Levels of β-galactosidase activity expressed from the fusion plasmids in strain BWG1-7a were determined as described in Materials and Methods and displayed in the column headed UAS_{HEM1}. Levels of β-galactosidase activity expressed from galactose-inducible fusion plasmids are shown in the columns headed UAS_{GAL}. These values were determined in strain BWG1-7a bearing the respective plasmids grown either in synthetic glucose or synthetic galactose medium. Bars indicate values not determined.

assayed. Thus, the lower levels of β-galactosidase activity exhibited by yeast cells containing longer fusions is partly due to reduced synthesis or stability of the fusion proteins.

All the fusion proteins detected in the immunoblot were of the expected sizes. Cells bearing plasmid pHZGC18-28 expressed the largest fusion protein, of approximately 165,000 daltons, whereas cells bearing plasmid pHZG2C expressed a fusion protein of approximately 140,000 daltons and cells containing pHZG4-16 expressed a fusion gene product of approximately 120,000 daltons. The sizes of the fusion proteins confirm that they are synthesized with the proper δ-ALA synthase amino terminus.

Inhibition of growth by *HEM1-lacZ* fusion proteins. We wanted to examine whether overproduction of any of the fusion proteins might cause host cells to exhibit a slow-growth phenotype. Such phenotypes have been observed in bacteria and yeast cells bearing targeted fusions (3, 11, 47). A *gal80* constitutive strain, *S. cerevisiae* 8D, was trans-

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          10          20          30          40          50          60
          *           *           *           *           *           *
ATG CAA CGC TCC ATT TTT GCG AGG TTC GGT AAC TCC TCT GCC GCT GTT TCC ACA CTG AAT
Met Gln Arg Ser Ile Phe Ala Arg Phe Gly Asn Ser Ser Ala Ala Val Ser Thr Leu Asn

          70          80          90          100         110         120
          *           *           *           *           *           *
AGG CTG TCC ACG ACA GCC GCA CCA CAT GCG AAA AAT GGC TAT GCC ACC GCT ACT GGT GCT
Arg Leu Ser Thr Thr Ala Ala Pro His Ala Lys Asn Gly Tyr Ala Thr Ala Thr Gly Ala

          130         140         150         160         170         180
          *           *           *           *           *           *
GGT GCC GCT GCT GCC ACT GCC ACA GCG TCA TCA ACA CAT GCA GCA GCA GCA GCA GCC GCT
Gly Ala Ala Ala Ala Thr Ala Thr Ala Ser Ser Thr His Ala Ala Ala Ala Ala Ala Ala

          190         200         210         220
          *           *           *           *
GCT GCC AAC CAT TCC ACC CAG GAG TCG GGT TTC GAT TAC GAA GGC C
Ala Ala Asn His Ser Thr Gln Glu Ser Gly Phe Asp Tyr Glu Gly
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FIG. 4. DNA sequence and deduced amino acid sequence of *HEM1* coding region found in plasmid pHZ4-16.

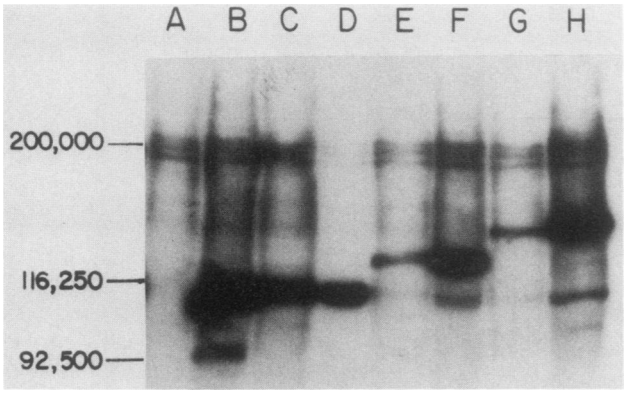

FIG. 5. Immunoblot of crude extracts prepared from strain BWG1-7a transformed with galactose-inducible fusion plasmids. The fusion proteins were detected with anti-β-galactosidase antibodies as described in Materials and Methods. Lanes: A and B, pLGSD5; C and D, pHZG4-16; E and F, pHZG2C; G and H, pHZGC18-28; A, C, E, and G, cells grown in lactate in the absence of galactose; B, D, F, and H, cells grown with galactose in the medium. Lane D is underloaded.

formed with plasmids containing the galactose-inducible fusions pHZG4-16, pHZG4L, pHZG2C, pHZGC18-28, and the control plasmid pLGSD5. The transformants were streaked onto synthetic glucose, synthetic galactose, and synthetic lactate plates. The plates were incubated at 30°C, and after 2 days the growth phenotypes of the different transformants were scored. In lactate medium, the UAS_{GAL} is fully derepressed in a *gal80* strain (52), whereas glucose represses the UAS_{GAL} (10, 36, 52).

After 2 days at 30°C, strain 8D transformed with the two longer fusion plasmids pHZG2C and pHZGC18-28 showed a galactose-sensitive phenotype. The colonies formed on the synthetic galactose plates were smaller than those formed by the same strain transformed with pHZG4-16, pHZG4L, or the control plasmid pLGSD5 (Fig. 6). On synthetic lactate plates, strain 8D transformed with all four galactose-inducible fusion plasmids, pHZG4-16, pHZG4L, pHZG2C, and pHZGC18-28, exhibited slow growth compared with cells bearing the control plasmid pLGSD5 (Fig. 6). No growth inhibition was observed with cells bearing any of the fusion plasmids on synthetic plates containing glucose.

Cellular location of the *HEM1-lacZ* fusion gene product. Subcellular fractionation was performed to localize the fusion proteins; β-galactosidase enzyme activity was used to locate the fusion proteins.

TABLE 1. Localization of fusion proteins in subcellular fractions[a]

Fusion plasmid	% β-galactosidase activity in:	
	Mitochondria	Postmitochondrial supernatant
pLGSD5	0.3	99.7
pHZG328	15.1	84.9
pHZG329	31.6	68.4
pHZG233	18.7	81.3
pHZG4-16	24.0	76.0
pHZG2C	76.0	24.0
pHZGC18-28	74.1	25.9

[a] Cells were grown in a rich lactate medium. The cells were converted to spheroplasts and fractionated as described (9). Mitochondria prepared in this manner contain <0.5% of whole-cell glyceraldehyde 3-phosphate dehydrogenase activity and >99% of whole-cell cytochrome b_2 activity.

BWG1-7a transformants bearing plasmids with galactose-inducible *HEM1-lacZ* fusions and pLGSD5 were grown separately in a rich lactate medium (9). Galactose was added to induce high levels of synthesis of the β-galactosidase fusion proteins, and the cells were harvested and fractionated into a cytoplasmic supernatant and a mitochondrial pellet (40). The fractions were assayed for the level of β-galactosidase activity. The results are summarized in Table 1. With the cytoplasmic β-galactosidase control pLGSD5, only 0.3% of the enzyme activity was associated with the mitochondrial pellet, whereas 99.7% of the activity was found in the cytoplasmic fraction. Cells bearing fusion plasmid pHZG328, pHZG329, pHZG233, or pHZG4-16, with 9, 35, 47 and 75 amino acid residues of the amino terminus of δ-ALA synthase attached to β-galactosidase, respectively, had approximately 15 to 30% of the enzyme activity associated with the mitochondria, whereas cells bearing the two longer fusion genes pHZG2C (232 amino acid residues) and pHZG18-28 (404 amino acid residues) had approximately 75% of the β-galactosidase activity associated with the mitochondria. Assays for glyceraldehyde 3-phosphate dehydrogenase, a cytoplasmic marker enzyme, indicated that in the subcellular fractionation of cells bearing the different plasmids, not more than 0.2% of the cytoplasmic enzyme activity was associated with the mitochondrial pellet. Therefore, in all cases examined the β-galactosidase activity associated with the mitochondrial pellet was not due to contamination of the pellet by cytoplasmic activity.

To rule out the possibility that the cofractionation of the β-galactosidase activity with the mitochondria was due to the formation of large aggregates of fusion protein, the

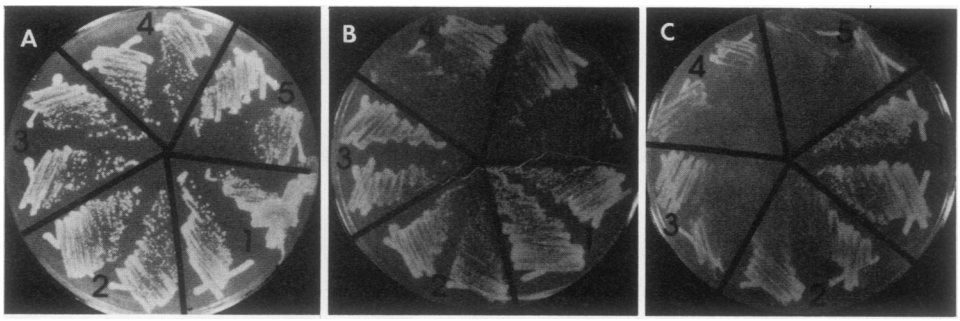

FIG. 6. Inhibition of growth by *HEM1-lacZ* fusion proteins. *S. cerevisiae* 8D cells bearing galactose-inducible fusion plasmids were streaked onto synthetic plates with glucose (A), galactose (B), or lactate (C) as the carbon source. Cells were incubated for 2 days at 30°C and photographed. Segments: 1, pLGSD5; 2, pHZG4-16; 3, pHZG4L; 4, pHZG2C; 5, pHZGC18-28.

mitochondrial pellets isolated from fusion-bearing strains were further analyzed on sucrose density gradients (11). Mitochondria do not pellet in such gradients. Under these conditions, the β-galactosidase activity expressed from the fusion plasmid pHZG4-16 was found to comigrate with the activity of the mitochondrial marker enzyme fumarase (Fig. 7A and C). Identical results were obtained with mitochondria prepared from strain BWG1-7a bearing fusion plasmids pHZG328, pHZG329, pHZG2C, and pHZGC18-28 (data not shown). When mitochondria isolated from a strain bearing plasmid pLGSD5, coding for a cytoplasmic fusion protein, were examined on these sucrose gradients, >90% of the low level of β-galactosidase activity was found on top of the gradient (Fig. 7B and D). These results demonstrate a specific association of the fusion proteins with the mitochondria. Results of immunofluorescence studies with anti-β-galactosidase antibodies also indicate specific localization of the fusion proteins to the mitochondria (data not shown).

Thus, in all cases examined, the *HEM1* encoding sequences present in the fusion plasmids were sufficient to direct the β-galactosidase moiety to the mitochondria.

Localization of the *HEM1-lacZ* gene product to within the mitochondria. δ-ALA synthase is located in the matrix of the mitochondria (48). We wished to determine whether the fusion proteins encoded by plasmids pHZG328, pHZG329, pHZG4-16, pHZG2C, and pHZGC18-28 were also located in the matrix. Hase et al. (20) and Schleyer et al. (44) showed that any protein inside the mitochondria would be protected from protease digestion by the inner and outer membranes of the organelle. We performed a similar experiment with the mitochondrial pellets and found that in all cases, over 80% of the β-galactosidase activity was protected from added proteinase K (Table 2). However, over 95% of the activity in each case was inactivated by proteinase K in the presence of 0.5% Triton X-100, which solubilizes the mitochondrial membranes; addition of 0.5% Triton X-100 alone in each case did not result in any net change of β-galactosidase activity. Identical results were observed in each case when levels of activity of fumarase, a matrix enzyme, were measured (data not shown). These results showed that the fusion gene products in cells bearing plasmid pHZG328, pHZG329, pHZG233, pHGZG4-16, pHZG2C, or pHZGC18-28 were associated with the mitochondria in such a way as to be protected from protease digestion by at least one membrane barrier.

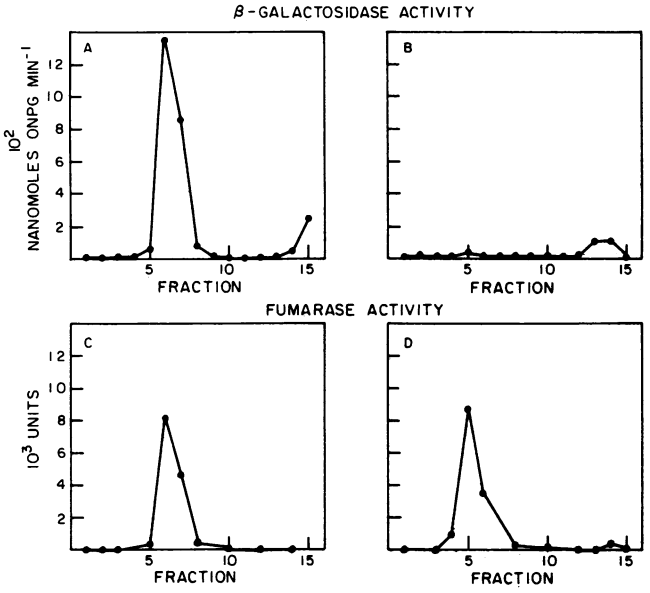

FIG. 7. β-galactosidase activity encoded by *HEM1-lacZ* fusion plasmid cofractionating with fumarase activity on a sucrose gradient. Mitochondria were prepared from BWG1-7a bearing fusion plasmids as described previously (40). Mitochondria (2.5 mg [pHZG4-16] and 3.8 mg [pLGSD5]) were layered onto a 20 to 70% sucrose gradient and centrifuged. Fifteen fractions (approximately 1 ml each) were collected from each gradient and assayed for the indicated enzymes. The bottoms of the gradients are to the left. (A and C) pHZG4-16; (B and D) pLGSD5.

TABLE 2. Protection of β-galactosidase activity in mitochondria and mitoplasts from proteinase K digestion

Plasmid[a]	Proteinase K added	Triton X-100 added	% β-Galactosidase activity remaining[b] in:	
			Mitochondria	Mitoplasts
pHZG328	−	−	100	ND[c]
	+	−	128	ND
	−	+	224	ND
	+	+	0.1	ND
pHZG329	−	−	100	ND
	+	−	139	ND
	−	+	180	ND
	+	+	0.1	ND
pHZG233	−	−	100	100
	+	−	104	127
	−	+	137	135
	+	+	0.1	0.1
pHZG4-16	−	−	100	100
	+	−	87.4	71.6
	−	+	136	111
	+	+	0.7	7.5
pHZG2C	−	−	100	100
	+	−	78.4	88.8
	−	+	128	87.5
	+	+	0.2	0.9
pHZGC18-28	−	−	100	100
	+	−	100	72.3
	−	+	135	84.6
	+	+	0.4	9.8

[a] Host strain is *S. cerevisiae* BWG1-7a.
[b] All values are expressed as percentages of β-galactosidase activity measured in the absence of Triton X-100 or proteinase K.
[c] ND, Not determined.

To determine whether the hybrid proteins were within the inner mitochondrial membrane, mitoplasts were prepared from strain BWG1-7a containing either plasmid pHZG233, pHZG4-16, pHZG2C, or pHZGC18-28. The mitoplasts consist of mitochondrial matrix surrounded by the inner mitochondrial membrane. Outer membranes were removed by osmotic lysis in 0.06 M sorbitol (20). This treatment released the intermembrane space enzyme cytochrome b_2. In all cases examined, the β-galactosidase activity was quantitatively recovered with the mitoplasts. Moreover, as with the intact mitochondria, over 90% of the β-galactosidase activity in each case was protected from externally added proteinase K (Table 2). Addition of 0.5% Triton X-100 rendered the β-galactosidase activity protease sensitive. The fate of the matrix enzyme fumarase paralleled that of the β-

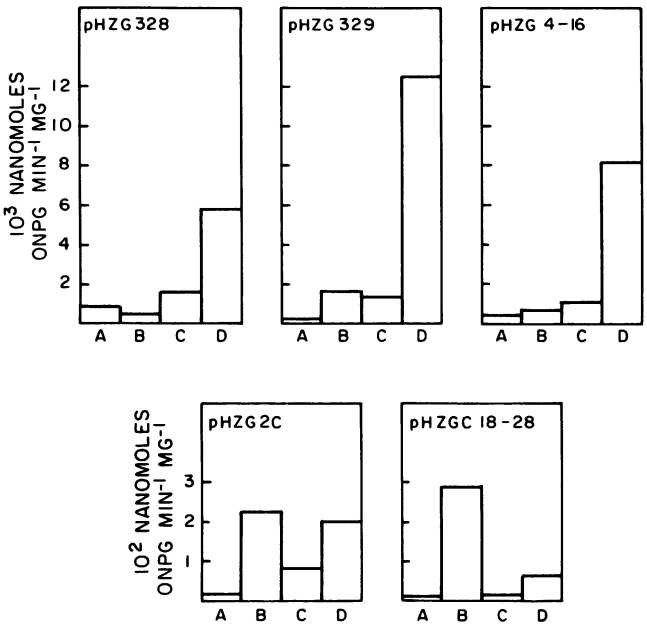

FIG. 8. Distribution of β-galactosidase activity in subfractions of the mitochondria. Yeast mitochondria from strain BWG1-7a bearing the indicated plasmids were subfractionated as described previously (9). The ordinates mark the specific activities of β-galactosidase. (A) cytoplasm; (B) membranes; (C) intermembrane space; (D) matrix. The membranes fraction represent 44 to 58% of mitochondrial proteins, the intermembrane space represents 13 to 24%, and the matrix represents 24 to 34%.

galactosidase activity (data not shown). The results demonstrate that the fusion proteins in all the cases examined were protected from protease inactivation by the inner membranes of the mitochondria. Thus, the fusions appear to reside in the matrix.

To verify the matrix location of the fusion proteins, the mitochondria were fractionated into their various compartments: membranes, intermembrane space, and matrix (9). Measurements of specific activities of cytochrome *c* oxidase (inner membrane), cytochrome b_2 (intermembrane space), and fumarase (matrix) indicated that there was no substantial contamination of one compartment with any other. Specific-activity measurements of β-galactosidase showed that in strains bearing plasmids pHZG328, pHZG329, and pHZG4-16, the fusion proteins are located in the matrix, whereas in strains bearing plasmids pHZG2C and pHZGC18-28, the fusion proteins are localized to both the membranes and matrix compartments (Fig. 8).

DISCUSSION

Amino-terminal fragments containing as few as nine residues of the *HEM1* gene product δ-ALA synthase direct β-galactosidase into the mitochondrial matrix. The location of the fusion proteins was determined by using the method of subcellular fractionation. The fusion proteins were found to be specifically associated with the mitochondria in sucrose density gradients. They were protected from protease digestion both in whole mitochondria and in mitoplasts, in which the outer membranes of the mitochondria were disrupted, and subfractionation of the mitochondria clearly showed that the fusion proteins were located in the matrix. Interestingly, the two longest fusion proteins tested (containing 232 and 404 *HEM1* codons) also showed a substantial fraction of their activity located in the membrane fractions. The protease-resistant property of these fusion proteins in mitoplast preparations suggests that they are associated with the inside of the inner membrane. The possibility that δ-ALA synthase itself displays an association with the inside of the inner membrane is currently being tested.

Gene fusion as a method to study import has been successfully used by Douglas et al. (11), Horwich et al. (25), and Hurt et al. (26–28). Of particular interest is the finding that as few as 12 amino-terminal residues of cytochrome oxidase subunit IV will direct mouse dihydrofolate reductase, a cytosolic protein, to the mitochondrial matrix (28). It would therefore appear from our data and those of Hurt et al. that mitochondrial signal sequences are, in general, very short. A comparison of the first 9 residues of δ-ALA synthase with the first 12 residues of cytochrome oxidase subunit IV indicates that both sequences are largely hydrophobic, with a few basic residues interspersed (28) (Fig. 4). Precise homology between these signals is not apparent.

The amino-terminal 35 residues of δ-ALA synthase consist largely of uncharged amino acids with a few basic residues (Fig. 4). This region is followed by an alanine-rich stretch highlighted by a run of nine consecutive alanine residues between positions 54 and 62. A similar run of 10 consecutive alanines is found in the cytochrome *c* peroxidase pre-piece (29). For δ-ALA synthase, the alanine-rich region appears not to be required to direct import.

Because of the low level of activity expressed from the original fusion plasmids, we fused the *HEM1-lacZ* fusions to a UAS_{GAL} and induced 10-fold-higher levels of synthesis of fusion proteins with galactose. Douglas et al. (11) reported that an inability of the host cells to grow on a nonfermentable carbon source is associated with the production of high levels of F_1-ATPase-β-subunit-β-galactosidase fusion gene product. Similarly, a partial inhibition of growth is caused by all of our fusions when induced cells are grown in lactate media. The longer fusion proteins also inhibit growth on galactose media. We are currently attempting to exploit this phenotype to obtain mutations which define both *cis*- and *trans*-acting components mediating import.

In several instances in which a β-galactosidase moiety is fused to signals directing secretion through a bacterial membrane, the hybrid protein becomes "stuck" in the membrane (3, 47). It is unclear whether β-galactosidase contains sequences which block the secretion machinery or which prevent passage through the lipid bilayers in these cases. We have shown that fusions of δ-ALA synthase sequences to β-galactosidase via a 90-amino-acid "bridge" of the *lac* repressor can result in matrix localization. The ability of the mitochondrial machinery to import β-galactosidase through the outer and inner membranes of the organelle may reflect unique aspects of the import process.

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