SHY1, the Yeast Homolog of the Mammalian SURF-1 Gene, Encodes a Mitochondrial Protein Required for Respiration*

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C173 and W125 are pet mutants of Saccharomyces cerevisiae, partially deficient in cytochrome oxidase but with elevated concentrations of cytochrome c. Assays of electron transport chain enzymes indicate that the mutations exert different effects on the terminal respiratory pathway, including an inefficient transfer of electrons between the bc1 and the cytochrome oxidase complexes. A cloned gene capable of restoring respiration in C173/U1 and W125 is identical to reading frame YGR112w of yeast chromosome VII (GenBank Z72897). The encoded protein is homologous to the product of the mammalian SURF-1 gene. In view of the homology, the yeast gene has been designated SHY1 (Surf Homolog of Yeast). An antibody against the carboxyl-terminal half of Shy1p has been used to localize the protein in the inner mitochondrial membrane. Deletion of part of SHY1 produces a phenotype similar to that of G91 mutants. Disruption of SHY1 at a BamHI site, located approximately 2/3 of the way into the gene, has no obvious phenotypic consequence. This evidence, together with the ability of a carboxyl-terminal coding sequence starting from the BamHI site to complement a shy1 mutant, suggests that the Shy1p contains two domains that can be separately expressed to form a functional protein.

Respiratory defective pet mutants1 of Saccharomyces cerevisiae have been useful in understanding some of the processes underlying the biogenesis of mitochondria. In earlier studies, pet mutants have been grouped into different phenotypic classes based on their spectral properties and their respiratory and ATPase activities (1, 2). A substantial number of complementation groups consist of mutants displaying defects in single enzymes of the respiratory chain (e.g. cytochrome oxidase, ubiquinol-cytochrome c reductase) or ATPase. Such strains have been exploited in different laboratories to identify and isolate genes coding for the protein constituents of the respiratory complexes (3, 4) and for ancillary factors acting at different stages of their assembly (5–7). Another commonly encountered phenotype is characterized by the pleiotropic absence of oligomycin-sensitive ATPase and the respiratory chain complexes, whose synthesis depends in part on the expression of the mitochondrial gene products. This phenotype is frequently elicited by mutations in constituents of the mitochondrial translational machinery (8).

In addition, numerous mutants exhibit the presence of all the respiratory chain components but at levels below those found in wild type yeast. This class of mutants has received little attention because the gross biochemical phenotypes do not provide obvious clues about the primary lesions responsible for the respiratory defect. To enlarge on current information about the contribution of the nuclear genome toward the maintenance of respiratory competent mitochondria, we have begun studies of pet mutants with partial pleiotropic phenotypes. In this communication we report on mutants from complementation group G91 of our collection of pet strains (9). Enzyme assays have revealed that the depression of mitochondrial respiration in this group of mutants cannot be explained in any simple way by the activities of different segments of the electron transport chain but rather appears to be related to inefficient transfer of electrons in the span between the bc1 and cytochrome oxidase complexes. The mutations responsible for the respiratory defect have been localized to the yeast homolog of the SURF-1 gene, previously shown to be in a highly conserved gene cluster of several mammalian (10) and chicken genomes (11). We present evidence that the product of the yeast SHY1 gene is a membrane constituent of mitochondria and therefore is likely to function directly in some aspect of mitochondrial organization and function.

MATERIALS AND METHODS

Yeast Strains and Media—The genotypes and sources of the strains of S. cerevisiae used in this study are listed in Table I. The media used to grow yeast have been described elsewhere (8).

Cloning of SHY1—SHY1 was cloned by transformation of the pet mutant C173/U1 (a ura3-1 shy1-2) with a yeast genomic plasmid library by the method of Beggs (13). The library used for the transformation was constructed from partial Sau3A fragments of nuclear DNA (averaging 7–15 kb) cloned into the BamHI site of the shuttle vector YEp24 (14). This library was kindly provided by Dr. Marian Carlson, Department of Genetics and Development, Columbia University. Approximately 1 × 10^5 cells were transformed with 10 μg of plasmid DNA. The transformation mixtures were plated on minimal glycerol medium to select clones complemented for the uracil auxotrophy and respiratory defect. The transformation yielded two uracil-independent and respiratory competent clones. The transformed phenotypes of both clones were verified by segregation tests to be plasmid-dependent.

Preparation of Yeast Mitochondria and Enzyme Assays—Wild type and mutant yeast were grown to stationary phase in YPGal (2% galactose, 1% yeast extract, and 2% peptone), and mitochondria were prepared by the procedure of Faye et al. (15), except that Glusulase was replaced by Zymolase 20,000 (ICN Biomedicals, Inc.) during preparation of spheroplasts. ATPase activity was assayed at 37 °C by the colorimetric determination of inorganic phosphate released from ATP (16). NADH oxidase was measured polarographically with a Clark electrode. The reactions were done in 20 mM potassium phosphate, pH 7.5, containing 2 mM NADH, NADH-cytochrome c reductase, succinate-cytochrome c reductase, and cytochrome oxidase were all assayed at 25 °C by previously described procedures (2).

Construction of W303ΔSHY1/H and W303ΔSHY1/U—Two different mutant alleles of SHY1 were introduced into a respiratory competent...
Strain | Genotype | Source |
|---|---|---|
| CB11 | a ade1 | (12) |
| W303-1A | a ade2-1 his3-1,15 leu2-3,112 trp1-1 ural3-1 | a |
| W303-1B | a ade2-1 his3-1,15 leu2-3,112 trp1-1 ural3-1 | a |
| W303 | a ade2-1 ade2-1 his3-1,15 his3-1,15 leu2-3,112 leu2-3,112 trp1-1 trp1-1 ural3-1 ural3-1 | W303-1A × W303-1B |
| W125 | a ade2-1 his3-1,15 leu2-3,112 trp1-1 ural3-1 shy1-1 | This study |
| C173 | a met6 shy1-2 | (9) |
| B173 | a ade1 shy1-2 | C173 × CB11 |
| C173/U1 | a ura3-1 shy1-2 | C173 × W303-1A |
| W303ASHY1/H | a ade2-1 his3-1,15 leu2-3,112 trp1-1 ural3-1 shy1-1:HIS3 | This study |
| aW303ASHY1/H | a ade2-1 his3-1,15 leu2-3,112 trp1-1 ural3-1 shy1-1:HIS3 | This study |
| W303ASHY1/U | a ade2-1 his3-1,15 leu2-3,112 trp1-1 ural3-1 shy1-1:URA3 | This study |
| W303ASHY1/U | a ade2-1 his3-1,15 leu2-3,112 trp1-1 ural3-1 shy1-1:URA3 | This study |

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strain of yeast. The first was a simple disruption of the SHY1 coding sequence at the internal BamHI site, with a fragment containing the yeast HIS3 gene. This allele was made by cloning the 630-base pair EcoRI-SphI fragment of pG91/S5T6 in YEpl52 (17). The resultant plasmid (pG91/S5T6) was linearized with BamHI and ligated to HIS3 on a 1.7-kb BamHI fragment to yield shy1::HIS3. In the second construct, the sequence extending from the SpII site to the ClaI site was removed and substituted by a 1-kb fragment with the yeast URA3 gene. This deletion allele (Δshy1::URA3) was made by linearizing pG91/S12 at the unique ClaI site 215 nucleotides downstream of SHY1. The ClaI site was blunted with Klenow polymerase, ligated to an eight nucleotide long SpI linker, and recircularized. After digestion of this plasmid with SpII, the linear plasmid, deleted for more than two-thirds of SHY1, was ligated to a 1-kb fragment with the yeast URA3 gene. Both the shy1::HIS3 and Δshy1::URA3 alleles were recovered as linear fragments and used to disrupt the corresponding wild type genes by the one-step gene replacement method (18) in the respiratory competent diploid strain W303. Haploid progeny with the mutant alleles were recovered following sporulation of diploid transformants.

Preparation of Antibodies to Shy1p—To obtain antibodies against Shy1p, the sequence coding for the carboxyl-terminal half of the protein was expressed as a fusion protein in Escherichia coli. The 1.5-kb BamHI-HindIII fragment containing the region of SHY1 coding for 166 carboxy-terminal residues of the protein was ligated to pATH22 (19). The resultant plasmid expressed a 55-kDa hybrid protein consisting of the amino-terminal half of anthranilate synthetase component I fused in-frame to the Shy1p sequence. The fusion protein constituted most of the insoluble protein fraction of the E. coli cells harboring the pATH22 construct. This fraction was dissolved in a 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 buffer, containing 2% SDS, 5 mM β-mercaptoethanol, and 20 μg/ml phenylmethylsulfonyl fluoride and was further purified on a Bio-Gel A0.5 column developed with a buffer containing 10 mM Tris-HCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol. Fractions enriched for the fusion protein were pooled, concentrated by acetone precipitation, and used to raise antibodies in rabbits.

Miscellaneous Procedures—Standard procedures were used for the preparation and ligation of DNA fragments and for transformation and recovery of plasmid DNA from E. coli (20). The preparation of yeast nuclear DNA and the conditions for the Southern hybridizations were as described by Myers et al. (8). DNA probes were labeled by random priming (21). DNA was sequenced by the method of Maxam and Gilbert (22). Proteins were separated by polyacrylamide gel electrophoresis in the buffer system of Laemmli (23), and Western blots were treated with antibodies against the Shy1p fusion protein followed by a second reaction with 125I-protein A (24). Protein concentrations were determined by the method of Lowry et al. (25).

RESULTS

Phenotype of Pet Mutants from Complementation Group G91—C173 is one of two independent pet isolates previously assigned to complementation group G91 of a collection of respiratory deficient mutants of yeast (9). The respiratory defect of the mutants is complemented by ρ0 testers (cytoplasmic petite mutants lacking mitochondrial DNA), indicating that each has a recessive mutation in a nuclear gene. The G91 mutants are genetically stable and, unlike some nuclear pet mutants (8), do not acquire deletions in mitochondrial DNA at any appreciable frequency. In vivo labeling of the mitochondrial gene products indicated that mitochondrial protein synthesis is not affected in the mutants (data not shown).

The cytchrome composition of mutant mitochondria was determined from the visible spectrum of extracts obtained under conditions known to quantitatively solubilize all the respiratory components of the organelle. A comparison of the spectra obtained from various mutants showed a reduction in cytochromes a and a3, and an increase in the a-absorption bands at 550 nm corresponding to c-type cytochromes (Fig. 1). To distinguish between cytochromes c and c1, mitochondria were first treated with high salt to remove cytochrome c and were then extracted with deoxycholate to solubilize the remaining cytochromes. Spectra of the salt and detergent extracts (Fig. 2) show that the increase in absorption at 550 nm in the shy1 null mutant is due to cytochrome c. Its concentration, based on the difference spectra, was estimated to be 0.57 nmol/mg mitochondrial protein in wild type and 0.9 nmol/mg protein in the mutant. The spectra of the cytochromes remaining after the salt wash confirm the lower concentration of c-type cytochromes but do not show any appreciable differences in cytochromes b and c1.

The mutants were also assayed for electron transport activities representing different spans of the respiratory chain. These, as well as mitochondrial ATPase activity, were measured in two independent G91 mutants and in two different mutant constructs, only one of which expressed a respiratory defective phenotype (see below). These assays indicated mutant mitochondria to have reduced cytochrome oxidase activity, consistent with their spectral properties (Table II). No significant differences were found in the oligomycin-sensitive ATPase. All the mutants, however, had approximately two times higher NADH-cytochrome c reductase and succinate c reductase activities (Table II).

The presence in W125, C173/U1, and W303ASHY1/H of higher than normal NADH- and succinate-cytochrome c reductase and of lower but nonetheless respectable cytochrome oxidase activity was not consistent with their growth phenotype. All three mutants fail to show any appreciable growth on glycerol. To further assess their respiratory potential, mutant and wild type mitochondria were assayed for NADH and succinate oxidase. As expected, these assays revealed both activities to be lower in the mutant mitochondria. The decrease, however, is quantitatively greater than predicted from the individual rates of cytochrome c reduction and oxidation by the terminal oxidase. The 70% decrease of NADH oxidase measured in W125 mitochondria is difficult to reconcile with the 2-fold enhancement in NADH-cytochrome c reductase and a reduction of only 23% in the cytochrome oxidase rate. A similar trend is evident in the results obtained with C173/U1 and W303ASHY1/U, although the discrepancies between the reductions in NADH oxidase and cytochrome oxidase were not as large.
**SHY1, the Yeast Homolog of the Mammalian SURF-1 Gene**

![Figure 1](image1.png)

**FIG. 1. Spectra of mitochondrial cytochromes in wild type and mutant yeast.** Mitochondria at a protein concentration of 5 mg/ml were extracted with potassium deoxycholate under conditions that quantitatively solubilize all the cytochromes (2). Difference spectra of the extracts reduced (sodium hydrosulfite) versus oxidized (potassium ferricyanide) were recorded at room temperature. The genotypes of the parental strain W303-1A and of the different mutants are described in Table I. The absorption bands corresponding to cytochromes $a$ and $a_3$ have maxima at 603 nm. The corresponding maximum for cytochrome $b$ is 560 nm and for cytochrome $c$, 550 nm.

The different respiratory activities of the mutant mitochondria were also difficult to relate to their growth properties on non-fermentable substrates. For example, even though W303ΔSHY1/U and W125 retain 20 and 30% of the wild type NADH oxidase activity, respectively, neither mutant shows any appreciable growth on glycerol or ethanol.

**SHY1 Confers Respiratory Competence to G91 Mutants—** To clone the wild type gene mutated in G91 mutants, C173/U1 was transformed with a yeast genomic library. Two respiratory competent clones obtained from the transformation were analyzed for their plasmid content. Plasmids isolated from the transformants were amplified in E. coli and their nuclear DNA inserts characterized. The restriction maps of the two plasmids indicated both to have the same inserts. This plasmid was designated as pG91/T1 (Fig. 3).

The gene responsible for restoring respiration in G91 mutants was identified by transfecting different regions of the pG91/T1 insert into YEplac32 and transforming C173/U1 with the new plasmids (Fig. 3). These experiments indicated that the 2.3-kb SpH1 fragment internal to the pG91/T1 insert restored respiration (pG91/ST4), whereas the smaller plasmid pG91/ST3 lacking the 400 base pairs of DNA from the BamHI to the SpH1 site did not, suggesting the BamHI site to be internal to the gene. The gene was identified by sequencing the span between the two SpH1 sites. This region has a reading frame matching open reading frame YGR112w on chromosome VII (GenBank accession number Z72897). The calculated molecular weight of the primary product encoded by this reading frame is 45,062. Even though the insert of pG91/ST4 lacks the sequence coding for the amino-terminal 104 residues of the encoded protein, this plasmid restores respiration as efficiently as pG91/T1, containing the entire gene (see also below).

The predicted sequence of the YGR112w product is homologous to the proteins encoded by the human and mouse SURF-1 genes (10, 11) (Fig. 4). The sequence similarity is highest in the central portion of the protein. This region of the yeast protein (residue 81 to 191) shares 50 identities (44%) with the mouse homolog. A shorter conserved region occurs near the carboxyl terminus where there are 12 identities in a span of 23 residues. The yeast and mammalian proteins have two potential membrane spanning domains close to their amino and carboxyl termini. The sequence of the amino-terminal 20–30 residues has a preponderance of basic residues conforming to the composition generally found in cleavable mitochondrial import signals (26). Because of its homology to SURF-1, the yeast gene and proteins will henceforth be referred to as SHY1 (Surf Homolog of Yeast) and Shy1p, respectively.

**Properties of Shy1 Null Mutants—** Restoration of respiratory activity in G91 mutants by SHY1 could be mediated either by complementation or extragenic suppression. To distinguish between these mechanisms, SHY1 was disrupted/deleted in a respiratory competent strain of yeast. Two different mutant alleles were made. The first was constructed by insertion of the HIS3 gene at the BamHI site in the SHY1 reading frame. Introduction of shy1::HIS3 into the chromosomal DNA of haploid yeast did not affect the transformant’s ability to grow on glycerol. The second mutant construct was made by replacing...
specific activity refers to \( \mu \text{mol of ATP hydrolyzed or of cytochrome c reduced or oxidized per min per mg of mitochondrial protein. The specific activity of NADH oxidase is expressed as microatoms of oxygen used per min/mg of protein. The assay conditions for the three activities are described under "Materials and Methods." The concentration of oligomycin (oligo) in the assay was 5 \( \mu \text{g per ml. The rates measured in two independent assays did not differ by more than 10%. The values reported are averages of the two assays.}

| Strain | ATPase \(-\text{Oligo}\) | ATPase \(+\text{Oligo}\) | NADH-c reductase | Succinate c reductase | Cytochrome oxidase | NADH oxidase |
|--------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| W303-1A | 6.4             | 1.4             | 1.39            | 0.45            | 1.72            | 1.11           |
| W125   | 5.6             | 1.4             | 3.44            | 0.92            | 1.32            | 0.33           |
| C173/U1 | 6.6             | 1.3             | 3.24            | 0.81            | 1.04            | 0.18           |
| W303ΔSHY1(H) | 5.7         | 1.4             | 1.36            | 0.32            | 1.29            | 1.09           |
| W303ΔSHY1(U) | 6.1         | 1.3             | 3.31            | 0.88            | 0.67            | 0.21           |

**FIG. 3.** **Restriction maps of pG91/T1 and derivative subclones.** The locations of the restriction sites for BamHI (B), HindIII (H), BglII (G), and SpfI (S) are shown above the nuclear DNA insert in pG91/T1. The unique SpfI site in the vector is marked for orientation purposes. The regions of pG91/T1 subcloned in YEp352 are represented by the bars in the upper part of the figure. The plus and minus signs enclosed by the brackets indicate complementation and lack thereof, respectively, of the respiratory defect of C173/U1. The SHY1 reading frame and the direction of transcription of the gene is indicated by the solid arrow.

**Fig. 4.** **Homology of Shy1p and the mammalian SURF-1 product.** The upper panel shows a dot matrix comparison of the yeast and mouse proteins. The dot matrix was obtained by the dot plot program of the GCG Sequence Analysis Package with the stringency set at 15. The hydropathy profile of the yeast protein obtained with the Kyte-Doolittle algorithm is shown in the lower panel. The locations of the BamHI (B) and SpfI (S) sites in the corresponding sequence of SHY1 are marked. The regions of the two putative membrane anchoring domains are highlighted by the cross-hatches.

W125. Most of the Ura+ clones obtained from the transformation of the mutants were respiratory competent suggesting that the integration had occurred at the locus of the mutations. This was verified by crosses of a respiratory competent clone from each transformation to the wild type haploid strain W303-1A or W303-1B and to the corresponding mutants with the \( \Delta \text{shy1::URA3} \) allele. Diploid cells issued from each cross were sporulated and ascii dissected. The meiotic spore progeny from the back crosses to the wild type W303 strain were respiratory competent (total of 18 complete tetrads). Analysis of the tetrads obtained from the crosses to the \( \text{shy1} \) mutants confirmed a 2:2 segregation of the respiratory competent phenotype (total of 16 complete tetrads). The respiratory competent progeny were all Ura+. Transformation of W125 with pG91/ST17 linearized at the NruI site of SHY1 also yielded respiratory competent Leu+ clones. One such clone was crossed to W303-1B and also to W303ΔSHY1/U. The meiotic segregation results obtained with the diploid cells were identical to those discussed above, except that the respiratory competent phenotype of the spores obtained from the cross to W303ΔSHY1/U cosegregated with the LEU2 marker, as expected.

Localization of \( \text{shy1} \)p—The respiratory defect resulting from mutations in \( \text{SHY1} \) suggested that the encoded protein was likely to be a mitochondrial constituent. This was confirmed with an antibody prepared against a fusion protein consisting
The probe detected the 2.3-kb fragment in W303-1A and a fragment approximately 4 kb in the two independent transformants. The size increase of the fragment detected in the transformants is consistent with the size of the HIS3 fragment (1.7 kb) used to disrupt SHY1. B, in the second construct most of the gene was deleted and replaced by a 1-kb fragment containing URAT1. Genomic DNA from the wild type haploid strain W303-1A and two independent transformants, W303-1A and SHY1/U and a W303-1A SHY1/U, was digested with HindIII and PstI. The probe used for the hybridization was a 1-kb HindIII fragment (probe). The probe detected the 2.7-kb HindIII fragment in the wild type strain and a smaller fragment of 1.9 kb corresponding to the PstI-HindIII fragment resulting from the presence of a PstI site in the fragment containing URA3. SHY1 is depicted by the solid bar and the disrupters HIS3 or URA3 by the open bars. bp, base pairs: E, EcoRI; S, Spal; C, ClaI; B, BamHI; H, HindIII.

Mitochondrial localization of Shy1p. A, mitochondria (MIT) and the post-mitochondrial supernatant fraction (PMS) from the respiratory competent strain W303-1A were separated on a 12% polyacrylamide gel. Following transfer to nitrocellulose the blot was first incubated with antiserum against the trpE/Shy1p fusion protein followed by a second reaction with 125I-Protein A. The prominent band seen in the lane containing mitochondrial proteins migrates on the same level as a 45-kDa size standard (indicated in the margin). The higher and lower molecular weight bands seen in the lane with the post-mitochondrial supernatant fraction are cross-reacting proteins. B, mitochondria were prepared from the wild type (WT) haploid strain W303-1A and from the transformant C173/U1/T1 harboring SHY1 on a multicopy plasmid. Total mitochondrial proteins (20 μg) were separated electrophoretically and treated as in A. The native and overexpressed protein migrate at the position of the 45-kDa marker. C, mitochondria from the wild type haploid strain W303-1A were sonically irradiated under conditions leading to a disruption of the membranes. The homogenate was centrifuged at 105,000 g for 20 min. The pellet fraction was resuspended in the starting volume of buffer. Equal volumes of mitochondria (MIT), the supernatant fraction consisting of the soluble mitochondrial proteins (SUP), and the pellet submitochondrial particles (SMP) were applied to a 12% polyacrylamide gel and processed as in A. The 45-kDa protein identified as Shy1p is seen in the lane with submitochondrial membrane proteins but not the soluble matrix proteins. D, mitochondria of the wild type strain W303-1A were suspended in 10 mM Tris-HCl, pH 7.5, at a protein concentration of 12 mg/ml. Samples were adjusted to 1 M KCl and the indicated concentrations of potassium deoxycholate (DOC). The mixtures were centrifuged at 140,000 × g, for 15 min. The supernatants were collected and the pellets were resuspended in the starting volume of buffer. Equal volumes of pellets and supernatants were separated on a 12% polyacrylamide gel and treated as in A. Shy1p in the different fractions is identified by the 45-kDa marker in the margin.

The solubility of Shy1p was determined by fractionation of sonically disrupted mitochondria into soluble matrix proteins and membrane vesicles after disruption of wild type mitochondria by sonic irradiation. Western blot analysis of the two fractions indicate that Shy1p is enriched in the membrane fraction. Extraction of Shy1p is effected by concentrations of deoxycholate that are generally used to solubilize hydrophobic proteins, providing additional support for its membrane association (Fig. 6). To localize Shy1p more precisely, the outer and inner mitochondrial membranes were separated by isopycnic centrifugation. The gradient was fractionated with an antibody against subunit 5 of cytochrome oxidase (an inner membrane marker) as well as antibodies against total outer membrane proteins (the serum used detects chiefly porin), and with the antibody against Shy1p. The procedure used to separate the two membranes yields outer membrane relatively free of inner membrane; fractions enriched in inner membrane,
except that the isopycnic gradient was centrifuged at 365,000 x g, for 5 h in a Beckman SW65 rotor. The gradient was collected in 19 equal fractions. Fractions 7–19 were separated on a 12% polyacrylamide gel, transferred to nitrocellulose paper, and probed with the antibody against Shy1p. The same fractions were reacted with a mixture of antibodies against the outer membrane and against subunit 5 of cytochrome oxidase. Shy1p, subunit 5, and porin are identified in the margin. However, contain significant amounts of the outer membrane. The results of the Western analysis indicate that Shy1p fractionates with the inner membrane. This is evidenced by the cosedimentation of Shy1p with the cytochrome oxidase subunit 5 marker and its absence in the less dense fractions of the gradient that are enriched in the outer membrane marker (Fig. 7). Localization of Shy1p in the inner membrane was confirmed by the difference in its susceptibility to proteinase K in mitoplasts and mitochondria (Fig. 8). The results of this experiment indicate that Shy1p is protected against proteinase K, Subunit 5 of cytochrome oxidase, being almost entirely embedded in the inner membrane (29), is resistant to proteinase K in both mitochondria and mitoplasts. Sco1p, an inner membrane protein facing the intermembrane space (30), behaves analogously to Shy1p.

Shy1p does not appear to be a component of a larger complex. Sedimentation in sucrose gradients calibrated with size standards ranging from 60 to 500 kDa indicates Shy1p to have a size consistent with the monomer molecular weight (data not shown).

**Confrerral of Respiration by Partially Deleted Copies of SHY1**—The complete SHY1 reading frame and the regions starting from the SpA1 or BamHI sites of the gene were inserted in single copy either at the URA3 locus of C173/U3 and at the URA3 or LEU2 loci of W125. The same constructs were also integrated at the LEU2 locus of W303ΔSHY1/U, a respiratory defective strain with a partial deletion of SHY1. Transformants harboring the different constructs were tested for growth on the non-fermentable substrate glycerol as the carbon source. These experiments indicate that the entire gene is required to complement the respiratory defect of W303ΔSHY1/U. This, however, is not true of the point mutants. A construct containing the sequence coding for the carboxyl-terminal 286 codons (pG91/ST21) restored respiration in both C173/U1 and in W125 (Fig. 9). Truncation of an additional 120 codons, leaving only the sequence coding for the carboxyl-terminal 166 residues (pG91/ST18), complements the respiratory defect of W125 but not C173/U1. The same results were obtained when the different constructs were introduced into the mutants on the multicopy plasmids, pG91/ST3 and pG91/ST4 (see also Fig. 3).

The differential effect of the partially deleted SHY1 genes on the growth phenotypes of C173/U1 and W125 could indicate that the two strains have mutations in different genes. This was excluded by the tight linkage of the two mutations. Diploid cells issued from the cross of C173/U1 to the respiratory competent transformant W125/T1 were selected on minimal glucose and were sporulated. Meiotic progeny from 13 complete tetrads were cured of the pG91/T1 plasmid on glucose medium containing 5-fluoroorotic acid. All the spores from the 13 tetrads tested became respiratory defective after loss of the plasmid.

The ability of pG91/ST3 and pG91/ST4 (and their integrative counterparts) to complement W125 implies that the partial SHY1 sequences present in these plasmids express proteins which, in conjunction with the mutant Shy1p, are capable of performing the function of the normal protein. The requirement for the mutant protein produced by W125 is evident from the failure of either plasmid to complement W303ΔSHY1/U. The presence of truncated forms of Shy1p in W125/ST3 and W125/ST4 was confirmed by Western analysis of the proteins in mitochondria and the post-mitochondrial supernatant fractions of the two transformants. The antibody to the fusion protein detected novel lower molecular weight proteins in the mitochondrial but not post-mitochondrial supernatant proteins of the transformants (Fig. 10). The 30- and 21-kDa proteins observed in W125/ST4 and W125/ST3, respectively, approximate the sizes expected of the truncated products based on the sequences retained in the two plasmids. The first in-frame methionine codon in pG91/ST4 corresponds to residue 124 of the protein. The predicted molecular weight of the product translated from this codon is 31,600. In pG91/ST3, the first methionine and 10 additional codons are contributed by the sequence upstream of the BamHI site in the multiple cloning region of YEp352. The molecular weight of the product translated from this frame is 20,350, a value in close agreement with the size estimated by SDS.

Since both truncations retain only one of the two putative
transmembrane domains, it was of interest to determine whether the shorter polypeptides behave as intrinsic membrane proteins. This was tested by sonic disruption of mitochondria and sequential extraction of the resultant submitochondrial membranes with salt and with detergent. The results of this experiment show that the 31- and 20-kDa peptides have properties similar to that of Shy1p (Fig. 10). Shy1p and the two truncated proteins are absent in the soluble fractions released from the parental strain W303-1A, from the shy1 mutant W125, W125/ST3 (W125 transformed with pG91/ST3), and W125/ST4 (W125 transformed with pG91/ST4). Equal amounts of each fraction (30 μg of protein) were separated electrophoretically on a 15% polyacrylamide gel, transferred to nitrocellulose, and reacted with antibody against the Shy1p fusion protein as in the legend to Fig. 6. The migration of molecular weight markers are indicated in the margin. A less prominent band is seen at the level of the 45-kDa marker in the lanes containing mitochondrial proteins of the mutant. This is probably a cross-reacting protein since it also appears as a weak band in the null mutant. B, mitochondria of W303-1A were sonically irradiated and submitochondrial particulates separated from the matrix proteins by centrifugation. The pelleted membranes were resuspended in the starting volume of 1 M NaCl, 20 mM Tris-Cl, pH 7.5. Equal volumes of mitochondria (lane 4), the matrix proteins (lane 2), the NaCl extract (lane 3), the deoxycholate extract (lane 4), and of the final protein pellet (lane 5) were separated on a 12% polyacrylamide gel and immunoblotted as in A, C, same as B except that the mitochondria were isolated from W125/ST3. D, same as A except that the mitochondrial were isolated from W125/ST4.

Product of the mutant gene. The absence of the protein in W125 may indicate stabilization of the protein by the polypeptides expressed from pG91/ST3 and pG91/ST4.

**DISCUSSION**

**SURF-1** has been identified in mammalian (10), chicken (11), *Drosophila melanogaster* (31), and *S. cerevisiae* (GenBank, Z7297) genomes. Human, mouse, and chicken *SURF-1* are part of a conserved locus that includes several other genes (10, 11). The absence of this organizational feature in the fly and yeast genomes casts some doubt on its functional significance (27). Even though the *SURF-1* gene appears to be widely, if not universally, distributed in eucaryotic organisms, nothing is known about its function. In our studies, the yeast *SURF-1* homolog was identified because of its ability to restore respiration in mutants previously placed in complementation group.
G91 of a pet mutant collection (9). Several lines of evidence indicate that the yeast homolog of SURF-1 (SHY1) confers respiratory competence to G91 mutants by complementation rather than suppression. The biochemical properties of a shy1 null mutant construct mimic those of point mutants. The mutant alleles in two independently isolated members of complementation group G91 were found to be genetically linked to the shy1 null mutation.

The partial pleiotropic phenotype of shy1 mutants is representative of a substantial number of pet complementation groups. Mutants of this phenotypic class have not been studied in any systematic way for the principal reason that their biochemical properties are not conducive to any easy interpretation of the primary lesion. To learn more about the basis of the respiratory defect in shy1 mutants, we have compared the partial activities associated with different spans of the electron transport chain to the net flux through this pathway with either NADH or succinate as electron donors. The enzyme assays revealed consistently higher succinate- and NADH-cytochrome c reductase and lower cytochrome oxidase activity in the mutant mitochondria. The 2- to 3-fold increase in reductase activity may be related to the higher content of cytochrome c in the mutant mitochondria. The presence of more endogenous cytochrome c in the membrane probably allows for a more rapid equilibration/reduction of the free cytochrome c used as the electron acceptor in the assay. The reduction in cytochrome oxidase correlates with a lower mitochondrial concentration of cytochromes a/a3, although the reason for this is not clear. Significantly, the loss of both succinate and NADH oxidase is greater than can be accounted for by the reduction in cytochrome oxidase. This discrepancy suggests that overall electron transport in shy1 mutants is also impaired, as a result of less efficient transfer of electrons from the bc1 complex to cytochrome oxidase. The interaction of these complexes could be affected by their organization in the membrane or by the efficiency with which cytochrome c mediates electron transfer between the two complexes. The absence of detectable growth on non-fermentable substrates, even when the oxidation of succinate or NADH is 20–30% of wild type, points to still other effects of the mutations on mitochondrial function.

Shy1p has been localized to mitochondria and is stably associated with the inner mitochondrial membrane. Release of Shy1p from the inner membrane requires detergent in the concentration range normally used to solubilize intrinsic membrane proteins. The primary sequence suggests the presence of two hydrophobic sequences of sufficient length to act as membrane anchoring domains. They are located near the amino and carboxyl termini of the protein. The membrane anchoring property of the carboxyl-terminal sequence is supported by the membrane association of the partial proteins expressed from pG91/ST3 and ST4, both of which lack the amino-terminal region of Shy1p.

Notwithstanding its relatively small size, Shy1p appears to be composed of at least two separate functional domains which, when expressed separately, are capable of providing the function of Shy1p. The two domains can be dissected at the BamHI site as evidenced by the absence of a phenotype in W303ΔSHY1/H, a strain in which SHY1 was disrupted at the BamHI site. The ability of the sequence 3′ of the BamHI site to act as a separate functional domain is also supported by complementation of W125 by pG91/ST3 and pG91/ST18 whose inserts start at the BamHI site and detection of the corresponding truncated protein in the transformant W125/ST3. Restoration of respiratory growth in C173/U1 is observed with pG91/ST4 and pG91/ST19 both of whose inserts start from the Spel site. This mutant is not complemented by pG91/ST3 or pG91/ST18 suggesting that the mutation is located between the BamHI and Spel sites. The mutation was also localized to this region by cloning the gene from C173/U1 and swapping the Spel-BamHI fragment between the wild type and mutant genes. Only plasmids with the wild type Spel-BamHI fragment complemented the mutant. The failure of the polypeptides expressed from the truncated genes to complement W303ΔSHY1/U containing only the amino-terminal coding region up to the Spel site suggests that the amino-terminal domain must include at least part of the sequence encoded by the region between the Spel and BamHI sites.

The restoration of respiratory function in C173/U1 and W125 by the amino-terminal truncated polypeptides suggests that their orientation in the membrane is the same as the native protein. In the absence of information about the transport route for Shy1p, how this is accomplished can only be speculated. In the most general sense, it is possible that by virtue of their interaction (perhaps through contact points in the central hydrophilic region), the two separate domains insert into the membrane in a manner analogous to that of the native protein. The simplest way to visualize this is if membrane insertion of the carboxyl-terminal transmembrane segment occurs from the intermembrane space. The sensitivity of Shy1p in mitochondrial fractions to proteinase K digestion suggests that the hydrophilic central region faces the intermembrane space, an orientation consistent with insertion from that compartment. Even though the partial proteins with the carboxyl-terminal domain (whether expressed from the two partially deleted genes or from the gene disrupted at the BamHI site) lack the putative amino-terminal import signal they may fortuitously contain sequences capable of targeting them to mitochondria. This would not be unusual in view of the rather loose sequence requirements of targeting signals (32).

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