Isolation and Nucleotide Sequence of the *Saccharomyces cerevisiae* Gene for the Succinate Dehydrogenase Flavoprotein Subunit*

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Succinate dehydrogenase (EC 1.3.99.1) of the mitochondrial inner membrane is a four-subunit membrane-bound enzyme that catalyzes the oxidation of succinate to fumarate and the transfer of electrons into the electron transport chain to oxygen. The catalytic domain of the enzyme is composed of a flavoprotein subunit which contains a covalently attached FAD cofactor and an iron-sulfur subunit with three nonidentical iron-sulfur clusters. We have isolated a complete genomic clone for the flavoprotein subunit of the succinate dehydrogenase from *Saccharomyces cerevisiae* and determined its nucleotide sequence. The sequence predicts a protein of 70,185 Da (640 amino acids) that shows more similarity to the *Escherichia coli* succinate dehydrogenase than to the only other mitochondrial homologue, the human flavoprotein subunit. The yeast flavoprotein subunit precursor was synthesized in a cell-free translation system and shown to possess a mitochondrial targeting sequence that directs its import into isolated, energized mitochondria where it is processed by the matrix-localized protease. The genes for the flavoprotein and the iron-sulfur subunits reside on different chromosomes and hence form different transcriptional units.

Succinate dehydrogenase is a membrane-bound flavoenzyme of the bacterial or mitochondrial inner membrane that catalyzes the oxidation of succinate to fumarate in the tricarboxylic acid cycle and donates reducing equivalents to the electron transport chain. Similarly, the bacterial fumarate reductases are also membrane-bound iron-sulfur flavoenzymes, but catalyze the reduction of fumarate to succinate (Cole et al., 1985). The succinate dehydrogenases and fumarate reductases from a number of sources show many structural and functional similarities. They invariably contain two catalytic subunits: a large flavoprotein subunit of 64–73 kDa containing covalently attached FAD, and a smaller iron-sulfur protein subunit of 27–30 kDa. In addition, these subunits become membrane-bound and coupled to the electron transport chain by associating with either one or two hydrophobic polypeptides which may contain heme.

The yeast succinate dehydrogenase provides an excellent model system for the study of the biogenesis of mitochondrial membrane protein complexes. All four subunits are nuclear-encoded, cytosolically synthesized, and transported into the mitochondrion for assembly. The succinate dehydrogenase flavoprotein subunit will provide an opportunity to investigate the mechanism and temporal sequence of cofactor attachment with respect to transmembrane transport. It has been suggested that mammalian mitochondria may contain an enzyme responsible for the modification of several cofactor flavoproteins (Lang et al., 1991). Alternatively, the covalent modification of a mitochondrial precursor in the cytosol has been shown not to inhibit import (Taroni and Rosenberg, 1991).

Recently, the role of covalent flavin attachment has been investigated by the isolation of site-directed mutants of His44 of the *Escherichia coli* fumarate reductase (Blaut et al., 1989). The covalent linkage of the FAD considerably raises its redox potential with respect to free FAD. Fumarate reductase containing noncovalently bound FAD loses the ability to act as a succinate dehydrogenase while retaining considerable fumarate reductase activity. The covalent interaction between the enzyme and its cofactor appears to be necessary to modulate the redox properties of the FAD and enable the enzyme to act as a succinate dehydrogenase.

In this paper, we describe the isolation of a complete genomic clone for the flavoprotein subunit gene, *SDHA*, by screening a plasmid library and by complementation of a mutant containing a targeted gene disruption of this gene. This is the first complete nucleotide sequence of a eucaryotic *SDHA* gene to be determined; it predicts a hydrophilic protein of 70,185 Da bearing strong resemblance to the flavoprotein subunits of other succinate dehydrogenases and fumarate reductases. The *in vitro* expressed protein is synthesized as a precursor that can be imported into isolated, energized mitochondria. Gene mapping indicates that at least two of the succinate dehydrogenase subunits are located on different chromosomes.

**EXPERIMENTAL PROCEDURES**

**Strains, Vectors, and Media**—The *Saccharomyces cerevisiae* and *E. coli* strains, as well as media, have previously been described (Robinson et al., 1991) except *D273-10B* (MATα; ATCC 2567). The vectors pRS416 and pBluescript11 were obtained from Stratagene (La Jolla, CA).

**Cloning and Sequencing**—A 0.95-kb1 polymerase chain reaction product (Roinson et al., 1991) was labeled with digoxigenin-dUTP as described by the manufacturer (Boehringer Mannheim), and used as a probe to screen 4000 colonies of a yeast genomic library in the vector YCp50 (Rose et al., 1987) using both single- and double-stranded template DNA (Mierendorf and Pfeffer, 1987). Exonuclease digestions for the production of nested deletions were performed according to the supplier (Stratagene). All

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1 The abbreviations used are: kb, kilobase; SDS, sodium dodecyl sulfate.
sequences were determined using information obtained from both strands of the DNA.

Mitochondrial Protein Import—Mitochondria were isolated and import experiments performed as described (Gasser et al., 1982). Coupled in vitro transcription and translation in rabbit reticulocyte lysate was performed as described by the supplier (Promega Corp., Madison, WI) using Trans'N-S Label (ICN Biomedicals, St. Laurent, Quebec). Published procedures were used to assay inaccessibility to externally added proteinase K (Hurt et al., 1985) and for polycrylamide gel electrophoresis and fluorography (van Loon et al., 1986).

Other Methods—Enzyme assays, Southern and Western blot analyses, and the determination of covalently bound flavin, transformations, and recombinant DNA methods have been described (Robinson et al., 1991).

RESULTS AND DISCUSSION

Isolation and Subcloning of the SDHA Gene—A yeast genomic library was screened by colony hybridization with a previously isolated partial gene fragment that had been labeled with digoxigenin-dUTP in a polymerase chain reaction (Robinson et al., 1991). Of approximately 4000 colonies, three demonstrated strong hybridization. Plasmid DNA from each of the positives was isolated and further examined by restriction mapping; the three plasmids were identical. The SDHA gene was localized to a 3.5-kb BamHI fragment by Southern analysis. This fragment was cloned into the vector, pRS416, an autonomously replicating, single-copy yeast-E. coli shuttle vector carrying the yeast-selectable marker, URA3, to produce the plasmid, pSDHA. In the following experiments, pRS416 and pSDHA were found to be retained by at least 70% of the cells in each culture.

Complementation of an SDHA Mutant—To determine whether pSDHA encoded a functional gene, sdhA6L, a mutant constructed by targeted gene disruption of the SDHA gene, was transformed with the plasmid. sdhA6L grows very slowly on glycerol; however, sdhA6L transformed with pSDHA displayed growth similar to MH125, the parental wild type strain (data not shown).

Western Blot Analysis—Mitochondria from sdhA6L transformed with either pSDHA or the pRS416 vector were isolated and analyzed by Western blot analysis. The SDHA polypeptide was not detectable in the mutant transformed with pRS416, but was clearly evident as a 67-kDa protein in the cells bearing pSDHA (Fig. 1A). Expression of the flavoprotein subunit from pSDHA was also accompanied by the reappearance of the iron-sulfur subunit (Fig. 1B).

pSDHA Restores Succinate Dehydrogenase Activity to sdhA6L—Membranes were isolated from the parental wild-type strain, MH125, and from sdhA6L transformed with either pRS416 or pSDHA. Succinate-dichlorphenol indophenol reductase activity is not detectable in membranes from the mutant plus vector, but is comparable in wild type membranes and membranes from the mutant plus pSDHA (Table I).

pSDHA Restores Trichloroacetic Acid-precipitable Flavin Levels in sdhA6L—The only yeast protein with a covalently attached flavin is the succinate dehydrogenase flavoprotein subunit (Singer et al., 1965). Therefore, the amount of trichloroacetic acid-precipitable flavin is a direct measure of succinate dehydrogenase content. While membranes from the mutant plus vector have no trichloroacetic acid-precipitable flavin above background levels, membranes from the mutant plus pSDHA contain levels comparable to those of wild type membranes (Table I).

The genomic DNA insert in pSDHA contains a functional copy of the succinate dehydrogenase flavoprotein subunit gene; the plasmid hybridizes to a partial clone we had previously isolated (Robinson et al., 1991), is able to complement an SDHA mutant, and restores succinate dehydrogenase activity, polypeptides recognized by antisera to the SDHA and SDHB subunits, and the levels of covalent FAD detected in submitochondrial membranes.

Nucleotide Sequence Determination—The nucleotide sequence of the pSDHA insert was determined by sequencing cloned restriction fragments and nested deletions constructed by exonuclease digestion. Sequence determination was performed with M13 universal primers and on one occasion, with a specifically designed oligonucleotide primer. Sequence was determined from both strands for the entire open reading frame of the SDHA gene (Fig. 1).

Table I

| Strain     | sdhA6L + pRS416 | sdhA6L + pSDHA |
|------------|-----------------|----------------|
| MH125      | 287 (100%)      | 232 (87%)      |
| sdhA6L     | ND <2%          | ND <10%        |

"Activities are expressed in nanomoles of dichlorphenol indophenol reduced min⁻¹ mg⁻¹.

ND, not detectable.

Picomoles of flavin/mg.

Fig. 2. Sequencing strategy for SDHA. The top line represents part of the 3.5-kb BamHI fragment of pSDHA. The SDHA open reading frame is represented by the box. The arrows indicate directions and lengths of sequences obtained from various subcloned fragments. The restriction sites indicated are: B, BglII; H, HindIII; N, NdeI; R, EcoRI.
**Fig. 3.** Nucleotide sequence of SDHA and flanking regions. The sequence extends over 2.64 kb of the genomic insert in pSDHA. The reading frame identified as SDHA starts with a methionine initiation codon at nucleotide +1 and terminates with an ochre codon at nucleotide +1921. Only the sequence of the sense strand is shown. The amino acid sequence of the succinate dehydrogenase flavoprotein subunit is shown below the nucleotide sequence. Shown in **bold** type are two potential TATAA boxes and **underlined** is a possible polyadenylation signal.
frame and flanking regions (Fig. 2). The sequence begins at a BamHI site 272 nucleotides upstream of the initiator methionine codon, proceeds through an open reading frame of 1920 nucleotides, and ends nearly 500 nucleotides downstream. Two potential TATAAA boxes in the promoter region (positions -137 to -141 and -155 to -161) are shown in bold type (Fig. 3). A possible polyadenylation signal, TTTTAT (2143–2149), in a region of sequence with high A + T content is underlined (Fig. 3; Irriger et al., 1991).

The predicted size of the SDHA polypeptide precursor is 70,185 Da, and as expected, it displays considerable sequence similarity with other flavoprotein subunits of this family (Table II). The yeast SDHA subunit is most closely related to its E. coli homologue. Surprisingly, it is least similar to the only other mitochondrially derived flavoprotein subunit, a partial sequence from a human cDNA library (Malcovati et al., 1991). This is in marked contrast to the case for the iron-sulfur subunits of yeast and human where extensive sequence identity (68.7%) occurs (Gould et al., 1989). However, the human sequence does not contain the amino terminus of the polypeptide with the highly conserved regions involved in flavin attachment and binding. Despite the low similarity of the yeast sequence to its human counterpart, it is clear that pSDHA encodes the S. cerevisiae SDHA gene.

The alignment of the yeast SDHA polypeptide sequence with related flavoprotein subunits is displayed in Fig. 4. The FAD cofactor of the bovine succinate dehydrogenase is covalently bound via the â-methyl group of the isoalloxazine ring to the N(3) of a histidine (Singer and Edmondson, 1974). The sequence of a 23-residue flavopeptide from this enzyme was determined (Singer et al., 1973), and comparison with the yeast sequence reveals that 17 of 23 positions are identical; this identifies His-90 as the probable site of flavin attachment in the yeast protein. In contrast to the other flavoprotein subunits listed in Fig. 4, this histidine residue is not the first in the yeast sequence, but is preceded by His-47 and His-51. This observation is consistent with the notion that FAD is attached cotranslationally to the first histidine residue encountered, but rather suggests that some minimal tertiary structure is necessary to accommodate the cofactor (Hamm and Decker, 1978) or that the process is posttranslational (Cecchini et al., 1985). The role of the presequence in flavin attachment to a mitochondrially localized protein is under investigation.

From comparisons with glutathione reductase, a noncovalent flavoprotein for which three-dimensional structural data exist, the succinate dehydrogenase and fumarate reductase flavoprotein subunits are believed to interact noncovalently with the AMP moieties of the FAD in two regions (Schulz et al., 1982; Wierenga et al., 1983; Cole et al., 1985). One such region contains a Rossmann nucleotide binding fold which contacts the bottom of the AMP and is located near the amino terminus. The second region appears in the center of the polypeptide and forms the top of the AMP binding domain (Fig. 4). The residues in these regions, along with those near the site of FAD attachment, are among the most conserved.

It is believed that a histidine residue plays an important role in the function of the fumarate-succinate oxidoreductases. This residue is conserved in the sequence His-Pro-Thr (where this sequence is His-Pro-Ser (residues 287–289)). It is in marked contrast to the case for the iron-sulfur subunits (Wood et al., 1984). The analogous cysteine is conserved in the Proteus vulgaris FRDA (Cys-248; Cole, 1987) and the Wollinella succinogenes FRDA (Cys-272; Lauterbach et al., 1990), but not in the B. subtilis or the human SDHAs where alanines are located; it is for this reason that the B. subtilis enzyme is believed to be insensitive to thiolreactive reagents (Phillips et al., 1987). In the yeast sequence, the analogous residue is Ala-302; however, there is a cysteine residue nearby (Cys-296) that may account for the enzyme’s sensitivity to p-chloromercuribenzoate (Singer et al., 1957).

Import into Isolated Mitochondria—The SDHA gene was transcribed in vitro with T7 RNA polymerase and the mRNA translated in a cell-free reticulocyte lysate in the presence of 35S-labeled methionine. As expected, the SDHA polypeptide is able to bind to mitochondria (Fig. 5, lanes 2 and 5). Import into mitochondria is membrane potential-dependent (lanes 5 and 6); only in the presence of energized mitochondria is a faster migrating species seen (lanes 2 and 3). This species is resistant to externally added protease (lane 3) except when mitochondrial integrity is disrupted with detergent (lane 4).

As is commonly observed with in vitro synthesized precursor proteins, a fraction is present in an insoluble form (lane 7). This fraction is however, totally sensitive to protease (lane 8).

The amino terminus of the SDHA precursor contains the consensus sequence for cleavage upon import by two separate prosequences RX(F)XX(S) where R is arginine; X is any amino acid; (F) is phenylalanine or other hydrophobic residues; and (S) is serine, threonine, or glycine (Hendrick et al., 1989). In the yeast SDHA subunit, this motif is composed of Arg-19, Phe-21, and Ser-24 and would predict that the yeast SDHA precursor is first cleaved between Thr-20 and Phe-21 and that a subsequent cleavage by the matrix processing protease to a
T7 RNA polymerase, and the mRNA was translated in a cell-free mitochondria from D273-10B were isolated as described (Daum et al., 1982). Plasmid pSDHA was linearized and transcribed in vitro with T7 RNA polymerase, and the mRNA was translated in a cell-free system to yield SDHA precursor. Mitochondria were reisolated, disrupted by the addition of 10 mM succinate and 2 mM NADH. The mitochondria were reisolated and analyzed by SDS-gel electrophoresis and fluorography. Lane 1, 10% of the amount of lysate added to each import reaction; lane 2, mitochondria that have imported the SDHA precursor; lane 3, same as lane 2 but treated with 250 μg of proteinase K/ml; lane 4, mitochondria incubated with the SDHA precursor, reisolated, disrupted by the addition of 1% Triton X-100, and treated with proteinase; lane 5, mitochondria incubated with the SDHA precursor in the presence of 10 μg of valinomycin; lane 6, same as lane 5 but treated with proteinase; lane 7, a mock import reaction in the absence of mitochondria; lane 8, same as lane 7 but treated with proteinase. p, precursor form; m, mature form.

**Fig. 5. Import of SDHA into isolated mitochondria.** Mitochondria from D273-10B were isolated as described (Daum et al., 1982). Plasmid pSDHA was linearized and transcribed in vitro with T7 RNA polymerase, and the mRNA was translated in a cell-free system to yield SDHA precursor. Mitochondria were reisolated, disrupted by the addition of 10 mM succinate and 2 mM NADH. The mitochondria were reisolated and analyzed by SDS-gel electrophoresis and fluorography. Lane 1, 10% of the amount of lysate added to each import reaction; lane 2, mitochondria that have imported the SDHA precursor; lane 3, same as lane 2 but treated with 250 μg of proteinase K/ml; lane 4, mitochondria incubated with the SDHA precursor, reisolated, disrupted by the addition of 1% Triton X-100, and treated with proteinase; lane 5, mitochondria incubated with the SDHA precursor in the presence of 10 μg of valinomycin; lane 6, same as lane 5 but treated with proteinase; lane 7, a mock import reaction in the absence of mitochondria; lane 8, same as lane 7 but treated with proteinase. p, precursor form; m, mature form.

**Fig. 6. Mapping of the SDHA gene onto chromosome XI.** Chromosomai DNA from *S. cerevisiae* that had been separated by contour-clamped homogeneous electric-field electrophoresis and transferred to a nylon membrane (Chromo-blot from Clontech Laboratories, Palo Alto, CA) was probed. The roman numerals on the left indicate the positions of the individual chromosomes. A, probed with a fragment of the SDHA gene; B, probed with the iron-sulfur subunit gene; *URA*, probed with the *URA3* gene; *ORI*, origin.

The mature species occurs between Arg-28 and Gln-29. The purpose of such a dual cleavage event has not been elucidated. The latter cleavage can be inhibited in vitro by the addition of o-phenanthroline, a metal chelating agent (data not shown). We have not detected an intermediate in our in vitro import reactions, and are currently determining the mature amino terminus of the imported precursor.

**Chromosomal Mapping**—The chromosomal location of the SDHA gene was mapped by probing a nylon membrane to which had been transferred *S. cerevisiae* chromosomal DNA separated by contour-clamped homogeneous electric-field electrophoresis. As controls, two known genes, the succinate dehydrogenase iron-sulfur subunit (SDHB) gene on chromosome VII and the *URA3* gene on chromosome V were also mapped (Lombardo et al., 1990). For the SDHA gene, a strong signal corresponding to chromosome number XI was observed (Fig. 6). A weaker signal corresponding to chromosome XIV may indicate the presence of a related gene in the genome.

The SDHA and SDHB genes are located on different chromosomes in the *S. cerevisiae* genome. In all procaryotic organisms in which fumarate reductases or succinate dehydrogenases have been examined, the enzymes are expressed from operons encoding all of the subunits. Therefore, mitochondrial localization, multisubunit enzymes likely have evolved from the translation of polycistronic messages to the expression of individual subunits from different transcriptional units within the nucleus. This evolution requires that each subunit gene must not only acquire transcriptional signals but also an in-frame mitochondrial targeting sequence. It is improbable that each of the succinate dehydrogenase subunit genes would simultaneously undergo these changes. Gene transfer as an evolutionary process is a basic tenet of the endosymbiotic theory which proposes that mitochondria and chloroplasts were once free-living organisms (Gray and Doolittle, 1982). Regardless of whether the nuclear, precursor-encoding mitochondrial dehydrogenase genes originated from the mitochondrial progenitor or not, it is probable that at some time during evolution, the enzyme was composed of both nuclear and mitochondrially encoded subunits in a situation similar to those of complexes I, III, IV, and V in most modern eukaryotes (Attardi and Schatz, 1988).

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