Mitochondrial and Cytoplasmic Fumarases in Saccharomyces cerevisiae Are Encoded by a Single Nuclear Gene FUM1*

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The existence of mitochondrial and cytosolic fumarase has been documented in cultured human (1, 2) and mouse (3, 4) cells and in a liver tissue of pig and rat (5). Based on genetic and biochemical data, the two enzymes have been proposed to be products of a single gene (1, 5). In the present communication we report evidence for the presence of cytosolic and mitochondrial fumarase in the yeast Saccharomyces cerevisiae. Unlike the situation in mammals, the two yeast fumarases are encoded by a single gene (FUM1). This gene has been cloned and its sequence determined.

There are currently a number of examples of yeast enzymes that are shared between different compartments, but are encoded by the same gene (6-12). The mechanism ensuring differential intracellular targeting of such enzymes was first clarified for sucrose, a product of the SUC2 gene (6, 7). The SUC2 gene gives rise to two different transcripts, only one of which contains the sequence coding for the amino-terminal secretion signal (6, 7). The second transcript lacking this sequence encodes a shorter primary translation product, corresponding to cytoplasmic sucrose. A similar mechanism has recently been shown to direct histidyl-tRNA synthetase either to mitochondria or the cytoplasm (8). Based on the results of the present study we propose that the FUM1 gene for yeast fumarase also gives rise to multiple transcripts some of which lack the sequence coding for the amino-terminal mitochondrial import signal.

MATERIALS AND METHODS

Strains and Growth Media—The genotypes and sources of S. cerevisiae strains used in this study are described in Table I. Respiratory-deficient pet* strains including the fumarase mutants reported in this study were obtained by mutagenesis of the wild type haploid strain S. cerevisiae D273-10B/A1 as described previously (15). The following media were used for routine cultivation of yeast: YPD (2% glucose, 1% yeast extract, 2% peptone), WO (2% glucose, 0.67% nitrogen base without amino acids (Difco), YEPG (3% glycerol, 2% ethanol, 1% yeast extract, 2% peptone), YPgal (2% galactose, 1% yeast extract, 2% peptone). All solid media contained 2% agar. Other specialized media are described in the legends to the tables.

Manipulation and Sequence Analysis of DNA—Transformation of Escherichia coli, isolation of plasmid DNA, digestion of DNA with restriction endonucleases, electrophoresis of DNA fragments on agarose for analytical and preparative purposes, and ligation of DNA fragments were performed according to standard procedures (16). All DNA sequences were obtained by the method of Maxam and Gilbert (17) using single-stranded DNA fragments labeled at the 5' ends.

Northern and S1 Nuclease Analysis of Fumarase Transcripts—Total RNA from wild type yeast was fractionated on poly(U) Sepharose 4B (Pharmacia P. L. Biochemicals). The fraction enriched for poly(A)* RNA was separated electrophoretically on 1% agarose and transferred to DBM paper. Northern blots were hybridized to different nick-translated probes as described by Alwine et al. (18).

S1 nuclease mapping of the 5' ends of the fumarase transcripts was performed by the method of Berk and Sharp (19) using 5'-end-labeled single-stranded DNA fragments as protection probes. Poly(A)* RNA (10 µg) or total RNA (50 µg) and labeled complementary DNA (approximately 20 ng) were mixed, denatured at 65 °C for 5 min and further incubated at 42 °C for 3 h. Following dilution with S1 buffer, the hybridization mixture was treated with different amounts of S1 nuclease for 30 min. The products formed were sized on 7 or 20% polyacrylamide sequencing gels. The abbreviations used are: pet, respiratory-defective mutants of S. cerevisiae with mutations in nuclear DNA; kb, kilobase pair(s); bp, base pair(s); Pipes, 1,4-piperazinediethanesulfonic acid.
FUM1 Codes for Yeast Mitochondrial and Cytoplasmic Fumarase

| TABLE I | Genotypes and sources of Saccharomyces cerevisiae strains |
|---------|----------------------------------------------------------|
| Strain  | Genotype | Source                        |
| D273-10B/A1 | α sp′, met6          | Ref. 13                      |
| CB11    | α sp′, ade1         | Ref. 14                      |
| W303-1A | α sp′, ade2-1, his3- | R. Rothstein*                |
| W303-1B | α sp′, ade2-1, his3- | R. Rothstein*                |
| C5      | α sp′, met6, fum1-1  | This study                   |
| B5      | α sp′, ade1, fum1-1  | C5 × CB11                    |
| C5/U3   | α sp′, ura3-1, fum1-1 | C5 × W303-1A                |
| W303VFUM1 | α sp′, ade2-1, his3- | This study                   |
|         | 11,15, leu2-        |                             |
|         | 3,11, trpl-1, ura3-1 |                             |
|         | 1, FUM1::LEU2       |                             |

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gels. A sample of the probe derivatized by the method of Maxam and Gilbert (17) for A+G-specific reactions was used as a sequencing ladder.

Transformation of Yeast—To clone the gene coding for yeast fumarase, C5/U3 (α ura3-1, fum1-1) was transformed with a yeast genomic library consisting of partial Sau3AI fragments of yeast nuclear DNA averaging 7–10 kb ligated to the BamHI site of the shuttle vector YEp24 (20). The plasmid bank was kindly supplied by Dr. Marian Carlson of the Department of Human Genetics, Columbia University, New York. Approximately 106 cells grown to early log phase in 2% galactose medium (YPGal) were transformed with 2 µg of plasmid DNA as described previously (21). Yeast clones complemented for the uracil auxotrophy and respiratory deficiency were selected on minimal glycerol medium. Out of 107 transformants tested, 10 were also respiratory-competent.

Enzyme Assays—Fumarase was assayed spectrophotometrically at 37°C by the method of Lowry et al. (24).

Results

Phenotype of Fumarase Mutants—The fum1-1 mutant C5 was found in a collection of respiratory-deficient pet mutants selected for their inability to grow on glycerol medium supplemented with yeast extract and peptone (YPEG) (25). Based on the spectral properties of isolated mitochondria and in vivo labeling experiments, this mutant was also found to be deficient in mitochondrial protein synthesis. C5 was assigned to complementation group G5 which includes two other independent isolates. A fourth mutant allele (FUM1::LEU2), constructed by in situ disruption of the gene (see below). The mutant with the gene disruption has been designated W303VFUM1.

The growth phenotype of C5 is clearly expressed on YPEG in the first 2 days. The mutant, however, grows slowly on this medium and when incubated for longer periods forms small colonies. In contrast, W303VFUM1 with a long deletion in this gene shows substantial growth on rich glycerol medium (Table II). This discrepancy in the growth properties can be accounted for by the genetic backgrounds of the two mutants. C5 was isolated by mutagenesis of the respiratory competent haploid strain D273-10B/A1. The disruption of the FUM1 gene was introduced into the wild type haploid strain W303-1A. We have observed that meiotic progeny with the fum1-1 mutation obtained from a cross of C5 to W303-1A show a range of growth properties on rich glycerol medium. Some haploid spores grow as well as W303VFUM1, while other spores have a phenotype similar to that of C5. Whether this is due to some subtle differences in the intermediary metabolism of the two strains or some other characteristic (e.g., amino acid uptake) is not clear at present.

Neither C5 nor W303VFUM1 show any growth on minimal glycerol even after prolonged incubation. Both strains, however, grew slowly on this medium when supplemented with either aspartic acid, asparagine, or serine; other amino acids were ineffective in promoting growth on the nonfermentable substrate (Table II).

Fumarase activity of wild type yeast is found in both the mitochondrial and postribosomal supernatant fractions. Although the specific activity is higher in mitochondria, approximately 70% of total enzyme units are associated with the soluble fraction (Table III). Both activities are essentially absent in C5 and in W303VFUM1. This observation suggests that the mitochondrial and cytoplasmic enzymes are products of the same gene. The absence of fumarase in C5 and in W303VFUM1 with the FUM1::LEU2 allele further argues that the differences in the growth characteristics of the two strains are not due to any allele-specific variations in their fumarase activity.

Cloning of the Yeast Fumarase Gene—The fumarase mutant C5/U3 (α ura3-1, fum1-1) was transformed with a yeast genomic library consisting of partial Sau3AI fragments of yeast nuclear DNA cloned in the shuttle vector YEp24 (20). Transformants were selected on minimal glycerol medium. The transformation yielded two clones, C5/T1 and C5/T2, which were complemented for both the uracil auxotrophy and respiratory deficiency. Analysis of C5/T2 indicated that complementation of the ura3 and fum1 mutations is a function of an autonomously replicating plasmid as evidenced by the co-loss and co-retention of the glycerol* and ura* phenotypes among vegetative progeny of the transformant grown on nonselective medium.

The complementing plasmid was isolated from C5/T2 and was amplified in E. coli. The restriction map of this plasmid (pG5/T2) indicated a nuclear DNA insert of approximately 6 kb (Fig. 1). To localize the gene, several regions of the insert in pG5/T2 were subcloned in the shuttle vector YEp352 (26). The derivative plasmids were tested for their ability to complement the respiratory defect of C5/U3. As shown in Fig. 1, the shortest region of DNA (pG5/ST2) capable of complementing the fumarase mutant consisted of a 2.8-kb fragment defined by an XhoI and a HindIII site. The lack of complementation by pG5/ST9, pG5/ST4, and pG5/ST5 suggested that the gene crosses the BamHI and one of the two internal EcoRI sites.

Sequence of the Yeast Fumarase Gene—The 2.9-kb HindIII fragment of pG5/T2 and some additional 350 bp lying up-stream of the leftmost HindIII site (see Fig. 1) were sequenced by the method of Maxam and Gilbert (17). All the restriction sites used for 5′-end labeling were crossed from neighboring sites, and approximately 70% of the sequence was confirmed from the complementary strands. The restriction fragments used for 5′-end labeling are shown in Fig. 2. The region sequenced includes two open reading frames both of which are in the same strand of DNA (Fig. 3). The first reading frame is 1464 nucleotides long and can code for a protein with a molecular weight of 53,212. This gene starts with a methionine initiation codon 51 nucleotides upstream of the leftmost
HindIII site and spans the BamHI and EcoRI sites, both of which were found to be in the region necessary for complementation of C5/U3 (see Fig. 1). Since the XhoI-HindIII fragment cloned in pG5/ST2 lacks the amino-terminal 51 residues of the product encoded by the gene, this part of the protein does not appear to be needed for complementation of the fumarase deficiency. An alignment of the protein sequence encoded by the yeast gene with the reported sequence of Bacillus subtilis fumarase (27) shows 56% of the residues to be identical (Fig. 4). The homology to the bacterial fumarase constitutes strong evidence for the identity of the reading frame as the structural gene of yeast fumarase (FUM1). The primary translation product of FUM1 has an extra 25 residues at the amino-terminal end that are absent in the bacterial enzyme. Evidence to be discussed later in this paper suggests that at least part of this sequence is necessary for transport of the protein into mitochondria.

The second reading frame is initiated by an ATG codon approximately 500 bp downstream of the termination codon of FUM1. A computer search of the partial sequence encoded by this reading frame against the Claverie library (29) has failed to provide clues about the identity of this gene.

**In Situ Disruption of the FUM1 Gene**—To confirm that the respiratory deficiency of C5 is the consequence of a mutation in FUM1, the one-step gene replacement procedure (30) was used to introduce a disrupted copy of the gene in the respiratory competent haploid strain W303-1A. The 2.9-kb HindIII fragment of pG5/ST1 was transferred to the unique HindIII site of YEpl5H (this vector is identical to YEpl5 (24) except that the multiple cloning region of the latter is replaced by a single HindIII site). The new construct was digested with a combination of Sphi and EcoRI to remove 970 bp internal to the FUM1 gene (Fig. 5). The deleted sequence was replaced

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### Table II

**Growth properties of fumarase mutants**

The compositions of rich media (YPD, YEPG) are described under "Materials and Methods." Minimal media contained the indicated carbon sources, 0.67% yeast nitrogen base without amino acids (Difco), plus the auxotrophic requirements of the strains. Aspartic acid was added to a final concentration of 100 µg/ml. All media contained 2% agar. Growth on the various media was scored after 1 day, 3 days, and 5 days of incubation at 30°C. Most rapid growth is indicated by three pluses. Minus signs indicate absence of growth even after more than a week of incubation. +/− signifies appearance of visible patches after 3 days.

| Strain   | Genotype    | Rich media | Minimal media |
|----------|-------------|------------|---------------|
|          |             | YPD        | YEPL | Glycerol | Glycerol + Aspartate |
| W303-1A  | FUM1        | +++        | ++  | +       | +                  |
| W303VFUM1| FUM1::LEU2  | +++        | +   | −       | −                  |
| C5       | fum1-1      | ++         | +/− | +       | +/−                |
| C5/U3    | fum1-1      | ++         | +/− | +       | +/−                |

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### Table III

**Cytosolic and mitochondrial fumarase in wild type and mutant yeast**

| Strain   | Specific activity | Homogenate | Mitochondria | Supernatant | Total units |
|----------|-------------------|------------|--------------|-------------|-------------|
|          |                   |            |              |             |             |
|          | µmol/min/mg       |            |              |             |             |
| D273-10B | 0.36              | 2.36       | 0.66         |             |             |
| C5       | 0.01              | 0.08       | 0            | 1.04        | 0.46        |
| W303VFUM1| <0.01            | 0          | 0            | <1          | 0           |

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**Fig. 2. Sequencing strategy.** The restriction sites used for 5′-end labeling and the approximate lengths of sequences obtained are depicted by the arrows. The restriction map shows the locations of recognition sites for EcoRI (Φ), HindIII (Φ), BglII (Φ), EcoRI (Ο), BamHI (Ο), Kpn (Ο), SphI (Β), Hinfl (Ψ), Tagl (Ψ), and DdeI (Ο). The open bar in the upper part of the figure shows the position of the open reading frame proposed to code for fumarase.
FUM1 Codes for Yeast Mitochondrial and Cytoplasmic Fumarase

FIG. 3. Sequence of FUM1 and flanking regions. The sequence shown spans 2.75 kb of the genomic fragment in pG5/T2 starting approximately 700 bp from the left side of the insert and ending some 500 bp short of the most distal HindIII site (see Fig. 1). The protein encoded by the FUM1 gene starts with the methionine codon at nucleotide +1 and terminates with an ochre codon at nucleotide +1465. The 5' termini of the major transcripts protected by S1 nuclease mapping are marked with asterisks. The partial sequence of a second gene coding for an unknown product has also been translated.

with a 3-kb SphI-EcoRI fragment containing the yeast LEU2 gene. This plasmid was digested with HindIII, and the linear 4.9-kb fragment was used to integrate the disrupted allele FUM1::LEU2 into W303-1A. Transformants prototrophic for leucine were selected on minimal glucose medium supplemented with all the auxotrophic requirements of W303-1A except leucine. Several leu+ clones obtained from the transformation failed to grow on minimal glycerol and were not complemented by C5 indicating that the mutations induced as a result of transformation with the disrupted copy of FUM1 are allelic to the fuml-1 mutation.

Southern analysis of genomic DNA from both wild type yeast and from two independent transformants (W303VFUM1) confirmed that the wild type gene had been replaced by the deleted copy. As shown in Fig. 5, the 2.9-kb HindIII probe detects the homologous fragment in genomic blots of DNA isolated from W303-1A. In the transformants, the same probe hybridizes to a HindIII fragment of approximately 4.9 kb, commensurate with the size increase expected by the presence of the 3-kb insert with the LEU2 gene.

Intracellular Distribution of Fumarase in Yeast Transformed with pG5/T2 and pGS/ST1—The absence of fumarase in mitochondria and in the postribosomal fraction of fuml mutants (Table II) is consistent with the idea that both enzymes are products of FUM1. This observation, however, does not exclude the possibility that the extramitochondrial fumarase seen in wild type yeast was released from mitochondria during the fractionation.

The significance of the two yeast fumarases was examined by measuring enzyme activity in the mitochondrial and soluble fractions of a yeast fumarase mutant transformed with

FIG. 4. Homology of yeast and bacterial fumarases. The primary sequences of B. subtilis fumarase (27) have been aligned with the protein sequences of the FUM1 product by the MFALGO program (28). Positions in the two sequences with identical residues have been boxed.
either pG5/T2 or pG5/ST1. Based on the sequences of the inserts in the two recombinant plasmids only the former was ascertained to contain the complete coding sequence of FUM1. The 2.9-kb HindIII fragment present in pG5/ST1 lacks the sequence coding for the amino-terminal 17 residues of the FUM1 product. The results of the enzyme assays, summarized in Table IV, show that the specific activity of fumarase in a mutant harboring pG5/T2 is 5 times higher in the mitochondrial and 20 times higher in the postribosomal supernatant fraction when compared to the wild type. The increase of mitochondrial and cytoplasmic fumarase in the transformant over that seen in wild type yeast is presumably due to the presence of the gene on a multicopy episomal plasmid. The same mutant transformed with pG5/ST1 shows a 10-fold increase in the specific activity of fumarase in the postribosomal supernatant. Although the specific activity of mitochondrial fumarase was also higher in C5/ST1 than in the mutant, it was less than 20% of the wild type activity. Furthermore, based on total units, mitochondrial fumarase in C5/ST1 represented less than 1% of the activity recovered in the combined fractions. The comparable value of mitochondrial fumarase in C5/T2 was 6%. This difference in localization of the enzyme in the two transformants is also reflected in the distribution of total enzyme units recovered in the two fractions.

The 40-fold lower specific activity of mitochondrial fumarase in cells transformed with pG5/ST1 as compared to pG5/T2 suggests a requirement of the amino-terminal 17 residues of the FUM1 product for import into mitochondria. The high fumarase activity measured in the postribosomal fraction of C5/ST1 also shows that the deletion at the amino-terminal 17 residues does not compromise the activity of the enzyme. Although the specific activity of fumarase in the postribosomal supernatant was two times higher when the transforming plasmid contained the entire FUM1 gene this could be due to less efficient transcription of the gene from a plasmid lacking the natural promoter. We have not, however, compared the mRNA levels in C5/T2 and C5/ST1. The increase of fumarase activity in the postribosomal fraction of cells transformed with pG5/ST1 also suggests that the enzyme in this fraction is distinct from mitochondrial fumarase.

Northern Analysis and S1 Nuclease Mapping of RNAs Transcribed from the FUM1 Gene—To ascertain whether the intracellular location of fumarase is determined by differential transcription of the gene, the 5' termini of the fumarase transcripts were characterized by S1 nuclease mapping and by Northern hybridization analysis. To map the 5' termini, poly(A)+-enriched RNA from wild type yeast was hybridized to a 5'-end-labeled single-stranded DNA probe spanning the sequence from the XhoII site at nucleotide +193 to the Accl site at -213. This probe was found to protect a major 5'-site near the Accl site. A better resolution of this region was obtained by using a short HindIII fragment extending from -161 to -302. As shown in Fig. 6, the major protected 5'-end centers at -185. Other less prominent protected sites were also observed both 5' and 3' to the -185 site (data not shown). Transcripts starting at these sites should code for a protein with the putative signal sequence for import into mitochondria and would, therefore, correspond to the mRNA(s) for mitochondrial fumarase. By the same reasoning the mRNA for the cytoplasmic fumarase should have a 5' terminus after the first ATG at nucleotide +1 of the sequence shown in Fig. 3. This was tested with two different probes, one starting at the BglII site at nucleotide +211 and the second at the XhoII site at nucleotide +153. Both probes included the sequence up to the AhaIII site at nucleotide -94. These probes identified a cluster of 5' termini spanning the region from +57 to +68 between the first and second ATGs of the gene (Fig. 6). Additional transcripts were detected with 5'-ends between +68 and +98. These transcripts lacking either part or all of the sequence coding for the amino-terminal import sequence should correspond to the mRNAs for the cytoplasmic fumarase.
FIG. 6. S1 nuclease mapping the 5' termini in fumarase transcripts. The single-stranded 5'-end-labeled probes used for protection against S1 nuclease are shown at the bottom of the figure. The location of restriction sites for HindIII (F), AccI (C), Aha111 (A), HindIII (H), and XhoII (O) used for the preparation of the probes is indicated on the map. Only the results obtained with the upstream HindIII and the XhoII-Aha111 fragments are presented. The left panel shows the major 5'-end protected by the upstream HindIII fragment (probe 1). The S1 nuclease digestion was carried out at 37°C for 30 min and the products separated on a 20% polyacrylamide gel. Lane 1, 10 units/ml S1 nuclease; lane 2, 300 units/ml S1 nuclease; lane 3, probe 1 derivatized by the A+G-specific reactions. The right panel shows the most prominent 5'-ends protected by the XhoII-Aha111 fragment (probe 2). The S1 digestions were done at 23°C for 30 min and the products separated on a 7% polyacrylamide gel. Lane 1, DNA probe alone digested with 50 units/ml S1 nuclease; lane 2, DNA probe alone digested with 500 units/ml S1 nuclease; lane 3, hybridization mixture digested with 50 units/ml S1 nuclease; lane 4, hybridization mixture digested with 500 units/ml S1 nuclease; lane 5, probe 2 derivatized by the A+G-specific reaction. The sequence ladders have been numbered according to the convention used in Fig. 3.

The results of the S1 mapping experiments indicate that the major transcript coding for mitochondrial fumarase should exceed the shorter mRNA(s) for cytoplasmic fumarase by at least 200 bp assuming identical 3'-ends. Furthermore, only the mRNA for the mitochondrial enzyme should hybridize to DNA probes from the region upstream of the first ATG. This was supported by the results of Northern hybridization analyses of poly(A)+ RNA from wild type yeast and from a transformant harboring the pG5/T2 plasmid. As shown in Fig. 7 the 2.9-kb HindIII fragment containing most of the FUM1 gene plus substantial 3'-flanking sequences detects three different transcripts in RNA preparations of both wild type and the transformant. Since the longest of the three transcripts does not hybridize to the smaller HindIII-BamHI probe internal to the FUM1 gene, it is probably derived from the downstream gene present in the 2.9-kb HindIII fragment. To ascertain whether the two transcripts detected by the FUM1-specific probe differ in their 5'-flanking sequences, the hybridizations were repeated using the upstream BglII-

FIG. 7. Northern analysis of fumarase transcripts. Total RNA from the wild type strain D273-10B/A1 and from the transformant C5/T2 were enriched for mRNA by passage through poly(U)-Sepharose. The poly(A)+ fraction from each strain (5-4 μg) was separated by electrophoresis on 1% agarose and transferred to diazobenzoyloxyethyl paper. The diazobenzoyloxyethyl blots were hybridized to nick-translated restriction fragments inclusive of the sequence between the two HindIII sites (probe 1), a BglII-PstI fragment from the 5'-flanking region of the gene (probe 2), and a short HindIII-BamHI fragment completely internal to the FUM1 gene (probe 3). Probe 1 encompassing almost the entire FUM1 gene and part of the downstream unidentified reading frame (ORF) hybridized to three different transcripts. Probe 3 containing only a short region of FUM1 hybridized to two of the transcripts of which only the larger was detected by the upstream probe (probe 2). The migration of DNA size standards is marked in the margin. The locations of the HindIII (H), BamHI (B), PstI (P), and BglII (G) sites are indicated.

PstI fragment encompassing the region from approximately nucleotide -820 (BglII) to nucleotide -72 (PstI). As seen in Fig. 7 this probe hybridizes only to the larger and more abundant transcript. This RNA must have a transcriptional start site upstream of the ATC at nucleotide +1 and, therefore, probably codes for the mitochondrial fumarase. Since the smaller transcript does not hybridize to the 5'-flanking probe we presume that it is the mRNA for the cytoplasmic enzyme.

DISCUSSION

Fumarase is an enzyme of the tricarboxylic acid cycle responsible for the reversible conversion of fumarate and malate. Like most tricarboxylic acid cycle enzymes, fumarase is located in the matrix compartment of mitochondria. A number of studies indicate, however, that in mammalian cells there is a second fumarase in the cytoplasm (1-5). The function of the cytoplasmic isoenzyme has not been established. The fumarase mutants of S. cerevisiae reported in the present study suggest the existence of two compartmentally distinct forms of fumarase in this organism as well.
Measurements of fumarase activity in the mitochondrial and postribosomal fractions of wild type yeast indicate that while approximately 30% of total enzyme units are associated with the mitochondrial fraction the remaining 70% are recovered in the postribosomal supernatant. Even though there is more extramitochondrial fumarase, the specific activity is 3-4 times higher in mitochondria. Both activities are absent in respiratory-deficient strains with recessive mutations in a single nuclear gene. This gene, designated FUM1, has been cloned by complementation of a fumarase-deficient mutant with a yeast genomic library. Several lines of evidence confirm that the cloned gene codes for both mitochondrial and cytoplasmic fumarase. 1) The amino acid sequence derived from the nucleotide sequence of FUM1 is homologous to the sequence of bacterial fumarase. 2) The episomal plasmid pG5/T2 containing the entire FUM1 gene restores the respiratory defect of fumarase mutants, and transformants harboring the plasmid exhibit elevated fumarase activity in mitochondria and in the postribosomal fraction. 3) Disruption of the chromosomal copy of FUM1 induces a respiratory defect with a concomitant loss of mitochondrial and cytoplasmic fumarase. Strains carrying the disrupted gene FUM1::LEU2 do not complement the fumarase mutant C5 used to select the gene from the genomic library.

Although the above results suggest a dual localization of fumarase in yeast, they do not exclude the possibility that the activity measured in the postribosomal fraction may be the result of leakage from mitochondria during fractionation of the cells. The latter seems a less tenable explanation based on the results of the distribution of fumarase activity in yeast transformed with two different plasmids, one containing the whole gene and flanking sequences (pG5/T2) and the other lacking the sequence coding for the amino-terminal 17 residues (pG5/ST1). Fumarase mutants transformed with pG5/T2 show a substantial increase of fumarase in the mitochondrial and postribosomal supernatant fractions. Compared to the wild type strain total fumarase units in the transformant were 4-5 times higher in mitochondria and 18 times higher in the postribosomal supernatant fraction. In contrast, transformants harboring pG5/ST1 had less than 20% of the wild type levels of fumarase in mitochondria even though there was a 10-fold elevation of fumarase in the postribosomal fraction.

Additional evidence favoring the notion of a distinct cytoplasmic fumarase in S. cerevisiae was obtained by analyzing the transcripts of the FUM1 gene in wild type and in yeast transformed with pG5/T2. Northern hybridization analysis of poly(A)+ RNA revealed that a probe from the FUM1 coding region hybridizes to two different transcripts. Based on their electrophoretic migration in agarose the two transcripts are estimated to differ in length by several hundred nucleotides. Only the longer and more abundant RNA, however, was detected by a probe from the 5'-flanking region of the gene (-929 to -72). These results suggested that the shorter RNA is transcribed from a more downstream initiation site.

Based on S1 nuclease mapping experiments, the longer transcript(s) has a 5' terminus upstream of the first ATG initiation codon. The major protected end in this region was mapped near nucleotide -185. This and other minor transcripts that include variable regions 5' to the first ATG are the most likely candidates to be mRNAs for the mitochondrial fumarase since their translation products would include the amino-terminal sequence corresponding to the mitochondrial import signal. Although we have no information about the primary sequence of the mature yeast mitochondrial fumarase, a comparison of the proteins encoded by the bacterial and yeast genes indicates that the primary translation product of FUM1 has 25 extra amino acids at the amino terminus. This sequence contains basic (3 arginine, 3 lysine) and hydroxylated (3 serine, 2 threonine) amino acids and lacks acidic residues. Since the signal peptides of proteins targeted for import into mitochondria commonly have an excess of basic and hydroxylated amino acids it is not unreasonable that part of the amino-terminal sequence encoded by FUM1 is proteolytically cleaved during transport to the mitochondrial matrix.

In addition to the termini mapped to the 5'-flanking region, S1-protected sites were also found in the coding sequence downstream of the first ATG. Such sites were observed between the first and second ATGs of the gene and other minor sites beyond the second ATG. These shorter transcripts lack part or all of the mitochondrial import signal and, therefore, are likely to code for the cytoplasmic enzyme. Taken together with the results of the intracellular distribution of fumarase activity in mutants transformed with pG5/T2 and pG5/ST1, these data constitute strong evidence that mitochondrial fumarase is translated from a longer mRNA encompassing the first in-frame ATG of the FUM1 gene and that the cytoplasmic isoenzyme is translated from a shorter transcript(s) lacking either part or all of the amino-terminal sequence needed for transport into mitochondria. Fumarase, therefore, represents one of several recently described mitochondrially and cytoplasmically shared enzymes encoded by the same gene whose targeting to its appropriate compartment is decided at the transcriptional level. Among the presently known examples of such compartmentally shared proteins are histidyl-tRNA synthetase (8), tRNA modification enzymes (9-11), and a-isopropylmalate synthase (12). At present it is not clear why functionally similar enzymes are in some instances encoded by distinct genes and in other instances by one gene. Fumarase catalyzes a nonregulated reaction of the tricarboxylic acid cycle and has no known cofactor requirements. Under these circumstances a single gene coding for identical proteins may be the most efficient means of supplying fumarase with optimal catalytic properties for both subcellular compartments. Other enzymes, however, whose activities are regulated may necessitate different primary sequences and hence separate genes to accommodate for differences in substrate or cofactor concentrations in the extra- and intramitochondrial phases.

Judging by the intensity of the signals seen in the Northern hybridization, the longer transcript for mitochondrial fumarase appears to be 3-4 times more abundant. This is inconsistent with the relative distribution of total fumarase units in mitochondria (30%) and the postribosomal supernatant (70%). This discrepancy could be due to 1) differences in the efficiency of translation or stability of the two classes of mRNAs, 2) a higher turnover number of the cytoplasmic enzyme, or 3) more quantitative recovery of the mRNA for the mitochondrial enzyme in the poly(A)+-enriched fraction.

The fumarase mutants exhibit several interesting phenotypic characteristics. Both C5 and W303VFUM1, the latter with a sizable deletion in the gene, grow slowly on the nonfermentable substrate glycerol in the presence of yeast extract and/or peptone. The mutants also grow at a slow rate when grown on minimal glycerol provided the medium is supplemented with aspartate, asparagine, or serine. None of the other amino acid constituents of proteins can be substituted for the above. The requirement for these particular amino acids suggests that one of the consequences of a block in the conversion of fumarate to malate may be a depletion of oxalacetate, thereby preventing replenishment of the tricarboxylic acid cycle intermediates needed for synthesis of certain amino acids and...
for other biosynthetic pathways. Addition of exogenous aspartic acid or related amino acids that can be converted to oxalacetate could ameliorate the metabolic block by stimulating forward flux of the cycle up to fumarate. This explanation is not totally satisfactory since glycerol can be glycolytically metabolized to pyruvate which in turn can be carboxylated to form oxalacetate. Formation of oxalacetate from glycerol, however, requires a functional respiratory chain to permit reoxidation of the extra NADH. Since fumarase mutants are pleiotropically deficient in respiratory chain components (cytochromes a, a3, b) it is conceivable that conversion of glycerol to oxalacetate is severely limited in the mutants. The deficiency in respiratory chain cytochromes appears to be the consequence of a secondary effect of mutations in fumarase. The reason for the absence of mitochondrial protein synthesis is not totally satisfactory since glycerol can be glycolytically metabolized to pyruvate which in turn can be carboxylated to form oxalacetate. Formation of oxalacetate from glycerol, however, requires a functional respiratory chain to permit reoxidation of the extra NADH. Since fumarase mutants are pleiotropically deficient in respiratory chain components (cytochromes a, a3, b) it is conceivable that conversion of glycerol to oxalacetate is severely limited in the mutants. The deficiency in respiratory chain cytochromes appears to be the consequence of a secondary effect of mutations in fumarase on mitochondrial translation which is required for the synthesis of cytochrome b and subunits of cytochrome oxidase. The reason for the absence of mitochondrial protein synthesis is also not clear at present but could be a reflection of a lower pool of intramitochondrial amino acids such as aspartic and glutamic acid in the mutant.

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REFERENCES
1. Tolley, E., and Craig, I. (1975) Biochem. Genet. 13, 867-883
2. Edwards, Y. H., and Hopkinson, D. A. (1979) Ann. Hum. Genet. 43, 103-198
3. O'Hare, M. C., and Doonan, S. (1985) Biochem. Biophys. Acta 827, 127-134
4. Kobayashi, K., and Tuboi, S. (1983) J. Biochem. (Tokyo) 94, 707-713
5. Zinn, A. B., Kerr, D. S., and Hoppel, C. L. (1986) N. Engl. J. Med. 315, 469-475
6. Perlman, D., Halverson, H. O., and Cannon, L. E. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 781-785
7. Carlson, M., and Botstein, D. (1982) Cell 28, 145-154
8. Natsoulis, G., Hilger, F., and Fink, G. R. (1986) Cell 46, 235-243
9. Ellis, S. R., Moraies, J., Li, J.-M., Hopper, A. K., and Martin, N. C. (1986) J. Biol. Chem. 261, 9703-9709
10. Ellis, S. R., Hopper, A. K., and Martin, N. C. (1987) Proc. Natl. Acad. Sci. U. S. A., in press
11. Dihanich, M. E., Najarian, D., Clark, R., Billman, E. C., Martin, N. C., and Hopper, A. K. (1987) Mol. Cell. Biol. 7, 177-184
12. Buetzer, J. P., Chang, L.-P. L., Hinkkanen, A. E., and Kohkah, G. R. (1986) J. Biol. Chem. 261, 5169-5167
13. Tzagoloff, A., Akai, A., and Fourny, F. (1976) FEBS Lett. 65, 391-396
14. ten Berge, A. M. A., Zoutewelle, G., and Needleman, R. B. (1974) Mol. Gen. Genet. 131, 113-121
15. Tzagoloff, A., Akai, A., and Needleman, R. B. (1975) J. Biol. Chem. 250, 8228-8235
16. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Mazza, E., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 560-564
18. Alwine, J. C., Kemp, D. J., and Stark, G. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5350-5354
19. Berk, A. J., and Sharp, P. A. (1977) Cell 12, 721-732
20. Botstein, D., and Davis, R. W. (1982) in The Molecular Biology of the Yeast Saccharomyces cerevisiae: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W., and Broach, J. R., eds) pp. 607-636, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Dieckmann, C. L., and Tzagoloff, A. (1983) Methods Enzymol. 97, 255-360
22. Faye, G., Kujawa, C., and Fukuhara, H. (1974) J. Mol. Biol. 88, 185-203
23. Faye, G., and Bradshaw, R. A. (1969) Methods Enzymol. 13, 91-99
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
25. Dieckmann, C. L., Bonitz, S. G., Hill, J., Homison, G., McGrow, P., Jape, L., Thalenfeld, B. E., and Tzagoloff, A. (1982) in Mitochondrial Genes (Slonimski, P. P., Bortz, P., and Attardi, G., eds) pp. 213-223, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) Yeast 2, 163-167
27. Miles, J. S., and Guest, J. R. (1985) Nucleic Acids Res. 13, 131-140
28. Woodbury, N. W., and Doolittle, R. F. (1980) J. Mol. Evol. 15, 129-148
29. Claverie, J. M., and Bricault, L. (1986) Proteins 1, 60-65
30. Rothstein, R. J. (1983) Methods Enzymol. 101, 202-211