Isolation and Characterization of the *Saccharomyces cerevisiae* MIS1 Gene Encoding Mitochondrial C1-Tetrahydrofolate Synthase*

(Received for publication, November 16, 1987)

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C1-Tetrahydrofolate synthase is a trinuclear poly-peptide found in eukaryotic organisms that catalyzes 10-formyltetrahydrofolate synthetase (EC 6.3.4.3), 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), and 5,10-methyltetrahydrofolate dehydrogenase (EC 1.5.1.5) activities. In *Saccharomyces cerevisiae*, C1-tetrahydrofolate synthase is found in both the cytoplasm and the mitochondria. The gene encoding yeast mitochondrial C1-tetrahydrofolate synthase was isolated using synthetic oligonucleotide probes based on the amino-terminal sequence of the purified protein. Hybridization analysis shows that the gene (designated *MIS1*) has a single copy in the yeast genome. The predicted amino acid sequence of mitochondrial C1-tetrahydrofolate synthase shares 71% identity with yeast C1-tetrahydrofolate synthase and shares 39% identity with clostridial 10-formyltetrahydrofolate synthetase. Chromosomal deletions of the mitochondrial C1-tetrahydrofolate synthase gene were generated using the cloned *MIS1* gene. Mutant strains which lack a functional *MIS1* gene are viable and can grow in medium containing a nonfermentable carbon source. In fact, deletion of the *MIS1* locus has no detectable effect on cell growth.

The transfer of one-carbon units is essential in several major aspects of cellular metabolism. In many of these processes, one-carbon transfers are mediated by the coenzyme tetrahydrofolate (THF). Derivatives of THF supply the activated one-carbon units required for the biosynthesis of purines, thymidylate, methionine, histidine, pantothenate, and formylmethionyl-tRNA<sub>Met</sub> (Fig. 1) (1–3). Various catabolic reactions generate specific one-carbon derivatives of THF, which are then interconverted between different oxidation states by 10-formyltetrahydrofolate synthetase (EC 6.3.4.3), 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), and 5,10-methyltetrahydrofolate dehydrogenase (EC 1.5.1.5). In eukaryotes, synthetase, cyclohydrolase, and formylase (18, 19). Although the existence of many of these folate-dependent enzymes in the mitochondria is not yet well-documented, this has not discouraged workers from proposing potential functions for these enzymes in mitochondrial metabolism. However, the physiological role of folate-dependent enzymes in the mitochondria has yet to be established.

We have taken advantage of the genetic manipulations possible in the yeast *S. cerevisiae* to investigate the function of mitochondrial C1-THF synthase. We report here the isolation and the disruption of the gene encoding mitochondrial C1-THF synthase, which we have designated *MIS1*. We have found, however, that a functional *MIS1* gene is completely dispensable in yeast.

**EXPERIMENTAL PROCEDURES**

*Materials—*Restriction enzymes, the large fragment of DNA polymerase I (Klenow fragment), and T4 DNA ligase were purchased from Boehringer Mannheim. T4 polynucleotide kinase, DNA polymerase I, and synthetic linkers were obtained from Bethesda Research Laboratories. All enzymes were used as recommended by the supplier. Radiochemicals were purchased from Amersham Corp. Common reagents were commercial products of the highest grade available.

*Yeast Strains, Genetic Techniques, and Cell Growth—*The *S. cerevisiae* strains used in this study are listed in Table 1. The preparation of growth media and the techniques used for diploid construction, sporulation, and tetrad dissection have been described (20). Yeast were grown aerobically at 30 °C. Growth was monitored by measuring turbidity at 600 nm with a Zeiss PMQ II spectrophotometer. Unless otherwise indicated, cells were grown in YPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose) or, to maintain selective pressure for plasmids, in minimal medium (0.67% yeast nitrogen base, 2% glucose, plus appropriate auxotrophic supplements). Cells were harvested in late log phase (OD<sub>580</sub> = 10). Extracts were prepared by disrupting washed cells with glass beads (0.45-mm diameter) in buffer containing 50 mM Tris-Cl, pH 7.5, 10 mM KCl, 10 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonfyl fluoride. Homogenates were centrifuged 30 min at 16,000 × g. The supernatant fractions were used for enzyme and protein assays.

*Recombinant DNA Techniques—*The techniques used for isolation of plasmid DNA, attachment of synthetic linkers, DNA blot analysis, nick translation, and transformation of *Escherichia coli* were as described by Maniatis et al. (21). Published procedures were used for the isolation of yeast genomic DNA (20) and for the transformation of yeast spheroplasts (22).

*Cloning of the MIS1 Gene—*Oligonucleotides 1 (5'-AAYGAYGARRCC-3') and 2 (5'-AARCAYCCNAAYTTYAARCC-3'), which correspond to the amino-terminal sequence of mitochondrial C1-THF synthase (10), were prepared with an Applied Biosystems Model 280A DNA synthesizer and purified by silica gel thin-layer chromatography (23). The oligonucleotides were end-labeled with γ-
The yeast genomic library were plated and transferred onto nitrocellulose (24), and the filters were probed with the oligonucleotides and enzymes shown are: 1, 10-formyl-THF synthetase; 2, 5,10-methylene-THF cytochrome; 3, 5,10-methylene-THF dehydrogenase; 4, serine hydroxymethylase (EC 2.1.2.1); 5, glycine cleavage system (EC 2.1.2.10); and 6, thymidylate synthase (EC 2.1.1.45). DHP, dihydrofolate.

Table I

S. cerevisiae strains

| Strain                | Genotype                  | Source                      |
|-----------------------|---------------------------|-----------------------------|
| 3-5281                | MATa ade3-130 ura1 ser1   | E. W. Jones (Carnegie-Mellon University) |
| YNN281                | MATa trpl his3 ura3-52 his2 ade2 | YGSC^* |
| KSY1                  | MATa ade3-130 ura3-52 ser1 | Spore from cross of 3-5281 × YNN281 |
| KSY2                  | MATa ade3-130 ura3-52 ser1 | Spore from cross of 3-5281 × YNN281 |
| KSY3                  | MATa trpl ura5-52 his2-801 | Spore from cross of 3-5281 × YNN281 |
| KSY4                  | MATa ade3-130 ura3-52 ser1 | Diploid of KSY1 × KSY2 |
| KSY5                  | MATa ade3-130 ura3-52 ser1 mis1::URA3 | See "Experimental Procedures" |
| KSY6                  | MATa ade3-130 ura3-52 ser1 mis1::URA3 | See "Experimental Procedures" |
| KSY7                  | MATa ade3-130 ura3-52 ser1 mis1::URA3 | See "Experimental Procedures" |
| KSY8                  | MATa ade3-130 ura3-52 ser1 mis1::URA3 | See "Experimental Procedures" |
| KSY9                  | MATa ura5-52 mis1::URA3 | From cross of KSY3 × KSY6 |
| KSY10                 | MATa ura5-52 mis1::URA3 | From cross of KSY3 × KSY6 |

^*YGSC, Yeast Genetic Stock Center, Department of Biophysics and Medical Physics, University of California, Berkeley, CA.

**Results**

Isolation of the Gene Encoding Mitochondrial C1-THF Synthase—We designed two degenerate oligonucleotide probes based on the amino-terminal sequence of mitochondrial C1-THF synthase (10). The probes were used to screen a yeast genomic library in an episomal vector (YPEp24) by the colony hybridization technique (24). A single plasmid (YPEpKS6) carrying a 10.1-kb genomic insert was isolated which hybridized to both oligonucleotides (Fig. 2). An ade3 deletion strain which lacks cytoplasmic C1-THF synthase (36) was transformed with YEpKS6 DNA. The YEpKS6-transformed strain had levels of synthetase and dehydrogenase activities approximately 10-fold greater than the same strain transformed with vector DNA alone (Table II). These levels of synthetase and dehydrogenase activities are comparable to those found in ADE3 wild-type strains; however, transformation with YEpKS6 DNA did not alleviate the adenine and histidine requirement of ade3 parent strain. We purified synthetase.
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FIG. 2. Restriction map of the yeast MIS1 gene. The positions of restriction sites within the inserts of plasmids YEpKS6, YEpKS7, YEpKS14, and YEpKS17 are indicated. The boxed region represents the MIS1 protein coding sequence. ORF, open reading frame.

TABLE II
Comparison of mitochondrial C_{1}-THF synthase activities in an ade3 deletion strain (KSY1) transformed with plasmid YEp24 and plasmid YEpKS6

| Plasmid   | Synthetase (mIU/mg) | Dehydrogenase (mIU/mg) | Overexpression SYN* | DH |
|-----------|---------------------|------------------------|---------------------|----|
| YEp24     | 13.8                | 4.07                   | 1.0                 | 1.0|
| YEpKS6    | 140                 | 46.3                   | 10.1                | 11.4|

* SYN, synthetase; DH, dehydrogenase.

activity from the YEpKS6-transformed strain and found that the purified protein is indistinguishable from mitochondrial C_{1}-THF synthase with respect to its purification properties and its subunit molecular weight (data not shown).

We performed deletion analysis to localize the boundaries of the mitochondrial C_{1}-THF synthase gene on YEpKS6. Various fragments generated by partial Sau3A digestion of YEpKS6 were cloned into YEp24; and the resulting plasmids, YEpKS7, YEpKS14, and YEpKS17 (Fig. 2), were used to transform an ade3 deletion strain. Only the strain containing plasmid YEpKS17 had levels of synthetase and dehydrogenase activities comparable to those found in the YEpKS6-transformed strain (Fig. 3). These data suggest that sufficient information to overexpress mitochondrial C_{1}-THF synthase is carried on the PvuII fragment of YEpKS6. DNA blots probed with a plasmid carrying this PvuII fragment confirmed the presence of the cloned insert in yeast genomic DNA. The fragments observed in these blots were identical to those predicted from the restriction map of the cloned insert (data not shown). These data indicate that mitochondrial C_{1}-THF synthase is encoded by a single copy gene which we have designated MIS1 for mitochondrial C_{1}-THF synthase.

Nucleotide Sequence of the MIS1 Gene—The nucleotide sequence of the MIS1 gene is shown in Fig. 4. Analysis of the DNA sequence revealed one open reading frame which encodes a protein of 975 amino acids with a calculated molecular weight of 106,235. The predicted amino acid sequence of residues 35-74 is identical to the amino-terminal sequence of mitochondrial C_{1}-THF synthase (10). The sequence predicted for residues 1-34 is highly enriched for arginine, leucine, and serine, which is typical of mitochondrial targeting sequences (37). The removal of these initial 34 residues yields a "mature" protein with a calculated molecular weight of 102,251, which
Isolation of the Gene Encoding Mitochondrial C_{1}-THF Synthase

AAAGTAATCCTGAGTTCAGTTCCAATTCCAATTCCGGTTCAGAGTACGAATCGGAGGAAGAAGTAGTCCCAAGATCAGCCACAGTCACACA
ACTCCAAAGCAGACCAGAGCCATACTACAAGAATAATGGAATGCCCTACTCACTCTCCAAAGTACGAGGAAGGCCCATGTATCCAAGACCT
GCTGAAGATGCTTACAATGCCAATTATATATTCAAGGTCTGCCCCAGTACCAAACATCTTATTTTTCGCAGCTGTTATTATCATCACCCCAGC
ATTACGAACATTCTCCACATCAAAGGAACTTTACGCCATCCAACCAATCGCATGGGAACTTTTATTAAATGTCTACATACATACATACATC
TCGTACATAAATACGCATACGTATCTTCG~AGTAAGAACCGTCACAGATATGATTGAGCACGGTACAATTATGTATTAGTCAAACATTAC
CAGTTCTCGAACAAAACCAAAGCTACTCCTGCAACAACACTCTTCTATCGCACATGTATGGTTCTTATTGTTTCCCGAGTTCTTTTTTACTGACG
CGCCAGAACGAGTAAGAAAGTTCTCTaAAAATA

FIG. 4. Nucleotide sequence of the MISI gene. The DNA sequence of the MISI gene and the predicted amino acid sequence of mitochondrial C_{1}-THF synthase are shown. The amino acid residues which were directly sequenced from the purified protein are indicated in bold-face type (residues 35–74).
closely matches the subunit molecular weight of mitochondrial C_{1}-THF synthase estimated by sodium dodecyl sulfate-polycrylamide gel electrophoresis ($M_r = 100,000$) (10). These results indicate that the cloned DNA is the structural gene for yeast mitochondrial C_{1}-THF synthase.

**Homology of the Protein Encoded by MIS1 to C_{1}-THF Synthase and 10-Formyl-THF Synthetase**—We compared the predicted amino acid sequence of mitochondrial C_{1}-THF synthase to that of C_{1}-THF synthase from yeast (38) and of 10-formyl-THF synthetase from *Clostridium acidurici* (2). The mitochondrial and cytoplasmic C_{1}-THF synthases are 71% identical to each other.
Disruption of the MIs1 Gene—Mitochondrial C1-THF Synthase is present in ade3 mutants that lack C1-THF synthase, yet the mitochondrial isoenzyme cannot satisfy the requirements for adenine and histidine that is characteristic of most ade3 mutant strains. This observation suggests that C1-THF synthase and mitochondrial C1-THF synthase have different metabolic roles. To determine how the lack of mitochondrial C1-THF synthase affects cell growth, we disrupted the MIs1 gene in vitro and introduced the nonfunctional gene into the yeast chromosome. A 1.7-kb fragment of the MIs1 coding sequence was replaced with a DNA fragment carrying the URA3 gene (Fig. 6A). The disrupted gene was excised from the plasmid and was used to transform a diploid ura5-32/ura5-32 ade3-130/ade3-130 ser1/ser1 strain (KSY4) to uracil prototrophy. Hybridization analysis of genomic DNA isolated from a Ura+ transformant confirmed that the disrupted gene had integrated into the MIs1 locus (Fig. 6B). The pattern of fragments seen in these blots suggests that the disrupted gene represents a wild-type copy of the MIs1 gene and one disrupted copy.

To determine whether a functional MIs1 gene is required for cell growth, we sporulated a URA5/mis1::URA3 diploid (KSY5) to generate haploid segregants. Four spores in each tetrad were Ura+, and two were Ura-. To test whether or not mitochondrial C1-THF synthase is produced from the disrupted MIs1 locus, we measured synthetase and dehydrogenase activities found in single tetrad. Extracts prepared from the Ura- spores had levels of synthetase and dehydrogenase activities found in a ura5-32/ura5-32 ade3-130/ade3-130 ser1/ser1 strain (KSY4) to uracil prototrophy. Hybridization analysis of genomic DNA isolated from a Ura+ transformant confirmed that the disrupted gene had integrated into the MIs1 locus (Fig. 6B). The pattern of fragments seen in these blots suggests that the disrupted gene represents a wild-type copy of the MIs1 gene and one disrupted copy.
Fig. 6. Analysis of MIS1 gene disruption. A, physical map of the MIS1 gene disruption. The construction of the mis1::URA3 allele is described under “Experimental Procedures.” The MIS1 coding sequence and the fragment containing the URA3 gene are represented by boxes. The arrow indicates the direction of transcription of URA3. B, hybridization analysis of the MIS1 gene disruption. DNA (20 μg/lane) isolated from the parent diploid strain, KSY4 (lanes 1 and 3), and a Ura" transformant, KSY5 (lanes 2 and 4), were digested with PvuII (lanes 1 and 2) or PstI (lanes 3 and 4), fractionated by electrophoresis on a 0.8% agarose gel, and transferred onto nitrocellulose. The filter was probed with plasmid pKS31 radiolabeled by nick translation. The molecular weight standards used were λ DNA/HindIII fragments and φX174 replicative form DNA/HaeIII fragments.

spores had no detectable activities (Table III). These results indicate that mis1 mutant strains are viable and that mitochondrial C1-THF synthase is dispensable in yeast.

Initiation of protein synthesis in mitochondria occurs via a unique tRNA species, tRNAN"(18), which functions as a formylated methionylated derivative. The formyl group is transferred to methionyl-tRNAN" from 10-formyl-THF (19), a product of mitochondrial C1-THF synthase. Thus, a plausible function for this enzyme may be to supply 10-formyl-THF for the synthesis of formylmethionyl-tRNA^Met. Reduced folates are required for mitochondrial function in yeast (39), possibly because mitochondrial protein synthesis is dependent on the formylation of methionyl-tRNA^Met (40). However, we found that strains containing the disrupted MIS1 gene which completely lack mitochondrial C1-THF synthase grew on medium containing a nonfermentable carbon source (YPG me-
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**Table III**

| Strain     | Spore Phenotype | Genotype   | Synthetase (mU/mg) | Dehydrogenase (mU/mg) |
|------------|-----------------|------------|--------------------|------------------------|
| KSY4       | 1 | Ura          | a MIS1 ura3       | 24.9                  | 6.26                   |
|            | 2 | Ura          | a MIS1 ura3       | 24.1                  | 6.67                   |
|            | 3 | Ura          | a MIS1 ura3       | 23.6                  | 6.94                   |
|            | 4 | Ura          | a MIS1 ura3       | 24.1                  | 6.90                   |
| KSY5       | 1 | Ura          | a mis1:Ura3 ura3  | 0.00                  | 0.08                   |
|            | 2 | Ura          | a mis1:Ura3 ura3  | 19.1                  | 5.39                   |
|            | 3 | Ura          | a mis1:Ura3 ura3  | 0.00                  | 0.02                   |
|            | 4 | Ura          | a mis1:Ura3 ura3  | 26.7                  | 7.27                   |

**DISCUSSION**

We have isolated the gene encoding mitochondrial C₅-THF synthase from *S. cerevisiae*. In yeast, mitochondrial C₅-THF synthase is encoded by a single gene which we have designated *MIS1*. The protein encoded by *MIS1* shows extensive homology with yeast cytoplasmic C₅-THF synthase and clostridial 10-formyl-THF synthetase. Genetic and physical evidence indicates that C₅-THF synthase has two functionally independent domains (4, 36, 43). Synthetase activity is catalyzed on a 70-kDa carboxyl-terminal domain, and dehydrogenase and cyclohydrolase activities are catalyzed on a 30-kDa amino-terminal domain. The alignment of the amino acid sequence predicted for monofunctional 10-formyl-THF synthetase with mitochondrial and cytoplasmic C₅-THF synthetases may define the synthetase domains of the trifunctional proteins. In both mitochondrial and cytoplasmic THF synthase, the putative synthetase domain is immediately preceded by a proline-rich region. These proline-rich regions probably have disordered and extended conformations that could act to separate the two domains. Proteolysis studies in our laboratory show that yeast C₅-THF synthase can be cleaved with chymotrypsin to generate 70- and 30-kDa peptides. The combined proteolytic fragments have each of the activities of the intact enzyme. However, we cannot isolate an active dehydrogenase and cyclohydrolase fragment. Here we show that a fragment of the *MIS1* gene which carries sufficient information to encode the dehydrogenase and cyclohydrolase domain (YeEpKSI14) does not express a protein with dehydrogenase activity. These data may suggest that an intact synthetase domain is necessary to stabilize the dehydrogenase and cyclohydrolase domain in yeast C₅-THF synthases.

Mitochondria use active one-carbon units for the formulation of the initiator tRNA, methionyl-tRNA⁹₅₅₅. The formyl donor in this reaction is 10-formyl-THF, a product of C₅-THF synthase and mitochondrial C₅-THF synthase. We found that mutant strains that lack both C₅-THF and mitochondrial C₅-THF synthase can grow on nonfermentable carbon sources, showing that these enzymes and their products are not required for mitochondrial function. These results can best be explained if mitochondrial protein synthesis can be initiated with formylated methionyl-tRNA⁹₅₅₅.

Although it is generally believed that the formulation of the initiator tRNA is required for protein synthesis in bacteria (44) and in eukaryotic organelles (40, 45), it has been established that this requirement is not absolute. *Streptococcus faecalis* can initiate protein synthesis in the absence of formylmethionyl-tRNA⁹₅₅₅. This organism cannot synthesize folate and normally requires this vitamin for growth. However, the requirement for folate can be entirely replaced by the addition of serine, methionine, thymine, a purine base, and pantothenate to the growth medium (46). Under these folate-deficient conditions, formylmethionyl-tRNA⁹₅₅₅ is not synthesized, and initiation of protein synthesis proceeds with non-formylated methionyl-tRNA⁹₅₅₅ (47-49). The ability of this bacteria to initiate protein synthesis without formylated methionyl-tRNA⁹₅₅₅ is due to a single modification in the initiator tRNA. In folate-sufficient cells, the uracil residue in loop IV is methylated to ribothymine; whereas in folate-deficient cells, this methylation does not occur (50). It was later shown that in *S. faecalis* a folate derivative, 5,10-methylene-THF, serves as the methyl donor in this methylation (61).

Similarly, certain *E. coli* mutants can initiate protein synthesis with unf ormylated methionyl-tRNA⁹₅₅₅. Baumstark et al. (52) isolated mutants that grew in the absence of p-aminobenzoate from a strain that required p-aminobenzoate for growth. These mutants cannot synthesize folate, and the cells contain no 10-formyl-THF. Extracts of the mutant strain support protein synthesis from exogenous mRNA; however, the proteins synthesized are initiated with methionine rather than with formylmethionine. The mutant strain is deficient in tRNA methyltransferase; and as a result, tRNA, from these cells have reduced levels of ribothymidine. Thus, in both folate-deficient *S. faecalis* and the mutant *E. coli* strain, the lack of ribothymidine in the tRNA allows initiation of protein synthesis with unf ormylated methionyl-tRNA⁹₅₅₅. We are now directing our efforts toward determining whether the initiator tRNA⁹₅₅₅ from our *ade3 mis1* mutant strain is formylated *in vivo* to investigate whether a similar mechanism can occur in yeast mitochondria.

Because reduced folates and their derivatives are not transported across the inner mitochondrial membrane (53), it is generally believed that active one-carbon units are independently generated in the mitochondria and the cytoplasm for use in each cellular compartment. However, there is evidence to suggest that one-carbon units generated in the mitochondria are used in the cytoplasm. Yeast *tmp3* mutants, which lack the mitochondrial form of serine hydroxymethyltransferase, require dTMP, methionine, histidine, and adenine (13, 54). Also, a glycine-requiring Chinese hamster cell line was found to be deficient in the mitochondrial form of serine.
hydroxymethyltransferase (42). More recently, Barlowe and Appling found that isolated rat liver mitochondria can utilize serine or sarcosine to generate one-carbon units for purine synthesis in the cytoplasm. They propose that a significant fraction of the cell’s one-carbon units are generated from serine in the mitochondria via mitochondrial serine hydroxymethyltransferase. The 5,10-methylene-THF generated in this reaction is converted to formate via mitochondrial C1-THF synthase, which can exit the mitochondria to be activated via cytoplasmic C1-THF synthase for use in biosynthetic reactions in the cytoplasm. This pathway for generating cytoplasmic one-carbon units depends on the integrity of mitochondrial C1-THF synthase; however, we found that the presence of mitochondrial C1-THF synthase offers no advantage to cells growing on minimal medium. Our results indicate that the mitochondrial folate pathway is not essential in yeast, although they do not rule out the possibility that this pathway can act as an alternate route for the synthesis of activated one-carbon units.

Acknowledgments—We thank Jasper Rine and Jeremy Thorner for their helpful suggestions and their critical reading of the manuscript, Morgan Park for his assistance in generating some of the sequencing templates, and Charles Barlowe and Dean Appling for sharing a preprint of their manuscript prior to publication.

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