Cloning and Molecular Characterization of Three Genes, Including Two Genes Encoding Serine Hydroxymethyltransferases, Whose Inactivation Is Required to Render Yeast Auxotrophic for Glycine*

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The genes encoding both the cytosolic and mitochondrial serine hydroxymethyltransferases (SHM2 and SHM1, respectively) and a third unidentified gene of the yeast Saccharomyces cerevisiae have been isolated and their nucleotide sequences determined. Analysis of the predicted amino acid sequence of the amino-terminal regions, sequence comparison with other genes encoding SHMT enzymes, and subcellular fractionation studies all suggested that the SHM1 gene encodes the mitochondrial SHMT, while the SHM2 gene encodes the cytosolic enzyme. The SHM2 gene but not the SHM1 gene has putative GCN4 sites upstream of the putative TATA box, suggesting regulation of its transcription by the general amino acid control system. Yeast mutants with disruptions at each SHM gene and in both genes were constructed and all mutants had the same growth requirements as the parental strains. Mutagenesis of the double-disrupted, shm1 shm2 yeast yielded strains of a single complementation group that are auxotrophic for glycine. Complementation of the glycine auxotrophy using a yeast genomic library retrieved the SHM1 and SHM2 genes and a third gene designated GLY1. Gene disruption studies demonstrated that inactivation of SHM1, SHM2, and GLY1 is required to yield yeast that are completely auxotrophic for glycine.

The primary source of C1 units for tetrahydrofolate-dependent cellular biosynthetic reactions is from the β-carbon of serine. These C1 units enter the tetrahydrofolate (H4PteGlu₄) pool as 5,10-CH₂-H₄PteGlu, in a reaction catalyzed by the enzyme serine hydroxymethyltransferase (SHMT; EC 2.1.2.1). This reaction is also the principal source of cellular glycine (reviewed in Ref. 1). The 5,10-CH₂-H₄PteGlu₄ is utilized for thymidylate biosynthesis and can be reduced to 5-CH₃-H₄PteGlu₄, the form used for methionine biosynthesis or oxidized to 10-CHO-H₄PteGlu₄, the form used for purine biosynthesis.

Genes encoding SHMT (glyA) have been isolated and sequenced from Escherichia coli (2, 3) and several other bacteria (4–6). Eukaryotes have both mitochondrial and cytosolic SHMT isoforms, encoded by separate nuclear genes. The cytosolic gene from Neurospora crassa has been cloned and sequenced (7) and both isoforms from rabbit liver have been purified and their amino acid sequences determined (8, 9). The rabbit cytosolic gene has been cloned and sequenced (10) and a plant mitochondrial gene has been cloned from pea (11). Recently, the cDNA sequences of the human cytosolic and mitochondrial genes have been published (12). There is a high degree of homology among all these proteins, particularly in the vicinity of residues known to be important for catalysis, such as the lysine residue which binds the pyridoxal phosphate cofactor.

Mutants of the E. coli glyA gene are auxotrophic for glycine (2). In Chinese hamster ovary cells, loss of SHMT activity in the mitochondria has been correlated with glycine auxotrophy (13, 14). CHOAuxB1 mutants lacking folate pools as a result of a mutation in folylpolyglutamate synthetase have a requirement for glycine, adenine, and thymidine (14–16). Complementation of the mutant with a bacterial folylpolyglutamate synthetase gene or a human cDNA lacking a mitochondrial leader sequence restores the adenine and thymidine deficiency but not the glycine deficiency (17), suggesting that mitochondrial folate metabolism is required for glycine synthesis in mammalian cells. In N. crassa, the for gene encoding the cytoplasmic SHMT complements a mutant which requires formate for growth (7). This finding suggests that in the absence of SHMT activity, C1 units enter the cytoplasmic folate pool as 10-CHO-H₄PteGlu₄, in a reaction catalyzed by formyltetrahydrofolate synthetase, as had been proposed by Barlowe and Appling (18). The Neurospora for gene is under the control of the amino acid cross-pathways control system, regulated by the transcription factor cpc-1. The analogous yeast regulatory system is the general amino acid control system regulated by GCN4.

In this report, we describe the cloning, DNA sequence determination, and chromosomal disruption of the genes encoding both the mitochondrial and cytoplasmic SHMT isoforms, SHM1 and SHM2 and of a gene, whose disruption is required in a shm1 shm2 strain to yield a glycine auxotroph of the yeast Saccharomyces cerevisiae.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]dATP (7000 Ci/mmol), [α-32P]dCTP (7000 Ci/mmol), and [α-32P]dGTP (1000 Ci/mmol) were obtained from Amerham Corp. [G-3H]Serine (23 Ci/mmol) and Tran32P-label were from ICN. Tetrahydrofolate was from Sigma or Schircks Laboratories, Switzerland. Modified T7 DNA polymerase and all deoxy- and dideoxynucleotides were purchased as a Sequenase kit from U. S. Biochemical Corp.
Restriction endonucleases, ribonucleases, and DNA modifying enzymes were purchased from Boehringer Mannheim, Pharmacia, or Life Technologies Inc. Oligonucleotide primers were synthesized and purified by Dalton Chemical Inc. or the HSC/Pharmacia Biotechnology Service Centre, University of Toronto.

Strains, Plasmids, and Media—E. coli strain DH5α (F- φ80 lacZ  AM15 [lacZYA-argF] U169 recA1 endA1 hsdR17[r- m+]) supE44 thi1 gyrA relA; Life Technologies Inc.) was the recipient for the plasmids used in this study. Strain GS245 (pHEA905 ara1D1989uc167 glyA::mu strA thy11) (2) was the recipient strain for bacterial expression of the yeast genes. DR1984 (recA uraB) (19) was used for maxicell experiments. S. cerevisiae strains used are listed in Table 1.

The yeast SHM and GLY1 genes were isolated from genomic libraries constructed in the shuttle vector Yep13 (20) and Yep24 (21), respectively. The PCR-amplified fragments were cloned into pEMBL10” (22). The genes were subcloned into pUC18 and pUC19 (23) for DNA sequencing and bacterial expression studies. E. coli strains containing plasmids were grown in 2YT (24) medium, supplemented with 50 μg/ml ampicillin. Strain GS245 was grown on M9 minimal medium supplemented with 50 μg/ml phenylalanine and 10 μg/ml vitamin B, to select for functional SHM gene products or supplemented with 50 μg/ml glycine. Yeast strains were grown routinely on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) or on SD medium (0.67% Bacto-yeast nitrogen base, 2% dextrose, pH 6.5, with 20 μg/ml each uracil, adenine, histidine, and tryptophan, 30 μg/ml leucine, 10 μm glycine, and formate or without selected supplements, depending on the selection required).

PCR Amplification of SHM Gene-Specific DNA Fragments—Degrade oligonucleotides were used during PCR amplification of SHM gene-specific probes. Oligonucleotides GLY1 (5'-CCGGTGAAATTGCAAAATATCCGCCGAGG-3') and GLY3 (5'-CCGGTTGAAATTGCAAAATATCCGCATG-3') were synthesized based upon conserved regions of SHM that were identified after sequence analysis of SHMT enzyme isolated from various eukaryotes (7). Degenerate positions are indicated by “Y” for pyrimidines, “R” for purines, and “N” for all bases.

Polymerase chain reaction amplification was performed using Taq polymerase (Boehringer Mannheim) as described (25) with the following modifications. To a microcentrifuge tube 0.1 μg of plasmid DNA from the pool of a yeast genomic library (20) was added. Samples were incubated in a DNA thermal cycler at 94°C for 2 min before commencement of 30 cycles of amplification. Each cycle included 1 min denaturation (94°C), 1.5 min annealing (65°C), and 1.5 min of chain elongation (72°C). After completion of the last cycle, the PCR products were purified by polyacrylamide gel electrophoresis, digested with EcoRI, and cloned into the phagemid vector pEMBL10”.

Plasmid Isolation, DNA Modification, Southern Analysis, and Infectious Transformation—Plasmid preparations were made by the alkaline lysis procedure as described in Sambrook et al. (25). Restriction endonuclease digestions, nuclease digestions, and ligations were carried out according to the manufacturer’s instructions. Colony blots were made as described (25). DNA was transferred to nitrocellulose or nylon membranes and hybridized by the Southern method as described in Ref. 25 with the appropriate probes at 65°C. DNA restriction fragments used as probes were isolated from low melting temperature agarose gels and labeled with [α-32P]dCTP or [α-32P]dATP by the random primer procedure of Feinberg and Vogelstein (26) using Klenow fragment of DNA polymerase 1. Transformation was by the method of Hanahan as described in Ref. 25.

RNA Isolation and Northern Analysis—Total yeast RNA was isolated as described (27). Oligonucleotides SHM1 (5'-GAAATTGGCGTTCCTTACCGGTCTTAGGG-3') and SHM2 (5'-CTTCCCTACCGGTCTTAGGG-3') are specific for SHM1 and SHM2 mRNAs, respectively, and were used as probes. Northern analysis was performed as described (28) using yeast cells synchronized by the α-factor arrest procedure (29).

Gene Disruption—The PCR-generated fragments were used to construct null mutants at the SHM1 and SHM2 loci using gene replacement (28). The 700-bp DNA fragments generated by PCR that were digested from the SHM1 and SHM2 genes were inserted into the EcoRI site of pEMBL10” to form pCRSHM1 and pCRSHM2, respectively. The yeast HIS3 gene was inserted into pCRSHM1 at the XhoI site within the SHM1 gene fragment and the LEU2 gene was inserted into pCRSHM2 at the BglII site within the SHM2 gene fragment (see Fig. 3). The resulting plasmids, pCRSHM1::HIS3 and pCRSHM2::LEU2, were digested with EcoRI and HindIII, respectively, to release the HIS3 and LEU2 genes flanked by SHM1 and SHM2, respectively. The linear DNA was used to transform recipient yeast W3031A to histidine and W3031B to leucine prototrophy (30). Gene disruption at the GLY1 locus was achieved through replacement of the 0.8-kb EcoRI fragment located within the cloned 3.0-kb SalI-XhoI yeast genomic fragment that includes GLY1 (see Fig. 8). The resultant plasmid, pGly1::URA3, was digested with SalI and BamHI and used to transform yeast to uracil prototrophy. Construction of the various null mutant strains was confirmed by Southern analysis. The probe used for Southern analysis of GLY1 disrupted strains was the 1.3-kb segment of DNA consisting of two HindIII fragments, as indicated in Fig. 8.

DNA Sequencing—Plasmid DNA was isolated by the boiling method (25) and prepared for double-stranded sequencing by the method of Hsiao (32). The dideoxynucleotide termination method of Sanger et al. (33) was used with [α-35S]dATP and T7 DNA polymerase.

Chemical Mutagenesis—The SHM double-disrupted yeast strain YM09 was treated with the mutagen ethyl methanesulfonate as described (34). Survivors were plated on SD medium supplemented with uracil, adenine, tryptophan, glycine (10 μm), and formate (10 μm). Colonies were replica-plated onto SD medium supplemented with uracil, adenine, and tryptophan. Those yeast strains which appeared to be auxotrophic were replated to confirm their requirement for glycine and/or formate.

Cell Extraction—Crude extracts of E. coli cells were prepared by sonication. For whole cell enzyme assays, yeast cells were either disrupted using glass beads or converted to spheroplasts using gluosulase (Du Pont). The pelleted spheroplasts were resuspended in 25 mM Tris-HCl, pH 7.6, 2 mM EDTA, 10 μM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μM benzanilide followed by homogenization with a Teflon homogenizer. Extracts were centrifuged at 1,900 × g to pellet whole cells and debris. The supernatants were centrifuged at 13,000 × g to pellet mitochondria and this supernatant was taken as the suitable extract. Mitochondrial pellets were resuspended in extraction buffer and sonicated for use in enzyme assays.

Enzyme Assay—SHMT activity was measured by a modification of the method of Geller and Kotb (35). [35S]Cysteine as described by Sancar et al. (37) was added to the assay mixture containing 5 mM D-[14C]serine, 1 mM H₃PO₄, and 2.5 mM pyridoxal phosphate. The labeled 5,10-CH₂-H₄PteGlu was separated from unreacted serine using minicolumns of DRAE-cellulose (DE52, Whatman) according to the procedure of McGuire (36).

Analysis of Plasmid-encoded Gene Products in Maxicells—E. coli strain DR1984 was transformed with various plasmids, UV irradiated, and plasmid-encoded proteins were labeled with [35S]methionine and disrupted with 1% Triton X-100. 5% of the extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography.
Cloning of Genes Inactivated in a Yeast Glycine Auxotroph

RESULTS

Isolation and Cloning of the S. cerevisiae SHM Genes—DNA fragments of 700 bp were amplified by PCR using primers GLY1 and GLY3 (DNA sequences are described under "Experimental Procedures"). GLY1 has a degenerate sequence coding for the amino acids NKVSEG, residues 63–68 in the Neurospora cytosolic SHMT (7), while GLY3 codes for PEFKKEY, residues 315–320 of the Neurospora enzyme. The residues are shown in Fig. 6. The amplified fragments were digested with EcoRI and ligated into EcoRI-digested pEMBL10* Plasmids containing inserts were identified and the insert DNA was sequenced from both ends to verify homology between the predicted amino acid sequence encoded by the insert DNA and the SHMT enzyme characterized from other sources. Two distinct sequences were found, each containing an open reading frame with a predicted amino acid sequence homologous to SHMT enzymes and the corresponding genes were designated SHM1 and SHM2. Probes were prepared from these amplified fragments and used to screen a yeast genomic library in the plasmid Yep13. One clone, pSH36 was found containing the intact SHM1 gene on a 5.3-kbp insert (see Fig. 1). Two clones were found, pSH2 and pSH3, containing the SHM2 gene (Fig. 1). Clone pSH2 contained a 6.3-kbp insert of yeast genomic DNA. Subcloning and DNA sequence analysis revealed that pSH2 contained an incomplete SHM gene, lacking the initiator methionine. Rescreening of the yeast genomic library using a 0.5-kbp DNA fragment from the 5' end of the pSH2 insert led to the isolation of pSH3, which contains the intact SHM2 gene within a 7.5-kbp insert.

Subcloning and Expression Studies—The pSH3 insert was subcloned into pUC18 as a 9-kbp PstI-BamHI fragment to form p3BP. Although the gene was in the correct orientation for transcription from the lac promoter, this plasmid failed to complement the GS245 glyA strain. A deletion of 3.2 kbp from the 5' end of the gene was done by digestion with Spel and religation. The resultant plasmid, termed p3BPS, complemented the glycine auxotrophy of the E. coli GS245 strain and the insertion of the HIS3 gene into the XhoI site of pSH3 and the insertion of LEU2 into the BglII site of SHM2 are shown. Restriction endonuclease abbreviations are as described in the legend to Fig. 1 with the following additions: N, NcoI; P, PstI; S, SacI.

Cloning into pUC18 and 19. The HindIII site was derived from the pBR322 portion of the Yep13 vector, while the BamHI site appears to be at the insert-vector boundary. The pSH36 subclones in pUC (p36BH and p36BH3) did not complement the GS245 strain of E. coli in either orientation nor did a derivative of the pUC19 subclone (p36BH3BglI) deletion removing 600 bp of upstream noncoding region and bring-
Cloning of Genes Inactivated in a Yeast Glycine Auxotroph

in yeast (39). The ATG codon at +34 does not have these features, suggesting that it is not the in vivo start codon. There is a sequence of 5'-TATAA-3' located at position -154 to -149 relative to the initiation codon, which conforms to a consensus TATA box sequence.

Fig. 5 shows the nucleotide sequence of the SHM2 gene, 327 nucleotides of 5'-noncoding sequence and 186 nucleotides of 3'-noncoding sequence. All sequences reported have been determined on both strands. The SHM2 gene has an open reading frame of 1407 bp encoding a predicted protein of 469 amino acids and a 55,000 but if so, it is inactive.

DNA Sequence Determination of the SHM Genes—The sequencing strategies for the SHM1 and SHM2 genes are shown in Fig. 3. Subclones were obtained for sequencing by restriction enzyme digestion and, in some cases, exonuclease III/alkaline phosphatase digestions, as indicated in the figure legend. The nucleotide sequence of the SHM1 gene was determined on both strands, except the first 250 bp. The SHM1 gene includes an open reading frame of 1407 bp encoding a predicted protein of 490 amino acids with a M, of 52,260. As is the case with the SHM1 gene, there is a A at the -3 position and a dT at the +6 position. The putative TATA box sequence of the gene is the sequence 5'-TATAA-3' at position -154 to -149 relative to the initiation codon, which conforms to a consensus TATA box sequence.

Fig. 4. Nucleotide sequence of the S. cerevisiae SHMI gene and flanking regions. The deduced amino acid sequence of the yeast mitochondrial SHMT is shown above the nucleotide sequence. The predicted termination codon is indicated with a dash. A putative TATA box sequence is underlined.
Cloning of a Genes Inactivated in a Yeast Glycine Auxotroph

The cytoplasmic SHMT is shown to be functional in general amino acid control of other yeast genes. The amino acid sequence identity with each product is the mitochondrial enzyme. The similarity of the residues include the mitochondrial leader sequence. The first 20 amino acids of the fungal enzymes. These results agree well with the phylogenetic scheme shown by Garrow et al. (12), which shows that the mammalian SHMT isoforms are more closely related to each other than to plant or fungal enzymes.

The predicted SHM1 product has 16 additional amino acids at its amino-terminal than does the predicted SHM2 product. This amino-terminal sequence is also more extended than the corresponding regions in the rabbit SHMT enzymes. The sequence of the rabbit mitochondrial enzyme was determined from amino acid sequencing of the mature protein and does not include the mitochondrial leader sequence. The first 20 amino acids of the SHM1 gene product have features typical of mitochondrial leader sequences: the presence of neither negatively charged amino acids nor extensive hydrophobic stretches of amino acids. Amino acids 51–20 of the yeast SHM1, VHRGL,
Cloning of Genes Inactivated in a Yeast Glycine Auxotroph

Conform to the consensus sequence of hydrophobic-polar-Lys-Arg-small-bulkily hydrophobic, proposed by Schmidt et al. (41) to be present at the cleavage sites of some proteins having mitochondrial leader sequences. Since Leu21 aligns with leucine residues conserved in 4 out of 5 eukaroytic SHMT enzymes, we would predict that the cleavage site of the mitochondrial SHMT is immediately following Leu.

**Characterization of SHM Null Mutants**—The chromosomal SHM1 and SM2 genes were inactivated by gene disruption. The 700-bp PCR-generated DNA fragments derived from the SHM1 and SM2 genes were cloned into pEMBL10* and designated pCRSHM1 and pCRSHM2, respectively. A DNA fragment containing the *sceiviria HIS3 genes was inserted into a *XhoI site within the SHM1 gene-specific DNA in pCRSHM1 and the SM2-specific DNA fragments, simultaneously.

**a** XhoI site within the SHM1 gene-specific DNA in pCRSHM1 (see Fig. 3A). A linearized DNA fragment containing the HIS3 gene flanked by SHM1 DNA was used to transform yeast strain W3031A to histidine prototrophy. Similarly, a LEU2 gene flanked by SHM2 DNA, as a linearized DNA fragment, was used to transform strain W3031R to leucine prototrophy. Southern analysis confirmed the insertion of the HIS3 and LEU2 sequences into the coding regions of chromosome SHM1 and SHM2 (Fig. 7). In panel A, lanes 2–5...
all show mutation disruptions at SHM1. In panel B, lanes 2 and 4 show disruptions of SHM2, while lanes 3 and 5 have wild type SHM2, presumably due to integration of the selectable marker at the LEU site. There are faint bands in both panels A and B, indicating cross-hybridization of the probe with the heterologous SHM gene and showing that those genes are wild type. Both YM06 (MATα shm1::HIS3) and YM07 (MATα shm2::LEU2) possessed phenotypes indistinguishable from the parental strains, indicating that disruption of a gene that codes for one of the yeast SHMT isozymes does not yield a glycine or formate auxotroph. Northern analysis was performed to confirm that insertion of the HIS3 and LEU2 genes obviated the expression of wild type SHM1 and SHM2 mRNA (not shown).

A yeast strain containing a double disruption at the SHM1 and SHM2 loci was constructed through mating of YM06 and YM07 and sporulation of the resultant diploid (YM08). YM09 is a haploid product of YM08 sporulation, which is prototrophic for histidine and leucine, indicating disruption at the SHM1 and SHM2 genes. This was confirmed by Southern (Fig. 7, panel C, lanes 5–7) and Northern analysis (not shown). Inactivation of both SHM1 and SHM2 did not result in any additional growth requirements for YM09.

Isolation of Glycine Requiring Yeast—The glycine prototrophic nature of SHM double-disrupted YM09 indicated that inactivation of a third gene may be required to render yeast a glycine auxotroph. Treatment of YM09 with ethyl methanesulfonate allowed the isolation of a collection of yeast Gly− mutants. Two classes of Gly− yeast were observed. The two classes are distinguishable by the ability or inability to grow when exogenous glycine is replaced with 10 mM formate. Those yeast which remain Gly+ in the presence of 10 mM formate were studied further. Mating and complementation studies indicated that among eight independent Gly− yeasts examined a single complementation group exists (data not shown). The mutant allele for this complementation group has been designated gly1.

Isolation of the S. cerevisiae GLY1 Gene—To isolate yeast sequences that complement the gly1 allele, Gly− yeast (YM09–10a, see Table I) were transformed with the Yep24 (URA3) yeast genomic library (21). Plasmid DNA isolated from Ura+ Gly+ transformants was used to retransform YM09–10a to glycine prototrophy. Based upon restriction enzyme and Southern analysis three types of yeast sequences were isolated (data not shown). Two of the sequences retrieved were the yeast SHM1 and SHM2 genes. This observation infers that the isolation of a Gly− yeast requires inactivation of both yeast genes that encode SHMT. The third yeast sequence isolated is not homologous to either SHM gene, and a partial restriction map of the yeast fragment in pGLY1–10a3 is shown in Fig. 8. Various plasmids were constructed by cloning portions of the yeast genomic fragment retrieved from the complementing plasmid pGLY1–10a3 into Yep24. Each derivative was used to attempt complementation of YM09–10a to glycine prototrophy (Fig. 8). A 1.5-kb SphI-SalI fragment was shown to be sufficient to complement the Gly− strain. The DNA sequence of this portion of the yeast genome is shown in Fig. 9. This sequence is identical to a sequence present in the EMBL Library data bank, which represents a portion of yeast chromosome 5 (accession no. L10380). Although portions of the sequence were determined on only one strand, all initial discrepancies with the EMBL sequence were resolved by sequencing both strands and running the labeling reactions with dITP to remove compressions. The fragment contains one open reading frame (designated ORF 35 in the EMBL citation) beginning at nucleotide 1437, which encodes a protein of 387 amino acids and a predicted molecular mass of 42,797 daltons, extending beyond the SalI site for an additional 41 base pairs. Our complementation studies suggest that the product is functional with the COOH-terminal 14 codons deleted. An inspection of GenBank did not identify any known gene products with significant homology to the GLY1 gene product.

Characterization of Yeasts with a Chromosomal Deletion at GLY1—Yeast with disruptions at GLY1 were constructed to confirm that inactivation of the chromosomal GLY1, SHM1, and SHM2 genes, is required to render yeast auxotrophic for glycine (Fig. 9). Strains YM09 (shm1 shm2 gly1::URA3) and YM12 (SHM1 shm2 gly1::URA3) are partial glycine auxotrophs and grow at similar reduced rates, indicating that both SHM genes are functional and can synthesize glycine. Strain YM10 (SHM1 shm2 gly1::URA3) is prototrophic for glycine but with a reduced doubling time compared to the parental yeast strain, W3031B. Interestingly, all strains disrupted at the SHM2 gene saturated at a higher cell concentration than those containing the intact gene, however, those shm2 strains also disrupted in GLY1 required glycine supplementation to show this increased growth (Fig. 10).
**Cell Cycle Analysis**—Because of its role in the generation of 1-carbon units for DNA precursor biosynthesis, SHMT plays an important role in DNA replication. In *S. cerevisiae*, most DNA replication genes are subject to transcriptional regulation during the G1 to S phase transition in the cell cycle (reviewed in Ref. 42). This particular pattern of transcription is conveyed by *Mlu1* cell cycle box elements, which have a consensus sequence between the SHM2 protein and known cytoplasmic SHMT proteins, particularly the cytoplasmic enzyme is the predominant SHMT in the yeast cell. Specific activities obtained were 2040 nmol/h/mg for wild type or the SHM2 genes. 2) The greater similarity in pre-processed mRNA for the SHM2 gene and flanking regions. These assignments were based on: 1) the deduced primary amino acid sequence for the SHM2 protein as the cytoplasmic enzyme. We have assigned the SHM2 protein as the mitochondrial enzyme, while the SHM2 protein as the cytoplasmic enzyme.

**Assays were done to determine SHMT activity in whole cell crude extracts of the null mutants and their parental strains. The shm2 extracts had 2–15% SHMT activity, compared to the parental strain, while the shm1 extracts had SHMT activity comparable to that of the parental strain, indicating that the cytoplasmic enzyme is the predominant SHMT in the yeast cell. Specific activities obtained were 2–40 nmol/h/mg for wild type and shm1 strains and 1–5 nmol/h/mg for shm2 strains. Spheroplasts were made and homogenates were subjected to differential centrifugation to separate the mitochondrial and cytoplasmic fractions. The shm1 extracts lacked SHMT activity in the mitochondrial fraction, while the shm2 extracts lacked SHMT activity in the cytoplasmic fraction and no SHMT activity was detected in extracts of the double mutants (Table II).**

**Discussion**

We have assigned the SHM1 protein as the mitochondrial SHMT and the SHM2 protein as the cytoplasmic enzyme. These assignments were based on: 1) the distribution of activities in the cytoplasmic and mitochondrial fractions after differential centrifugation of extracts of mutants disrupted at the *SHM1* or the *SHM2* genes. 2) The greater similarity in predicted primary amino acid sequence between the SHM2 protein and known mitochondrial SHMT proteins, particularly the *N. crassa* enzyme. 3) The extended amino-terminal sequence found in the SHM1 protein, which is consistent in amino acid sequence. The predicted termination codon is indicated with a dash. A putative TATA box sequence is underlined.
FIG. 10. Growth of wild type and mutant yeast strains disrupted in the SHM1, SHM2, and GLY1 genes. Yeast strains were grown in minimal SD medium supplemented with uracil, adenine, histidine, tryptophan, leucine, and in A, without glycine, or B, with 10 mM glycine. The designation SHM2 indicates that all the cultures that reach saturation at the lower growth level are strains that are wild type for the SHM2 gene, while shm2 indicates that all cultures reaching saturation at the higher growth level are disrupted in the SHM2 gene. A similar correlation of shm2/SHM2 state with growth level at saturation is seen in panel A for those strains that are wild type in GLY1. The symbols represent the following strains: V, wild type; ▽, shm1; ◇, shm2; △, shm1 shm2; ■, gly1; □, gly1 shm1; ●, gly1 shm2; ○, gly1 shm1 shm2.

FIG. 11. Northern analysis of SHM transcription. A culture of strain W3031A was synchronized by the α-factor release method (25), and samples were taken at 10-min intervals after α-factor release as indicated. RNA was prepared from each sample and equal amounts were electrophoresed through a native agarose gel. The gels were dried and hybridized with probes specific for SHM1, SHM2, TMP1, and LEU2 transcripts as described under "Experimental Procedures." The TMP1 and LEU2 determinations serve as controls for cell synchronization and equal loading of RNA, respectively.

The putative mitochondrial gene, SHM1, did not complement the E. coli glyA strain and does not express SHMT activity in E. coli. It is unlikely that this represents an inactive or pseudogene in yeast for several reasons. Repeated PCR amplifications did not yield sequences homologous to a third gene, representing the active mitochondrial gene but did consistently amplify the SHM1 gene. The shm2 disruption mutant, YM07, has measurable SHMT activity, however, the double disrupted shm1 shm2 yeast, YM09, does not. The SHM1 gene transformed into the glycine-requiring triple mutant strains complemented the glycine auxotrophy, indicating that it encodes a functional product. An alternate explanation for the lack of activity may be that the mitochondrial leader sequence, which would not be cleaved in E. coli, interferes with enzyme activity. Expression would then require the use of in vitro mutagenesis to delete the leader sequence codons and insert a Shine-Dalgarno sequence followed by an ATG codon at the predicted site of cleavage. The cytosolic enzyme is also relatively poorly expressed in E. coli. The level of expression we obtained is very similar to that found for the human SHMT enzymes using plasmids of similar copy number (12). We are conducting mutagenesis experiments to place the SHM2 gene under the con-
control of a more efficient bacterial promoter and include a bacterial ribosome binding site to optimize expression of this gene product in E. coli.

Surprisingly, inactivation of either one, or both yeast SHM genes did not result in any additional auxotrophic requirement. This is in contrast with results from other systems. Bacterial strains deficient in SHMT, and also CHO cells defective in mitochondrial SHMT exhibit an auxotrophy for glycine (2, 13, 14), while Neurospora strains lacking cytoplasmic SHMT require formate for growth (7). The S. cerevisiae gene disruption mutants are not leaky, since no enzyme activity can be detected in the double mutants, and both single mutants, particularly shm2, show decreased enzyme activity. Northern blots showed that the mRNA specific for the disrupted SHM gene was not detected in the shm1 or shm2 mutant strains. Mutagenesis of the double disruption mutants led to the isolation of glycine-requiring mutants, some of which could grow with formate in place of glycine. The GLY1 gene which complements the glycine auxotrophs is at a separate locus from the SHM genes. These studies indicate that yeast has an additional pathway, besides the SHMT reactions for glycine synthesis. One possible pathway for glycine synthesis is from glyoxalate using an aminotransferase. Our GenBank search for proteins homologous to GLY1 did not detect homology to transaminases. This does not exclude the possibility that GLY1 encodes an amino transaminase since few residues are conserved in all known transaminiases (48, 49), or the reaction in yeast may be catalyzed by a different family of aminotransferase. The glycine produced by this pathway could meet cellular requirements for protein synthesis and supply C1 units for the mitochondrial folate pool via the glycine cleavage system. The cytoplasmic folate pool would be provided with C1 units from formate using the 10-CHO-H4folate synthetase activity of the cytoplasmic C1-H4folate synthase. Unlike Neurospora, yeast must be able to generate sufficient formate from sugars to supply the C1 pool, since shm1 shm2 double mutants do not require exogenous formate. The production of this formate may be mediated by the product of the gene inactivated in our formate or glycine-requiring mutants. In this mutant, the glycine produced by the GLY1 pathway is insufficient to supply the C1 pools, since the supply of formate is now restricted. Exogenous glycine can be cleaved to form 5,10-CHO-H4folate in the mitochondria and the C1 units converted to formate by the mitochondrial C1-H4folate synthase, through reversal of its 10-CHO-H4folate synthetase activity. The formate can pass into the cytoplasm and be incorporated into the cytoplasmic C1 pool as proposed by Barlowe and Appling (18). We are presently characterizing the formate or glycine-requiring mutants and will study the effect of all our mutations in strains that also lack functional ADE3 or the MIS1 products to verify their roles in supplying C1 units in the absence of SHMT activity.

The growth studies suggest that the GLY1 pathway is the major source of glycine in yeast, since disruption of GLY1 alone affects the growth rate, whereas disruption of both SHM genes does not. The equal growth rates observed with the YM11 (shm1 shm2 gly1::URA3) and YM12 (SHM1 shm2 gly1::URA3) (Fig. 10) strains show that in yeast glycine synthesis is not confined to the mitochondria but the cytoplasmic SHMT makes a significant contribution to glycine synthesis.

SHM1 and SHM2 belong to a small class of S. cerevisiae genes that are involved in DNA precursor biosynthesis but are not subject to cell cycle regulation at the transcriptional level. Included in this class are DFR1, encoding dihydrofolate reductase, DCD1, encoding dCMP deaminase, and DUT1, encoding dUTP pyrophosphatase (45). Our results, however, do not preclude cell cycle regulation of SHM gene expression by post-translational processes.

Analysis of the 5'-noncoding regions of the SHM genes suggested that expression of SHM2 but not SHM1 may be regulated by the GCN4 transcription factor. This result was anticipated, since the Neurospora cytosolic SHMT is under the control of the amino acid cross-pathways control system (7), which is homologous to the yeast general amino acid control. The GCN4 factor-mediated general amino acid control pathway regulates amino acid biosynthesis. SHMT may be regulated by this pathway since the enzyme is involved in glycine biosynthesis and can be used to generate serine. GCN4 control is also likely involved in regulating the expression of two other folate-dependent yeast enzymes, NAD-dependent 5,10-methylenetetrahydrofolate dehydrogenase (46) and dihydrofolate reductase (47). It is somewhat surprising that the mitochondrial enzyme is not under general amino acid control, since the major site of glycine biosynthesis is mitochondrial in other systems and our studies with the glycine auxotroph triple mutants discussed above suggest that deficiency of the mitochondrial SHMT, combined with the second site mutation results in growth stimulation by glycine. We are presently performing deletion and mutagenesis studies to identify the sequences required for expression of both genes.

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