Polyglutamylation of Folate Coenzymes Is Necessary for Methionine Biosynthesis and Maintenance of Intact Mitochondrial Genome in Saccharomyces cerevisiae*

Received for publication, January 10, 2000, and in revised form, February 25, 2000

Hélène Cherest, Dominique Thomas, and Yolande Surdin-Kerjan‡
From the Centre de Génétique Moléculaire CNRS 91198 Gif-sur-Yvette cedex, France

One-carbon metabolism is essential to provide activated one-carbon units in the biosynthesis of methionine, purines, and thymidylate. The major forms of folates in vivo are polyglutamylated derivatives. In organisms that synthesize folate coenzymes de novo, the addition of the glutamyl side chains is achieved by the action of two enzymes, dihydrofolate synthetase and folylpolyglutamate synthetase. We report here the characterization and molecular analysis of the two glutamate-adding enzymes of Saccharomyces cerevisiae. We show that dihydrofolate synthetase catalyzing the binding of the first glutamyl side chain to dihydrofolate yields dihydrofolate is encoded by the YMR113w gene that we propose to rename FOL3. Mutant cells bearing a fol3 mutation require folinic acid for growth and have no dihydrofolate synthetase activity. We show also that folylpolyglutamate synthetase, which catalyzes the extension of the glutamate chains of the folate coenzymes, is encoded by the MET7 gene. Folylpolyglutamate synthetase activity is required for methionine synthetase and for maintenance of mitochondrial DNA. We have tested whether two folylpolyglutamate synthetases could be encoded by the MET7 gene, by the use of alternative initiation codons. Our results show that the loss of mitochondrial functions in met7 mutant cells is not because of the absence of a mitochondrial folylpolyglutamate synthetase.

In contrast to enteric bacteria or fungi such as Neurospora crassa and Aspergillus nidulans, Saccharomyces cerevisiae possesses a complete set of enzyme activities, which allows its growth on a large number of inorganic or organic sulfur sources. Because of the arrangement of the metabolic pathway for sulfur amino acids biosynthesis (1), a mutant strain unable to assimilate sulfate is capable of growing in the presence of either homocysteine, methionine, cysteine, or S-adenosylmethionine. In contrast, mutations impairing the conversion of homocysteine into methionine are the only ones that are expected to lead to strains that cannot grow on homocysteine or cysteine and require strictly methionine or S-adenosylmethionine for growth. In this reaction, catalyzed by homocysteine 5-methyltetrahydrofolate methyltransferase (methionine synthetase), the methyl group is supplied by 5-methyltetrahydrofolate, one of the products of the one-carbon metabolism (1).

One-carbon metabolism, in which one-carbon units are carried and donated by tetrahydrofolate derivatives, is essential for providing activated one-carbon groups to the biosynthesis of methionine, but also to those of purines and thymidylate (Fig. 1). There is no simple nomenclature system for naming tetrahydrofolate (H4 folate) derivatives but it is usually admitted to use H4 folate as an equivalent of tetrahydropteroylmonoglutamate (i.e. bearing one glutamate) (for nomenclature see Ref. 2). In S. cerevisiae, as well as in other eukaryotes, both the cytoplasmic and mitochondrial compartments possess a set of enzymes that catalyzes the interconversion of folate coenzymes, which differ by the oxidation state of their one-carbon unit (3). However, the reactions involving folate coenzymes and folate-dependent enzymes differ from one subcellular compartment to the other (Fig. 1). In the cytoplasm, the folate coenzymes participate in the synthesis of methionine, purines, and thymidylate. In mitochondria, folate coenzymes are required for the formylation of the initiator tRNA for mitochondrial protein synthesis. The interaction of cytoplasmic and mitochondrial one-carbon metabolism is not yet completely understood (3), but it has been shown in yeast that one-carbon donors such as serine, glycine, and formate are able to cross the mitochondrial membrane (4) (5).

The major cellular forms of folate coenzymes contain polyglutamate tails attached to the para-aminobenzoate moiety. Polyglutamylation of folates is catalyzed by the folylpolyglutamate synthetase (FPGS), which appears to exist in virtually all organisms. Accordingly, polyglutamylated folates are the preferred substrates of folate-dependent enzymes (for review, see Ref. 2). The importance of polyglutamylation in one-carbon metabolism is supported by the study of cell lines bearing a mutation-impairing FPGS activity, which exhibit auxotrophies for the products of one-carbon metabolism (2). In yeast, according to the one-carbon metabolism currently accepted, two reactions are specifically dedicated to methionine biosynthesis catalyzed respectively by methylene tetrahydrofolate reductase and by methionine synthetase. A strict requirement for methionine or S-adenosylmethionine is thus expected to arise from mutations inactivating one of the two genes encoding these enzymes (Fig. 1). However, mutant strains of S. cerevisiae unable to grow on homocysteine had been classified into five complementation groups defining genes MET6, MET7, MET13, MET23, and MET24 (6). More recent genetic analysis has shown that met6 and met24 mutations on the one hand and met7 and met23 on the other hand were allelic.2 The MET13

* This work was supported by the Centre National de la Recherche Scientifique and the Association de la Recherche sur le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 33 1 69 82 31 76; Fax: 33 1 69 82 43 72; E-mail: kerjan@cgm.cnrs-gif.fr.

1 The abbreviations used are: H4 folate, tetrahydrofolate; FPGS, folylpolyglutamate synthetase; ORF, open reading frame; PCR, polymerase chain reaction; 2-ME, 2-mercaptoethanol; DHFS, dihydrofolate synthetase; MM, YNB medium containing all the growth requirements except methionine.

2 H. Cherest, unpublished results.
gene, which corresponds to the ORF YGL125w, has been shown to encode methylene tetrahydrofolate reductase (7, 8). The MET6 gene has already been shown to encode methionine synthetase (9). It is moreover noteworthy that S. cerevisiae cells possess only one methionine synthetase, which functions without vitamin B_{12} as a cofactor.

The work presented here shows that the MET7 gene encodes FPGS. In addition, it shows that FPGS activity is absolutely required for methionine biosynthesis and for the maintenance of mitochondrial DNA. We show also that the gene YMR113w, displaying the capacity to specify a protein closely related to Met7p, encodes dihydrofolate synthetase.

MATERIALS AND METHODS

Strains, Media, and Microbiological Techniques—Escherichia coli strain JM103 was used as the host for plasmid maintenance. Yeast strains used in this work are listed in Table I. Standard yeast media were prepared as described by Cherest and Surdin-Kerjan (10). YPEA medium contains 0.5% yeast extract (Difco), 0.5% bacto-peptone (Difco), 2% ethanol, and 40 mg/l adenine). S. cerevisiae was transformed after lithium acetate treatment as described by Gietz et al. (11). Genetic crosses, sporulation, dissection, and scoring of markers were as described by Sherman et al. (12).

Recombinant DNA Methods—Plasmids pEMBLYe23, -25, and -31 as well as pRS313 and -314 were used as shuttle vectors between S. cerevisiae and E. coli (13, 14). The S. cerevisiae genomic library used for the cloning of the gene MET7 was constructed by inserting the product of a partial HindIII digest of chromosomal DNA from the wild type strain X2180–1A in the HindIII site of plasmid pEMBLYe23.

Gene Disruptions—The disruption of gene MET7 was performed by the one-step gene disruption method (15), as follows: the HindIII-HindIII fragment containing the MET7 region was cloned in the HindIII site of plasmid pBR322. The resulting plasmid was deleted of the BglII-EcoRV fragment containing most of the ORF of gene MET7 that was replaced by gene TRP1 from plasmid pFL35 (Fig. 2). In plasmid pFL35, the HindIII site of the TRP1 gene has been removed allowing the met7::TRP1 construct to be excised using the digestion by HindIII and SacI. The fragment was used to transform the diploid strain CC831 to tryptophan prototrophy yielding strain CD180. Correct integration was verified by Southern blot (data not shown).

Disruption of ORFs YMR113w and YKL132c were obtained as described in Ref. 16. In both cases, the HIS3 gene from Saccharomyces kluverii was used to replace the ORF. Cells from diploid CC831 were transformed with the amount of DNA generated by one PCR, and histidine prototrophs were selected. Correct replacement of the targeted gene was verified by analytical PCR using DNA extracted from one transformant for each gene.

Met7p Derivatives—First, the HindIII-XhoI fragment of pM7–1 was inserted in the EcoRV site of plasmid pRS313, yielding plasmid pM7–2. This eliminates the unique EcoRV site of pRS313. Five modified met7 alleles encoding Met7 derivatives with different translation initiation starts, beginning at methionine residue numbers 1, 17, 63, 86, and 98, respectively, were constructed. In these modified genes, the DNA fragments spanning the nucleotides 2190 to 21 (for a beginning at Met residue 1), 2190 to +48 (for a beginning at Met residue 17), −190 to +186 (for a beginning at Met residue 17), −190 to +255 (for a beginning at Met residue 86), and −190 to +291 (for a beginning at methionine residue 98) of the MET7 region were replaced by the promoter region of gene MET25 following a procedure similar to the one described by Muhlrad et al. (17). Briefly, bifunctional primers were designed that comprised 44 nucleotides homologous to the target sequence on the MET7 gene followed by 25 nucleotides homologous to the flanking
region of the \textit{MET25} promoter region. These primers were used to amplify the \textit{MET25} promoter region from plasmid pRS-pro\textit{MET25} (plasmid pRS314 bearing the \textit{2600 to 2600} promoter region of gene \textit{MET25}). For each construction, the resulting PCR product was co-transformed with plasmid pM7–2 linearized by \textit{EcoRV} into strain CD180–3B (\textit{met7}D). Transformants were selected on a medium lacking histidine but containing methionine. For each \textit{MET7} construct, plasmid DNA was recovered from six His\textsuperscript{+} transformants. Restriction analysis allowed the selection of the plasmid bearing the correct \textit{MET7} construction.

\textbf{Enzyme Assays—} Cells were grown in minimal medium complemented to meet their auxotrophic requirements and harvested by centrifugation when the cell concentration reached 10\textsuperscript{7} cells/ml (exponential phase). They were washed with 50 mM Tris-HCl buffer, pH 8.0, suspended in the same buffer containing 1 mM phenylmethylsulfonyl fluoride, and broken by passage through an Eaton Press at 9000 p.s.i. (18). Cell lysates were cleared by centrifugation at 10,000 \(\times\) g for 15 min and were used for enzymatic assays. The assay for folylpolyglutamate synthetase was essentially as described in Ref. 19. Assay mixtures contained 200 mM Tris-HCl, pH 8.5, 5 mM ATP, 10 mM MgCl\textsubscript{2}, 20 mM KCl, 100 mM 2-mercaptoethanol (2-ME), 0.5 mM aminopterine, L-[\textsuperscript{14}C]glutamate (5 mM, 2 \(\times\) \(10^6\) cpmp), and extract (about 2 mg of protein) in a total volume of 0.5 ml. The reactions were stopped by the addition of 20 \(\mu\)l of 2-ME, the mixture was centrifuged to discard the precipitated proteins, and 400 \(\mu\)l of the supernatant were applied to DE52 columns (20 \(\times\) 8 mm) equilibrated in

\begin{table}
\centering
\caption{Yeast strains}
\begin{tabular}{lll}
\hline
Strains & Genotype & Origin \\
\hline
W303–1A & MAT\textalpha, his3, leu2, ura3, ade2, trp1 & R. Rothstein \\
W303–1B & MAT\textalpha, his3, leu2, ura3, ade2, trp1 & R. Rothstein \\
CC788–2D & MAT\textalpha, his3, leu2, ura3, trp1 & This study \\
CC788–2B & MAT\textalpha, his3, leu2, ura3, trp1 & This study \\
CC831 & MAT\textalpha, his3, leu2, ura3, trp1, met7 & This study \\
CC704–18D & MAT\textalpha, his3, leu2, ura3, trp1, met7 & This study \\
CC927 & MAT\textalpha, his3, leu2, ura3, trp1, met7 & This study \\
 & MET7::URA3 MET7::met7 & This study \\
CD180–3A & MAT\textalpha, his3, leu2, ura3, trp1, met7::TRP1 & This study \\
CD180–3B & MAT\textalpha, his3, leu2, ura3, trp1, met7::TRP1 & This study \\
CD180–3D & MAT\textalpha, his3, leu2, ura3, trp1 & This study \\
CD181–1D & MAT\textalpha, his3, leu2, ura3, trp1, met7::TRP1, ymr113w::HIS3 & This study \\
CD202 & MAT\textalpha, his3, leu2, ura3, trp1, met7::TRP1, ymr113w::HIS3 & This study \\
CD200 & MAT\textalpha, his3, leu2, ura3, trp1, met7::TRP1, YMR113W::HIS3 & This study \\
CD214–1D & MAT\textalpha, his3, leu2, ura3, trp1, ymr113w::HIS3, ymr113w::HIS3 & This study \\
CD212–61 & MAT\textalpha, his3, leu2, ura3, trp1, ymr113w::HIS3, ymr113w::HIS3 & This study \\
CD210–1B & MAT\textalpha, his3, leu2, ura3, trp1, ykl132c::HIS3 & This study \\
CH2–16B & MAT\textalpha, his3, leu2, ura3, trp1, ykl132c::HIS3 & This study \\
CH1–6A & MAT\textalpha, his3, leu2, ura3, trp1, met7::TRP1 ykl132c::HIS3 & This study \\
\hline
\end{tabular}
\end{table}
Polyglutamylation of Folate Coenzymes in S. cerevisiae

10 mM Tris-HCl buffer, pH 7.5 containing 30 mM 2-ME and 80 mM NaCl. Unreacted glutamate was eluted with the equilibrium buffer (3 x 5 ml), and the labeled folate product was eluted with 0.1 N HCl (2 x 500 μl). Dihydrofolate synthetase was measured essentially as described in Ref. 20. As expected, that showed, as expected, that met7, ura3, and absence of dTMP. On the contrary to what was observed for adenine, the addition of dTMP up to 100 μg/ml was without significant effect on the growth of dTMP synthesis, we inactivated the TUP1 gene, for instance. To test if the disruption of the MET7 gene resulted in an impairment of dTMP synthesis, we inactivated the TUP1 gene in a strain bearing a met7Δ mutation and analyzed the growth of a double tpd1Δ, met7Δ disrupted strain (CD181–10D) in the presence and absence of dTMP. yeast cells can transport extracellular dTMP provided that they bear a mutation in the TUP1 gene, for instance. To test if the disruption of the MET7 gene resulted in an impairment of dTMP synthesis, we inactivated the TUP1 gene in a strain bearing a met7Δ mutation and analyzed the growth of a double tpd1Δ, met7Δ disrupted strain (CD181–10D) in the presence and absence of dTMP. On the contrary to what was observed for adenine, the addition of dTMP up to 100 μg/ml was without significant effect on the growth of tpd1Δ, met7Δ cells (Fig. 3B). Therefore, we concluded that dTMP synthesis can be made with monoglutamylated folate coenzymes. met7 mutant cells had been described as respiration-deficient (23), and we have verified that all the strains bearing met7 mutations from our collection were indeed “petites,” i.e. were unable to use ethanol as a carbon source. When the diploid strain CC827 (met7::MET7::URA3–3D) was sporulated (see above), we noted that a high number of methionine auxotroph spores were unable to use ethanol. Among the 78 methionine auxotrophs examined, only 32 spores were capable of using ethanol. In contrast, all the methionine prototroph spores could use ethanol as a carbon source. However, when diploid strain CC827 (met7::MET7::URA3–3D) was sporulated and tetrads were dissected, we tested that two spores grew quickly and two gave small colonies (Fig. 4). The quickly growing spores were methionine prototrophs. The small colonies were methionine auxotrophs and could not use ethanol as a carbon source. These experiments suggested that met7 mutant cells lose progressively their capacity to use ethanol and that met7 disrupted cells are respiration-deficient. Plasmid pM7–1 was the only one to transform both a haploid met7Δ strain and a heterozygous met7Δ/MET7 diploid strain. In the haploid strain, plasmid pM7–1 corrected the methionine requirement but not the respiration-deficient phenotype. Sporulation of diploid cells bearing the pM7–1 plasmid and analysis of the progeny showed that all the Trp+, Ura+ spores (met7::TRP1, harboring the Table II

| Strain       | Relevant genotype | Specific activity |
|--------------|------------------|------------------|
|              |                  | DHPS             | FPGS             |
| W303–1A      | met7::TRP1       | 0.18             | 0.29             |
| CD180–3A     | met7::TRP1       | 0.09             | <0.01            |
| CD208–2B     | ymr113w::HIS3    | <0.01            | 0.22             |
| CD210–1B     | ykl132w::HIS3    | 0.11             | 0.29             |
| CH2–1B       | ymr113w::HIS3    | <0.01            | 0.22             |

Met7p Is Essential Only for Methionine Synthesis and Mitochondrial DNA Integrity—In mammalian cells, polyglutamylated folates have been reported to serve as one-carbon unit donors in the synthesis of methionine as well as in that of purines and of thymidylate (dTMP). Moreover, it has been reported that met7 mutant cells require both methionine and adenine for growth (23). We found that the met7 mutants from our collection did not display the adenine requirement. To address more precisely the role of polyglutamylation of the folate coenzymes, we wanted to characterize more precisely the growth requirements of strains bearing a met7 null allele. As the MET7 gene was disrupted in a homozygous ADE2 diploid, which does not require adenine, growth of strain CD180–3B (met7, ADE2) was studied, and results show that its mean generation time was reduced from 5.5 to 3.25 h when adenine was added to the minimal synthetic medium containing methionine, indicating that the absence of polyglutamylation impairs modestly the biosynthesis of adenine (Fig. 3A).

RESULTS

Cloning of the MET7 Gene Encoding Polyglutamylate Synthetase—Strains bearing a met7 mutation had been described as methionine auxotrophs (6, 23). To clone the MET7 gene, strain CC704–18D (met7) was transformed by the gene library described under “Materials and Methods.” Among 150,000 transformants 15 were able to grow in the absence of methionine. They all bore the same plasmid with a 5-kilo-base pair insert (Fig. 2A). To confirm that we have indeed cloned the MET7 gene, we verified that the cloned insert is capable of targeting integration at the MET7 locus. The insert was recloned in a plasmid lacking an autonomous replicating sequence bearing the URA3 gene. The resulting plasmid was linearized by XhoI used to transform strain W303–1B (MET7, ura3), and stable uracil prototroph transformants were selected. One of these transformants was then crossed to CC704–18D (met7, ura3); the resulting diploid (CC827) was sporulated, and 39 tetrads were analyzed. In all cases a 2/2 segregation of the methionine auxotrophy was observed, and the Met+ spores were also Ura+, indicating that the insert had directed the integration to the MET7 locus. The position of the MET7 gene within the cloned DNA of pM7–1 was determined by subcloning. The subclones were tested for their ability to restore growth of strain CC704–18D (met7) on a medium lacking methionine (Fig. 2A).

The comparison of the restriction map of the insert of pM7–1 (Fig. 2A) with that of the region of chromosome XV where gene MET7 had been mapped (24) showed that the original cloned insert bore four ORFs, YOR239w–YOR242w and that pM7–3, which restores the growth of a met7 mutant on a medium lacking methionine, bears only one ORF, YOR241w. The polypeptide deduced from YOR241w shows extensive similarities with folylpolyglutamate synthetases that catalyze the extent of the glutamate chains of polyglutamylfolates in an ATP-dependent reaction. YOR241w was disrupted (Fig. 2B) in a diploid strain yielding strain CD180, and disruption was verified by Southern blotting (not shown). Diploid CD180 was sporulated, and tetrads were dissected. In the 18 tetrads tested, a 2/2 segregation of a methionine auxotrophy was observed, and the Met+ spores were also Trp+. To confirm that the YOR241Δ bearing strains were methionine auxotrophs, as are the met7 mutants. Strain CD180–3B (yor241Δ) was crossed to strain CC704–18D (met7), and analysis of the progeny showed, as expected, that YOR241w and MET7 are the same gene. To confirm that the MET7 gene does encode the yeast FPGS, the activity was assayed in both wild type and met7Δ bearing strains. As shown in Table II, the disruption of the MET7 gene results in an undetectable level of FPGS activity.

Taken together, all these results show that the MET7 gene corresponds to ORF YOR241w and encodes the yeast FPGS. The Met7p protein deduced from the nucleotide sequence of MET7 has a molecular mass of 62,157 and a calculated isoelectric point of 9.04.

References

1. Aigle, M., Kieffer, J. L., and Simon, M. (1983) Biochem. J. 208, 365–371.
2. Araki, H., Akazawa, H., and Goto, A. (1983) J. Biol. Chem. 258, 9950–9955.
The small colonies are those bearing the gene giving rise to two different proteins with the longer one containing the aminoterminal targeting signal for mitochondria. The second protein, lacking the sequence, is shorter and located in the cytoplasm (27–30).

In the amino-terminal sequence of MET7 five in frame ATG codons are found at positions 49, 164, 187, 256, and 292 corresponding to the methionine residues 17, 55, 63, 86, and 98 (Fig. 5). To test if MET7 could encode both a mitochondrial and a cytoplasmic enzyme, we constructed five different Met7p derivatives beginning at methionine residues 1, 17, 63, and 98, hoping that one derivative would lack the targeting signal and would thus encode only a cytoplasmic form of FPGS. We expected that a strain bearing a Met7p derivative without the targeting signal would not require methionine for growth and would not grow on ethanol as a carbon source. The constructions were made as described under “Materials and Methods.”

The modified genes were placed under the control of the MET25 promoter region using the GAP repair technique and the centromeric plasmid pM7–2. Plasmids bearing modified Met7p were used to transform strain CD180–4C (met7Δ, respiration-deficient, as expected. To test the ability of the mutants bearing each Met7p derivative to maintain the mitochondrial genome, one transformant for each met7 allele was crossed to strain CC788–2D (MET7, respiration-competent), the resulting diploid was sporulated, and in the progeny, the Trp⁺, His⁺ strains (i.e. bearing the met7 disruption and carrying the modified Met7p) were examined. In the crosses involving the met7 alleles encoding the Met7p beginning at the Met residues 1, 17, and 63, all the Trp⁺, His⁺ spores were methionine prototrophs and could use ethanol as a carbon source. In
regions of high similarities are boxed. They were aligned using the CLUSTAL V version 1.8 program (50). The results show that all the Met7p derivatives that led to a Met thionine requirement of methionine auxotrophs. These results medium. It is noteworthy that YPEA medium meets the me-
source and that the derivatives resulting in a Met
22% identities and 25% conservative replacements. Met7p and Ymr113p share
FIG. 5. Alignment of Met7, Ymr113w, and Ykl132c proteins. They were aligned using the CLUSTAL V version 1.8 program (50). The regions of high similarities are boxed.

the crosses involving the met7 allele encoding the Met7 protein beginning at Met-86 and Met-98 all the Trp, His spores were methionine auxotrophs and could not use ethanol on YPEA medium. It is noteworthy that YPEA medium meets the methionine requirement of methionine auxotrophs. These results show that all the Met7p derivatives that led to a Met phenotype resulted also in the ability to use ethanol as a carbon source and that the derivatives resulting in a Met phenotype led also to a respiration deficiency. Taken together, these results argue for the existence of only one cytosolic form of FPGS. As a consequence, these results favor the hypothesis that mitochon-
drial integrity depends on the presence of a metabolite whose synthesis takes place in the cytosol and is strictly de-
pendent on polyglutamylation of folate coenzymes.

**Met7p Belongs to a Family of Three Proteins Similar to Polyglutamate Synthetases**—In organisms that generate folates de novo, the addition of glutamyl side chains to cellular folates is achieved by two reactions catalyzed by dihydrofolate synthetase (DHFS) and FPGS, respectively. DHFS catalyzes the binding of the first glutamyl side chain to dihydropteroate yielding dihydrofolate, and then FPGS catalyzes the exten-
tion of the glutamate chains of polyglutamates in an ATP-de-
pendent reaction. In *E. coli*, which is able to synthesize folates, it has been shown that DHFS and FPGS are borne by a bifunc-
tional protein encoded by the *FolC* gene. Mammalian cells that do not synthesize folates de novo depend exclusively on intake of exogenously supplied folic acid and thus synthesize only FPGS, which does not exhibit a DHFS activity (31, 32). *S. cerevisiae* is able to synthesize folate coenzymes de novo; we thus wanted to determine if Met7p could have the DHFS ac-
tivity or if there were two different enzymes in this organism.

A search in the *Saccharomyces* Genome Data base revealed that Met7p shows significant similarities with the products of the two ORFs, YMR113w and YKL132c (Fig. 5). Ymr113p and Ykl132p are very closely related, sharing 43% identities and 32% conservative replacements. Met7p and Ymr113p share 22% identities and 25% conservative replacements. In addition, sequence alignments revealed that Met7p is more closely related to the human FPGS (59% identities and conservative replacements) than to the *E. coli* FPGS/DHFS enzyme (45% identities and conservative replacements), whereas the YMR113w and the YKL132c products are more closely related to the *E. coli* enzyme (55% identities and conservative replacements between FolCp and Ymr113wp). To decipher the relationship existing between the members of the Met7 protein family, we decided to inactivate each gene and to test the resulting strains for their phenotype and for the FPGS and DHFS activities.

**YMR113w Encodes Dihydrofolate Synthetase**—The ORF YMR113w was disrupted as described under “Materials and Methods” in the diploid strain CC831. The disruption was verified by PCR, and the disrupted diploid strain, CD208, was sporulated. When germination was performed on complete YPGA medium, only two spores were viable in each tetrad. However, if the complete YPGA medium was complemented by folic acid, the four spores of each tetrad germinated, and it was verified that the two spores requiring folic acid were the spores bearing the ymr113Δ allele. Folic acid is a folate compound (5-formyl-THF) that has been shown to be metabolized in higher eukaryotes by methenyl-THF synthetase yielding methenyl-THF, a folate coenzyme of the C1-metabolism that can be converted into the other folate coenzymes (see Fig. 1) (33). Further study of the growth requirements of the ymr113Δ mutant cells showed that they are able to grow on minimal synthetic medium when supplied with folic acid but that growth is better if adenine is added to the folic acid-containing medium (Fig. 6A). In contrast, the growth of ymr113Δ cells is not improved by the addition of methionine to the medium already containing folic acid. To test further the ability of ymr113Δ cells to grow on the final products of the C1 metabolism, the TUP1 gene was disrupted in the diploid strain CD208 (ymr113Δ/YMR113w) yielding a strain permeable to thymidylate (CD214). Diploid CD214 was sporulated, and tetrad were dissected. One recombinant strain CD214–1D (ymr113Δ, tup1Δ) was tested for growth. As its parental strain ymr113Δ, TUP1, the ymr113Δ, tup1 strain is capable to grow in the presence of folic acid, but it is also able to grow on a medium containing methionine, adenine, and thymidylate. The phenotype of the ymr113Δ strain is thus in accord with this gene encoding DHFS (see Fig. 1).

To confirm this result, we next assayed the glutamate-add-
ing enzymes FPGS and DHFS in the parental strain and in different mutant strains, as described under “Materials and Methods.” Results reported in Table II indicate that DHFS activity was below detection in strain CD208–2B (ymr113Δ), which exhibits a FPGS activity comparable to that strain W303–1A. In strain CD180–3B, impaired in FPGS activity, the DHFS activity was below detection in strain CD208–2B (ymr113Δ). Further study of the growth requirements of the ymr113Δ mutant cells showed that they are able to grow on minimal synthetic medium when supplied with folic acid but that growth is better if adenine is added to the folic acid-containing medium (Fig. 6A). In contrast, the growth of ymr113Δ cells is not improved by the addition of methionine to the medium already containing folic acid. To test further the ability of ymr113Δ cells to grow on the final products of the C1 metabolism, the TUP1 gene was disrupted in the diploid strain CD208 (ymr113Δ/YMR113w) yielding a strain permeable to thymidylate (CD214). Diploid CD214 was sporulated, and tetrads were dissected. One recombinant strain CD214–1D (ymr113Δ, tup1Δ) was tested for growth. As its parental strain ymr113Δ, TUP1, the ymr113Δ, tup1 strain is capable to grow in the presence of folic acid, but it is also able to grow on a medium containing methionine, adenine, and thymidylate. The phenotype of the ymr113Δ strain is thus in accord with this gene encoding DHFS (see Fig. 1).

To confirm this result, we next assayed the glutamate-add-
ing enzymes FPGS and DHFS in the parental strain and in different mutant strains, as described under “Materials and Methods.” Results reported in Table II indicate that DHFS activity was below detection in strain CD208–2B (ymr113Δ), which exhibits a FPGS activity comparable to that strain W303–1A. In strain CD180–3B, impaired in FPGS activity, the DHFS activity is comparable to that of the parental strain. Taken together, all our results show that YMR113w encodes DHFS. Therefore, we propose that the YMR113w ORF be named *FOL3* according to the standard yeast genetic nomenclature.

We then wanted to determine if the fol3Δ cells were respiration-competent. We thus tested growth of the fol3Δ mutant cells on a medium containing folic acid and a nonfermentable carbon source, and results show that growth in these conditions was very slow and was dependent on the concentration of folic acid added (Fig. 6B). This slow growth is perhaps not surprising as folic acid is known to be an inhibitor of several folate-dependent enzymes (34, 35). Although slow, growth on ethanol in the presence of folic acid indicates that the disruption of YMR113w did not result in strains bearing inactive mitochondria.
The pathway of folic acid utilization being still not completely deciphered, we then recombined the \( \text{met7} \) and the \( \text{fol3} \) mutations to study the phenotype of the resulting strains. The strains CD180–4C (\( \text{met7} \)) and CD208–2B (\( \text{fol3} \)) were thus mated yielding the diploid strain CD209. It was sporulated, and the tetrads were allowed to germinate on YPGA medium containing folic acid. This resulted in poor germination with only a few complete tetrads growing. In most cases, only two or three spores/tetrad were able to germinate. The analysis of the progeny showed that strains bearing both the \( \text{met7} \) and the \( \text{fol3} \) alleles were not viable, even in the presence of methionine and folic acid. This could be accounted for if the enzymes that allow the metabolism of folic acid require polyglutamylation. In that case, the double mutants \( \text{met7}, \text{fol3} \) would grow on the final products of the one-carbon metabolism. To test this hypothesis, gene \( \text{MET7} \) was disrupted in the diploid strain CD214 (\( \text{fol3}:\text{FOL3}, \text{tup1}:\text{TUP1} \)). The resulting diploid (CD212) was sporulated, and its progeny was analyzed. Strains bearing disruptions both in the \( \text{FOL3} \) gene and in the \( \text{MET7} \) gene along with a disruption in the \( \text{TUP1} \) gene were able to germinate on YPGA medium containing thymidylate and to grow, although poorly on synthetic medium containing methionine, adenine, and thymidylate, indicating that, indeed, the use of folic acid required polyglutamylation of folic coenzymes (Fig. 7). This is an indication that at least one enzyme implicated in the utilization of folic acid requires a polyglutamylated folic coenzyme.

As already stated, the product of the \( \text{YKL132c} \) gene was very similar to \( \text{Fol3p} \), but the growth requirement for folic acid of a \( \text{fol3} \) mutant strain was a first indication that the product of the \( \text{YKL132c} \) gene is not redundant with \( \text{Fol3p} \). The gene \( \text{YKL132c} \) was disrupted as described under “Materials and Methods” in the diploid strain CC831. The disruption was verified by PCR, and the resulting strain (CD210) was sporulated. The germination was performed on YPGA. In all tetrads, the four spores were able to germinate on this medium, showing that a strain bearing a \( \text{ykl132A} \) allele was viable. It was verified that mutants bearing a \( \text{ykl132A} \) allele were not petites and exhibited no growth requirements. In addition, as shown in Table II, the DHFS and the FPGS activities of the \( \text{ykl132c} \) null mutants are comparable to the activities found in the parental strain W303–1A. Different recombinants were constructed and showed that \( \text{ykl132A}, \text{fol3A} \) cells (strain CH2–16A) display the same phenotype as \( \text{fol3} \) cells and that \( \text{ykl132A}, \text{met7A} \) cells (strain CH1–6A) exhibit the same phenotype as the \( \text{met7} \) cells. We were thus not able to determine the function of the \( \text{YKL132c} \) gene.

**DISCUSSION**

One-carbon transfers mediated by folate coenzymes play an essential role in several major cellular processes. In the cytosol, folate coenzymes are implicated in purine and thymidylate synthesis as well as in the biogenesis of the methyl group of methionine. In mitochondria and chloroplasts, it is admitted that 10-formyltetrahydrofolate is necessary for the formulation of the initiator tRNA and thus for mitochondrial protein synthesis. This diversity of reactions stems from the ability of tetrahydrofolate to carry activated one-carbon units at different oxidation states and the ability of the cell to interconvert these different forms.

Plants, bacteria, and fungi (\( \text{N. crassa} \) and \( \text{S. cerevisiae} \)) synthesize folic cofactors de novo. In these organisms, two enzymes are responsible for the binding of glutamate to folate derivatives. Dihydrofolate synthetase adds the first glutamate to dihydropteroic acid yielding dihydrofolate, whereas polyglutamatase synthetase adds sequentially other glutamates. In \( \text{E. coli} \), these two activities are carried by the same protein encoded by the \( \text{folC} \) gene. In \( \text{N. crassa} \), as well as in plants, the two enzymes are probably encoded by distinct genes, although this has not been formally shown (20, 36, 37). In the present work we have brought evidence that, in \( \text{S. cerevisiae} \), DHFS is an enzyme distinct from FPGS and is encoded by the \( \text{YMR113w} \) gene that we propose to rename \( \text{FOL3} \). The requirement for folic acid of \( \text{fol3} \) mutant cells is in agreement with their enzymatic deficiency and results from the inability of the \( \text{fol3} \) mutant cells to synthesize de novo folic coenzymes.

In mammals, polyglutamylation was shown to lead to the synthesis of more efficient substrates for many folate-dependent enzymes. Indeed, Chinese hamster ovary mutant cells lack-
Polyglutamylation of Folate Coenzymes in S. cerevisiae

1. Thomas, D., and Surdin-Kerjan, Y. (1997) Microbiol. Mol. Biol. Rev. 61, 503–532
2. Schirch, V., and Strong, W. B. (1989) Arch. Biochem. Biophys. 269, 371–380
3. Appling, D. R. (1991) FASEB J. 5, 2645–2651
4. McNeil, J. B., Bognar, A. L., and Pearlman, R. E. (1996) Genetics 142, 371–381
5. Kastanos, E. K., Woldman, Y. Y., and Appling, D. R. (1997) Biochemistry 36, 14956–14964
6. Masselon, M., and De Robichon-Szulmajster, H. (1975) Mol. Gen. Genet. 139, 311–312
7. Tizón, B., Rodríguez-Torres, A. M., and Cerdan, M. E. (1999) Yeast 15, 145–154
8. Raymond, R. K., Kastanos, E. K., and Appling, D. R. (1999) Arch. Biochem. Biophys. 372, 300–308
9. Mountain, H. A., Bystrom, A. S., Larsen, J. T., and Korch, C. (1991) Yeast 7, 781–803
10. Charest, H., and Surdin-Kerjan, Y. (1992) Genetics 130, 51–58
11. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425–1426
12. Sherman, F., Fink, G. R., and Hicks, J. B. (eds) (1979) Methods in Yeast Genetics: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Baldari, C., and Cesaroni, G. (1985) Gene (Amst.) 35, 27–32
14. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
15. Rothstein, R. J. (1983) Methods Enzymol. 101, 202–213
16. Lorenz, M. C., Muir, R. S., Lim, E., McElvear, J., Weber, S. C., and Heitman, J. (1995) Gene (Amst.) 158, 113–117
17. Margad, D., Hunter, A., and Parker, R. (1992) Yeast 8, 79–82
18. Thomas, D., Charest, H., and Surdin-Kerjan, Y. (1989) Mol. Cell. Biol. 9, 3292–3298
19. Bognar, A. L., and Shane, B. (1986) Methods Enzymol. 122, 349–359
20. McDonald, D., Atkinson, I. J., Cossins, E. A., and Shane, B. (1995) Phytochemistry 38, 327–333
21. Lowry, O. H., Rosebrough, J. H., Farr, A. L., and Randall, J. (1951) J. Biol. Chem. 193, 265–275
22. Stover, P., and Schirch, V. (1992) Anal. Biochem. 202, 82–88
23. Jones, E. W., and Fink, G. R. (1982) The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W., and Broach, J. R., eds) pp 181–299, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Mortimer, R. K., Contopoulou, C. C., and King, J. S. (1992) Yeast 8, 817–820
25. Schatz, G. (1996) J. Biol. Chem. 271, 31763–31766
26. Schatz, G. (1997) Nature 388, 121–122
27. Chatton, B., Walter, P., Ebel, J.-P., Lacroute, F., and Fasiolo, F. (1988) J. Biol. Chem. 263, 52–57
28. Ellis, S. R., Hopper, A. K., and Martin, N. C. (1988) Mol. Cell. Biol. 9, 1611–1620
29. Wu, M., and Tzagoloff, A. (1988) J. Biol. Chem. 263, 12275–12280
30. Chiu, M. I., Mason, T. L., and Fink, G. R. (1992) Genetics 132, 987–1001
31. George, S., Cichowicz, D. J., and Shane, B. (1987) Biochemistry 26, 522–529
32. Cichowicz, D. J., and Shane, B. (1987) Biochemistry 26, 513–521
33. Dayan, A., Bertrand, B., Beauchemin, M., Chabbi, D., Mamo, A., Filion, M., Skup, D., Massie, B., and Jolivet, J. (1995) Gene (Amst.) 165, 307–311
34. Bertrand, R., and Jolivet, J. (1989) J. Biol. Chem. 264, 8834–8846
35. Giri, S., Suh, J. H., Jolivet, J., and Stover, P. J. (1997) J. Biol. Chem. 272, 4729–4734
36. Iwai, K., Ibeda, M., and Kobashi, M. (1980) Methods Enzymol. 66, 581–585
37. Inoue, M., and Cossins, A. E. (1997) Methods Enzymol. 281, 141–145
38. McBurney, M. W., and Whitmore, G. F. (1974) Cell 7, 173–182
39. Taylor, R. T., and Hanna, M. L. (1977) Arch. Biochem. Biophys. 161, 331–334
40. Foo, S. K., and Shane, B. (1982) J. Biol. Chem. 257, 3587–3592
41. Whitfield, C. D., Steers, E. J., Jr., and Weisbach, H. (1970) J. Biol. Chem. 245, 390–401
42. Myers, A. M., Pape, L. K., and Tzagoloff, A. (1985) EMBO J. 4, 2087–2092
43. Tzagoloff, A., and Dieckmann, C. L. (1990) Microbiol. Rev. 54, 211–225
44. Vambsatias, A., Ackerman, S. H., and Tzagoloff, A. (1991) Eur. J. Biochem. 201, 643–652
45. Tzagoloff, A., and Sitkanko, A. (1995) Eur. J. Biochem. 230, 582–586
46. Williamson, D. H., Maroudas, N. G., and Wilkie, D. (1971) Mol. Gen. Genet. 111, 209–223
47. Wintersberger, U., and Hirsch, J. (1973) Mol. Gen. Genet. 126, 61–70
48. Freudenthal, S. J., Taylor, S. M., Krystal, G., and Moran, R. G. (1995) J. Biol. Chem. 270, 9579–9584
49. Little, J. G., and Haynes, R. H. (1979) Mol. Gen. Genet. 168, 141–151
50. Higgins, D. G. (1994) Methods Mol. Biol. 25, 307
Polyglutamylation of Folate Coenzymes Is Necessary for Methionine Biosynthesis and Maintenance of Intact Mitochondrial Genome in *Saccharomyces cerevisiae*

Hélène Cherest, Dominique Thomas and Yolande Surdin-Kerjan

*J. Biol. Chem.* 2000, 275:14056-14063.
doi: 10.1074/jbc.275.19.14056

Access the most updated version of this article at [http://www.jbc.org/content/275/19/14056](http://www.jbc.org/content/275/19/14056)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 16 of which can be accessed free at [http://www.jbc.org/content/275/19/14056.full.html#ref-list-1](http://www.jbc.org/content/275/19/14056.full.html#ref-list-1)