Functional Characterization of Human Methylenetetrahydrofolate Reductase in *Saccharomyces cerevisiae*

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Human methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-Methyltetrahydrofolate is a major methyl donor in the remethylation of homocysteine to methionine. Impaired MTHFR can cause high levels of homocysteine in plasma, which is an independent risk factor for vascular disease and neural tube defects. We have functionally characterized wild-type and several mutant alleles of human MTHFR in yeast, *Saccharomyces cerevisiae*. We have shown that yeast MET11 is a functional homologue of human MTHFR. Expression of the human MTHFR cDNA in a yeast strain deleted for MET11 can restore the strain's MTHFR activity in vitro and complement its methionine auxotrophic phenotype in vivo. To understand the domain structure of human MTHFR, we have truncated the C terminus (50%) of the protein and demonstrated that expressing an N-terminal human MTHFR in *met11* yeast cells rescues the growth phenotype, indicating that this region contains the catalytic domain of the enzyme. However, the truncation leads to the reduced protein levels, suggesting that the C terminus may be important for protein stabilization. We have also functionally characterized four missense mutations identified from patients with severe MTHFR deficiency and two common missense polymorphisms found at high frequency in the general population. Three of the four missense mutations are unable to complement the auxotrophic phenotype of *met11* yeast cells and show less than 7% enzyme activity of the wild type in vitro. Both of the two common polymorphisms are able to complement the growth phenotype, although one exhibited thermolabile enzyme activity in vitro. These results shall be useful for the functional characterization of MTHFR mutations and analysis structure/function relationship of the enzyme.

Elevated plasma homocysteine levels, or hyperhomocysteinemia, is an independent risk factor for vascular disease and neural tube defects (1, 2). In humans, homocysteine can be metabolized in one of two ways, transsulfurated to form cysteine or remethylated to form methionine. Disturbance in either of these pathways can lead to an accumulation of homocysteine in plasma.

Methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme in the remethylation pathway. It catalyzes the formation of a methyl donor, 5-methyltetrahydrofolate (5-methylTHF), for converting homocysteine to methionine. Human *MTHFR* gene has been localized to 1p36.3. It encodes a protein of 656 amino acids, with a predicted molecular mass of 74.5 kDa (3). The enzyme is a flavoprotein that utilizes NADPH as an electron donor and FAD as a cofactor in mammalian cells (5). Human and other eukaryotic MTHFR exist as homodimers, whereas *Escherichia coli* MTHFR is a homotetramer (6, 7). Porcine MTHFR has been shown to be allosterically inhibited by S-adenosylmethionine (AdoMet) (5, 8). This inhibition was proposed to play a role in preventing the depletion of methylenetetrahydrofolate in cells (5).

Mutations in human *MTHFR* are known to cause clinical MTHFR deficiency, an autosomal recessive disorder (4). The clinical symptoms of patients with MTHFR deficiency are highly variable including developmental delay, motor and gait abnormalities, seizures, and premature vascular disease (9). The reductase activity from cultured fibroblasts of patients with severe MTHFR deficiency ranges from 0 to 20% of the control (10). The severity of the disease tends to correlate with the residual MTHFR activity (9). Several mutant alleles of *MTHFR* have been identified in patients with MTHFR deficiency (10, 11). In addition, two common polymorphisms in *MTHFR*, C677T (A222V) and A1298C (E428A), have also been described (12, 13). The C677T variant has an allele frequency of 35% in the North American population, with about 12% of the population being homozygous and 40–45% heterozygous for the mutation (14). The homozygotes appear to have a slightly increased risk of coronary heart disease and of giving birth to children with neural tube defects (15, 16). The A1298C mutation has an allele frequency of 33%. Neither homozygosity nor heterozygosity of this allele is associated with high plasma homocysteine levels or lower plasma folate concentration (13). However, this allele is reported to interact with the C677T allele in *trans* to cause reduced MTHFR activity and decreased plasma folate level, which could be a genetic risk factor for neural tube defects (13).

So far human MTHFR and its mutant alleles have not been individually expressed and characterized at molecular levels. We have taken advantage of the yeast, *Saccharomyces cerevisiae*, as a model organism to characterize wild-type and mutant alleles of *MTHFR*. This organism serves as a good model system for studying human biology because of the evolutionary conservation of human and yeast genes. Over 30% of yeast open reading frames share significant sequence similarity with known human genes, and more than 70 human cDNAs have been shown to complement yeast mutations (17). This structural and functional conservation has allowed the functional...
characterization of human genes in yeast and identification of mutations in human genes. Some examples of characterizing mutations and polymorphisms of human genes in yeast include p53 (18), cystathionine β-synthase (19), galactose-1-phosphate uridylyltransferase (20), and the Wilson disease gene (21). In addition, yeast systems, due to their genetic tractability, have proven useful in structure-function analysis of human proteins. For example, yeast systems have been used to identify functional domains of the human melatonin receptor (22), p53 (18), and cystathionine β-synthase (23).

In this paper we describe the development of a yeast system for functionally and structurally characterizing human \textit{MTHFR}, and we show the utility of the yeast system in both mutation analysis and structure-function analysis.

**MATERIALS AND METHODS**

**Strains**

\textit{E. coli} strain XL-1 Blue was used for all DNA manipulations. Yeast strains used were as follows: W303-1A, \textit{Mat a, ade2-1, can1-100, ura3-1, leu2-3, 112, trp1-1, his3-11,15, met11}.

**Plasmids**

\textit{pMV2.1—Human MTHFR} cDNA was obtained from a human cDNA library (27) by PCR amplifications using Klentaq (CLONTECH). The final PCR product containing the entire coding region of \textit{MTHFR} was ligated to PCR 2.1 (Invitrogen). The amplified cDNA carries the common C677T mutation.

\textit{pC677T—An XhoI/KpnI fragment (about 1.8 kb) containing the entire MTHFR coding region from pMV2.1 was isolated and ligated to Sa/I/KpnI-digested pBBP-GAL (28)}. This generates a plasmid carrying the 5′ end (hemagglutinin A) epitope-tagged MTHFR open reading frame under the control of the inducible yeast GAL1 promoter and the yeast UR3 gene as a selectable marker.

**pMTHFR**—The C677T mutation in \textit{pMV2.1} was reverted to wild-type (C677T) by site-directed mutagenesis using the Transformator Mutagenesis kit (CLONTECH). A 20-bp oligonucleotide with the altered sequence was used to introduce the desired mutation. The resulting plasmid, \textit{pMA2.1}, was digested with 

**pMTHFR**—The C677T mutation in \textit{pMV2.1} was reverted to wild-type (C677T) by site-directed mutagenesis using the Transformator Mutagenesis kit (CLONTECH). A 20-bp oligonucleotide with the altered sequence was used to introduce the desired mutation. The resulting plasmid, \textit{pMA2.1}, was digested with \textit{XhoI} and \textit{KpnI}, and a 1.8 kb DNA fragment containing wild-type \textit{MTHFR} cDNA (C677T) was gel-purified. To incorporate the wild-type cDNA into the expression plasmid, the yeast gap repair technique was used. A 1.8 kb DNA fragment from \textit{pMA2.1} was co-transformed into \textit{XSY3–1A} strain with an \textit{Smal}I (partial)/EcoRI-linearized \textit{pC677T} with a region containing T677 being removed. The transformants carry a gap repaired \textit{pMTHFR} identical to \textit{pC677T} except C-677 (wild-type). 

**pSalI**—\textit{SalI-linearized pC677T} was used for gap repair as above. The resulting yeast transformants carry the \textit{MTHFR} promoter.

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**pG458T and pA1298C**—These two plasmids were generated through PCR mutagenesis. Briefly, for \textit{pA1298C}, primer sets 5′-CTGCTGAGCTCCAGTGGGTGACGGGACG-3′ (forward) and 5′-GGATATGCCAGGAAAGCTGGGCGTGTCCG-3′ (reverse). The reverse primer contains a point mutation, G1059A, that changes a Trp codon to an OPA. This DNA fragment was used to gap repair as above. The resulting yeast transformants carry plasmids that express HA-tagged \textit{N-terminal MTHFR} under the control of the yeast \textit{GAL1} promoter.

**Disruption of Yeast Met11**

The chromosomal copy of \textit{MET11} in strain W303-1A was disrupted using the one-step gene disruption method (29). The DNA fragment used for disruption was generated by the fusion PCR (30). In this fragment, 200 bp of 3′ and 5′ \textit{MET11} flanking regions were fused to the yeast \textit{TRP1} gene at each end. Briefly, a PCR fragment was generated that contained the 5′-flanking region of \textit{MET11} plus 20 bp at the 3′ end homologous to the 5′ end of the yeast \textit{TRP1} gene. Another PCR fragment containing the 3′-flanking region and 20 bp at the 5′ end complementary to the 3′ end of the \textit{TRP1} gene was also generated. These two DNA fragments and the \textit{TRP1} gene were connected by PCR. PCR primers for the 5′-flanking region were 5′-ACCCCGAGTCGACACTG (forward) and 5′-TCGTCGATCTGGGAAAAACCTCCTGGCGTGTCCGTTGTTG-TGC (reverse). For the 3′-flanking region, the primers were 5′-TCGTGTGGAAAATTGTTCCAATGATGGAC (forward) and 5′-TCTTT-GAACAGTGGAGTAGCT (reverse). The fusion PCR product was gel-purified and transformed into W303–1A. Ten Trp+ transformants were obtained, and their chromosomal DNA was isolated for PCR analysis to verify that \textit{TRP1} gene was integrated into the \textit{MET11} locus. One of the ten transformants was confirmed to be \textit{met11A::TRP1} (\textit{TRP1} integrated at \textit{MET11} locus). This strain, \textit{XSY3–1A}, was used for complementation studies.

**Complementation Assay and Growth Curve Measurement**

To test for growth, yeast cells were inoculated on SD-Met-Ura plates and SG-Met-Ura plates. The plates were then incubated at 30 °C for 3–4 days.

For growth curve measurement, yeast cells were inoculated into SG-Met-Ura liquid media and incubated at 30 °C overnight. The cultures were diluted to about 0.1 × 10^8 cells/ml the next day. The number of cells in 1 ml of media was counted using a hemocytometer every 2 h.

**Yeast Extract Preparation and Immunoblot Analysis**

Yeast extracts and immunoblot analysis were done essentially as described (31). Monoclonal antibody 12C5A (against HA) was purchased from Rocke Molecular Biochemicals, and alkaline phosphatase-conjugated goat anti-mouse secondary antibody was from Bio-Rad. Color developing reagents, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, were from Sigma.

**MTHFR Enzyme Activity Assay**

The assay is described by Rosenblatt and Erbe (32). The assay measures the reverse reaction that \textit{MTHFR} catalyzes under physiological conditions. Briefly, 250 μg of extract was used for each reaction. The reaction mix also contained 0.18 M potassium phosphate, pH 6.3, 3.6 mM menadione bisulfite, 1.4 mM EDTA, 7.2 mM ascorbic acid, 178 μM flavin adenine dinucleotide, 420 μM 5-[^14]C]MeTHF. The reaction temperature was 57 °C, and the reaction time was 1 h. For heat treatment, the reaction mixtures without 5-[^14]C]MeTHF were heated 50 °C for 20 min. 5-[^14]C]MeTHF was then added back to the mix and proceeded as the samples without heat treatment. The 5-[^14]C]MeTHF (barium salt) was from Amersham Pharmacia Biotech. All other chemicals were from Sigma.

**RESULTS**

**Yeast \textit{MET11} Encodes \textit{MTHFR}**—To characterize human \textit{MTHFR} in yeast, a strain lacking endogenous \textit{MTHFR} was constructed. We searched for yeast proteins with sequence similarities to human \textit{MTHFR} in the Saccharomyces Genome Database. Protein sequence alignments showed that a polypeptide encoded by yeast \textit{MET11} shared the highest degree of identity with the human \textit{MTHFR} (38.3%). To determine if \textit{MET11} encodes the yeast \textit{MTHFR} enzyme, we constructed a disruption allele of \textit{MET11} in yeast strain W303-1A (see Materials and Methods). We anticipated that yeast lacking \textit{MTHFR} activity would require methionine for growth since 5-MeTHF is required for the synthesis of methionine from homocysteine. As expected, the resulting \textit{met11A::TRP1} strain, \textit{XSY3–1A}, failed to grow in media lacking methionine (see Fig. 1). To confirm further that \textit{MTHFR} activity was depleted in the \textit{met11}
tional modification. This band could suggest that it is related to MTHFR protein. The apparent molecular mass of the upper band is about 78 kDa, which is very close to the extract from glucose-grown cells. The apparent molecular mass of the lower band in the extract of cells grown in glucose media but not in the cells. As shown in Fig. 3, a doublet band was observed in the extract made from cells grown in galactose media but not in the SG-Met-Ura (galactose) plates.

Human MTHFR Can Functionally Substitute for the Yeast MET11 Gene—We next determined whether expression of human MTHFR in the met11 strain could complement the methionine auxotrophic phenotype. We constructed a yeast centromere plasmid, pHMHFR, carrying human MTHFR cDNA controlled by the galactose-inducible GAL1 promoter (see "Materials and Methods"). In the presence of galactose, this plasmid expresses wild-type human MTHFR with a hemagglutinin A (HA) epitope at its N terminus. The HA epitope allowed us to detect the protein by immunoblot analysis of whole cell extract with a monoclonal anti-HA antibody, 12CA5. This plasmid was transformed into the met11 yeast cells. As shown in Fig. 3, a doublet band was observed in the extract made from cells grown in galactose media but not in the extract from glucose-grown cells. The apparent molecular mass of the upper band is about 78 kDa, which is very close to the predicted size (74.5 kDa) for human MTHFR. The absence of the lower band in the extract of cells grown in glucose media suggests that it is related to MTHFR protein. This band could be either a proteolytic product or the result of post-translational modification.

Yeast met11 cells carrying pHMHFR were then tested for growth on galactose and glucose media lacking methionine. As shown in Fig. 1, expression of human MTHFR, induced by galactose, supported the growth of met11 cells on media lacking methionine. No growth was observed when the expression of human MTHFR was repressed in a glucose media lacking methionine. In fact, yeast met11 expressing human MTHFR grow as well as the same strain expressing yeast MTHFR (MET11). The ability of human MTHFR to complement the auxotrophic phenotype indicates that human MTHFR and yeast MET11 encode functional homologues.

Complementation Analysis of MTHFR Alleles Found in Patients with MTHFR Deficiency—To detect biological consequences caused by mutations in human MTHFR gene, we constructed four expression plasmids carrying mutant alleles of MTHFR. Each of the alleles contains a single MTHFR mutation (G164C, G458T, T980C, and C1141T), previously found in patients with clinical MTHFR deficiency (see Table I) (11). These mutations were engineered into MTHFR cDNA by either site-directed or PCR mutagenesis (see "Materials and Methods"). The resulting plasmids are identical to pHMHFR, except each carries a mutant allele. The plasmids were then transformed into the met11 cells.

We ascertained that MTHFR proteins were expressed from the mutant MTHFR alleles by immunoblot analysis of yeast whole cell extracts using the anti-HA antibody. The extracts were made from yeast cells grown in galactose medium to induce the expression of the alleles (see "Materials and Methods"). Immunoblot analysis of the extracts indicates that all the alleles were expressed at a similar level to the wild type (see Fig. 3).

We next examined the ability of the mutants to complement the auxotrophic phenotype of the met11 yeast cells. As shown in Fig. 1, met11 cells expressing three of the mutant alleles, G164C, G458T, and T980C, were unable to grow in either glucose (repressed) or galactose (induced) media lacking methi-
onine, indicating that these cells lacked MTHFR activity. Since immunoblot analysis (Fig. 3) showed that the mutant proteins were present in the cells, we conclude that these mutant proteins are non-functional. Our data show that the inability of these mutants to complement met11− yeast cells correlates with their pathogenic effects in humans.

However, one of the alleles tested, C1141T, was able to complement the methionine auxotrophic phenotype of the met11− cells and was phenotypically indistinguishable from the wild-type MTHFR in yeast. It should be noted that this mutation was found in a patient heterozygous for C1141T, C677T, and T980C. Thus it is possible that C1141T might be deleterious only in the context of these other mutations (see “Discussion”).

Complementation Analysis of Two Common Variants of MTHFR—We next used this system to characterize two common polymorphisms found in the human MTHFR, C677T and A1298C. We constructed plasmids pC677T and pA1298C that express C677T and A1298C from the GAL1 promoter, similar to pHMTFR (see “Materials and Methods”). The two plasmids were transformed into met11− cells, and expression of the two alleles was confirmed by immunoblotting with anti-HA antibody (see Fig. 3). As shown in Fig. 1, these alleles were able to rescue the auxotrophic phenotype of the met11− cells. This is not unexpected for two reasons. First, human lymphocyte extracts from individuals homozygous for C677T have 40–50% MTHFR activity of the control (14), and those A1298C homozygotes have 62% MTHFR activity of wild-type (13). Second, in our yeast cells these alleles were expressed from the strong yeast GAL1 promoter, resulting in high levels of MTHFR protein. This would elevate the reductase activity in the cell and compensate for the low MTHFR activity of the mutant alleles.

Enzyme Activity in Extracts Made from Yeast Cells Expressing Various MTHFR Alleles—We then determined whether the ability of a human MTHFR allele to complement the yeast growth phenotype correlated with its enzymatic activity in vitro. We measured the MTHFR activities using yeast whole cell extracts containing various MTHFR mutants (see “Materials and Methods”). As a negative control, MTHFR activity was also measured using an extract made from cells grown in glucose media where the expression of an allele was repressed. Since the gene product of the C677T allele is thermolabile (12), we have also examined thermal stability of MTHFR variants that display detectable reductase activity. As shown in Fig. 2, the mutant alleles that failed to complement the yeast growth phenotype showed less than 7% of wild-type activity, whereas extracts from cells carrying the complementing alleles, C677T, A1298C, and C1141T, all exhibited high levels of enzyme activity. After heat treatment (55 °C for 20 min), we found wild-type human MTHFR retained about 40% of its original activity, whereas C677T lost almost all its activity (Fig. 2). Alleles A1298C and C1141T retained about 40% of their enzyme activity similar to that of wild-type MTHFR. Thus A1298C and C1141T are indistinguishable from the wild type in their thermal stability and MTHFR activity in vitro.

Catalytic Domain of Human MTHFR—Our results show that A1298C and C1141T mutations do not cause any dysfunction of the enzyme under our assay conditions. One common feature of these two mutations is that they are located in the C-terminal domain of the protein. Comparison of human MTHFR and E. coli MetFp (encoding MTHFR in E. coli) revealed that the C terminus of human MTHFR was absent in E. coli MTHFR (Fig. 4). This suggested that the catalytic domain of human MTHFR was located in the N terminus of the protein. To verify this, we constructed a plasmid (pNHMTFR) that expresses a truncated human MTHFR (residues 1–349). Yeast met11− cells carrying this plasmid were able to grow on galactose medium lacking methionine but not on glucose medium lacking methionine (see Fig. 5). Further analysis (Fig. 6) showed that these yeast cells expressing the truncated MTHFR grew slower than those expressing the full-length protein, suggesting that the truncated protein was less active or the protein level was low. We then determined MTHFR activity using extracts made from cells expressing truncated MTHFR. The result in Fig. 2 shows that the C-terminal truncated protein does have MTHFR activity but at a lower level compared with the full-length protein. Immunoblot analysis, shown in Fig. 3, indicated that cells expressing the C-terminal truncated MTHFR contain a significantly lower amount of protein, compared with cells expressing wild-type MTHFR. This result suggests that the truncated protein has lower stability, which might reflect a role of the C terminus in stabilizing the protein.

**DISCUSSION**

We have expressed and characterized wild-type and mutant human MTHFR in S. cerevisiae. The work is based on the fact that human MTHFR can functionally substitute for the yeast MET11 gene. Deletion of the chromosomal copy of MET11 results in a haploid yeast strain becoming a methionine auxotroph, due to the lack of MTHFR to produce 5-MeTHF for methionine biosynthesis. Expression of wild-type human MTHFR in met11 yeast cells restores MTHFR activity to the cells and complements the methionine auxotrophic phenotype. However, mutant alleles of human MTHFR with severely reduced enzyme activities cannot complement the growth phenotype of met11− yeast cells. Thus, the met11− strain, XSY3-1A, provides an experimental tool for functionally investigating the effect of mutations in human MTHFR and revealing the domain structure of the enzyme. For example, the catalytic domain of human MTHFR has been proposed to reside in the N terminus, based on structural comparison with the E. coli enzyme (33). By using the yeast system, we show that the N terminus, 50% of the protein, is capable of complementing the auxotrophic phenotype, confirming that the N-terminal domain
that T980C is a dominant-negative allele. When yeast extracts C677T in the patient's MTHFR. We have tested the possibility complicated by the presence of two other mutations (T980C and mutation alone may not lead to MTHFR deficiency in the rable thermal stability as that of wild type, indicating that this high levels of MTHFR activity. This variant also had compa-
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ald contains the catalytic site.
To characterize the effect of mutations in human MTHFR, six previously described mutations were engineered individu-
ally into wild-type MTHFR cDNA. These included four rare mutations G164C, G458T, T980C, and C1141T found in pa-
tients with severe MTHFR deficiency and two common poly-
morphisms, C677T and A1298C (12, 13). Mutation G164C was originally described in a patient who is a compound heterozy-
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tient has about 2% MTHFR activity of the control (11). Among the four suspected pathogenic alleles we tested, G164C, G458T, and T980C did not complement the methionine auxotrophic phenotype and show less than 7% MTHFR activity of the wild type expressed in yeast. These results are in agreement with their pathogenic effect on humans.
However, the C1141T allele did complement and displayed high levels of MTHFR activity. This variant also had compa-
rable thermal stability as that of wild type, indicating that this mutation alone may not lead to MTHFR deficiency in the patient. The cause of the MTHFR deficiency in the patient is complicated by the presence of two other mutations (T980C and C677T) in the patient's MTHFR. We have tested the possibility that T980C is a dominant-negative allele. When yeast extracts containing the gene products of T980C and wild-type MTHFR were mixed together at various ratios, no reduction of MTHFR activity was observed (data not shown). In addition, co-expression of wild-type and T980C in met11 cells did not result in any impaired growth of the strain, even when T980C was expressed at significantly higher levels than the wild type (data not shown). These results do not support the dominant negative hypothesis. However, it is possible that the T980C allele could interact specifically with C677T and/or C1141T to cause the detrimental effect on the MTHFR activity. This can be tested by our yeast system should the exact genotype (cis/trans arrangement of the mutations) of the patient become available. It is also conceivable that the C1141T alteration is only a rare benign polymorphism but is genetically linked to an undiscover-
ered mutation. The yeast system offers a powerful tool in an-
alyzing the contribution made by each single mutation toward the detrimental effect caused by a combination of mutations.

The common polymorphism C677T allele has been reported to be thermolabile and retains 40–50% enzyme activity at normal temperature (14). The point mutation causes an alanine to valine substitution. Both the heterozygous and the homozygous state of this mutation are associated with reduced specific activity (12, 34, 35). The corresponding mutation in E. coli MTHFR was shown to have reduced affinity for the cofactor FAD (33). We found that this allele was able to complement the methionine auxotrophic phenotype of the met11 strain. The enzyme activity of crude yeast extracts from cells expressing the C677T allele was about 50% of the wild-type level and was extremely thermolabile, as seen in human cells. Thus, our results are consistent with the clinical data that the C677T alteration has a modest effect on enzyme activity.

The A1298C is another commonly occurring mutation with an allele frequency of 33% (13). This mutation causes an amino acid substitution of a glutamate into an alanine. However, this mutation is not known to associate with either higher plasma homocysteine or a lower plasma folate concentration (13). Our data show that A1298C can functionally rescue the yeast growth phenotype, and the yeast extract containing A1298C allele gene product retains high levels of MTHFR activity. Unlike the C677T mutant, the A1298C has comparable thermal stability to that of the wild-type enzyme. Similar to C1141T, A1298C, by itself, does not cause obvious phenotype alterations in human and yeast, suggesting that A1298C is a benign polymorphism.

It is interesting that the two mutant alleles (C1141T and A1298C) are both located in the C-terminal domain. Based on the sequence similarity between human MTHFR and E. coli MTHFR (MetFp), a hypothesis stating that the N terminus contains the catalytic domain has been proposed by Guenther et al. (33). To verify this we have shown that expression of a C-terminal truncated human MTHFR in met11 yeast cells can complement the growth phenotype. In fact, the two alleles (C1141T and A1298C) with mutations located in the C terminus retain the ability to complement the growth phenotype of met11 cells as well. In addition, these two mutations do not result in thermolability of the enzyme, unlike the C677T mutation that is located in the N terminus of the protein. It is likely that the mutations in the C terminus do not produce as severe a phenotype as the N-terminal mutations because they do not affect the catalytic domain. Since E. coli MTHFR also requires FAD as a cofactor, the lack of the C-terminal domain in E. coli MTHFR argues that the C terminus of human MTHFR is not required for FAD binding.

It has been reported previously that porcine MTHFR can be easily cleaved with trypsin to produce two fragments of 36 and 39 kDa (6). This limited proteolysis abolishes the inhibitory
effect of AdoMet, but the enzyme activity remains unchanged suggesting that one of the domains functions as a regulatory region (6). Considering our data indicating that the N terminus is the catalytic domain, it is likely that the C terminus is the regulatory domain that allows AdoMet to carry out the inhibitory effect. The C terminus may also be involved in stabilizing the enzyme. We observed a major reduction of the amount of this protein, when the C-terminally truncated MTHFR was expressed in yeast. When *E. coli* MTHFR that naturally lacks the C terminus was expressed under the same conditions, the protein level was also low (data not shown). These results imply that the C-terminal domain could be important for the protein stabilization in eukaryotes.

Interestingly, *met 11*<sup>−</sup> yeast cells expressing N-terminal MTHFR grew significantly slower than the parental *MET11* strain (W303-1A) although the enzyme activity of N terminus human MTHFR was only moderately less (78%) than that of Met11p according to our *in vitro* assay using crude yeast extracts. Unless this level of enzyme activity happens to be just below a threshold, the growth rate of cells seems to depend on more than just the reductase activity. This observation cannot be easily explained by the AdoMet regulation or protein stabilization. The growth rate discrepancy of the two strains suggests that the C terminus may be involved in other regulatory functions in *vivo*.

In conclusion, we have shown that yeast *MET11* is a functional homologue of human *MTHFR*. The *met 11*<sup>−</sup> yeast strain serves as model system for characterizing the effects of sequence alterations in the human MTHFR cDNA and understanding the domain structure of the enzyme. Our findings should be helpful in understanding the molecular basis of MTHFR deficiency and the domain structure of the enzyme; this in turn could improve the strategy for treating the disorder.

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