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Clustering of Mutations in Methylmalonyl CoA Mutase Associated with *mut*$^-$ Methylmalonic Acidemia

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Summary

Mutations have been described in human methylmalonyl CoA mutase (MCM) that exhibit partial defects in enzyme activity, including cobalamin-dependent (i.e., *mut*$^-$) or interallelic complementation. This work describes mutations in cells from four patients, three of whom exhibit a cobalamin-dependent phenotype and all four of whom exhibit interallelic complementation. Four novel mutations (R694W, G648D, G630E, and G626C) are identified that cluster near the carboxyl terminus of the protein, a region close to another *mut*$^-$ mutation (G717V). Each of these mutations was shown to express a phenotype congruent with that of the parental cell line, after transfection into *mut*^{0} fibroblasts, and each exhibits interallelic complementation in cotransfection assays with clones bearing a R93H mutation. The activity of mutant enzymes expressed in *Saccharomyces cerevisiae* parallels the residual activity of the parental cell lines and exhibits novel sensitivities to pH and salt. The clustering of these mutations identifies a region of MCM that most likely represents the cobalamin-binding domain. The location of this domain, as well as the pattern of sequence preservation between the homologous human and *Probionobacterium shermanii* enzymes, suggests a mechanism for interallelic complementation in which the cobalamin-binding defect is complemented in *trans* from the heterologous subunits of the dimer.

Introduction

A variety of naturally occurring mutations in human methylmalonyl CoA mutase (MCM; E.C.5.4.99.2) have provided insights into the genetics and function of this enzyme. MCM is a mitochondrial matrix enzyme and is a homodimer of 78,489-dalton subunits that requires adenosylcobalamin (ado-cbl) as a cofactor (Retey 1982). Inherited deficiency of this enzyme causes a disorder of organic acid metabolism, termed "*mut* methylmalonic acidemia" (*mut* MMA; McKusick 251000) (McKusick 1990). Two classes of mutations in MCM are classically distinguished by studies of [^{14}C]-propionate metabolism in primary fibroblasts from patients with *mut* MMA (Rosenberg and Fenton 1989; Ledley 1990). *mut*^{0} mutations are those in which there is no detectable MCM activity and no detectable [^{14}C]-propionate metabolism in cultured cells. *mut*$^-$ mutations are those in which there is a low level of both residual enzyme activity and [^{14}C]-propionate incorporation. Commonly, this residual activity can be stimulated in cultured cells by administration of high concentrations of hydroxocobalamin (Willard and Rosenberg 1977, 1979*a*, 1979*b*). Studies of the enzyme activity in *mut*$^-$ fibroblasts suggest that MCM in these cells exhibits an increased $Km_{\text{ado-cbl}}$ (Willard and Rosenberg 1977, 1979*a*, 1979*b*).

A variety of mutations in MCM have been discovered by molecular cloning of cDNAs from primary fibroblasts. Initial studies demonstrated that 20%–30% of alleles associated with *mut*^{0} forms of the disease produced abnormally low levels of MCM mRNA, indicative of a primary defect in mRNA transcription or processing (Ledley et al. 1990*a*). Several different *mut*^{0} mutations have been described in the amino-terminal half of the protein, and gene-transfer studies using clones bearing these mutations have demonstrated that each of these changes eliminates enzyme activity (Jansen and Ledley 1990; Ledley et al. 1990*a*). The initial cloning of cDNAs from cells with the *mut*$^-$ phenotype revealed that cells from three patients (DS79, TS87, and SO82) had identical mutations (G717V) near the carboxyl terminus of the protein (Crane et al. 1992*a*, 1992*b*). Gene transfer of clones with the G717V mutation demonstrated that this mutation expressed a *mut*$^-$ phenotype with activity stimulated by high concentrations of cobalamin in culture cells. The enzyme containing the G717V mutation was overexpressed in *Saccharomyces cerevisiae* and was shown to exhibit a $Km_{\text{ado-cbl}}$ >1,000-fold higher than normal (Crane et al. 1992*a*).

Recently, somatic cell hybrid studies have identified additional classes of mutations in *mut* MMA that exhibit interallelic complementation (Raff et al. 1991). The prototype cell line in this class, WG1130, exhibits a *mut*^{0} phenotype with no detectable enzyme activity. Fusion of this cell with some other *mut*^{0} cells or with most *mut*$^-$ cells

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results in a significant restoration of [^{14}C]-propionate metabolism (Raff et al. 1991). The mutation in the WG1130 cell line (R93H) has been identified. Gene-transfer studies demonstrate that clones with the R93H mutation exhibit no activity when transfected into cells lacking MCM mRNA but that they do exhibit activity when transfected into cells known to complement WG1130 in somatic-cell fusion experiments (Raff et al. 1991; D. Rosenblatt, personal communication). In addition, cotransfection of clones bearing the G717V mutation together with clones bearing the R93H mutation produces an increased level of enzyme activity, demonstrating that the residual functions of these two mutant gene proteins can together constitute catalytic function of the enzyme (Crane et al. 1992*a*).

To investigate the nature of the defects exhibiting the cobalamin-responsive, *mut*⁻ phenotype as well as the phenomenon of interallelic complementation, we have cloned cDNAs from a series of cells that exhibit these properties. Three of the cells used in this study exhibit a *mut*⁻ phenotype. One exhibits a *mut*^{0} phenotype. All four exhibit interallelic complementation with clones bearing the R93H mutation. These studies identify a cluster of mutations that constitute a putative cobalamin-binding domain near the carboxyl end of MCM.

Material and Methods

Cells

Cells were provided to the laboratory from patients who had a clinical diagnosis of MMA. Complete clinical information was not available. GM1673 fibroblasts were obtained from the NIGMS Mutant Cell Repository. These cells express a *mut*^{0} phenotype with decreased mRNA and no detectable enzyme activity or [^{14}C]-propionate incorporation (Ledley et al. 1990*a*). The DS97 cell line exhibits a prototype *mut*⁻ phenotype (Crane et al. 1992*a*, 1992*b*). The MAS cell line is a compound heterozygote exhibiting a *mut*^{0} phenotype (Jansen and Ledley 1990). The phenotypes of STI, CHO, MM87, and REG fibroblasts have been partially described elsewhere (Ledley et al. 1990*a*).

Cells were determined to have a *mut* phenotype by using the DNA-mediated complementation assay described elsewhere (Wilkemeyer et al. 1991). Discrimination of *mut*− and *mut*^{0} phenotypes was made as described elsewhere (Ledley et al. 1990*a*). In brief, propionate incorporation was determined in the presence of varying concentrations of hydroxycobalamin that were 0–1 μg/ml. Regression analysis was performed. Cells in which there were a statistically significant correlation coefficient (R^2; $P<.01$) and a positive slope were considered to have a *mut*− phenotype. Cells in which there was no correlation between hydroxycobalamin and propionate incorporation were considered to have a *mut*^{0} phenotype. Complementation groups and phenotypes were confirmed independently by Dr. D. Rosenblatt (McGill University).

Cloning and Identification of Mutations in MCM cDNA

Total RNA was obtained using RNAzolII (Biotech Lab) (Chomczynski and Sacchi 1987) and was treated with DNAase, transcribed with avian reverse transcriptase (RT), and amplified by PCR (fig. 1), providing two overlapping fragments of the cDNA (Jansen and Ledley 1990; Crane et al. 1992*a*). The 5′ and 3′ ends of the gene were independently subcloned for sequencing (Jansen and Ledley 1990). Sequencing was performed on clonal isolates, as well as on pools of ≥15 independent clones, to confirm the authenticity of sequence variations and to eliminate PCR mistakes. Oligonucleotide sequences for cloning and sequencing have been described by Jansen and Ledley (1990).

Analysis of Genomic Sequences by Amplification and Allele-specific Probes

The structure of the MUT locus and the sequences of exon/intron boundaries have been described elsewhere (Nham et al. 1990; Crane et al. 1992*a*). Genomic DNA was purified (Sambrook et al. 1989), and exon/intron boundaries were amplified by PCR (fig. 2) (Scharf et al. 1986; Saiki et al. 1988). Allele-specific probes were synthesized to selectively recognize either normal or mutant sequences shown in table 1 (Kriegler 1990; Crane et al. 1992*b*). Oligonucleotide sequences are given in the legend to figure 2.

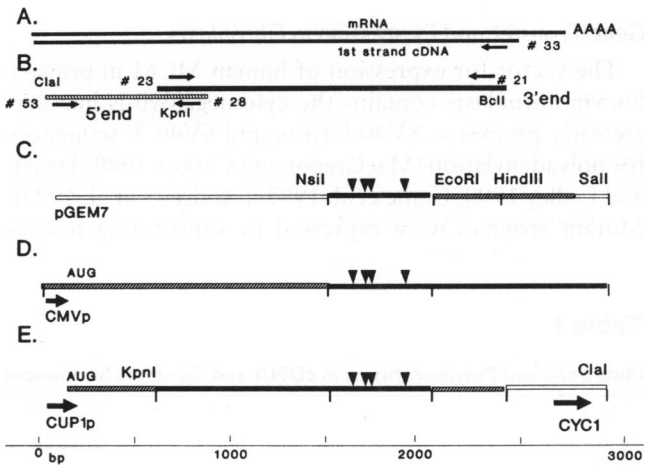

Figure 1 Strategy for cloning and subcloning in fibroblasts and yeast. First-strand MCM cDNA was synthesized by reverse transcription with oligonucleotide 33 (*A*). This product was used in two PCR reactions: one to amplify the 5′ end of the cDNA and the other to amplify the 3′ end of the cDNA. The PCR products were subcloned into the *Cla*I/*Kpn*I or *Kpn*I/*Bam*HI sites of the vector pGEM7zf(+) (*B*) and were sequenced as described elsewhere. The positions of identified mutations are identified by arrows. Restriction fragments containing a single point mutation were subcloned into intermediary MCM 3′ end subclone (*C*) and, later, into either normal eukaryotic expression vectors pCMV-hMCM (*D*) (*Nsi*I/*Eco*RI fragment) or the normal *Saccharomyces cerevisiae* expression vector pYEPV-hMCM (*E*) (*Kpn*I/*Eco*RI fragment), to study the effect of the putative mutations.

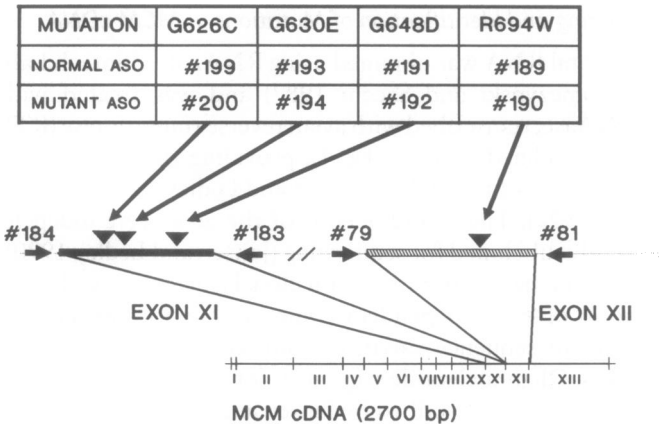

Figure 2 Identification of mutations and polymorphisms in genomic DNA. Genomic DNA containing exon 11 of the MUT locus was amplified by PCR using oligonucleotides within the flanking introns to produce an amplified fragment of 250 bp. Genomic DNA containing exon 11 was amplified by PCR using oligonucleotides within the flanking introns to produce a 181-bp fragment. The PCR products were analyzed by hybridization with specific oligonucleotides corresponding to each of the normal sequences. Oligonucleotide sequences are as follows: 184, TGCTTTTAATAACTTGAAGATTTGC; 183, GCAAGTCAGAGGCTACATACCAGTT; 79, CTTTCTGACTGTTTAGACTCCTC; 81, CTCAAGATTCCCATCACAGTACTAG; 191, GACATAGGCCCTCTTT; 193, TGACAGAGGAGCAAAAGT; 199, CAAGATGGCCATGAC; 189, CCTTGGACGGCCAGAT; 192, TGGACATAGACCCTCTT 194, TGACAGAGAAGCAAAAGTT; 200, ACAAGATTGCCATGAC; and 190, CCTTGGATGGCCAGAT.

Gene Transfer and Expression in Fibroblasts

The vector for expression of human MCM in primary human fibroblasts contains the cytomegalovirus immediate early promoter, SV40 intron, and SV40 3′ sequences for polyadenylation (MacGregor and Caskey 1989; Jansen and Ledley 1990; Crane et al. 1992*a*; Andrews et al. 1993). Mutant enzymes were expressed by substituting restriction fragments from clones containing a single point mutation into the normal vector. The *Nsi*I-*Eco*RI fragment from clones containing the R694W, G648D, G630E, or G626C mutations were cloned into the *Nsi*I/*Eco*RI sites of a vector containing a *Nsi*I-*Sal*I fragment from the 3′ end of the normal cDNA (Raff et al. 1991; Crane et al. 1992*a*). The *Nsi*I-*Sal*I segments from these clones were substituted for the normal sequence in the vector pCMV-hMCM (fig. 1). The integrity of cloning sites and the insert was confirmed by restriction mapping and bidirectional sequencing.

Fibroblasts were cultured in MEM (JRH Bioscience) with 20% fetal bovine serum. Electroporation was performed using conditions described elsewhere (Wilkemeyer et al. 1991; Crane et al. 1992*a*). [^{14}C]-propionate metabolism was assayed as the incorporation of [^{14}C]-propionate into TCA-precipitable material, with incorporation of [^{3}H]-leucine as a control (Ledley et al. 1990*a*; Crane et al. 1992*a*). Cotransfection was performed with equal concentrations of two plasmids. [^{14}C]-propionate incorporation is measured in the presence of either media alone or media containing 1 μg hydroxocobalamin/ml (Sigma). All experiments are performed in triplicate, with results expressed as the mean number of nanomoles of propionate incorporated per micromole of leucine incorporated per 18 h at 37°C.

Expression and Analysis of Gene Product Expressed in Saccharomyces cerevisiae

A vector for expression of normal MCM in *S. cerevisiae* has been described elsewhere (Andrews et al. 1993). This plasmid contains the human MCM open reading frame without the human 5′ or 3′ untranslated regions, the CUP1p promoter, 2μ origin of replication, and TRP-1 gene for tryptophan selection (McDonnell et al. 1989). Mutant clones were constructed by replacing the *Kpn*I/

Table 1

Mutations and Polymorphisms in cDNA and Genomic Sequences

			cDNA					
	Mutation			Polymorphism			Genomic	
Cell Line	Base	Amino Acid	Mutation	712	1671	2087	Mutation	*Hin*dIII
DS79	g2226t	G717V	+/+	+/+	+/+	+/+	+/−	+/+
CHO	g2019a	G648D	+/+	+/+	+/+	+/+	+/−	+/+
REG	g1965a	G630E	+/+	−/−	−/−	−/−	+/+	−/−
STI	g1952t	G626C	+/+	−/−	−/−	−/−	+/−	−/−
MM87	c2156t	R694W	+/+	+/+	+/+	+/+	+/+	+/+

Note.—Mutations and polymorphisms were identified in cDNA and genomic material after PCR amplification using allele-specific probes or restriction-enzyme digests. No evidence of heterozygosity was present in the cDNA of any cell line. Heterozygosity was evident in genomic sequences, indicating that one allele did not express amplifiable transcripts. Polymorphic sequences are those that have been described elsewhere, including 712—a712g (*Hin*dIII RFLP), silent change; 1671—a1671g, H532R; and 2087—g2087a, V671I.

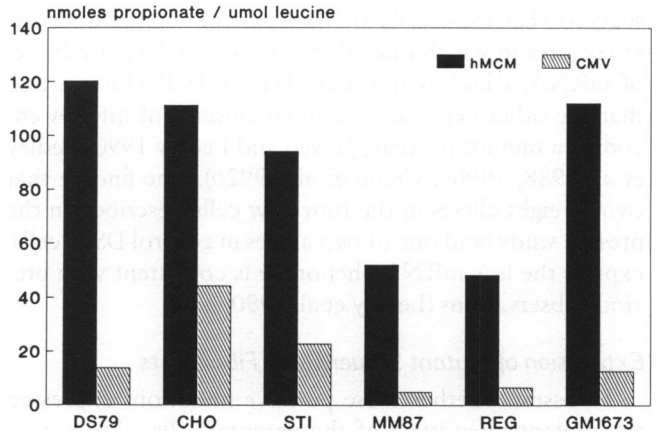

Figure 3 Diagnosis of *mut* MMA in fibroblasts, by gene transfer. Fibroblasts were electroporated with the pCMV-hMCM vector, which expresses normal MCM. The restoration of [^{14}C]-propionate incorporation by gene transfer indicates that the defect involves the apoenzyme rather than enzymes required for provision of the cobalamin cofactor. Data are shown for the four fibroblasts described in this report, for a control *mut*^{0} cell GM1673, and for a prototype *mut*$^-$ cell DS79. Blackened bars represent activity in cells transfected with the normal human MCM cDNA expression vector; and hatched bars represent cells transfected with an identical control vector lacking the MCM insert.

*Eco*RI of the normal sequence by an equivalent fragment of clones carrying R694W, G648D, G630E, or G626C (fig. 1). The integrity of cloning sites and the insert was confirmed by restriction mapping and bidirectional sequencing.

Clones were introduced into the proteinase-deficient *S. cerevisiae* strain BJ3505 (TRP-1$^-$, URA-3$^-$), and expression was induced with 200 μM $CuSO_4$ for 7 h (Crane et al. 1992*a*). Enzyme was assayed in cell extracts by using the potassium permanganate/perchloric acid (Kolhouse et al. 1988). All experiments were performed in triplicate, and results are expressed as the mean number of nanomoles of succinate formed per milligram of protein per hour in yeast cell extracts. Western blotting of yeast cell extracts was performed using rabbit antibodies against synthetic MCM peptide (Andrews et al. 1993), with standard detection methods using alkaline phosphatase–coupled anti-rabbit IgG. Protein was quantified using the Bradford assay (Bio Rad).

Results

Phenotype of the Parental mut$^-$ *and* mut^0 *Cells*

Fibroblasts from four patients that exhibited defective [^{14}C]-propionate incorporation were selected for analysis. These cell lines and mutations, which were identified in the course of this study, are listed in table 1. The *mut* phenotype was confirmed by gene transfer of a vector encoding the normal MCM cDNA, which resulted in a restoration of [^{14}C]-propionate incorporation (fig. 3) (Wilkemeyer et al. 1991). This indicated that these cells had a defect in the MCM apoenzyme rather than in the enzymes required for cobalamin metabolism (*cbl* defects).

To determine whether these fibroblasts exhibited a *mut*^{0} or *mut*$^-$ phenotype, [^{14}C]-propionate incorporation was measured in the presence of basal cobalamin and in the presence of 1 μg hydroxocobalamin/ml (fig. 4*A*). CHO, STI, and MM87 fibroblasts exhibited statistically significant increases in [^{14}C]-propionate incorporation in the presence of increasing concentrations hydroxocobalamin up to 1 μg/ml, by criteria described elsewhere (Ledley et al. 1991). This is characteristic of a *mut*$^-$ defect and is similar to that observed with the prototype *mut*$^-$ DS79 cell line (Crane et al. 1992*a*). Significantly, the magnitude of the response to hydroxocobalamin by different cell lines varied considerably. Cobalamin treatment of CHO increased [^{14}C]-propionate incorporation almost to normal levels. Cobalamin treatment of STI and DS79 produced a consistent but significantly smaller response. Cobalamin treatment of MM87 produced only a minimal, though statistically significant, response. REG and GM1673 cells exhibited no increase in [^{14}C]-propionate incorporation, which is characteristic of a *mut*^{0} defect.

Each of these fibroblasts has previously been reported to exhibit interallelic complementation in somatic cell hybrids with WG1130 (Raff et al. 1991; D. Rosenblatt, personal communication). To confirm this complementation, clones expressing mutant MCM with the R93H mutation

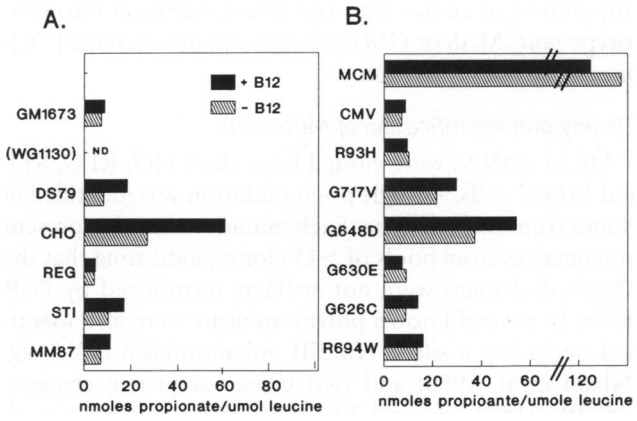

Figure 4 Cobalamin responsiveness of MUT fibroblasts and enzyme expressed from mutant clones. *A*, Characterization of parental cells. Three of the four cells described in this report (CHO, STI, and MM87) exhibit low levels of [^{14}C]-propionate incorporation, which is stimulated by the addition of hydroxocobalamin (1 μg/ml), similar to the activity in the prototype *mut*$^-$ DS79 cells. REG cells exhibit no response to cobalamin, which is similar to the situation in prototype *mut*^{0} GM1673 cells. *B*, Characteristics of activity expressed by mutant clones. Clones bearing the mutations identified in CHO, STI, and MM87 electroporated into the GM1673 cell line imparted the cobalamin-responsive phenotype of [^{14}C]-propionate incorporation characteristic of the parental cell. Clones bearing the mutations from REG express no activity. Data show incorporation of [^{14}C]-propionate into TCA-precipitable material.

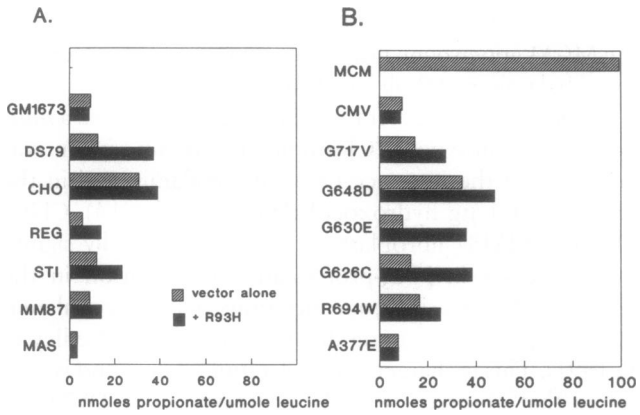

Figure 5 Interallelic complementation between clones with R93H mutation, parental cells, and mutations identified in these cells. *A*, Electroporation of the DS79, CHO, STI, and MM87 cells with clones bearing the R93H mutation, which leads to a partial reconstitution of [^{14}C]-propionate incorporation. Controls include electroporation into the prototype *mut*^{0} cells, GM1673 and MAS, which do not exhibit interallelic complementation. *B*, Cotransfection of clones bearing R93H, with clones containing mutations identified in parental cells, which produces a similar pattern of interallelic complementation. Controls include transfection of R93H with normal MCM, with the empty expression vector, and with clones having the A377E identified in MAS cells.

(Raff et al. 1991) were electroporated into each cell line. Electroporation resulted in an increase in [^{14}C]-propionate incorporation in the CHO, REG, STI, and MM87 cells that was similar to that seen in identical experiments performed with the prototype *mut*$^-$ DS79 cells (fig. 5*A*). Electroporation of clones with the R93H mutation into prototype *mut*^{0} MAS or GM1673 cells did not increase [^{14}C]-propionate incorporation.

Cloning and Identification of Mutations

MCM cDNAs were cloned from the CHO, REG, STI, and MM87 cells. A single point mutation was identified in clones from each cell line. Each mutation was also present in sequences from pools of >15 clones, indicating that the observed changes were not artifacts introduced by PCR (table 1). Several known polymorphisms were also identified, including a silent *Hin*dIII polymorphism (a712→g) (Nham et al. 1990) and two benign sequence changes, H532R (a1671→g) and V671I (g2087→a) (Crane et al. 1992*a*) (table 1). Each cell contained only a single species of mRNA, as indicated by the uniform presence or absence of both a single mutation and each of the three known polymorphisms (table 1).

To determine whether the parental cells were homozygous for the identified mutation or were compound heterozygous, genomic sequences were amplified by PCR and were analyzed using allele-specific probes (Crane et al. 1992*b*) and RFLPs (Nham et al. 1990). This analysis demonstrated that DS79, CHO, and STI cells were heterozygous at the site of the identified mutation (table 1). This suggests that these cells are derived from compound heterozygotes in which one allele expresses a decreased level of mRNA, which is not identified by PCR cloning, and that the other expresses a normal amount of mRNA encoding a mutant protein (Jansen and Ledley 1990; Ledley et al. 1988, 1990*b*; Crane et al. 1992*b*). The finding that two of eight alleles in the four new cells described in the present study (and one of two alleles in control DS79 cells) express the low mRNA phenotype is consistent with previous observations (Ledley et al. 1990*a*).

Expression of Mutant Sequences in Fibroblasts

To assess whether these putative mutations expressed the mutant phenotype of the parental cells, clones containing the mutant sequences were constructed and electroporated into *mut*^{0} fibroblasts (GM1673) (fig. 4*B*). Electroporation of clones containing the G648D mutation resulted in increased levels of [^{14}C]-propionate incorporation, which could be further increased by the addition of 1μg hydroxocobalamin/ml. It is interesting that the parental cell line containing this mutation (CHO) exhibited the highest level of residual activity and greatest stimulation with cobalamin. Electroporation of clones bearing the G630E mutation resulted in no increase in [^{14}C]-propionate incorporation and in no response to hydroxocobalamin. This *mut*^{0} phenotype is congruent with the phenotype of the parental cell (REG). Electroporation of clones bearing G626C or R694W mutations resulted in intermediate phenotypes in which there was no increase in [^{14}C]-propionate incorporation in the presence of basal concentrations of cobalamin but in which there was increased [^{14}C]-propionate incorporation in the presence of 1 μg hydroxocobalamin/ml. These phenotypes are also congruent with the characteristics of the parental cells (STI and MM87). Control experiments demonstrated that electroporation with clones expressing normal MCM restored [^{14}C]-propionate incorporation to normal levels (Wilkemeyer et al. 1993), that electroporation with either the empty vector CMV or clones containing R93H produced no increase in [^{14}C]-propionate activity, and that clones bearing the prototype *mut*$^-$ mutation G717V produced a *mut*$^-$ phenotype in which [^{14}C]-propionate incorporation was stimulated by hydroxocobalamin (fig. 4*B*) (Crane et al. 1992*a*).

To assess whether clones bearing these mutations exhibited interallelic complementation, cotransfections were performed with clones bearing the R93H mutation and with clones containing the novel mutations (fig. 5*B*). Cotransfection of clones bearing R93H, either with the CMV vector alone (CMV) or with clones bearing A377E (a prototype *mut*^{0} mutation from a cell line that does not complement with WG1130; Jansen and Ledley 1990), produced no increase in [^{14}C]-propionate incorporation, indicating that there was no interallelic complementation. Cotransfection of clones bearing R93H, with clones bearing

G648D, G630E, G626C, R694W or with the prototype *mut*– mutation G717V, produced significant stimulation of [^{14}C]-propionate incorporation, similar to that seen when clones bearing the R93H mutation were introduced into the parental cells.

Overexpression of Mutant Enzymes in Saccharomyces cerevisiae

The mutant enzymes were expressed *S. cerevisiae* for enzymatic studies. Western blotting of cell extracts demonstrated the expression of immunoreactive protein from each of the *mut*⁻ clones (G646D, G717V, G626C, and R6942W); from one of the *mut*^{0} clones, which exhibits interallelic complementation 3-14 (G630E); but not from another *mut*^{0} clone, which does not exhibit interallelic complementation (A377E) (fig. 6). The concentration of immunoreactive protein varied between preparations. All enzyme assays were performed using extracts in which the amount of immunoreactive MCM was higher than that in normal human liver.

No MCM activity is evident in extracts of either nontransformed *S. cerevisiae* or *S. cerevisiae* transformed with vector alone as described elsewhere (Ledley et al. 1991). No MCM activity is evident in extracts expressing the normal human MCM when they are assayed without exogenous ado-cbl. This is consistent with the previous observations that *S. cerevisiae* does not have endogenous MCM activity and that all of the recombinant enzyme produced in these organisms is apoenzyme (Andrews et al. 1993).

Assays for MCM activity in extracts expressing the normal human MCM revealed levels of enzyme activity 3–10 times higher than those in human liver extracts. No MCM activity was detectable in extracts from yeast transformed with any of the mutant clones assayed in the presence of 63 μM ado-cbl when standard reaction conditions were used (Kolhouse et al. 1988). To assess the cobalamin responsiveness, assays were performed with 1,260 μM ado-cbl in the presence of varying reaction conditions.

Enzymes bearing the G648D mutation were found to express high levels of enzyme activity with an apparent $Km_{\text{ado-cbl}}$ of ~25–30 μM when assayed at acid (pH 5.5) or neutral (7.5) pH values. When the *Km* was determined at basic pH (9.5), the apparent $Km_{\text{ado-cbl}}$ was ~200 μM. Significantly, there was no change in the apparent *Km* of the normal enzyme assayed under identical conditions with pH 5.5–9.5 (fig. 7). No significant activity was observed in extracts containing the other mutants, at either pH.

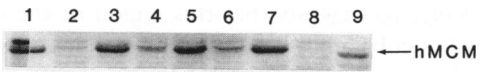

Figure 6 Immunoreactive MCM produced in *Saccharomyces cerevisiae*. Western blotting was performed on crude extracts of yeast expressing MCM with various mutations. Lane 1, Normal pYEPV-hMCM. Lane 2, Vector alone. Lane 3, G648D (*mut*⁻). Lane 4, G717V (*mut*⁻). Lane 5, G626C (*mut*⁻). Lane 6, R694W (*mut*⁻). Lane 7, G630E (*mut*^{0}). Lane 8, A377E (*mut*^{0}). Lane 9, Mouse liver. The two bands represent propeptide and mature enzyme.

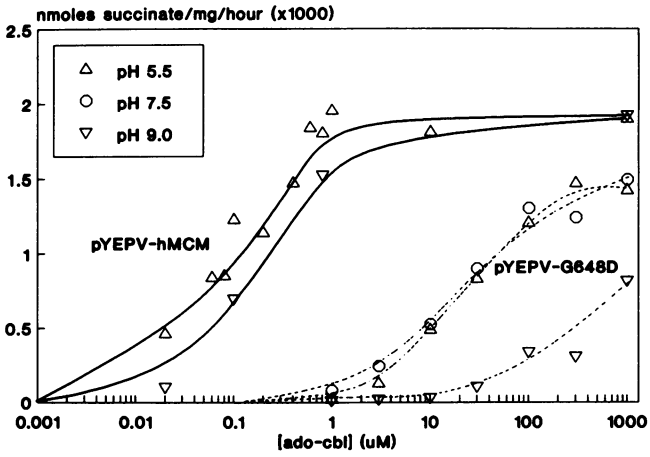

Figure 7 MCM activity in enzyme produced in *Saccharomyces cerevisiae*. MCM activity was assayed in the presence of different concentrations of ado-cbl and at different pH values. Activity is shown for extracts containing a normal MCM (i.e., pYEPV-hMCM) and enzyme bearing the G648D mutation.

The conditions of the enzyme assay were modified, in an effort to detect activity of the other mutant clones. Enzymes bearing the G626C mutation exhibited increased activity when assayed in the presence of 500 mM NaCl and 1,000 μM ado-cbl (table 2). Significantly, both the normal enzyme and enzymes bearing the G648D mutation were almost completely inhibited by NaCl at this concentration in parallel experiments. Enzymes bearing the R694W mutation were found to exhibit low-level activity when assayed in the presence of 1 mM β-mercaptoethanol and 1,000 μM adenosylcobalamin. Minor stimulation of enzyme activity was observed with the normal clone, as well as with clones bearing G626C and G648D, in parallel experiments (table 2). None of the conditions tested produced detectable activity of clones bearing the G630E mutation, which is derived from the *mut*^{0} cell line. The level of sensitivity of these assays would detect residual activity at levels 1%–2% of those in normal human liver.

Discussion

This study was undertaken to characterize the spectrum of mutations that are associated with defective, but not absent, MCM enzyme activity. Fibroblasts from four patients with *mut* MMA were selected for this study. These cells exhibited a range of phenotypes. CHO, STI, and MM87 cells exhibited the cobalamin responsiveness characteristic of the *mut*⁻ phenotype (Morrow et al. 1969; Willard et al. 1976; Willard and Rosenberg 1977, 1980*a*, 1980*b*). REG cells exhibited a characteristic *mut*^{0} phenotype. All four of these cells exhibited interallelic comple-

Table 2

MCM Activity in Extracts Containing Mutant Enzymes

Mutation	Reaction Conditions[a] (nmol/mg/h)		
	Normal	1 mM β-ME	500 mM NaCl
None	2,060	2,299	20
G648D	1,291	1,478	0
G630E	15	15	14
G626C	13	46	205
R694W	0	90	0
Vector	0	0	0

[a] MCM was assayed with different additives to the reaction to stimulate enzyme activity. All experiments were performed in the presence of 1,000 μM adenosylcobalamin. βME = β-mercaptoethanol.

mentation after either fusion with WG1130 cells or electroporation with clones having the R93H mutation.

A single missense mutation was identified in each of the four fibroblasts, and gene-transfer studies demonstrate that enzymes containing each of these mutations expressed the phenotype characteristic of the parental cells. Clones bearing the G626C, G648D, or R694W mutations express a *mut*$^-$ phenotype and complement with clones bearing R93H in cotransfection assays. This phenotype is equivalent to that expressed by the prototype *mut*$^-$ mutation G717V (Crane et al. 1992*a*). In contrast, clones bearing the G630E mutation express a *mut*^{0} phenotype and interallelic complementation with R93H.

The phenotype of the mutant enzymes was further analyzed by overexpressing the mutant proteins in *S. cerevisiae*. Each of the mutations produced an enzyme with distinct characteristics. One mutation, G648D, expresses high levels of enzyme activity, despite having a $Km_{ado\text{-}cbl}$ >100 times higher than normal. Significantly, the $Km_{ado\text{-}cbl}$ is further increased at high pH (9.5), whereas the *Km* of the normal enzyme is not pH sensitive. This suggests that the specific chemical interactions between ado-cbl and amino acids in the mutant enzyme may be distinct from the interaction between ado-cbl and the normal enzyme. The two remaining *mut*$^-$ mutations, G626C and R694W, exhibited surprisingly low enzyme activity, which was detectable only by altering the reaction conditions. The observation that 500 mM NaCl stimulates activity of enzymes bearing G626C while inhibiting either the normal enzyme or the enzyme with G648D provides further evidence that the chemical interaction between ado-cbl and the mutant enzymes may be significantly different from that which occurs with the normal enzyme.

It is intriguing that the level of enzyme activity observed in yeast extracts containing similar amounts of immunoreactive protein correlates with the level of [^{14}C]-propionate activity in transfected cells, as well as with the level of activity in the parental cell line. Clones bearing the G648D mutation were capable of high levels of enzyme activity, approaching normal; clones bearing the G626C or R694W mutation exhibited intermediate levels of activity and [^{14}C]-propionate stimulation; and clones bearing G630E consistently exhibited a *mut*^{0} phenotype with no detectable activity. We have previously observed that the amount of MCM in normal fibroblasts is not rate limiting on [^{14}C]-propionate incorporation and that a linear relationship between the amount of enzyme activity and the rate of [^{14}C]-propionate incorporation exists only at low concentrations of the enzyme. The present data suggest that the extremely low levels of activity in the cultured cells are in the linear range of activity required for [^{14}C]-propionate incorporation.

It is significant that the four novel mutations described in this study, as well as the previously described prototype *mut*$^-$ G717V mutation, cluster within a 90-amino-acid-segment region of the protein, between residues 626 and 717, near carboxyl terminus. Four of these mutations express activity that is stimulated by high concentrations of cobalamin, and for two of these mutations (G717V and G648D) we have been able to demonstrate a $Km_{ado\text{-}cbl}$ >1,000-fold higher than normal. These mutations appear to represent the *mut*$^-$ phenotype described elsewhere (Willard and Rosenberg 1977, 1979*a*, 1979*b*). At least one of these mutations does not exhibit enzyme activity even in the presence of 1,000 μM ado-cbl and appears to represent an authentic *mut*^{0} phenotype. Nevertheless, the fact that each of these mutations exhibits interallelic complementation with R93H suggests that they are associated with similar functional defects in enzyme activity. This is true as well for the G630E mutation, which does not express cobalamin-dependent activity but which does express stable immunoreactive protein that is capable of expressing the same functions as do the other mutants in interallelic complementation.

Each of the mutations that are identified in this report disrupt residues that are preserved in the homologous MCM sequences from *Propionobacterium shermanii* (fig. 8) (Leadlay and Ledley 1990). Both the preservation of these residues through evolution and the fact that each of these substitutions interferes with enzyme activity suggest that these residues occur in a region that is critical for the structure or function of the enzyme. The demonstration that several of these mutations are associated with an abnormal $Km_{ado\text{-}cbl}$ suggests that this region of the enzyme constitutes a cobalamin-binding domain. This cobalamin-binding domain may be bounded by sequences proximal to H532R, where this nonconservative substitution has no effect on enzyme activity (Crane et al. 1992*a*), as well as by sequences between residues 546 and 547, where there is an insertion/deletion between the otherwise highly preserved mammalian and *P. shermanii* MUT B sequences. Evolutionary insertions or deletions in charged regions of-

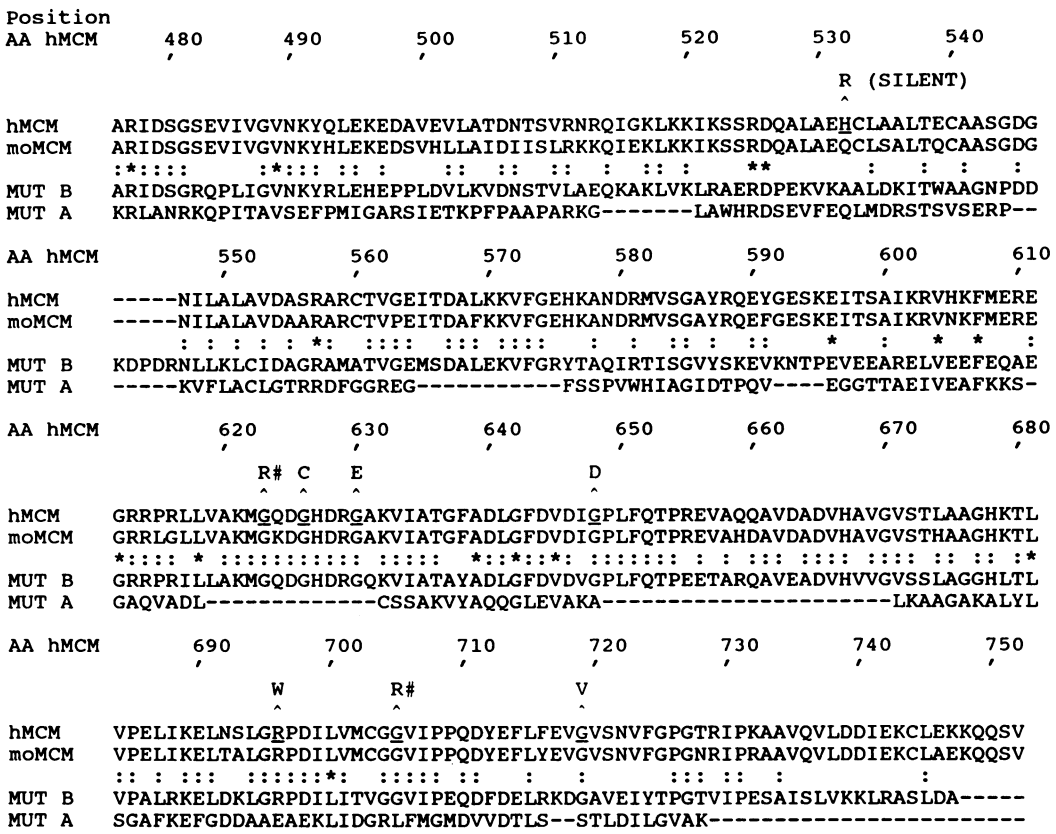

Figure 8 Amino acid alignment of eukaryotic and procaryotic MCM. Optimal alignment of the carboxyl-terminal end of human MCM (hMCM), murine MCM (moMCM), *Plasmodium shermanii* α subunit (MUT B), and *P. shermanii* β subunit (MUT A). The normal sequences of human and mouse MCM are shown in alignment with the sequences of *P. shermanii*. A caret (∧) denotes mutations identified in this report, with the mutant amino acid; a pound symbol (#) denotes mutations described by Qureshi et al. (in press); an asterisk (*) denotes amino acids identical in normal human, murine, and *P. shermanii* MUT A and MUT B sequences; and a colon (:) denotes amino acids identical in normal human and murine MCM and either MUT A or MUT B.

ten represent spacers between functional domains of a protein. It is significant that there is little sequence preservation between the MUT A chain of *P. shermanii* MCM and either the homologous MUT B or eukaryotic MCM sequences in this region of these proteins. The degeneracy of the MUT A sequence in this region may account for the different stoichiometry of ado-cbl binding, between the eukaryotic and prokaryotic enzymes. Eukaryotic MCM is a homodimer that will have two preserved binding domains and that binds 2 mol/homodimer. Prokaryotic MCM will have only one homologous domain and binds only 1 mol/MUT A–MUT B heterodimer (Cannata et al. 1965).

We have also identified both a cluster of natural mutations (R93H and W105R) and nonnatural mutation L153V at the amino-terminal end of the molecule, which interfere with enzyme activity, suggesting that there is another critical domain in this region. The function of this domain—and the mechanism by which R93H (but not the other mutations in this region) engages in interallelic complementation—remains to be elucidated in further studies. Ongoing efforts to characterize the tertiary structure of MCM (Marsh et al. 1989), together with further characterization of natural and site-specific synthetic mutants, may continue to elucidate the structure and function of this important enzyme.

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