Genetic Characterization of a MUT Locus Mutation Discriminating Heterogeneity in *mut*^{0} and *mut*$^-$ Methylmalonic Aciduria by Interallelic Complementation

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Abstract

Genetic complementation of fibroblasts from patients with methylmalonic aciduria (MMA) defines a unique class of allelic mutations arising from mutations at the locus encoding the methylmalonyl coenzyme A (CoA) mutase apoenzyme. Various phenotypes of MMA have been delineated including complete absence of enzyme activity (*mut*^{0}) and abnormal enzyme activity with an elevated K_m for adenosylcobalamin (*mut*$^-$). We describe genetic studies on a cell line (WG1130) from a patient with *mut*^{0} MMA which exhibited an unusual complementation phenotype, complementing with three of nine *mut*^{0} cell lines and four of five *mut*$^-$ cell lines. This suggests that interallelic complementation occurs between mutant alleles in WG1130 and subsets of alleles associated with both *mut*^{0} and *mut*$^-$ phenotypes. The methylmalonyl CoA mutase cDNA was cloned from WG1130 and found to contain a $G_{354} \rightarrow A$ ($Arg_{93} \rightarrow His$) mutation. Gene transfer of this mutant clone into primary fibroblasts from patients with MMA confirms that this mutation expresses a *mut*^{0} phenotype when transferred into a *mut*^{0} cell line with low levels of mRNA but can contribute to apoenzyme function when transferred into *mut* cell lines which show correction with WG1130 by somatic cell complementation. These results point to further heterogeneity within both *mut*^{0} and *mut*$^-$ and may enable identification of mutations affecting discrete components of apoenzyme function. (*J. Clin. Invest.* 1991. 87:203–207.). Key words: complementation • gene transfer • genetic heterogeneity • methylmalonic aciduria • vitamin B_{12}

Introduction

Methylmalonic aciduria may occur as a result of a block in the conversion of methylmalonyl coenzyme A (CoA) to succinyl CoA. Such a deficiency arises from either a defect in methylmalonyl CoA mutase (MCM),[1] the mitochondrial homodimeric enzyme that catalyses the reaction, or a defect in the pathway for the synthesis of 5′-deoxyadenosylcobalamin (AdoCbl), the Cbl-derived cofactor required by the MCM apoenzyme. Genetic complementation analysis of cultured fibroblasts from patients with methylmalonic aciduria without homocystinuria has defined three complementation classes that reflect the clinical response to Cbl supplementation: *mut*, which has a defective MCM apoenzyme; and *cbl*A and *cbl*B, which represent defects in the intramitochondrial synthesis of AdoCbl cofactor.

The *mut* class is divided into two subclasses: *mut*$^-$ lines have a MCM enzyme with detectable activity but exhibiting a greatly reduced affinity for the AdoCbl cofactor; *mut*^{0} lines show no detectable MCM activity even if AdoCbl is provided in excess. These two subclasses have never been observed to complement each another, suggesting that the mutations occur at the MUT locus (1). The recent cloning and sequencing of human MCM (2, 3) has enabled molecular characterization of mutations at this locus underlying the various phenotypes (4–6).

This investigation involved a primary fibroblast line, designated WG1130, obtained by skin biopsy from a patient with neonatal methylmalonic aciduria without homocystinuria who died at the age of 5 mo. The line exhibited a marked decrease in propionate (a precursor of methylmalonyl CoA) incorporation that was unresponsive to Cbl supplementation, suggesting a mutation in the MCM apoenzyme. Total Cbl distribution was within normal limits, also indicating a defect in the MCM apoenzyme.

To confirm the diagnosis of WG1130 as a member of the *mut* complementation class, cell-fusion complementation analysis was performed with WG1130 against a series of cultured fibroblast lines representing the *cbl*A, *cbl*B, and *mut* complementation classes. As expected, WG1130 complemented with the *cbl*A and *cbl*B cell lines. However, complementation was also observed with four out of five *mut*$^-$ lines and three out of nine *mut*^{0} lines. Such findings strongly suggest the possibility of interallelic complementation.

Methods

Materials. Cell culture media and fetal calf serum was obtained from Gibco Laboratories, Grand Island, NY; [1-^{14}C]propionate from New England Nuclear, Boston, MA; and polyethylene glycol 1000 from the S. T. Baker Chemical Co., Phillipsburg, NJ.

Cell culture. Skin fibroblasts were obtained from the proband with informed consent and designated WG1130. Fibroblasts from patients with documented *mut*^{0} and *mut*$^-$ mutations were available from the collections at Yale University, Baylor College of Medicine, and McGill University. The characteristics of cell lines from Yale University have been reported (1, 7, 8): WG1510 (Yale 418, *mut*^{0}); WG1511 (Yale 589, *mut*$^-$); WG1512 (Yale 663, *mut*^{0}); WG1599 (Yale 378, *mut*$^-$); WG1601 (Yale 438, *mut*^{0}); WG1602 (Yale 505). The characterization

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1. *Abbreviations used in this paper:* AdoCbl, 5′-deoxyadenosylcobalamin; CN-Cbl, cyanocobalamin; Cbl, cobalamin or vitamin B_{12}; CRM, cross-reacting material; OH-Cbl, hydroxocobalamin; MCM, methylmalonyl CoA mutase; MUT, locus encoding the gene for methylmalonyl CoA mutase; PCR, polymerase chain reaction.

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of enzyme, mRNA, and DNA of cells from Baylor College of Medicine have been reported (2, 4): WG1605 (Edg, *mut*^{0}); WG1607 (Mas, *mut*^{0}); WG1608 (Pou, *mut*^{0}); WG1609 (Reg, *mut*^{0}); WG1610 (TS87, *mut*$^-$); WG1611 (DS79, *mut*$^-$); WG1612 (GM50, *mut*^{0}); WG1613 (SO82, *mut*$^-$).

All strains were determined to be free of mycoplasma contamination. Cultures were maintained in Eagle's minimum essential medium with nonessential amino acids supplemented with 10% (vol/vol) fetal bovine serum. Cbl uptake and distribution and the whole cell incorporation of [^{14}C]methyltetrahydrofolate and [^{14}C]propionate were determined as previously described (9).

Cell fusion. Equal numbers of cells from two lines were mixed and plated into tissue culture dishes. 24 h after plating, one half of the cultures were exposed for 60 s to a 40% solution of polyethylene glycol 1000 in phosphate-buffered saline to induce cell fusion. After an additional 24 h, medium was replaced with Puck's F medium supplemented with 15% fetal bovine serum and 100 μM [1-^{14}C]propionate (to give a specific activity of 10 μCi/umol) and reincubated for 18 h. At the end of this period propionate incorporation was calculated as above (9). To determine whether complementation had occurred the incorporation of propionate from fused and unfused replicates were compared.

Cloning and expression of MCM cDNA. Total RNA was prepared from confluent fibroblasts and MCM cDNA was generated by primer extension and polymerase chain reaction (PCR) amplification as described (5, 6). The cDNA was amplified and subcloned into the vector pGEM7zf(+) (Promega Biotec, Madison, WI). Double-stranded dideoxy sequencing was performed using T7 polymerase (Pharmacia, Inc., Piscataway, NJ) with T7 (Promega Q5011), SP6 (Promega Q5121), or MCM-specific primers described in (6). The sequence of a clonal isolate of the MCM cDNA was compared to the sequence from a pool of 15 independent clones to distinguish authentic heterozygous or homozygous mutations from artifacts introduced by PCR.

Expression of recombinant MCM. A vector which expresses the normal consensus human MCM cDNA using the cytomegalovirus immediate early promoter in the vector pNAss-CMV (10) has been described (11). A clone containing the WG1130 mutation was constructed by PCR amplification of a region from the mutant cDNA spanning the *Msp*I site, the mutation site, and the *Kpn*I site, and recombining the *Msp*I/*Kpn*I fragment of this material with *Not*I/*Msp*I, *Kpn*I/*Nsi*I, and *Nsi*I/*Sal*I fragments in the *Not*I/*Sal*I–digested vector pNAss-CMV by sequential multipart ligations. The reconstitution of ligated sites was confirmed by restriction mapping and sequences generated by PCR were confirmed by sequencing. Clones were introduced via electroporation (12) into a *mut*^{0} primary fibroblast cell line GM1673 (National Institute of General Medical Services Mutant Cell Repository). Electroporation was performed using a Gene Pulser (BioRad Laboratories, Richmond, CA) after a 5-min preincubation of 0.6 ml of cells at a density of 3×10^6/ml with 20 μg of DNA using voltage pulses of 260 V at a capacitance of 960 μF. MCM activity was assayed 2 d after electroporation using a modified propionate incorporation assay which uses [^{3}H]leucine incorporation to normalize for cell number and constitutive protein synthesis (4). Results of this assay are expressed as the mean nanomoles of propionate per micromoles leucine incorporated in triplicate samples.

Results

Biochemical analysis of WG1130. Incorporation of [^{14}C]propionate into acid-precipitable macromolecules by WG1130 fibroblasts was decreased and not responsive to OH-Cbl in the culture medium: 0.57 nmol/mg protein per 18 h without OH-Cbl in the culture medium and 0.40 nmol/mg protein per 18 h with OH-Cbl in the culture medium (controls 10.8±3.7 and 10.9±3.5 nmol/mg protein per 18 h without and with OH-Cbl [n = 12, $\bar{x}$±SD]). Such a finding suggested that the defect was located in the MCM apoenzyme. The total accumulation and distribution of [^{57}Co]CN-Cbl in WG1130 fibroblasts after incubation in 25 pg/ml for 4 d was within the control range: 6.47 and 5.7 pg per 10^6 cells with 11.65% AdoCbl (controls: 4.58±2.0 pg per 10^6 cells with 15.3±4.2% AdoCbl [$\bar{x}$±SD, n = 12]). After incubation in 50 pg/ml [^{57}Co]CN-Cbl for 72 h, 6.8% of label was found bound to the MCM enzyme and 93.2% of label bound to methionine synthase after polyacrylamide gel electrophoresis as compared to 19.6% and 80.4%, respectively, in the controls. In vitro MCM activity was 5.3 nmol succinate formed/mg per h consistent with levels found for other *mut* cell lines [controls: 34.9±4.7 nmol/mg per h, $\bar{x}$±SD, n = 9] (4). MCM mRNA levels were ample and of normal size. In experiments performed by Dr. W. A. Fenton, Yale University, pulse-labeling with [^{35}S]methionine showed no difference in WG1130 MCM when compared with other *mut* cell lines. However, Western blot analysis with a MCM-specific antibody showed only 10% of the band intensity relative to a *mut*$^-$ cell line (WG1599).

Cell-fusion complementation of WG1130. To confirm the diagnosis of WG1130 as a *mut,* cell-fusion complementation analysis was performed with WG1130 against cultured cell lines representing *cbl*A (WG1191), *cbl*B (WG1185), and a *mut* line of the *mut*$^-$ subclass (WG1186). Positive complementation was demonstrated in all three cases by a severalfold increase in [^{14}C]propionate incorporation in the mixed and fused cultures relative to the mixed and unfused cultures. Self-fusion of WG1130 fibroblasts showed no stimulation of propionate uptake.

Further cell-fusion complementation analyses were performed with WG1130 to assess if complementation with *mut* would be consistent. Cell fusions were performed between WG1130 and a panel of fourteen different *mut* cell lines (five *mut*$^-$ lines and nine *mut*^{0} lines) each characterized either with respect to amount of cross-reacting material (CRM), as determined by radioimmunoassay, and newly synthesized protein (7, 8); or with respect to levels of hybridizable messenger RNA (2, 4). The data obtained, shown in Table I, demonstrate that WG1130 does not complement with six of nine *mut*^{0} cell lines nor with one of five *mut*$^-$ cell lines. As well, with the three *mut*^{0} lines which complement, the degree of complementation observed varies from a 10-fold increase in propionate uptake, seen with WG1609, to an increase of approximately 50% with WG1605. The increase with WG1601 is twofold.

The three *mut*^{0} cell lines that had demonstrated complementation with WG1130 were fused with each of the five *mut*$^-$ lines described above to see if further complementation within the *mut* complementation class might be observed. In no case was any suggestion of complementation noted (Table II).

Cloning and expression of MCM cDNA from WG1130. The MCM cDNA from WG1130 was sequenced and a single homozygous mutation ($G_{354} \rightarrow A$; $Arg_{93} \rightarrow$ His) was identified (Fig. 1 *A*).

In order to determine whether this sequence change was responsible for the phenotype of the WG1130 cells, a *Msp*I/*Kpn*I fragment containing this mutation was substituted into the expression vector containing the functional consensus MCM cDNA (pCMV-hMCM) to create the clone pCMV1130 (Fig. 1 *B*). The pCMV1130 clone was introduced into the *mut*^{0} cell line GM1673 (phenotypically equivalent to WG1612), the *mut*^{0} cell line WG1609, or the *mut*$^-$ cell line WG1611. Control experiments were performed with the normal MCM expression vector pCMV-hMCM, a vector containing the human MCM cDNA in an antisense orientation, or the vector without

Table I. Cell Fusion Complementation with WG1130

	Propionate uptake		
	Alone	+WG1130−PEG	+WG1130+PEG
	nmol/mg protein per 18 h		
mut° line			
WG 1510	0.36 (0.01)	0.90 (0.05)	0.66 (0.12)
WG 1512	0.30 (0.03)	0.38 (0.03)	0.38 (0.09)
	—	0.81 (0.14)	0.79 (0.04)
WG 1602	0.55 (0.02)	0.39 (0.03)	0.40 (0.13)
WG 1607	0.81 (0.12)	0.62 (0.03)	0.49 (0.15)
WG 1608	0.39 (0.02)	0.56 (0.01)	0.52 (0.05)
WG 1612	0.46 (0.05)	0.63 (0.02)	0.53 (0.08)
WG 1601	0.46 (0.04)	0.44 (0.03)	0.97 (0.10)
	—	0.40 (0.04)	0.84 (0.07)
WG 1605	0.49 (0.05)	0.45 (0.02)	0.60 (0.01)
	—	0.32 (0.01)	0.56 (0.02)
WG 1609	0.17 (0.01)	0.21 (0.01)	2.11 (0.29)
	—	0.37 (0.01)	3.98 (0.93)
mut$^-$ line			
WG 1511	0.58 (0.01)	0.55 (0.04)	3.10 (0.79)
	0.93 (0.04)	1.47 (0.71)	4.13 (0.09)
WG 1610	0.42 (0.02)	0.32 (0.01)	1.99 (0.46)
WG 1611	0.95 (0.02)	0.60 (0.05)	2.72 (0.25)
WG 1613	1.10 (0.09)	0.88 (0.07)	3.21 (0.55)
WG 1599	0.78 (0.01)	0.56 (0.02)	0.45 (0.04)
		0.38 (0.02)	0.43 (0.10)

Numbers in parentheses are the standard deviations of triplicate determinations.

insert (Fig. 2). Transformation of each cell line with the normal expression vector results in increased propionate incorporation, whereas transformation with the antisense vector or control vector results in no change of propionate incorporation. Transformation of the *mut*^{0} GM1673 cell line with the CMV1130 vector produced no increase in propionate incorporation. This demonstrates that the mutation accounts for the *mut*^{0} phenotype of the WG1130 cell line. In contrast, transformation of the *mut*^{0} cell line WG1609 or the *mut*$^-$ cell line WG1611 with the CMV1130 vector produced an increase in propionate incorporation. This is congruent with the complementation observed by cell fusion and demonstrates that the $Arg_{93} \rightarrow$ His mutation determines the characteristic phenotype of the WG1130 cell line.

The propionate incorporation assay is not a linear measure of apoenzyme activity. Other studies using MCM gene transfer indicate that the stimulation of propionate incorporation in the WG1611 and WG1609 cell lines after transformation with the CMV1130 vector corresponds to the level observed when < 0.5 μg of the normal MCM expression vector is electroporated into *mut*^{0} cells (M Wilkemeyer and F. D. Ledley, unpublished data). Thus the amount of MCM apoenzyme produced by complementation may be < 5% that in normal fibroblasts. This is consistent with the fact that we were unable to demonstrate an increase in apoenzyme activity in transformed cells using a relatively insensitive in vitro assay (data not shown).

Discussion

We have demonstrated an unusual pattern of complementation between a *mut*^{0} cell line (WG1130) and a panel of *mut* cell lines with various phenotypes of *mut* MMA. These studies demonstrate that fusion of WG1130, a *mut*^{0} cell line lacking any evidence of apoenzyme activity, with several *mut*$^-$ or *mut*^{0} cell lines which are similarly deficient in the MCM apoenzyme, produces hybrids with significant levels of propionate uptake activity.

Complementation between two *mut* cell lines has never previously been described. In fact, the singular nature of the MUT locus encoding the MCM apoenzyme was demonstrated by the presence of a discrete *mut* complementation group. This inference has been confirmed by molecular genetic studies which identified a single chromosomal locus (MUT) gene encoding the subunit of the MCM homodimer (13, 14). Since there is only a single MUT locus, constitution of MCM activity in the *mut* X WG1130 hybrids can only arise by interallelic (intragenic) complementation between the mutant allele in these cell lines.

Studies by Willard and Rosenberg (1) were able to delineate distinct classes of mutations within the MUT locus based on the phenotype of propionate incorporation in response to the addition of OH-Cbl to the culture medium. Cell lines with no

Table II. Cell Fusion Complementation among mut Lines

	Propionate uptake	
	+WG1601−PEG	+WG1601+PEG
	nmol/mg protein	
mut$^-$ line		
WG1511	0.59 (0.01)	0.47 (0.03)
WG1599	0.49 (0.04)	0.38 (0.04)
WG1610	0.49 (0.01)	0.39 (0.03)
WG1611	0.61 (0.03)	0.47 (0.09)
WG1613	0.63 (0.03)	0.50 (0.04)
	+WG1605−PEG	+WG1605+PEG
	nmol/mg protein	
mut$^-$ line		
WG1511	0.54 (0.01)	0.43 (0.04)
WG1599	0.48 (0.01)	0.41 (0.09)
WG1610	0.37 (0.01)	0.38 (0.04)
WG1611	0.57 (0.02)	0.45 (0.04)
WG1613	0.58 (0.02)	0.41 (0.05)
	+WG1609−PEG	+WG1609+PEG
	nmol/mg protein	
mut$^-$ line		
WG1511	0.46 (0.05)	0.33 (0.01)
WG1599	0.65 (0.03)	0.54 (0.06)
WG1610	0.27 (0.02)	0.24 (0.02)
WG1611	0.77 (0.06)	0.60 (0.03)
WG1613	0.41 (0.02)	0.36 (0.04)

Numbers in parentheses are the standard deviations of triplicate determinations.

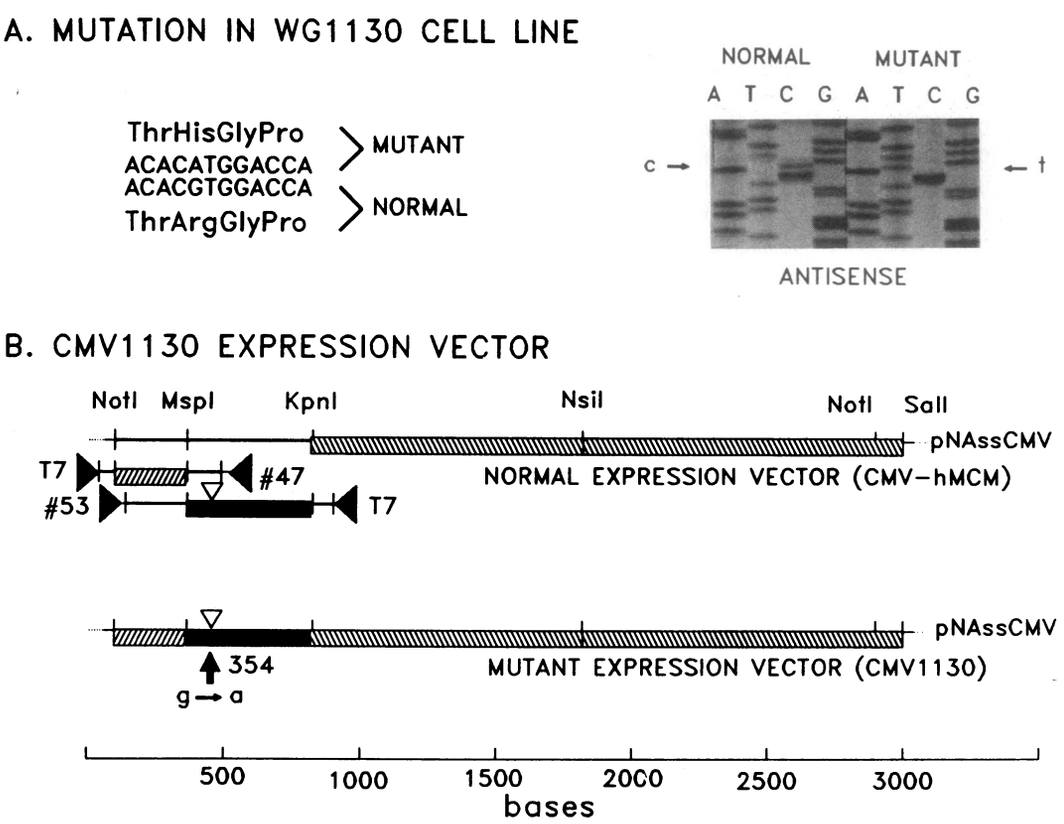

Figure 1. Identification of mutation in WG1130 cDNA and scheme for construction of expression vector. (*A*) A homozygous mutation $G_{354} \rightarrow A$ was identified by sequencing cloned cDNA. The sequencing gel shows the sequence in an antisense orientation. (*B*) The KpnI/NsiI and NsiI/SalI fragments derived from the normal consensus MCM expression vector (CMV-hMCM) were combined by sequential multipart ligations with a NotI/MspI fragment of the normal clone and a MspI/KpnI fragment of the mutant cDNA isolated by PCR amplification. The resulting vector, CMV1130 contains a single point mutation relative to the consensus sequence. All segments obtained by PCR were sequenced to rule out the possibility of new sequence artifacts and cloning boundaries confirmed by restriction digest.

evidence of propionate incorporation under any conditions were designated *mut*^{0}, whereas mutant cell lines in which propionate incorporation could be stimulated with Cbl were designated *mut*$^-$. The enzyme in at least some *mut*$^-$ cell lines has been shown to have a markedly elevated $K_{m_{(cobalamin)}}$ (1). Subsequent work by Kolhouse et al. (7) showed that all *mut*$^-$ cell lines showed immunochemically CRM to MCM antibody, whereas some *mut*^{0} lines showed CRM and others did not.

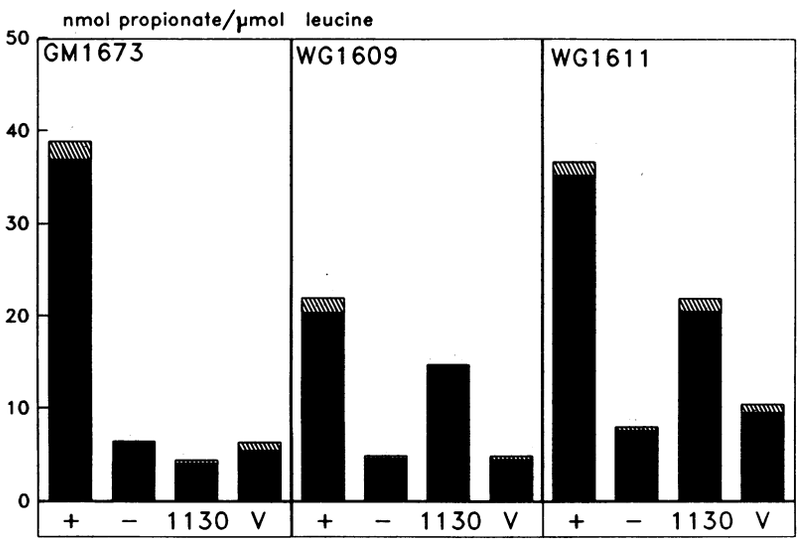

Figure 2. Complementation of propionate incorporation in *mut*^{0} and *mut*$^-$ cell lines by DNA mediated gene transfer of CMV1130. Cell lines with various *mut* phenotypes were transformed with the normal consensus MCM cDNA CMV-hMCM (+), an antisense cDNA (−), the CMV1130 vector (1130) containing the mutation from the WG1130 cell line, or the CMV vector without insert (V). Propionate incorporation was assayed during the transient phase of gene expression and is expressed as nanomoles propionate incorporated per micromole leucine. The GM1673 cell line is a *mut*^{0} cell line with low MCM mRNA phenotypically equivalent to cell line WG1612. WG1609 is a *mut*^{0} cell line and WG1611 is a *mut*$^-$ cell line. The mean (*solid bar*) and standard deviation (*hatched bar*) of triplicate samples is shown.

More recent studies have demonstrated that within the *mut*^{0} class some cell lines exhibit apparently normal levels of MCM mRNA and some exhibit specifically decreased levels of MCM mRNA (4).

The cell lines used in the present study represent a spectrum of *mut* phenotypes. Most of the *mut*$^-$ cell lines were found to complement WG1130. WG1599 (Yale 378), the one *mut*$^-$ line that did not complement with WG1130, has been shown to be a classic *mut*$^-$ on the basis of propionate uptake responsive to OH-Cbl, the presence of residual MCM activity with an elevated Km for AdoCbl, and the presence of CRM (1, 7). In contrast, most *mut*^{0} cell lines did not complement with WG1130. Three *mut*^{0} lines were found that did complement with WG1130 (Table I). The *mut*^{0} phenotype of these cells was confirmed by the absence of MCM activity and the absence of an increase in propionate incorporation in response to the addition of OH-Cbl to the culture medium.

WG1130 itself can be classified as a *mut*^{0} mutation on the basis of low MCM apoenzyme activity in cell extracts and low propionate incorporation in culture which does not respond to OH-Cbl. Cloning and sequencing of the cDNA from this cell line revealed a single point mutation which is shown to be capable of disrupting the activity of the recombinant MCM in a transfer assay which utilizes a *mut*^{0} cell line with reduced MCM mRNA and no immunoreactive protein (6, 11). In contrast to the inability of the CMV1130 expression vector to express MCM activity in the *mut*^{0} cell line GW1673, this vector was capable of stimulating propionate uptake in the *mut*$^-$ cell line WG1611 and the *mut*^{0} cell line WG1609. These data demonstrate that the $Arg_{93} \rightarrow$ His mutation is capable of expressing the complex phenotype identified the by cell fusion complementation studies described above. This mutation expresses a *mut*^{0} phenotype in the homozygous (or hemizygous) state but is apparently able to contribute to a functional MCM apoenzyme by interallelic complementation in certain heterozygous combinations.

It is significant that the ability of cells to complement with WG1130 is not predicted by their *mut*$^-$ or *mut*^{0} phenotype. The present studies reveal at least three subsets of *mut*^{0} alleles. The first is the $Arg_{93} \rightarrow$ His mutation of the WG1130 allele. The second is the allele present in the WG1609 line which will complement WG1130 but not other cell lines. The third includes the majority of alleles which do not complement WG1130 and remain classic *mut*^{0} alleles with no evidence of enzyme activity such as those in WG1612 or GM1673. Similarly, two fundamentally different defects within the *mut*$^-$ class can be distinguished by their ability to complement WG1130.

The observation that most *mut*$^-$ cells will complement WG1130 is particularly interesting and suggests a possible mechanism for this complementation. Since *mut*$^-$ cells are presumed to have a fundamental defect in Cbl binding, it may be presumed that the WG1130 gene product is capable of providing Cbl binding in a heterodimer. Conversely, the WG1130 gene product must be defective in other aspects of enzyme function which are compensated by the *mut*$^-$ gene products. The specific mechanism of complementation is not addressed by the present work. Future experiments will require higher levels of apoenzyme expression for biochemical analysis to address whether this complementation is caused by improved stability of the hybrid dimer, altered secondary or tertiary structure of the monomeric subunits, complementation of constituent functions required for holoenzyme activity, or a combination of these. It is our expectation that these studies may provide powerful insights into discrete and complementable aspects of enzyme structure and activity.

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