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Heterozygous Mutations at the *mut* Locus in Fibroblasts with *mut*^{0} Methylmalonic Acidemia Identified by Polymerase-Chain-Reaction cDNA Cloning

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Summary

Genetic defects in the enzyme methylmalonyl CoA mutase cause a disorder of organic acid metabolism termed "*mut* methylmalonic acidemia." Various phenotypes of *mut* methylmalonic acidemia are distinguished by the presence (*mut*$^-$) or absence (*mut*^{0}) of residual enzyme activity. The recent cloning and sequencing of a cDNA for human methylmalonyl CoA mutase enables molecular characterization of mutations underlying *mut* phenotypes. We identified compound heterozygous mutations in a *mut*^{0} fibroblast cell (MAS) line by cloning the methylmalonyl CoA mutase cDNA by using the polymerase chain reaction (PCR), sequencing with internal primers, and confirming the pathogenicity of observed mutations by DNA-mediated gene transfer. Both mutations alter amino acids common to the normal human, mouse, and *Propionibacterium shermanii* enzymes. This analysis points to evolutionarily preserved determinants critical for enzyme structure or function. The application and limitation of cDNA cloning by PCR for the identification of mutations are discussed.

Introduction

Methylmalonic acidemia/aciduria (MMA; McKusick 25100) is an inborn error of organic acid metabolism, resulting from functional deficiency of the enzyme methylmalonyl CoA mutase (MCM; E.C.5.4.99.2) (reviewed in Rosenberg and Fenton 1989). MCM catalyzes isomerization between methylmalonyl CoA and succinyl CoA during degradation of various metabolites via propionyl CoA to the Krebs cycle. MCM is a mitochondrial matrix homodimer which requires an adenosylcobalamin cofactor. Functional deficiency of MCM results either from defects in the apoenzyme (designated *mut*) or from defects in genes required for synthesis of adenosylcobalamin from dietary hydroxycobalamin (designated *cbl*) (Cooper and Rosenblatt 1987; Rosenberg and Fenton 1989).

We have reported cloning and sequencing of a human MCM cDNA (Ledley et al. 1988*a;* Jansen et al. 1989). The consensus cDNA (authors' unpublished data) comprises 2,798 bases and encodes a propeptide of 750 amino acids (83,007 daltons). This protein encompasses a 32-amino-acid mitochondrial targeting sequence and the 718-amino-acid mature apoenzyme (Jansen et al. 1989). DNA-mediated gene transfer of the consensus MCM cDNA into MCM-deficient cells restores MCM activity to normal levels (authors' unpublished data). The genomic locus encoding MCM (designated MUT) maps to human chromosome 6p12-21.1 (Ledley et al. 1988*b;* Zoghbi et al. 1988) and contains 13 exons spanning >35 kb (Nham et al., in press). Homologous genes have been cloned both from mouse (Wilkemeyer et al., in press) and from a prokaryote, *Propionibacterium shermanii* (Marsh et al. 1989). *Propionibacterium shermanii* MCM is a heterodimer with two subunits (MUTA and MUTB) which exhibit 22% identity to each other (Marsh et al. 1989). The human amino acid sequence is 94% identical to the

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murine sequence, 59% identical to MUTB, and 22% identical to MUTA, reflecting the preservation of critical structure-function determinants despite differences both in reaction kinetics and in quaternary structure (Leadlay and Ledley 1989; Wilkemeyer et al., in press).

Preliminary molecular analysis of cell lines from patients with MMA (Ledley et al. 1988*a*, 1990) revealed no gross structural alterations in the MUT locus, suggesting that most defects involved point mutations or small deletions or insertions. Northern blots demonstrated that some *mut*^{0} cells contain low levels of MCM mRNA, indicative of defects in transcription, processing, or stability of the mRNA. The majority of *mut*^{0} cells and all *mut*$^-$ cells expressed grossly normal mRNA, suggesting that the defects would be mutations within the open reading frame.

To identify these mutations, methods were developed for cloning the MCM cDNA by using the polymerase chain reaction (PCR), identifying mutations by sequencing with specific sequencing primers, and confirming that observed sequence variations eliminate the activity of the apoenzyme. This method is used to demonstrate compound heterozygosity in the *mut*^{0} MAS cell line. The complications of using PCR in heterozygous cell lines are discussed.

Material and Methods

Clinical History

The proband (MAS) was a male, 8-lb 1-oz product of an unremarkable pregnancy to a G3;P2-3 mother. A previous child had died at 2 wk of age of an undiagnosed illness. The proband was discharged at 24 h and was readmitted at 36 h of age with respiratory distress. MMA was diagnosed by urine organic-acid analysis. During the course of multiple hospitalizations, this child exhibited organic acidemia, hyperbilirubinemia, hypoglycemia, hyperammonemia, neutropenia, and thrombocytopenia. The patient was maintained on carnitine, B12, and dietary restrictions with parenteral fluids and nutrients for acute exacerbations. The patient died at 1½ years of age during a recurrent episode of metabolic acidosis.

The MAS cell line expresses no detectable enzyme activity (*mut*^{0}), and molecular analysis demonstrates that the MUT locus exhibits a grossly normal structure and expresses normal amounts of MCM mRNA (Ledley et al. 1990).

Cloning of MCM cDNA: Preparation of mRNA

Total RNA was prepared from confluent fibroblasts by using the hot-phenol method (Scherer 1969). Cells were harvested in 10 ml 50 mM NaAc/1% SDS (pH 5.5) and were incubated successively with phenol at 57°C for 15 min and at 4°C for 15 min with vigorous vortexing. The aqueous phase was removed and reextracted using an identical procedure. RNA was collected by ethanol precipitation, and residual DNA was removed by treatment with RNase free DNaseI (1 U of DNaseI added/μg RNA; Pharmacia) for 10 min at 37°C, followed by extraction with phenol chloroform (1:1) and by EtOH precipitation.

Cloning of MCM cDNA: Reverse Transcription and PCR for cDNA Synthesis

Double-strand cDNA was generated by a coupled reverse transcription and PCR reactions (Vrieling et al. 1988). Five micrograms DNAaseI-treated total RNA and 10^{-6} M oligo 33 were combined in 10 μl annealing buffer (250 mM KCl, 10 mM Tris-HCl [pH 8.3], and 1 mM EDTA) and were denatured at 80°C for 5 min. To this, 1 μl 50 mM DTT (final concentration 5 mM DTT) and 20 U RNAsin (Promega) were added, and the sample was allowed to anneal for 20 min at 50°C. The volume was increased to 25 μl with the addition of 15 μl of cDNA buffer (40 mM Tris-HCl [pH 8.3], 20 mM $MgCl_2$ 9.015% gelatin, 10 mM DTT, 40 U RNAsin, 0.85 mM each of dATP, dTTP, dGTP, and dCTP), and 20 U AMU reverse transcriptase (Stratagene), and the mixture was incubated 60 min at 40°C. Five microliters of this reaction was diluted to 25 μl with water, covered with light mineral oil, and denatured at 100°C for 5 min. After the mixture was cooled to 55°C, 25 μl of PCR buffer (100 mM Tris-HCl [pH 8.3], 100 mM KCl, and 5 mM $MgCl_2$), 0.4 mM each of dATP, dTTP, dCTP, and dGTP, 2 μM each of oligo 23 + 21 or of oligo 53 + 28, and 1 U *Taq* DNA polymerase (Perkin Elmer–Cetus) were added under the oil layer. This sample was incubated 1 min at 55°C and then 3 min at 74°C for synthesis of second-strand cDNA. Thirty-five cycles of PCR were then performed with denaturing at 92°C for 1 min, annealing at 55°C for 1 min, and extension at 74°C for 1.5 min (Scharf et al. 1986; Saiki et al. 1988).

The amplified fragments were extracted with 1:1 phenol/chloroform and were precipitated with 3 vol EtOH at −70°C and were washed. The 5′ segment of the cDNA was cloned into the *Cla*I/*Kpn*I sites of pGEM7zf(+) by using the *Cla*I site within oligo 53 and the internal *Kpn*I site at base 720. The 3′ segment of the cDNA was cloned into the *Kpn*I/*Bam*HI sites of

pGEM7zf(+) by using the *Kpn*I site and the *Bcl*I site at bp 2354.

Identification of Mutations in the MCM cDNA by DNA Sequencing

Double-stranded dideoxy sequencing was performed using the T7 polymerase sequencing kit (Pharmacia) with T7 (Promega Q5011), SP6 (Promega Q5121), or MCM-specific primers (table 1). Single isolates of the 5′ and 3′ clones were sequenced and compared with the sequence from a pool of 15 independent clones, to confirm the authenticity of the observed sequence variations (see below).

DNA-mediated Gene Transfer and Assay of MCM in Cultured Cells

An expression vector, containing the consensus human MCM cDNA transcribed from the cytomegalovirus immediate early promotor (MacGregor and Caskey 1989), will be described elsewhere (authors' unpublished data) (fig. 5*A*). Expression constructs containing the mutated sequences were generated by substituting cDNA fragments carrying the mutations into a subclone containing a *Not*I-*Nsi*I fragment of the cDNA (bases 1–1715) and then reconstituting the full-length mutant cDNA by three-part ligation of the *Not*I-*Nsi*I fragment with *Nsi*I-*Sal*I and *Sal*I/*Not*I fragments of the expression vector.

Clones were introduced, via electroporation (Shigekawa and Dower 1988), into a *Mut*^{0} primary fibroblast cell line GM1673 (NIGMS Mutant Cell Repository) which expresses abnormally low amounts of MCM mRNA (Ledley et al. 1990). Electroporation was performed using a Gene Pulser™ (BioRad) after a 5-min preincubation of 0.3 ml of cells, at a density of 3×10^6/ml, with 10 μg of DNA by using voltage pulses of 260 V at a capacitance of 960 μF. MCM activity was assayed 2 d following electroporation by using the propionate incorporation assay (Willard et al. 1976; Rosenblatt et al. 1984; Ledley et al. 1990). Incorporation of [^{14}C]-propionate into TCA-precipitable protein is a measure of MCM holoenzyme activity. [^{3}H]-leucine incorporation normalizes for cell number and constitutive protein synthesis. Results are expressed as the mean nanomoles propionate per micromoles leucine incorporated in triplicate samples.

Results

Identification of Mutations in MCM cDNA Clones

The scheme for cloning and sequencing the MCM cDNAs is shown in figure 1. The strategy was designed to distinguish (*a*) mistakes introduced by either reverse transcription or PCR from (*b*) true heterozygous or homozygous sequence variations. Artifacts introduced by reverse transcriptase or PCR were detected in the sequence of the single clones but should not be represented in the pooled clones (fig. 2*A*). These artifacts were identified with a frequency of approximately 1:300 bases. Heterozygosity was demonstrated by the pres-

Table 1

Sequence, Position, and Orientation of Oligonucleotides Used for Cloning and Sequencing of Mutant MCM from Fibroblasts

Number	MCM Bases	Orientation	Sequence
2	382–401	Antisense	5′-GGCGGATGGTCCAGGGCCTA-3′
15	1740–1758	Antisense	5′-AGGGCATCTGTGATTTCT-3′
16	1740–1748	Sense	5′-AGAAATCACAGATGCCCT-3′
17	2145–2160	Antisense	5′-CCTCCACACATGACAA-3′
21[a]	2350–2370	Antisense	5′-ACAGGGCCCTACTCTCTTCTTTGATCATAAC-3′
23	624–641	Sense	5′-CCGCTCGAGTTTCCATGACTATGA-3′
28	877–896	Antisense	5′-ATGCATCAGCCCCTGCTTCC-3′
33[a]	2377–2393	Antisense	5′-TCAGGGCCCGAAATTAAATTGAAGACA-3′
34	774–793	Sense	5′-TTCCTCCAGACCCATCCATG-3′
35	1246–1266	Sense	5′-TGATGAAGCTTTGGGTTTGCC-3′
37	1848–1867	Antisense	5′-ATGAACCCTCTTGATAGCAG-3′
48	644–663	Antisense	5′-GAACTGGTATAACTGCTCC-3′
53[b]	37–61	Sense	5′-AGTCAGTTCTTATATCGATTGGGTG-3′

[a] Clones contain an *Apa*I linker at the 5′ end, in addition to MCM sequences.
[b] Clone contains a *Cla*I linker at the 5′ end, in addition to MCM sequences.

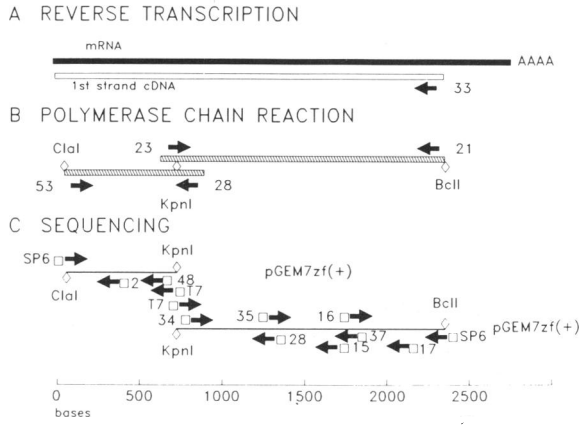

Figure 1 Scheme for cloning and sequencing MCM cDNA by using PCR. First-strand cDNA synthesis is performed with reverse transcriptase primed with oligo 33 (*A*), which is complementary to sequence in the 3′ untranslated region of the mRNA. The first strand cDNA is added to two PCR reactions which amplify the 5′ end of the cDNA and the 3′ end of the cDNA, respectively (*B*). PCR products are digested with *Cla*I and *Kpn*I (5′ end) or with *Kpn*I and *Bcl*I (3′ end) and are subcloned into the *Cla*I/*Kpn*I or *Kpn*I/*Bam*HI sites of the vector pGEM7zf(+). Clones are sequenced using the SP6 or T7 promotor primers or oligonucleotides complementary to the MCM sequence as indicated (*C*). Oligonucleotide sequences are given in table 1.

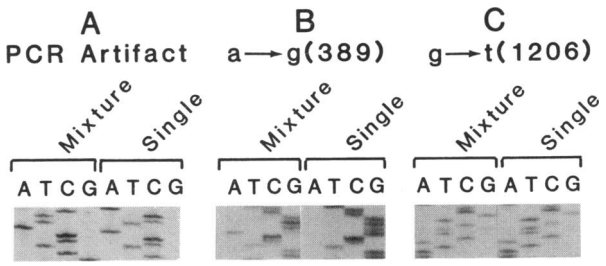

Figure 2 Delineation of homozygous, heterozygous, and artifactual sequence variations in MAS cDNA. Sequence variations observed in single isolates of cloned cDNA were confirmed by comparison of identical sequences of mixtures containing 15 separate cDNA clones. PCR artifacts were identified by the absence of the novel sequence in the mixture. Heterozygous variants were identified by the presence of both novel and normal sequences in the mixture. *A*, Example of a PCR mistake with a c residue in the single isolate not represented in the mixture. *B*, Heterozygous mutation at position 389 (antisense orientation). The a→g change is apparent in the single clone, and both the a and g residues are present in the mixture. This corresponds to a t→c change in the sense orientation. *C*, Heterozygous mutation at position 1206 (antisense orientation). The g→t change is apparent in the single clone, and both the g and t residues are present in the mixture. This corresponds to a c→a change in the sense orientation.

ence of two bands in the pooled sample (figs. 2*B* and 2*C*).

Two heterozygous sequence variants were identified (figs. 2*B*, 2*C*, and 3). The first change was identified, in a 5′ clone, as a t→c$_{389}$ which results in the change Trp$_{105}$→Arg. A second change was identified, in a 3′ clone, as an a→c$_{1206}$ which results in the change Ala$_{378}$→Glu. The cDNA clones were homozygous for the *Hin*dIII(−) polymorphism at position 712 (Ledley et al. 1988*b*; Nham et al., in press), which was consistent with previous Southern blotting data (Ledley et al. 1990).

Effect of Mutations on Expression of Enzyme Activity

To demonstrate that the 389 and 1206 sequence changes are causally associated with the *mut*^{0} phenotype, expression plasmids were constructed by substituting segments of the mutant cDNA clones (free of artifactual sequence variations) into a functional human MCM cDNA expression vector (fig. 4). The fidelity of these constructs was verified by restriction mapping the junctions and sequencing the inserts.

The *mut*^{0} fibroblast line GM1673 was transformed with the consensus MCM expression vector, vectors containing the 389 or 1206 mutations, control vectors containing no cDNA insert, or antisense MCM cDNA.

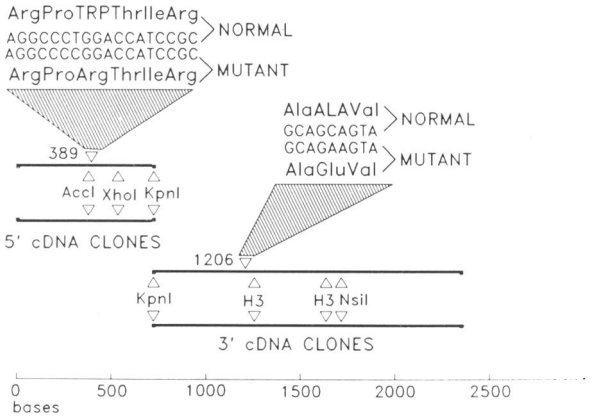

Figure 3 Mutations identified in cDNA clones. Two mutations were identified among the MCM cDNA clones from the MAS cell line. The first is a t→c change at position 389, which produces the change Trp→Arg. The second is a c→a change at position 1206, which produces the change Ala→Glu. These two mutations were present in the 5′ and 3′ cDNA clones, respectively, and were shown to be heterozygous among the cDNA clones. Restriction sites which were used for subsequent construction of the expression vector are shown.

MCM activity, measured by propionate incorporation, was restored to normal levels by transformation with the consensus MCM clone. No increase in MCM activity over the activity in controls was observed with

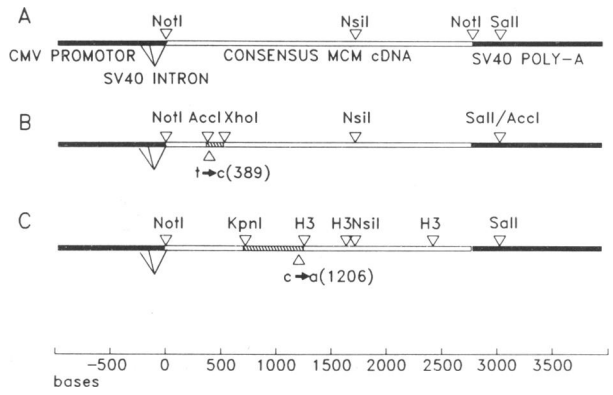

Figure 4 Vectors for expression of mutant cDNAs. *A*, Consensus human MCM sequence expressed in the vector pNAssCMV (MacGregor and Caskey 1989), which contains the cytomegalovirus Immediate Early Promotor, SV40 intron, and SV40 polyadenylation signals in PUC19. Segments of the cDNA containing the two mutations identified in the MAS cell line were placed in this construct. *B*, 389 Mutation, introduced by replacing an *Acc*I-*Xho* fragment of the cDNA representing bases 375–530 of the cDNA. *C*, 1206 Mutation, introduced by replacing a *Kpn*I-*Hin*dIII (H3) fragment of the cDNA representing bases 720–1251. Both mutant segments were first introduced into a *Not*I-*Nsi*I subclone of the cDNA and were reintroduced into the expression vector as a three-part ligation with the *Nsi*I-*Sal*I fragment and *Not*I-*Sal*I-digested vector.

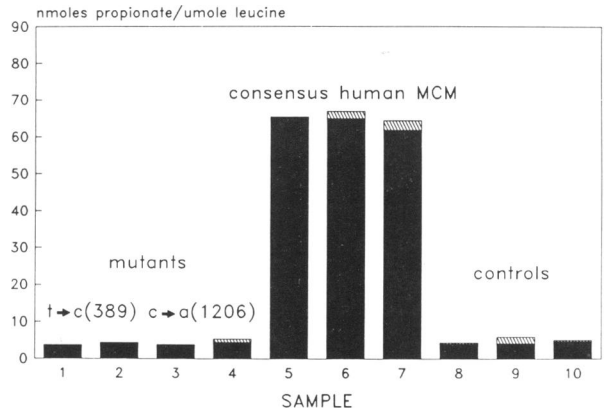

Figure 5 Expression of human MCM cDNA with 389 mutation and 1206 mutation. Clones containing the consensus human MCM cDNA, cDNA with the 389 or 1206 mutations (fig. 3), or controls were introduced into the GM1673 fibroblast cell line by electroporation, and MCM activity was measured by incorporation of [^{14}C]-propionate into TCA-precipitable material. [^{3}H]-leucine incorporation is used as a control for cell number and viability. This cell line exhibits a *mut*^{0} phenotype and low levels of mRNA on northern blots. Lanes 1 and 2, MCM cDNA with 389 mutation. Lanes 3 and 4, MCM cDNA with 1206 mutation. Lanes 5–7, Consensus MCM cDNA, sense orientation. Lane 8, Consensus MCM cDNA, antisense orientation. Lane 9, pNAssCMV vector (no insert). Lane 10, No transfection. These results demonstrate the constitution of normal propionate incorporation by the consensus MCM sequence and the elimination of this activity by the 389 and 1206 mutations.

clones containing either the 389 or 1206 mutations (fig. 5). Two separate clones with each mutation were tested and gave identical results.

Discussion

In the present paper two mutations have been described which eliminate the ability of the MCM cDNA to express enzyme activity in cultured cells. The first mutation, at base 389, results in the change Trp_{105}→Arg. It has been observed previously in the human and mouse proteins that Trp residues along with Tyr and Pro residues are nonrandomly distributed in the amino-terminal half of the molecule (Wilkemeyer et al., in press). The significance of this asymmetry in amino acid composition is unknown, though it is possible that such residues may be more resistant to attack by free radicals formed during the rearrangement of methylmalonyl CoA. During this reaction, the $-CH_3$ of methylmalonate is stripped by the deoxyadenosyl radical formed from the adenosylcobalamin cofactor.

The Trp residue at this position is conserved in the human, mouse (Wilkemeyer et al., in press), and *Propionibacterium shermanii* MUTB sequences (Leadlay and Ledley 1989) but not in MUTA. The MUTA sequence is highly divergent from the MUTB and eukaryotic sequences in this region. It is not known whether the MUTA gene contains all of the determinants of the eukaryotic monomer or whether these functions have been sorted between the heterologous subunits. Thus, there is no evidence to indicate what specific functions are disrupted by the Trp→Arg mutation at this position.

The second mutation, at base 1206 (amino acid position 378), results in the change Ala→Glu. The Ala at this position is conserved in mouse, MUTA, and MUTB sequences. This mutation occurs within a sharply hydrophobic segment (fig. 6). While Ala is unlikely to be directly involved in catalytic function, the insertion of the charged Glu in place of the hydrophobic Ala might significantly alter the secondary and tertiary structure of this hydrophobic segment. Future work will explore the dynamic consequences that these mutations have on the stability or kinetics of enzyme activity.

Preservation of these amino acids through the divergence of eukaryotic and prokaryotic MCM is consistent with strong pressure against evolutionary selection at these positions. The lethal phenotype of MMA in this compound-heterozygous patient is an exemplar of

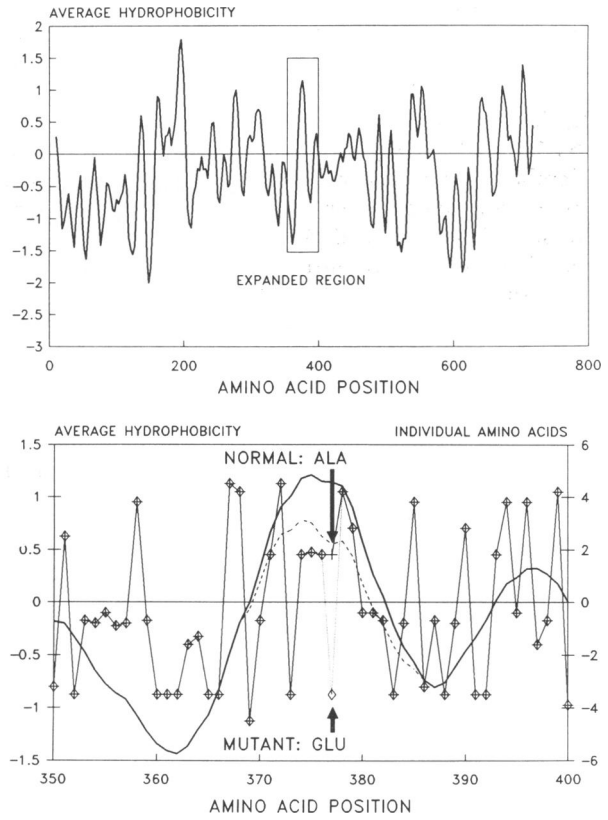

Figure 6 Hydrophobicity of human MCM and the consequences of the ALA-Glu mutation. *A*, Kyte-Doolittle values (Kyte and Doolittle 1982) of individual amino acids were interactively averaged and are plotted as the median position. The 1206 mutation changes the alanine at position 378 to glutamic acid. This change occurs in the midst of a significant local hydrophobic maximum. The boxed region is expanded in panel *B*. *B*, Averaged hydrophobicity and hydrophobicity of individual amino acids at positions 350–400. The effect of the Ala-Glu change at position 378 is shown.

this evolutionary selection. We have not yet explored the prevalence of these mutations among *mut* alleles. There is, however, no evidence from population genetics, biochemical analysis, or molecular cloning that mut^0 MMA represents a discrete set of common mutations. Our initial studies indicate that multiple alleles can be distinguished in *mut* cell lines by their RFLP haplotype, ability to encode detectable mRNA, and ability to encode detectable enzyme activity (Ledley et al. 1990).

The phase of these two mutations between the heterologous alleles was not unequivocally determined in this work. We presume that, since both mutations give a mut^0 phenotype after transfection, one allele bears the 389 mutation and the opposite allele bears the 1206 mutation. Our attempt to demonstrate that these mutations were, in fact, on opposite alleles revealed a fundamental limitation in the application of PCR to cDNA cloning.

We amplified and cloned a segment of cDNA spanning the 389 and 1206 mutations. We expected to find the two mutations either in separate clones if they were on heterologous alleles or in the same clones if they were on the same allele. We observed random association of the two mutations in a series of cDNA clones, suggesting that the phase of the mutations had been disrupted during amplification or cloning (data not shown). Similar phase disruptions in cloned PCR products have been noted by others (e.g., see Kobayashi et al., in press).

In separate work using a model construct with two restriction-endonuclease sites, we quantitated this disruption of phase (Jansen and Ledley, in press). The phase of the heterozygous variations is disrupted in approximately 1% of molecules after PCR, presumably because of incomplete chain elongations and subsequent priming of a heterologous template. Phase is disrupted in 25% of clones from the PCR products, presumably because of excision repair of heteroduplexes in the PCR product during cloning.

This disruption of phase which is inherent in the cloning of PCR products makes it difficult to unequivocally identify mutations in heterozygous cell lines. The exclusivity of mutations in "full-length" clones does not demonstrate that such mutations are pathogenic, since there is a statistical likelihood that additional sequence variations may be missed. Genetic data which demonstrate segregation of mutations with the disease phenotype or gene transfer data which demonstrate that the mutation eliminates enzyme activity is essential.

Acknowledgments

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