Cloning and Expression of Mutations Demonstrating Intragenic Complementation in *mut*^{0} Methylmalonic Aciduria

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Abstract

The *mut*^{0} mutation resulting in methylmalonyl CoA mutase (MCM) apoenzyme deficiency and methylmalonic aciduria is characterized by undetectable enzyme activity in cell extracts and low incorporation of propionate into cultured cells which is not stimulated by hydroxycobalamin. A *mut*^{0} fibroblast cell line (WG1681) from an African-American male infant complemented another *mut*^{0} cell line (WG 1130). Cloning and sequencing of cDNA from WG 1681 demonstrated compound heterozygosity for two novel changes at highly conserved sites: G623R and G703R. In addition, two previously described homozygous polymorphisms, H532R and V671I, were found. Hybridization of allele-specific oligonucleotides to PCR amplified MCM exons from the proband and family members identified a clinically normal mother, half-sister, and half-brother as carriers of the G703R change in *cis* with both polymorphisms. Transfection of each change into a *mut*^{0} cell line with very low MCM mRNA (GM1673) demonstrated a lack of stimulation of propionate uptake in the absence and presence of hydroxycobalamin. Cotransfection of each mutation with the previously identified R93H mutation of WG 1130 stimulated propionate uptake, indicating that G623R and G703R are independently capable of complementing the R93H mutation. (*J. Clin. Invest.* 1994. 93:1812–1819.) Key words: methylmalonyl coenzyme A mutase • vitamin B_{12} • cobalamin • inborn errors • interallelic complementation

Introduction

Methylmalonyl CoA mutase (MCM)[1] (EC 5.4.99.2) is a 5′deoxyadenosyl cobalamin (AdoCbl)-dependent mitochondrial matrix enzyme that is responsible for the isomerization of methylmalonyl CoA to succinyl CoA. A defect or deficiency of either apoenzyme or coenzyme results in methylmalonic aciduria (MMA) (1). Somatic cell complementation of cultured fibroblasts has defined the genetic defects causing MMA without homocystinuria into Cbl defects, which are mutations in the genes required for the synthesis of AdoCbl (*cblA* and *cblB*) (2) and mutase complementation class (*mut*) defects, which are mutations in the gene encoding the MCM enzyme (3). The latter class has been further subdivided and designated *mut*^{0} for mutations resulting in no detectable MCM enzyme activity, and *mut*$^-$ for mutations resulting in residual MCM enzyme activity with reduced affinity for the AdoCbl cofactor (4). Cross-reactive material to MCM has been detected in all *mut*$^-$ cells and in some *mut*^{0} cells (5).

The MUT locus encoding MCM has been mapped to chromosome 6p12-21.2 (6), and the MCM gene has been cloned and sequenced (7, 8). It contains 13 exons spanning 40 kb, and is transcribed in the nucleus as a 2.8-kb mRNA. A 750–amino acid propeptide (83 kD) containing a 32–amino acid mitochondrial targeting sequence is cleaved during transport into the mitochondrial matrix (9). The mature enzyme contains 718 amino acids (2,798 bases), and is a homodimer with ~ 78 kD subunits. 1 mol of AdoCbl is bound per mole of subunit. Very little is known about the tertiary structure, binding, and catalytic sites. Bacterial and murine MCM have an overall homology of 94% with human MCM (10).

Intragenic complementation within the *mut* subclasses has been previously described for a *mut*^{0} cell line WG 1130, (11) which presently is known to complement 5 of 13 *mut*^{0} cell lines and 6 of 8 *mut*$^-$ cell lines (Farah, R., and D. S. Rosenblatt, unpublished communication). Cloning and sequencing of MCM cDNA from WG 1130 characterized a homozygous R93H alteration, which was expressed in vitro and confirmed to be the pathogenic mutation. We describe the identification and expression of two novel mutations from a *mut*^{0} cell line, WG 1681, exhibiting intragenic complementation with WG 1130. We demonstrate that each novel mutation is independently capable of complementing the R93H mutation of WG 1130.

Methods

Cell culture. Skin fibroblasts from a control cell line (MCH 24) and patients (WG 1130 and WG 1681) were obtained from the Repository for Mutant Human Cells, Montreal Children's Hospital (Montreal, Quebec). Cultures were maintained in standard media consisting of Eagle's minimum essential medium with Earle salts, L-glutamine, and nonessential amino acids (Flow Laboratories, Mississuaga, Ontario), supplemented with 10% (vol/vol) fetal bovine serum (Gibco Laboratories, Grand Island, NY). Propionate incorporation and cell fusion experiments were conducted as previously described (11).

Portions of this work have been presented in abstract form at the Annual Meeting of Human Genetics, 9–13 November 1992, San Francisco, CA, and 5–9 October 1993, New Orleans, LA, as well as the Annual Meeting of the Canadian Society for Clinical Investigation, 9–13 September 1993, Vancouver, British Columbia.

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Received for publication 16 August 1993 and in revised form 1 December 1993.

1. *Abbreviations used in this paper:* AdoCbl, 5′deoxyadenosylcobalamin; ASO, allele-specific oligonucleotide; CMV, cytomegalovirus; MCM, methylmalonyl CoA mutase; MMA, methylmalonic aciduria; *mut,* mutase complementation class.

J. Clin. Invest.

0021-9738/94/04/1812/08 $2.00
Volume 93, April 1994, 1812–1819

Clinical history. Fibroblast cell line WG 1681 is from a male infant of parents of African-American origin with no family history of MMA. He was diagnosed with MMA after showing symptoms of lethargy, hyperammonemia, and acidosis in the first week of life. He had several readmissions because of acidosis, and at 3 ½ yr of age, he had ~ 6 mo delay in his verbal and fine motor development (12).

WG 1130 is a well-characterized *mut*^{0} cell line (11) from a French Canadian female who was diagnosed with MMA in the first week of life. She had recurrent vomiting, dehydration, metabolic episodes, and died at 5 mo of age.

Cloning of MCM cDNA from WG 1681: mRNA extraction. A method using acid guanidinium thiocyanate-phenol-chloroform was used to isolate RNA from confluent fibroblast cell monolayers (13).

Reverse transcription and polymerase chain reaction. The RNA was amplified separately in four overlapping fragments using a modified combined reverse transcription/ PCR procedure of Wang et al. (14) (Fig. 1 *A*). The primers used for amplification have been described before (15). Each 50-μl reaction contained 0.5–1 μg RNA, 40 U RNasin inhibitor, 1 μM of each flanking primer, 200 μM each dNTP (dATP, dGTP, dCTP, dTTP), 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% wt/vol gelatin; Perkin-Elmer Cetus Norwalk, CT), 2 U Taq polymerase (Pharmacia fine Chemicals, Piscataway, NJ), and 80 U Moloney's murine leukemia virus reverse transcriptase (BRL, Grand Island, NY). Samples were overlaid with 50 μl of mineral oil and placed in a thermal cyler (Tempcyler model 60; Coy Laboratory Products Inc., Ann Arbor, MI). Reverse transcription was carried out at 50°C for 10 min, followed by heat denaturation at 94°C for 3 min. The program then proceeded to PCR amplify for 10 cycles of 94°C for 1 min, 60°C for 1.5 min, 72°C for 1.5 min, and 20 cycles of 94°C for 1 min, 55°C for 1.5 min, 72°C for 1.5 min, followed by a final elongation at 72°C for 10 min.

The amplified fragments were then isolated from low melting agarose gel using a glassmilk method (Geneclean; BIO 101, Inc. La Jolla, CA) to eliminate unamplified and degraded RNA.

Cloning and sequencing. Each PCR fragment was ligated to the pCR1000 vector provided by the TA Cloning Kit (Invitrogen, San Diego, CA). Transformation of INVαF′ bacterial cells, provided with the kit, was carried out. A total of 24 white colonies for each fragment were separately grown overnight in 3.7% BHI (Bacto Brain Heart Infusion, Difco Laboratories, Detroit, MI) media containing 50 μg/ml kanamycin. A representative self-ligated vector (blue colony) was also grown. Slot lysis of each clone allowed for a quick selection of those containing the proper ligated product. 10 μl of each overnight culture were aliquoted, and cells were digested in 10 μl of a protoplasting mix with lysozyme (0.05 μg/μl) and RNase (0.096 μg/μl).

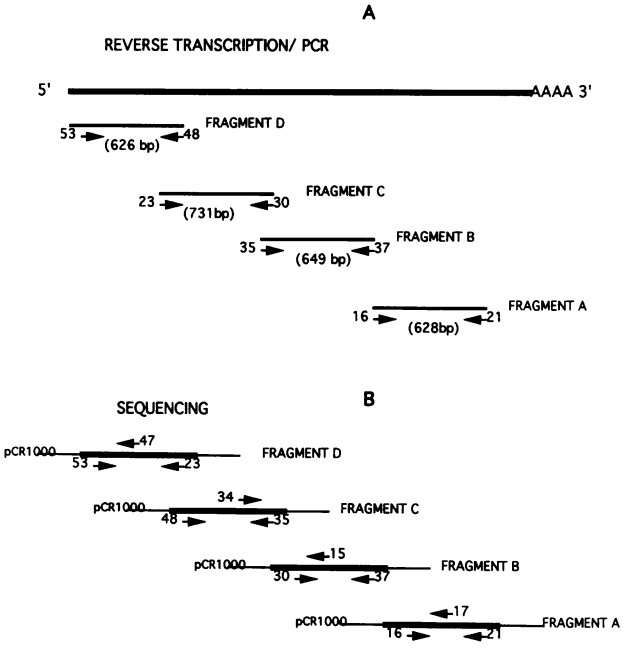

Figure 1. (*A*) Position of RT/PCR Primers. Numbers represent primers used. (*B*) Position of cDNA Sequencing Primers. Flanking and internal primers used are shown.

Table I. Genomic PCR Primers

Exon	Primer no.	Sequence	Anneal temp	No. of cycles
2	112	5′AGCTCCTATTCCACCCCTCTTC 3′	54°C	30
	70*	5′AAAATCTCACCTTAATGTTGTCC 3′		
9	9–1	5′CCTTTCCTTGACTTTTTC 3′	51°C	40
	9–2*	5′TGCCATTACCCTCTTTTG 3′		
11	11–1	5′ACTTGAAGATTTGCTGTG 3′	48°C	30
	11–2*	5′TGCTGTCATCATTTTACTAC 3′		
12	12–1	5′CAGGGTTTTTATAGTCATTA 3′	50°C	30
	12–2*	5′CAAGATTCCCATCACAGT 3′		

* Antisense primer. Primers were used in direct sequencing, and annealing temperatures were kept the same.

Plasmid DNA preparations from a single clone and a pool of 15 clones were subjected to double-stranded dideoxy sequencing according to the procedure recommended by the Pharmacia T7 Sequencing Kit. PCR and internal primers were used (Fig. 1 *B*). Double-stranded sequencing directly on PCR-amplified exons 9, 11, and 12 from genomic DNA of WG 1681 and control was also performed according to the instructions of the BRL dsDNA Cycle Sequencing System.

Family study: DNA extraction and amplification. SDS lysis and phenol/chloroform extraction methods were used to isolate genomic DNA from WG 1681 and from blood samples obtained from family members. Amplification of exons 9, 11, and 12 from genomic DNA was performed. Each 50-μl reaction contained 0.5 μg DNA, 1 μM of each 5′ and 3′ primers, 200 μM each DNTP (DATP, DGTP, DCTP, and DTTP), 1X Perkin Elmer Cetus reaction buffer (10 mM Tris-Hcl pH 8.3, 50 mM Kcl, 1.5 mM $MgCl_2$, 0.01% wt/vol gelatin), and 2 U Taq polymerase (Pharmacia Fine Chemicals) or 2 U Amplitaq (Perkin-Elmer Cetus). Samples were overlaid with 50 μl mineral oil and subjected to amplification in a Diamed Coy thermal cycler. After an initial incubation at 94°C for 4 min, each amplification consisted of 30 cycles of 94°C for 1 min, annealing temperature for 1.5 min, and 72°C for 1.5 min. Sequences of primers used, and respective annealing temperatures are listed (Table I). Because of nonspecific bands, amplification of exon 9 was performed according to a modification published by Yap and McGee (16). 10 cycles of amplification were performed as described above, followed by an additional 30 cycles using a denaturation temperature of 88°C for 1 min.

Allele-specific oligonucleotides. Allele-specific oligonucleotides were designed for the G703R, G623R changes, and the H532R, V671I polymorphisms. The oligo sequences, hybridization, and washing temperatures are shown (Table II).

Table II. Conditions for Allele-specific Oligonucleotides

Exon	ASO	Sequence	Hybridization temp.	Wash temp
9	PN532	5′GGCTGAACATTGTCTT 3′	42°C	56°C
	P532	5′GGCTGAACGTTGTCT 3′	42°C	47°C
11	623N	5′CAAAAATGGGACAAGAT 3′	42°C	46°C
	623M	5′GCAAAAATGAGACACAAGA 3′	42°C	54°C
12	703N	5′TGTGTGGAGGGGTGAT 3′	46°C	50°C
	703M	5′TGTGTGGACGGGTGAT 3′	46°C	50°C
12	PN671-2	5′TGTGGGCGTAAGCA 3′	38°C	44°C
	P671	5′CTGTGGGCATAAGCAC 3′	46°C	50°C

P, polymorphism, N, normal allele, M, mutant allele. Numbers of ASO correspond to the specific change being studied.

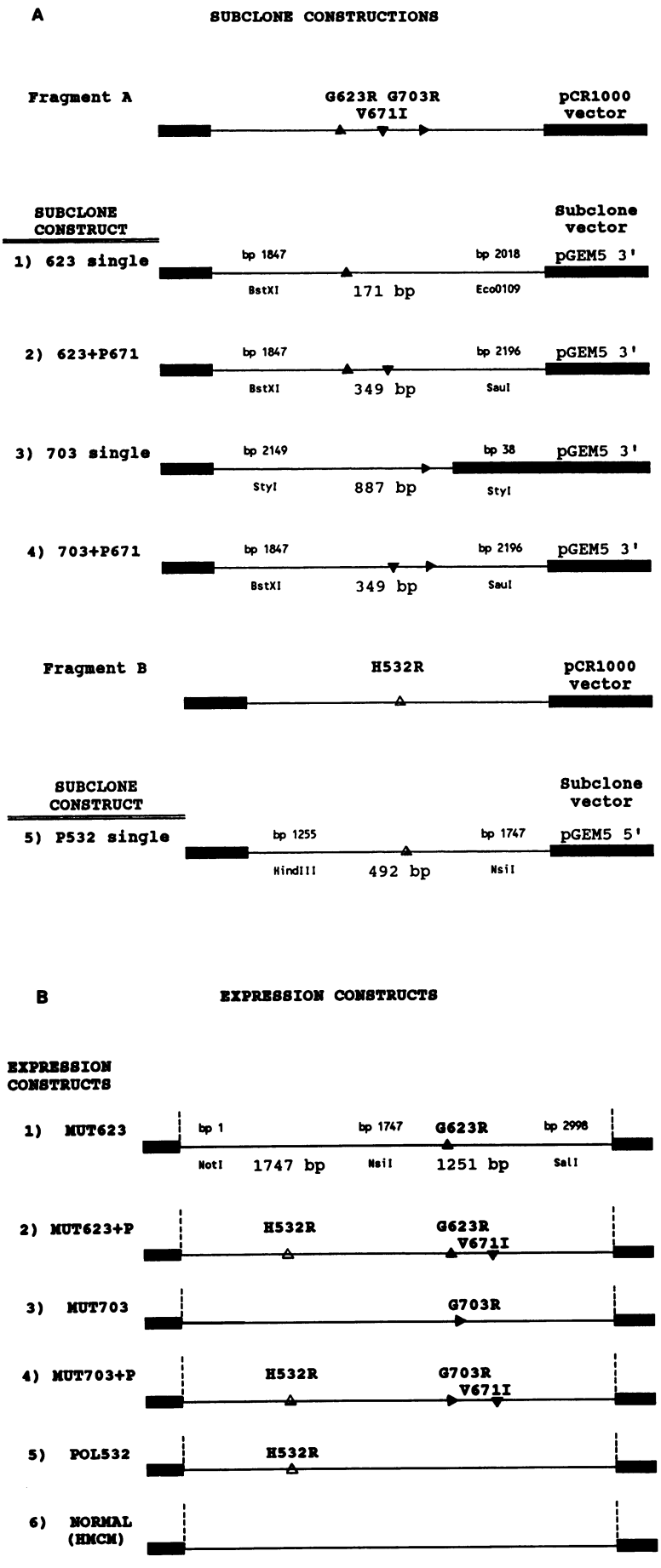

Figure 2. (*A*) Construction of subclones carrying the mutations and/or polymorphisms were performed by double digestions at the cDNA positions indicated above the enzymes used. Fragment A (carrying either G623R ▲, G730R ▶, and V671I ▼) changes was digested from the pCR1000 sequencing clone by the enzymes specified. The generated fragments (size indicated in center) were substituted into the pGEM5 3′ subcloning vector carrying the normal 3′ MCM cDNA. *1*) G623R change alone; *2*) G623R and V671I polymorphism; *3*) G703R change alone; *4*) G703R and V671I polymorphism. Fragment B (carrying the H532R polymorphism was digested from the pCR1000 sequencing clone by the enzymes specified. The 492-bp fragment was substituted into the pGEM5 5′ subcloning vector carrying the normal 5′ MCM cDNA. *5*) H532R polymorphism alone △. (*B*) Expression constructs were generated by NotI/NsiI or NsiI/SalI double digestions of the five different subclone constructs. Subclone constructs carrying single mutations (G623R ▲, or G703R ▶) or each mutation in *cis* with the V671I polymorphism (▼) were digested with NsiI/SalI, and the 1,251-bp fragments were substituted into the expression clone containing normal MCM cDNA. Subclone constructs carrying the H532R polymorphism (△) were digested by NotI/NsiI. This 1,747-bp fragment was substituted into the expression clone just described above to reconstitute MCM cDNA carrying a mutation and both polymorphisms. The normal MCM cDNA was reconstituted using similar digestions and multipart ligations. *1*) G623R change alone; *2*) G623R, as well as H532R and V671I polymorphisms; *3*) G703R change alone; *4*) G703R, as well as H532R and V671I polymorphisms; *5*) H532R polymorphism alone; *6*) normal MCM consensus sequence.

Table III A. Proprionate Uptake of WG 1681

Cell line	[^{14}C]Propionate uptake	
	Without OH-Cbl	With OH-Cbl
	nmol/mg protein/18 h	
MCH 24 (Control)	9.6	9.0
WG 1681	1.1	0.85

OH-Cbl, 1.5 μM.

Expression of mutations. The expression vector pCMV-hMCM was constructed by inserting human MCM cDNA into the pNassCMV plasmid (17). This vector uses the cytomegalovirus (CMV) immediate early promoter, simian virus 40 polyadenylation sequences and simian virus 40 late viral protein splice donor and acceptor signals (18). Subcloning cassettes were constructed by inserting either the 5′ (NotI/NsiI fragment) or the 3′ (NsiI/SalI fragment) end of human MCM cDNA into the pGEM-5Zf(+) plasmid (19).

Isolated fragments carrying each putative mutation singly or in combination with the V671I polymorphism were substituted for the equivalent normal segment in the 5′ or 3′ subclone cassette. A subclone carrying only the H532R polymorphism was also constructed (Fig. 2 *A*). Subclones were subjected to double-stranded dideoxy sequencing to confirm the absence of sequence changes resulting from artifacts.

SalI/NsiI fragments and NotI/NsiI fragments were substituted from mutant subclones into the normal expression vector by sequential multipart ligation. Expression constructs containing each putative mutation singly or in combination with both polymorphisms were thus made. An expression construct carrying only the H532R polymorphism and a reconstructed normal MCM expression vector were also made (Fig. 2 *B*). These served not only as experimental controls, but also as controls for construction methodology.

Expression studies were performed as described before (20). The mutant clones were introduced via electroporation into GM1673 cells (*mut*^{0} cell line with very low MCM mRNA). MCM activity of the transfected cells were assayed by [^{14}C]propionate incorporation into TCA-precipitable material, and [^{3}H]leucine incorporation was measured to normalize for constitutive protein synthesis. The effect of Cbl on expression of transfected cells was assayed by measuring uptake of [^{14}C]propionate in the presence of 1.0 μg/ml hydrocobalamin (OH-Cbl) (21). Results are expressed as the mean nanomoles propionate per micromoles of leucine incorporated in triplicate samples.

Analysis of intragenic complementation. Experiments were performed as described previously (19). The total amount of DNA was kept constant and different combinations of plasmids containing R93H, G623R, G703R, H532R, G717V, G623R and both polymorphisms, G703R and both polymorphisms, or empty CMV vector were used. DNA was electroporated into GM1673 *mut*^{0} cells or into WG1681 fibroblasts, and [^{14}C]propionate incorporation was measured in the absence of Cbl. In addition, the R93H and the G717V constructs were also electroporated independently into WG 1681 fibroblasts. The significance of the increase in [^{14}C]propionate incorporation was determined by paired *t* tests.

Table III B. Intragenic Complementation between WG 1681 and WG 1130

Cell line		Fusions with WG 1681	
		[^{14}C]Propionate uptake	
		Without PEG	With PEG
		nmol/mg protein/18 h	
WG 1681 (*mut*^{0})		0.69	0.53
WG 1607 (*mut*^{0})	*(1.7)	1.5	1.1
WG 1599 (*mut*$^-$)	*(0.86)	0.86	0.89
WG 1588 (*cb*1A)	*(1.5)	1.05	4.0
WG 1586 (*cb*1B)	*(0.93)	0.79	3.6
WG 1130 (*mut*^{0})	*(0.56)	0.74	2.4

* Uptake values by cell line itself. PEG, polyethylene glycol fusion.

Results

Analysis of WG 1681 fibroblasts. The incorporation of [^{14}C]propionate into trichloroacetic-precipitable material by WG 1681 fibroblasts was demonstrated to be decreased and unresponsive in the presence of OH-Cbl in culture medium. This suggested that the defect was located in the MCM apoen-

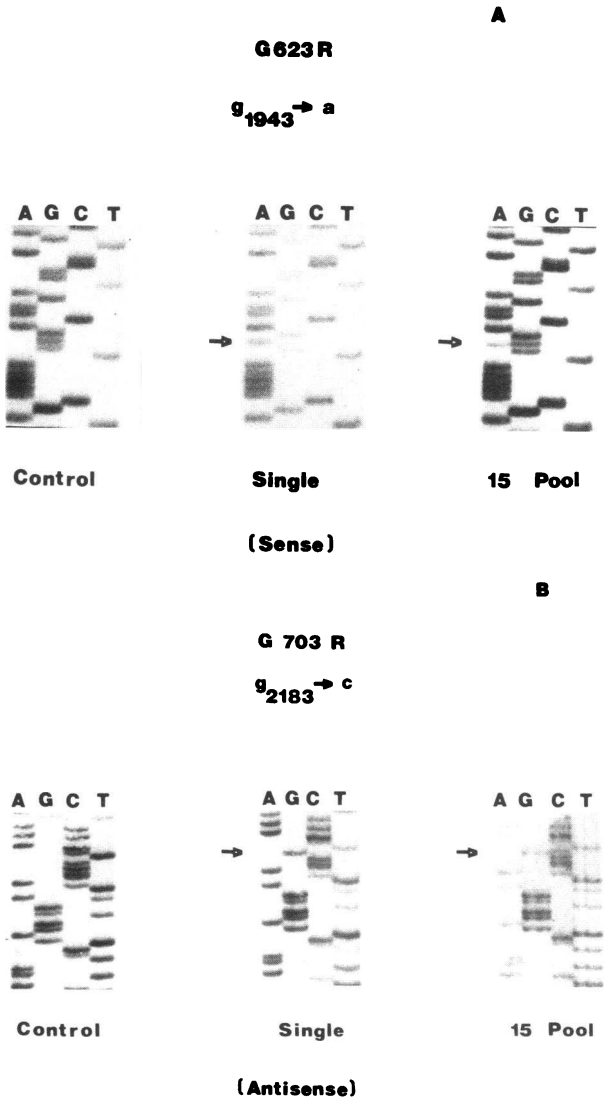

Figure 3. (*A*) cDNA sequencing results for G623R change. The sense strand of fragment A was sequenced. Arrows indicate band shift identified in a single clone, and comigration of normal and mutant alleles in the 15 pooled clones. (*B*) cDNA sequencing results for G703R change. The antisense strand of fragment A was sequenced. Arrows indicate band shift identified a single clone, and comigration of normal and mutant allels in the 15 pooled clones.

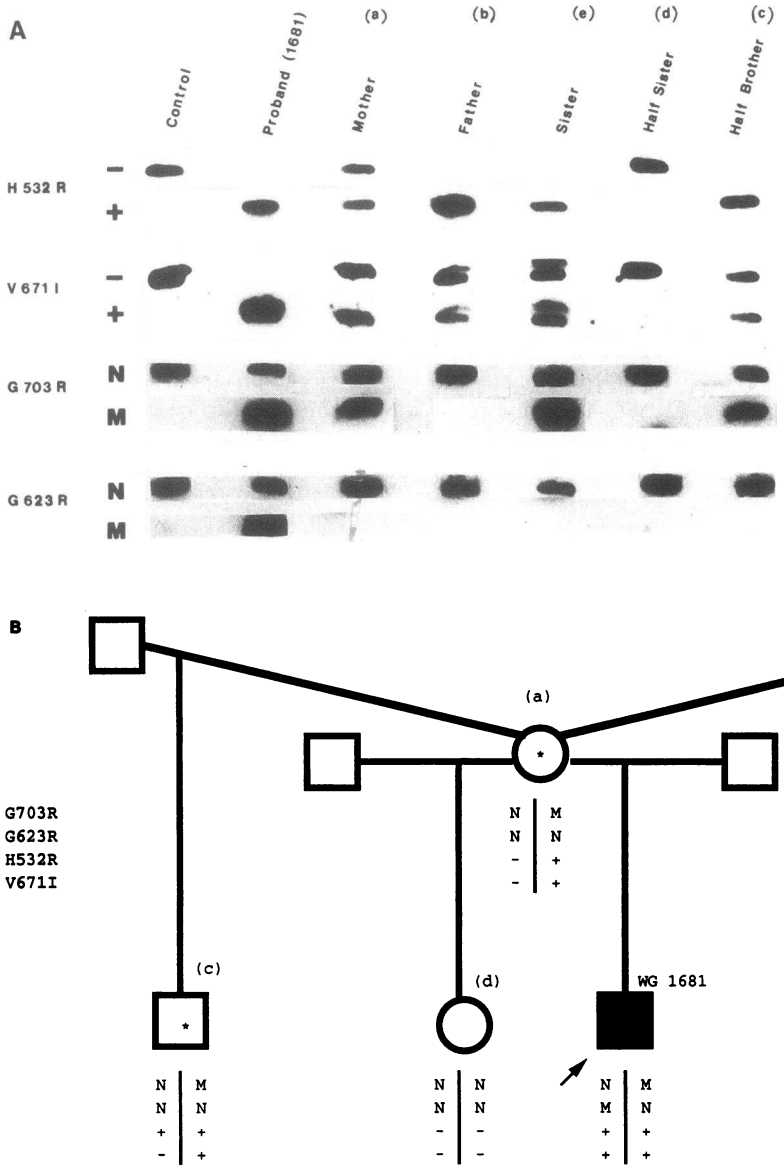

Figure 4. (*A*) Allele-specific oligonucleotide results. Letters correspond to individuals from the original pedigree (not shown). *N*, normal allele; *M*, mutant allele; +, presence of polymorphism; −, absence of polymorphism. (*B*) Haplotype of WG 1681 family. Pedigree drawn after ASO results. (*a*) mother; (*b*) father of individual (*e*); (*c*) half-brother; (*d*) half-sister; *arrow*, WG 1681 proband; (*e*), half-sister. * Carrier. The order of alleles for the haplotype is indicated at the left of the pedigree.

zyme resulting in a mut^0 phenotype (Table III *A*). Further analysis demonstrated that propionate uptake increased on polyethylene glycol fusion of WG 1681 with WG 1130 (Table III *B*). This indicated that these two mut^0 cell lines show intragenic (interallelic) complementation at the somatic cell level.

Identification of mutations in cDNA clones. With respect to the consensus MCM cDNA sequence, four differences were observed by sequencing the four overlapping cDNA fragments from WG 1681. An $a_{1671} \rightarrow g$ (H532R) change was identified in fragment B, and three changes were identified in the most 3′ fragment A: $g_{2087} \rightarrow a$ (V671I), $g_{1943} \rightarrow a$ (G623R), and $g_{2183} \rightarrow c$ (G703R). Sequencing the pooled clones authenticated any changes observed in the sequence of the single clone, minimizing errors caused by PCR or cloning artifacts (15). When two bands were observed to migrate at the same position when pooled clones were sequenced (implying heterozygosity) and the single clone showed a band of the normal allele, individual clones were prepared and sequenced until a clone was found that showed the changed allele. The normal allele was not present in the sequence of pooled clones for the H532R and V671I substitutions, indicating homozygosity. These two changes have been previously identified as normal MCM polymorphisms in three mut^- cell lines from three unrelated African-American patients (19). Putative heterozygosity was observed for the novel G623R and G703R changes (Fig. 3, *A* and *B*, respectively). The H532R, G623R, and both G703R and V671I changes are within exons 9, 11, and 12, respectively. The basepair changes observed in the cDNA were verified in genomic DNA by direct sequencing of PCR-amplified exons.

Family study. PCR-amplified exons 9, 11, and 12 from WG 1681 and family members were analyzed by allele-specific oligonucleotides (ASO) (Fig. 4 *A*). Haplotypes were then generated for each member as shown (Fig. 4 *B*). Allele-specific oligonucleotides, used in the family study, verified the sequencing results for WG 1681 by identifying the two polymorphisms and the two changes as homozygous and heterozygous, respectively. Analysis using ASO identified the mother as the carrier of the G703R change; the G703R change was also observed in a clinically normal half-sister (*e*) and half-brother (*c*) of the proband. Haplotyping results show that these half-siblings of WG 1681 also inherited at the H532R and V671I polymorphisms. This indicated that the G703R change segregated

in *cis* with both polymorphisms. This observation was further supported by the haplotype of the other clinically normal half-sister (*d*). She did not inherit the G703R change or the two polymorphisms from her mother. The ASO study demonstrated that none of the half-siblings carried the G623R change.

Expression studies. Expression of the G623R or G703R changes singly, and in combination with both H532R and V671I polymorphisms are shown (Fig. 5 *A*). Results in the blank column are for nonelectroporated cells, and thus denote the basal MCM activity of GM1673. As expected, transfection of the CMV expression vector alone (no insert) did not affect MCM activity in GM1673. However, the transfection of the normal MCM consensus sequence or the single H532R polymorphism increased [^{14}C]propionate uptake. In comparison to these positive controls, the singly transfected G623R and G703R clones did not result in a significant increase in [^{14}C]propionate uptake. In comparison to these positive controls, the singly transfected G623R and G703R clones did not result in a significant increase in [^{14}C]propionate uptake. This was also observed with those clones that carried both polymorphisms in addition to the putative mutations.

Expression studies in the presence and absence of 1 μg/ml OH-Cbl were performed for the single clones to ascertain whether the G623R and G703R mutations were responsible for the *mut*^{0} phenotype exhibited by WG 1681 fibroblasts (Fig. 5 *B*). The G623R or G703R clones did not individually significantly stimulate incorporation of labeled propionate in the presence of OH-Cbl. In addition, the cotransfection of the constructs carrying the G623R and the G703R mutation did not affect propionate uptake in the presence or absence of OH-Cbl.

Intragenic complementation. Somatic cell complementation analysis has demonstrated that WG 1681 is able to complement WG 1130. To ascertain which of the two alleles (G623R or G703R) is responsible for this phenomenon, each clone of WG 1681 was cotransfected with an R93H construct (11) of WG 1130. Results are shown in Fig. 6 *A*, and the level of significance is shown in the figure legend. The cotransfection of the construct carrying the H532R polymorphism with the R93H construct did not significantly increase propionate uptake when compared to cells transfected with only H532R construct alone. The cotransfections of the constructs carrying the

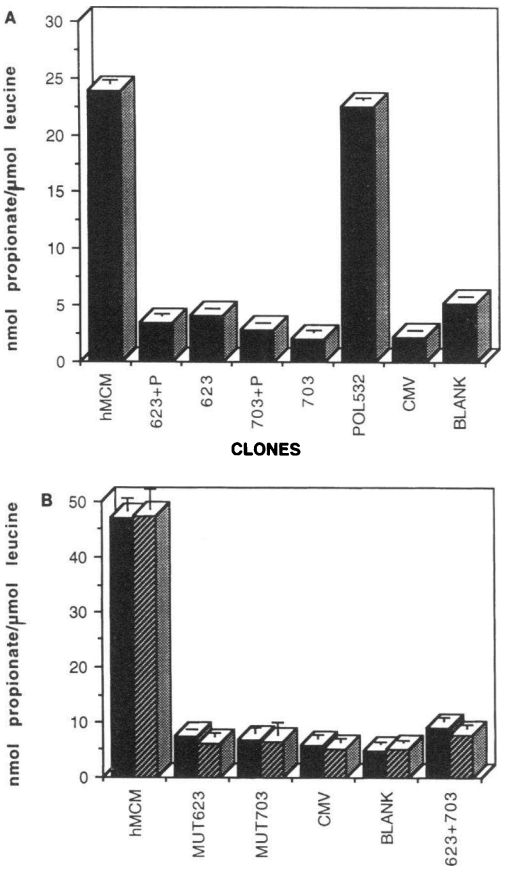

Figure 5. (*A*) Expression of constructs. Expression of constructs after electroporation into GM 1673 cells, then assayed for propionate incorporation in the absence of OH-Cbl. *HMCM,* normal MCM consensus sequence; *623,* G623R change alone; *623 + P,* G623R + V671I + H532R; *703,* G703R change alone; *703 + P,* G703R + V671I + H532R; *POL532,* H532R polymorphism alone; CMV, expression vector without insert; *BLANK,* non-electroporated cells; T bars, standard error. (*B*) Effect of OH-Cbl on expression. Expression of constructs after electroporation into GM 1673 cells. Propionate uptake was assayed in the absence and presence of 1 μg/ml OH-Cbl. *HMCM,* normal consensus sequence; *MUT623,* G623R change alone; *MUT703,* G703R change alone; *623 + 703,* cotransfection of MUT623 and MUT703 clones; ■, +OH-Cbl; ▨, −OH-Cbl.

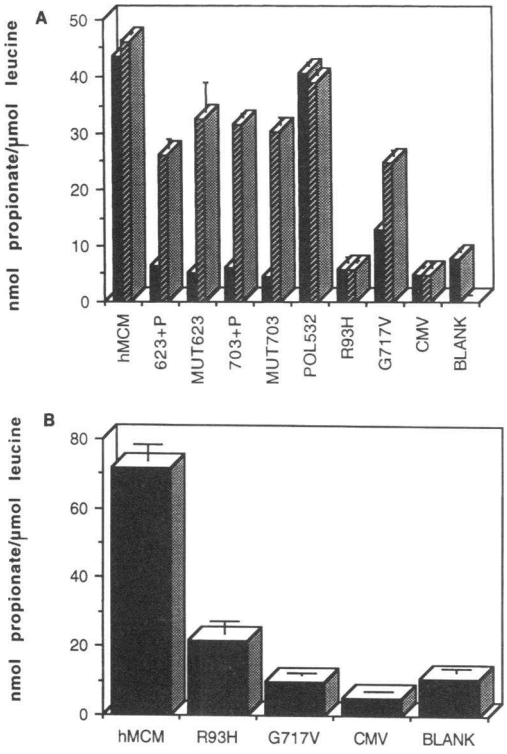

Figure 6. Intragenic complementation analysis. (*A*) Cotransfection of each clone with the R93H construct into GM 1673 cells in the absence of OH-Cbl. *hMCM,* normal consensus sequence (t = 5.59, P = 0.12); *623 + P,* G623R change with both polymorphisms (t = 22.44, P = 0.002); *MUT623,* G623R change alone (t = 7.01, P = 0.02); *703 + P,* G703R change with both polymorphisms (t = 64.39, P = 0.0002); *MUT703,* G703R change alone (t = 24, P = 0.002); *POL532,* H532R polymorphism (t = 1.19, P = 0.36); *R93H,* clone with WG 1130 mutation (t = 0.25, P = 0.82); *G717V,* clone with WG 1611 mutation (t = 7.25, P = 0.019); *CMV,* expression vector without insert (t = 0.092, p = 0.94); *BLANK,* nonelectroporated cells; *T bars,* standard error. ■, −R93H; ▨, + R93H. (*B*) Expression of clones in WG 1681 fibroblasts. Propionate uptake was measured in the absence of OH-Cbl.

G623R or G703R, or G717V mutations with the R93H construct significantly stimulated propionate incorporation. Transfection of the R93H construct into WG 1681 fibroblasts resulted in a significant increase in labeled propionate incorporation when compared to the expression vector without insert ($t = 6.12$, $P = 0.013$), to G717V ($t = 4.28$, $P = 0.025$), or to the nonelectroporated cells ($t = 3.67$, $P = 0.03$), but the transfection of the G717V construct into WG 1681 fibroblasts did not stimulate significant incorporation (Fig. 6 *B*).

Discussion

We have described two novel mutations involved in intragenic complementation at the MUT locus in fibroblasts from a single patient with MMA. The fibroblast cell line WG 1681 has been observed to complement a previously characterized *mut*^{0} cell line, WG 1130. WG 1681 is classified as a *mut*^{0} cell line on the basis of low propionate incorporation in culture that does not respond to OH-Cbl in medium. G703R and G623R were identified as the changes in WG 1681. These heterozygous changes result in the substitution of a large basic residue for a small hydrophobic amino acid near the carboxy terminus of the MCM polypeptide. Because phase could not be established by sequencing data alone, a family study was performed.

The mother was identified as the carrier of the G703R mutation in *cis* with two polymorphisms, H532R and V671I. A clinically normal half-sister and half-brother of the proband were also identified as carriers of this mutation. The ASO results supported the hypothesis that the G703R and G623R changes are inherited in *trans,* and as such, each change is encoded separately within either subunit of the MCM homodimer of WG 1681. Transfection of constructs with G703R or G623R singly, or in combination with the polymorphisms, resulted in much less incorporation of [^{14}C]propionate than that observed with control MCM constructs. In addition, both G703R and G623R constructs were unable to stimulate propionate uptake in the presence of OH-Cbl in medium after transfection. In contrast, Crane et al. (19) have previously shown that transfection of a *mut*$^-$ mutation, G717V, into GM 1673 cells resulted in a significant increase in propionate uptake in the presence of OH-Cbl in the culture medium. These observations indicate that the novel G623R and G703R are pathogenic *mut*^{0} mutations.

Because of the unknown tertiary or quaternary structures of the MCM apoenzyme, one can only speculate about the effects of the G623R and G703R mutations. The glycines at position 703 and 623 are conserved in human and murine MCM sequences, and in one subunit (MUTB) of the homologous enzyme from *Propionibacterium shermanii,* suggesting that these sites may be important to MCM structure and function. The most interesting aspect of the G623R and G703R mutations of WG 1681 is the correlation with the intragenic complementation observed at the somatic cell level. Intragenic complementation between mutations of the homodimeric MCM apoenzyme was first described by Raff et al. (11). As of yet, the *mut*^{0} cell line WG 1130 has been observed to complement 5 of 13 *mut*^{0} lines and 6 of 8 *mut*$^-$ lines. Interestingly, Raff et al. (11) reported that complementation ability between *mut* cell lines and WG 1130 did not depend on *mut*$^-$ or *mut*^{0} phenotypes. Table IV indicates that as of yet, WG 1681 has demonstrated complementation with the *mut*^{0} line WG 1130 and the *mut*$^-$ line WG 1618 only.

To ascertain whether both G623R and G703R mutations of WG 1681 or only one was responsible for interallelic complementation with the homozygous R93H mutation of WG 1130, cotransfection studies were performed. Results shown in Fig. 6 indicate that both G623R and G703R are separately capable of complementing the R93H mutation. A significant increase in propionate uptake was observed after cotransfection of different alleles, however, stimulation of propionate uptake was not observed after cotransfection of constructs carrying the G623R or G703R mutation (Fig. 5 *B*). This indicated that the novel mutations of WG 1681 do not complement each other, as predicted. The R93H construct transfected into WG

Table IV. Complementation between mut Cell Lines

	mut^0 WG 1130 R93H (hmz)	*mut*$^-$ WG 1511 H678R (hmz- cDNA)	*mut*$^-$ WG 1599 R369H (htz, cDNA)	*mut*^{0} WG 1607 W105R, A377E (htz)	*mut*$^-$ WG 1610 G717V (hmz)	*mut*$^-$ WG 1611 G717V (htz)	*mut*$^-$ WG 1613 G717V (hmz)	*mut*^{0} WG 1609 G630E	*mut*$^-$ WG 1618 UNKNOWN	*mut*^{0} WG 1681 G623R, G703R (htz)
WG 1130										
WG 1511	+									
WG 1599	−	ND								
WG 1607	−	ND	−							
WG 1610	+	ND	−	−						
WG 1611	+	+	ND	−	−					
WG 1613	+	+	ND	−	−	−				
WG 1609	+	−	−	−	−	−	−			
WG 1618	−	−	ND	−	+	+	+	ND		
WG 1681	+	ND	−	−	−	−	−	−	+	

ND, complementations not done; +, complementation; −, no complementation; htz, heterozygous mutation, heterozygosity observed at cDNA level; hmz, homozygous mutation, homozygosity observed at cDNA level. (Mutations of WG 1511 [Yale 589] and WG 1559 [Yale 378] were provided by N. Kogekar and W. A. Fenton, personal communication.)

1681 fibroblasts confirmed that the WG 1130 construct could complement the WG 1681 cell line (Fig. 6 *B*). Thus, intragenic complementation was demonstrated between these different mut^0 mutations at the somatic cell and molecular levels. In addition, the transfection of the G717V construct into WG 1681 demonstrated that this mutation could not complement this cell line. This is consistent with the observation that the mut^- cell lines, WG 1610, 1611, and 1613, which carry this mutation, do not complement WG 1681 (Table IV).

The *mut* mutations identified in cell lines that exhibit intragenic complementation to WG 1130 do not appear to exhibit any specific pattern of charge or amino acid structure. Furthermore, neither mut^0/mut^- phenotypes nor intragenic complementation can be predicted by the relative primary amino acid position. Ascertainment of tertiary and quaternary structures of the apoenzyme will assist in explaining the nature of MCM mutations, as will biochemical studies on cells expressing mutant protein.

Acknowledgments

We thank Rita Farah for performing intragenic complementation analysis on cells from the proband, and Estelle Lamothe and Angela Hosack for helpful discussions. Special thanks go to Wayne Fenton for providing information on the mutations in WG 1511 (Yale 589) and WG 1599 (Yale 378).

D. S. Rosenblatt is a principal investigator in the Medical Research Council of Canada Genetics Group. This is a publication of the Hess B. and Diane Finestone Laboratory In Memory of Jacob and Jenny Finestone. This work was supported in part by National Institutes of Health grants HD-24186 (F. D. Ledley) and HD-24064 (Mental Retardation Research Center, Baylor College).

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