THE MOLECULAR CHARACTERIZATION OF MUTATIONS AT THE METHYLMALONYL CoA MUTASE LOCUS INVOLVED IN INTERALLELIC COMPLEMENTATION

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July, 1993

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the Masters degree in Science

THE MOLECULAR ANALYSIS OF mut MUTATIONS IN INTRAGENIC COMPLEMENTATION

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ABSTRACT

Methylmalonic aciduria is an autosomal recessive metabolic disorder, which may be due to a defect in the methylmalonyl CoA mutase (MCM) apoenzyme. The mut^0 mutation is characterized by undetectable enzyme activity in cell extracts, and by the low incorporation of [^{14}C]propionate in the presence of hydroxocobalamin in culture. A mut^0 fibroblast cell line, WG 1681, from an African-American male infant was shown to complement another mut^0 cell line, WG 1130. Subsequent cloning and sequencing of cDNA from WG 1681 identified two previously described homozygous polymorphisms: H532R and V671I (1). In addition, compound heterozygosity was observed for two novel changes at highly conserved sites: G623R and G703R. Hybridization of allele specific oligonucleotides to PCR amplified MCM exons from WG 1681 and family members identified a clinically normal mother, sister and half-brother as carriers of the G703R change in cis with both polymorphisms. The putative father was not identified as a carrier of the G623R change. Transfection of each change, singly and in cis with both polymorphisms, into GM1673 cells demonstrated a lack of stimulation of [^{14}C]propionate uptake in the absence and presence of OH-Cbl, in comparison to controls. Co-transfection of each separate mutation with the previously identified R93H mutation of WG 1130 (2) stimulated propionate uptake. These results indicate that G623R and G703R are novel mutations responsible for deficient MCM activity and the mut^0 phenotype in WG 1681, and both mutations are independently capable of complementing the R93H mutation of WG 1130.

SOMMAIRE

L'acidurie méthylmalonique est un désordre métabolique de type autosomal récessif qui semble être causé par un défaut de l'apoenzyme mutase méthylmalonyl CoA (MCM). La mutation mut^0 est caractérisée par une activité non-détectable de l'enzyme dans les extraits cellulaires et par une faible incorporation en culture du propionate [^{14}C] en présence d'hydroxocobalamine. Il a été démontré que la lignée cellulaire fibroblaste, lignée mut^0, WG 1681, d'un enfant de race noire, complémente une autre lignée cellulaire mut^0, WG 1130. Les clônage et séquençage subséquents du cADN de WG 1681 ont permis d'identifier 2 polymorphismes homozygotes déjà décrits auparavant: H532R et V671 (1). De plus, de l'hétérozygosité composée à certains sites hautement conservés a été observée pour 2 nouvelles mutations: G623R et G703R L'hybridation d'oligonucleotides spécifiques aux allèles à des exons MCM, amplifiés par PCR a partir d'échantillons de WG 1681 et de membres de la famille, a permis d'identifier une mère, une soeur et un demi-frère cliniquement normale, et tous trois porteurs de la mutation G703R en "cis" avec les 2 polymorphismes. Le père présumé n'a pas été identifié comme porteur de la mutation G623R. La transfection des mutations, individuelles ou en "cis" avec les 2 polymorphismes, dans les cellules GM 1673 a démontré un manque de stimulation de l'incorporation du propionate [^{14}C] en l'absence ou en présence de OH-Cbl par rapport au contrôle. Les résultats indiquent que G623R et G703R sont de nouvelles mutations responsables d'une activité déficiente de MCM et du phénotype mut^0 de WG 1681. Les 2 mutations ont été capables indépendamment, de complémenter la mutation R93H, déjà identifiée chez WG 1130 (2).

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ABBREVIATIONS

Cbl	cobalamin, vitamin B_{12}
$Cbl^{+3,+2,+1}$	cobalamin in +3,+2,+1 oxidation states
cbl	cobalamin complementation group
AdoCbl.	5'deoxyadenosylcobalamin
AdoMet.	S-Adenosylmethionine
Ca^{+2}.	calcium in the +2 oxidative state
CN-Cbl.	cyanocobalamin
OH-Cbl.	hydroxocobalamin
GS-Cbl.	glutathionylcobalamin
IF.	intrinsic factor
TCI,II,III.	transcobalamin I, II, and III
MeTHF	methyltetrahydrofolate
MeCbl	methylcobalamin
MCM	methylmalonyl CoA mutase
MMA	methylmalonic aciduria
MUT	methylmalonyl CoA mutase locus
mut	defect in mutase apoenzyme
MUTA.	bacterial MCM gene encoding small subunit
MUTB.	bacterial MCM gene encoding large subunit
CRM	cross reactive material
PEG	polyethyiene glycol
MS.	methionine synthase
PBS	phosphate buffered saline
TAE	tris acetate
TBE	tris borate
BHI	brain heart infusion
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
IPTG.	isopropylthio-ß-D-galactoside
PCR	polymerase chain reaction
RT.	reverse transcription
ASO	allele specific oligonucleotide
bp.	base pair
a.a.	amino acid

TABLE OF CONTENTS

CHAPTER 3
INHERITED DISORDERS OF Cbl TRANSPORT AND METABOLISM

CHAPTER 4
MOLECULAR AND GENETIC CHARACTERIZATION OF METHYLMALONYL CoA MUTASE (MCM)

CHAPTER 5
MATERIALS AND METHODS

CHAPTER 6
RESULTS

LIST OF FIGURES AND TABLES

CHAPTER 1

STRUCTURE, DISTRIBUTION AND TRANSPORT OF COBALAMIN

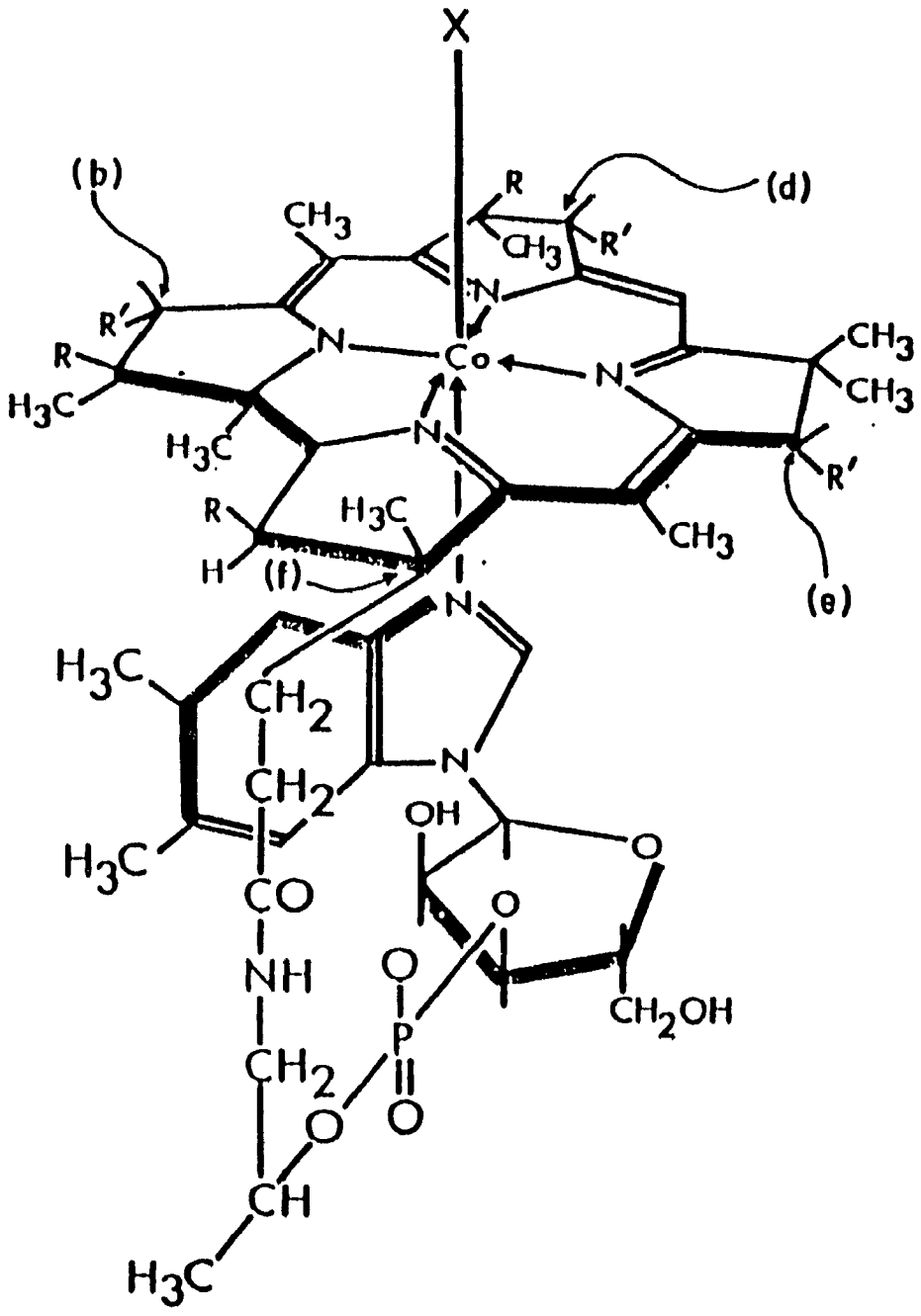
1.1 STRUCTURE OF COBALAMIN

Vitamin B_{12}, also known as cobalamin (Cbl), is a water soluble, organometallic molecule. It was first crystallized in 1947 by Karl Folkers and coworkers, and its three-dimensional structure was first revealed in 1956 by Dorothy Hodgkin's laboratory (3). Its core consists of a planar corrin ring surrounding a central cobalt atom which may exist in a +3, +2, or +1 oxidative state. A phosphoribo-5-6-dimethylbenzimidazolyl ligand is linked to the cobalt atom at the lower axial position, and the ligand at the upper coordination site determines the type of Cbl compound (Fig.1). The most common commercial form is cyanocobalamin (CN-Cbl) and does not occur naturally in plants, microorganisms and animal tissues. Hydroxy, methyl, and adenosyl ligands comprise the three forms of Cbl which have been frequently isolated from mammalian tissues: hydroxocobalamin (OH-Cbl), methylcobalamin (MeCbl), and adenosylcobalamin (AdoCbl), respectively (4). The latter two forms of Cbl fulfil an important role as cofactors in many organisms (5). Glutathionylcobalamin (GS-Cbl) has been recently isolated from mammalian tissues, and is presumed to be another naturally occuring form of Cbl (6).

1.2 DISTRIBUTION AND DIETARY SOURCES

Specific microorganisms possess the exclusive ability of Cbl biosynthesis (7). Cbl can be endogenously synthesized by certain fungi (*Streptomyces griseus*), certain actinomycetes (*Streptomyces aureofaciens*), and certain bacteria (*Propionibacterium shermanii, Clostridium tetanomorphum, Rhizobium meliloti*). Bacteria such as *E.coli* and *Lactobacillus leichmanii* cannot produce the corrin ring of Cbl (7). Yeast and man also cannot synthesize Cbl (8). Man is completely dependent on dietary Cbl due to his inability to produce the corrinoid structure. In addition, man is unable to absorb vitamin synthesized by the colonic flora as Cbl

FIG. 1 THE STRUCTURE OF COBALAMIN. R=-CH_2CONH_2; R'= -$CH_2CH_2CONH_2$; X= -CN for (cyanocobalamin), -OH (hydroxocobalamin), -CH_3 (methylcobalamin) or 5' deoxyadenosyl (adenosylcobalamin). Reprinted from Fenton and Rosenberg (4).


X
(b)
(d)
R
R'
CH3
N
Co
H3C
(f)
H
(e)
CH2
OH
O
CO
NH
P
CH2OH
CH

cannot be absorbed in the colon. All Cbl which is found in animal products or plants is derived from bacteria (8). Food products including meat, dairy, and fish are good dietary sources, whereas plants are a relatively poor source (8).

High concentrations of AdoCbl are found in the liver, kidney, and pituitary, while MeCbl predominates in the plasma. The established daily dietary requirement of cobalamin for adults is 2-5 μg, and the estimated daily rate of loss is approximately 0.1% of the total body pool (9). This implies that the development of a deficiency state will not occur for several years after the intake of Cbl has ceased (10).

1.3 ABSORPTION AND TRANSPORT OF COBALAMIN

Proteases and acids in the stomach remove Cbl from dietary proteins after ingestion. Absorption and transport of Cbl then depend on a rather complex system comprised of specific carrier proteins and cell surface receptors (11). This mechanism allows transport of 50% or more of carrier-bound Cbl. Only 0.1-1% of Cbl will cross the cell membrane in the absence of such carrier-receptor interactions (9).

1.3.1 R binders

The family of R proteins consists of R binders, transcobalamin I (TCI), transcobalamin III (TCIII), haptocorrins, and cobalophyllins. All R binders are the products of one genetic locus, differing in the number of glycosylations (12). Recently, the full length cDNA encoding human TCI has been isolated (13). The transcript is 1.5 kb, and the 433 amino acid sequence contains 9 glycosylation sites. Little is known about the physiological significance of TCI and TCIII except that almost 75% of total endogenous Cbl is bound to them (14). R binders are glycoproteins exhibiting high affinity for Cbl, and have been isolated from plasma, tissue extracts, secretions (eg. saliva and bile), and the cytoplasm of erythrocytes, granulocytes and platelets (4,9). Although the physiological significance has not yet been comprehended, it has been suggested that the role of R binders may be to

maximize Cbl utilization by removing potentially harmful Cbl analogues from circulation, and by preventing bacterial usage of Cbl (11).

Ingested Cbl binds to salivary and gastric R binders. In this form, Cbl cannot be absorbed or reabsorbed (8). Consequently, this complex is digested by pancreatic enzymes in the small intestine, where Cbl then binds to intrinsic factor (12). Pancreatic insufficiency results in the lack of tryptic digestion of R-binder from Cbl, thus inhibiting Cbl absorption. Diseases of the small bowel are particularly predisposed to acquired Cbl malabsorption (9).

1.3.2 Intrinsic Factor (IF)

IF is a glycoprotein produced in the parietal cells of the gastric mucosa, and mediates uptake of Cbl from R binders in the gastrointestinal tract (4). A cDNA clone encoding the rat gastric IF has been isolated and characterized (15). The deduced protein contains 421 amino acids with a molecular weight of 46 kD.

IF stringently binds Cbl, and does not bind or mediate uptake of Cbl analogues, thereby minimizing their accumulation (11). Upon binding of Cbl, the IF molecule shrinks, resulting in an increased affinity of the IF-Cbl complex for specific receptors located on the brush border membrane of the ileal cells (16). Binding occurs at neutral pH and requires the presence of Ca^{+2} ions (11). Enterocytes internalize the complex by an endocytotic process (9). Studies on intestinal uptake of rat IF-Cbl complexes have deduced that detachment of the complex from the receptor occurs within the enterocyte, and requires an acidic pH, indicative of lysosomal or endosomal localization (17). Thus, the release of Cbl from IF is presumed to be dependent upon lysosomal digestion. There does not seem to be any considerable recycling of IF to the brush border membrane (17).

Free Cbl is absorbed by traversing the basal membrane of the enterocyte, and appears in the portal circulation bound to transcobalamin II (4).

The most common acquired adult form of IF deficiency is caused by the atrophy of parietal cells of the stomach, resulting in pernicious anemia (18). In these patients, serum Cbl levels are markedly deficient and IF auto-antibodies are present

(4). This classical form of pernicious anemia occurs in both men and women over 60 years of age, and is a result of the destruction of the parietal cells by the autoantibodies causing atrophy with achlorhydria (18).

1.3.3 Transcobalamin II (TCII)

Transport of Cbl into mammalian tissue is mediated by a serum protein, TCII. This protein is also found in spinal fluid, plasma, semen, and extracellular fluid, and is synthesized by amniocytes, fibroblasts, endothelial cells, and enterocytes (9). All newly absorbed Cbl is complexed with TCII within the enterocyte, prior to entering the portal circulation. Although, TCII accounts for only a small percent of total Cbl binding, it is the only source of physiologically active Cbl (11,14). TCII undergoes conformational change upon binding of Cbl, increasing its affinity for plasma membrane receptors. Binding is dependent on the presence of Ca^{+2} ions (16,19). The complex is internalized via energy dependent endocytosis into the lysosome or endosome (9,19). Internalization occurs rapidly, and it may take several hours before TCII levels return to normal (11).

Human TCII has been linked to chromosome 22q (20), and the cDNA and amino acid sequences have been deduced (21). The full length cDNA is comprised of 1866 nucleotides, which encodes an 18 amino acid leader sequence and a 409 amino acid mature protein. Comparison of nucleotide sequence between TCII, TCI, and rat IF indicates more than 50% identity. Very recently, Li et al. (22) have isolated variant forms of human TCII. Two identified cDNA clones differed at codons 259 and 376 when compared to the previous isolated TCII sequence. The two clones differed from each other at codons 198 and 219, and in the length of the 5' and 3' non-coding regions. Although amino acid sequence analysis revealed an overall homology of 33% with TCI and rat IF, four identified regions had 80% homology while two other regions had 60% homology with TCI and rat IF. The Cbl-binding functional domain is predicted to reside within the six regions of high homology (22).

1.3.4 Alternative Cbl Transport

Cbl may be internalized into cells via other mechanisms, although the process already described is the most common. Free Cbl can be internalized by fibroblasts using a saturable, Ca^{+2}-independent system, requiring an intact electron transport chain (23). Patients deficient in TCII are able to transport Cbl in this manner when they are treated with high concentration of therapeutic Cbl. A second mechanism has been described in hepatocytes where asialoglycoprotein receptors are able to clear R protein bound Cbl from plasma into the bile (11). TCI and TCIII are able to recognize these membrane receptors, and upon internalization, lysosomal degradation releases free Cbl intracellularly.

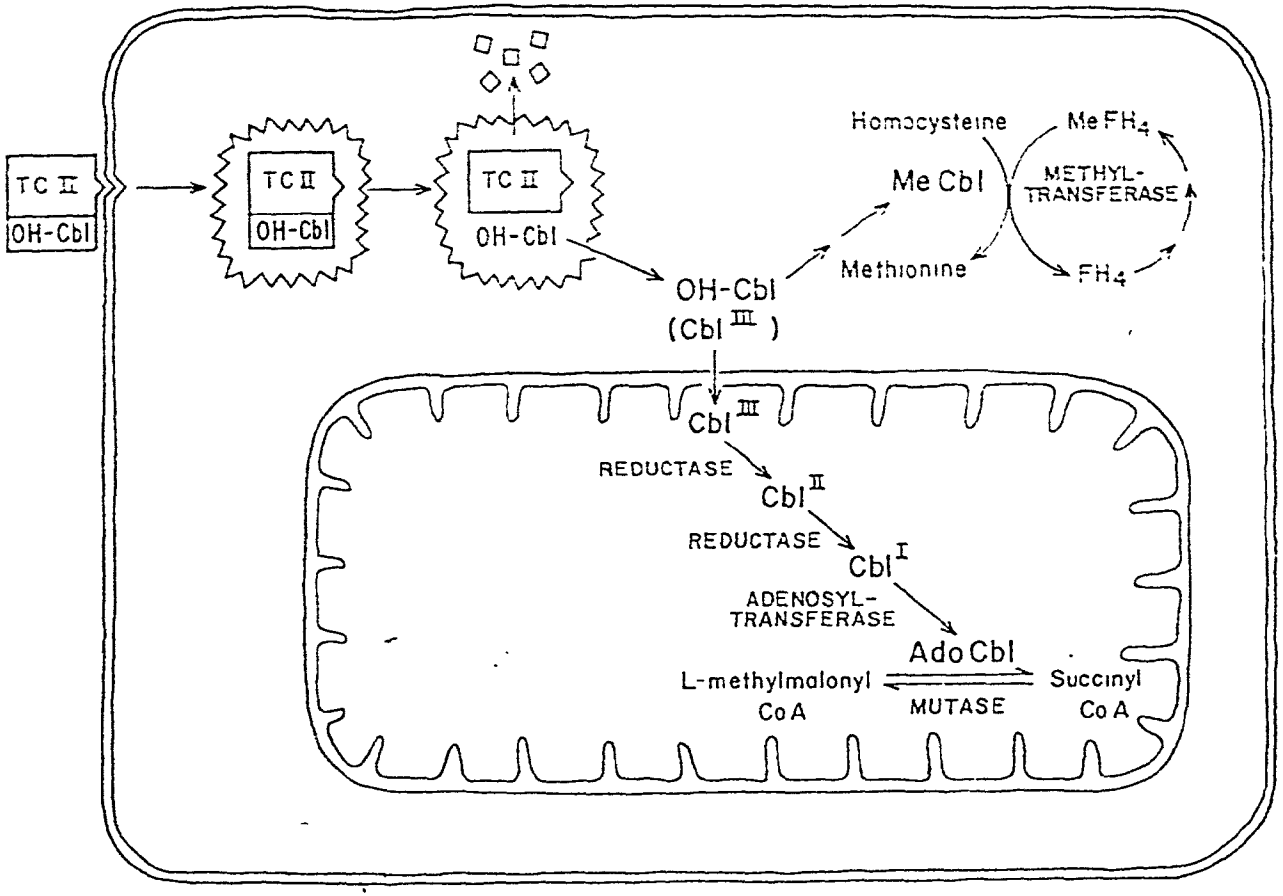
1.4 INTRACELLULAR Cbl METABOLISM

The TCII-Cbl complex is processed by the degradative action of proteases in the lysosome (24). Free cobalamin can efflux into the cytoplasm, while TCII leaves the cell in fragments (24). Studies on fibroblasts have demonstrated inhibition of TCII-Cbl degradation by choloroquine, leading to the accumulation of the complex in the lysosome (19). It is known that TCII is degraded in the lysosome, however, it is unclear whether the dissociation of Cbl from TCII occurs in the lysosome or the endosome. Although the mechanism of Cbl efflux is also unknown, recent work by Idriss and Jonas (25) has demonstrated Cbl transport by isolated rat liver lysosomal vesicles. The study of this system may help elucidate lysosomal transport and Cbl release. In the cytoplasm, cobalamin undergoes a reduction from cob(III)alamin to cob(II)alamin, which can then be methylated to form MeCbl and assist in the methionine synthase (MS) conversion of homocysteine to methionine (4). Alternatively, cob(III)alamin or cob(II)alamin can enter the mitochondrion and be further reduced to cob(I)alamin. It can then be adenosylated to AdoCbl and participate in the conversion of methylmalonyl CoA to succinyl CoA by methylmalonyl CoA mutase (MCM) (4). Almost 95% of intracellular Cbl is found bound to either MS or MCM (5,26).

1.5 MITOCHONDRIAL Cbl-UPTAKE

Although no specific mithochondrial transport system for Cbl has been described, OH-Cbl uptake in lysosomal-free preparations of rat liver mitochondria was observed to be dependent upon mitochondrial swelling (27). This process was concentrative, saturable and specific for OH-Cbl, and did not rely on energy metabolism or ion transport (28). Presumably, mitochondrial swelling increases mitochondrial permeability to OH-Cbl (28). Specificity of uptake was shown to result by high affinity binding of OH-Cbl to an intramitochondrial protein that comigrated with MCM (26). This suggests that mitochondrial Cbl uptake presumably occurs by passive diffusion followed by binding to MCM (28).

FIG. 2 INTRACELLULAR Cbl METABOLISM. TCII= transcobalamin II; OH-Cbl= hydroxocobalamin; $Cbl^{+I, +II, +III}$= Cbl with cobalt core at +1, +2, +3 oxidation states, respectively, MeCbl= methylcobalamin; $MeFH_4$= methyltetrahydrofolate; FH_4= tetrahydrofolate; AdoCbl= adenosylcobalamin. Reprinted from Rosenberg and Fenton (29).


TC II
OH-Cbl
TC II
OH-Cbl
TC II
OH-Cbl
OH-Cbl
(Cbl III)
Homocysteine
Me FH4
Me Cbl
METHYL-
TRANSFERASE
Methionine
FH4
Cbl III
REDUCTASE
Cbl II
REDUCTASE
Cbl I
ADENOSYL-
TRANSFERASE
Ado Cbl
L-methylmalonyl
CoA
MUTASE
Succinyl
CoA

CHAPTER 2
COBALAMIN-DEPENDENT ENZYMES

2.1 METHIONINE SYNTHASE (MS)

Methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase) (EC 2.1.1.13) is a cytosolic enzyme responsible for the conversion of homocysteine to methionine in mammalian cells. It participates in the transfer of a methyl group from 5-methyltetrahydrofolate (MeTHF) to homocysteine to form methionine, and requires MeCbl as a cofactor (9,29). Although the gene encoding MS has not yet been cloned, it has been assigned to chromosome 1 by measuring [^{57}Co]Cbl binding and MS activity in rodent-human cell hybrids (30). MS has been isolated from various animal tissues including porcine kidney (31) and liver (32), and human placenta (33). Analysis of MS from human placenta on a native polyacrylamide gel identified a single band of molecular weight 160,000. However, denaturing gels revealed three subunits of 90,000 Da, 45,000 Da, and 35,000 Da, indicating a complex protein structure (33). MS from human placenta contains 1 mole of Cbl per mole of apoenzyme, and 2 moles of iron per mole of holoenzyme. It has been postulated that the iron binds to the lower molecular weight subunits which can then participate in oxidation-reduction reactions (33).

In *E.coli* B, the MS enzyme is encoded by the MetH gene. Unlike mammalian MS, the bacterial product is monomeric. It contains 1 mole of Cbl per mole of enzyme, and it also contains 1 mole of copper per mole of MS-bound Cbl (34). Studies on the recombinant enzyme from *E.coli* K-12 revealed the lack of stoichiometric copper, thus suggesting that it may not play an important catalytic role (34).

2.1.1 Methylcobalamin (MeCbl)

MeCbl, the coenzyme form of Cbl, participates in the methionine synthase reaction, and is assumed to be synthesized in the cytosol (9). This coenzyme

is the major circulating Cbl species bound to TCI, and accounts for 60% to 80% of total plasma Cbl (4).

MeCbl is formed when MS bound cob(II)alamin is reduced to cob(I)alamin as it receives an initial methyl group from S-adenosylmethionine (AdoMet). This first methylation step is crucial in the catalytic activation of MS (35). MeCbl then serves as a methyl-transfer intermediate to homocysteine to generate methionine, and an MS-cob(I)alamin complex. Subsequent methyl groups are provided by 5-methyltetrahydrofolate (MeTHF), until there is a spontaneous oxidation of MS-cob(I)alamin to MS-cob(II)alamin (9). Experiments in *E.coli* demonstrate that methyl transfer from MeTHF does not generate the energy potential to initiate reduction of cob(II)alamin bound MS (34). Therefore, AdoMet, along with a reducing system, is unequivocally required to regenerate MeCbl.

In *E.coli*, a reducing system involving two flavoproteins called R and F component has been identified (36). FAD and FMN are noncovalently bound to each component, respectively, and operate as intermediates in electron transport from NADPH to MS (37).

Although intact human fibroblasts are able to synthesize MeCbl from dietary OH-Cbl (38), a specific reducing system has not yet been demonstrated in mammals. However, cell extracts from a patient described by Rosenblatt et al. (39), demonstrated normal MS activity under standard reducing conditions, but demonstrated low enzyme activity at decreased concentrations of reducing agents. This suggested that a defective MS-related reducing system was involved, and indicated that such a reducing system does exist in mammals (40).

2.1.2 5-Methyltetrahydrofolate (MeTHF)

The biosynthesis of purines or pyrimidines relies on the generation of tetrahydrofolate (THF) (34). MeTHF is the major circulating monoglutamyl form of folate, and is required for remethylation of cob(I)alamin bound to MS (9). Methylene tetrahydrofolate (CH_2-THF) reductase (E.C. 1.5.1.20) catalyses the irreversible NADPH-linked reduction of CH_2-THF to MeTHF. Functional MS-

cob(I)alamin transfers the methyl group from MeTHF to homocysteine and thus generates methionine and THF (41). Subsequently, THF is reconverted to CH_2-THF, which is required for thymidylate biosynthesis (34). Thus, diminished MS activity results in a decline of total intracellular folate levels. An accumulation of MeTHF, referred to as the MeTHF trap (42), occurs at the expense of other folate pools, and consequently results in the depletion of THF. The CH_2-THF levels are then exhausted, thus affecting thymidylate biosynthesis. The resulting impaired DNA synthesis appears to be the primary cause of megaloblastic anemia associated with either decreased MS activity or folate deficiency in man (34).

2.2 METHYLMALONYL CoA MUTASE (MCM)

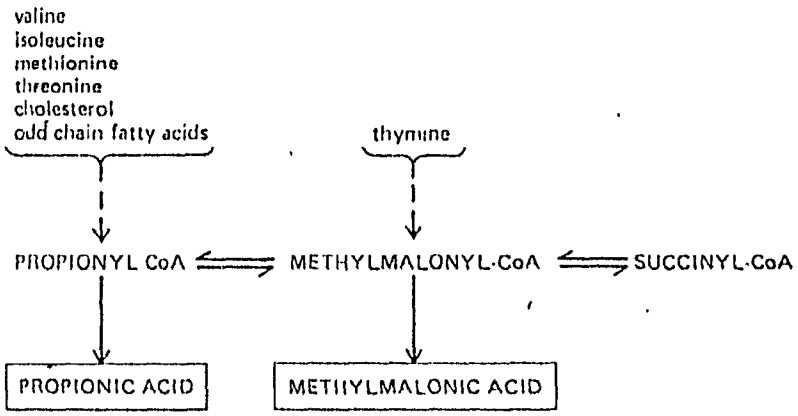
In 1955, an enzyme capable of converting methylmalonyl CoA to succinyl CoA was identified in sheep kidney and rat liver (43). Subsequently, this enzyme was termed methylmalonyl CoA mutase (MCM) (EC 5.4.99.2), and was shown to be involved as the final isomerization step in the metabolism of propionic acid in mammalian tissues (44). Although this pathway is not a predominant source of succinate in mammalian cells, it is required as an intermediate step in the degradative pathway for valine, isoleucine, threonine, methionine, thymine, odd chain fatty acids, and cholesterol in man (29) (Fig.3).

In other species, MCM is required for different functions. In ruminants, mutase is essential for gluconeogenesis from propionate (45), and in prokaryotes such as *Propionibacterium shermanii*, MCM is responsible for the isomerization of succinyl CoA to methylmalonyl CoA (the reverse reaction) during synthesis of propionate from tricarboxylic acid-cycle intermediates (46).

2.2.1 Enzymatic Properties

In 1962, Phares et al. (47), and Kellermeyer and Wood (48) independently demonstrated that the isomerization took place by an intramolecular shift of the CoA carboxyl group to the methyl moiety. Earlier studies on glutamate

FIG. 3 **CATABOLIC PATHWAY FOR PROPIONATE AND METHYLMALONATE.** Reprinted from Rosenberg and Fenton (29).


valine
isoleucine
methionine
threonine
cholesterol
odd chain fatty acids
thymine
PROPIONYL CoA
METHYLMALONYL-CoA
SUCCINYL-CoA
PROPIONIC ACID
METHYLMALONIC ACID

mutase from *Clostridium tetanomorphum* indicated that the isomerization of glutamate to mesaconate occurred by the same intramolecular rearrangement (49). Consequently, further studies on glutamate mutase (50) and MCM (51) from bacterial and rat liver mitochondrial extracts, respectively, demonstrated the high dependence of the transcarboxylation on the presence of a cofactor form of Cbl (see section 2.2.4).

2.2.2 MCM Enzyme Activity Assays

Two assays which directly measure MCM activity have been described. A spectrophotometric measurement of NADH consumption at 340nm examines the succinyl CoA-> methylmalonyl CoA reaction (10). The reaction mixture contains NADH, AdoCbl, succinyl CoA, glutathione, sodium pyruvate, oxaloacetate transcarboxylase, malate dehydrogenase, methylmalonyl CoA racemase and the MCM to be assayed. Decrease in absorbance measures the catalyzing ability of MCM as μmol succinyl CoA consumed per minute.

The second assay utilizes radioactivity and is fundamentally used when examining the methylmalonyl CoA-> succinyl CoA reaction in mammalian cells (44). MCM to be assayed is incubated with a reaction mixture containing AdoCbl, buffer, and [^{14}C]-L-methylmalonyl CoA. The reaction is terminated with perchloric acid, and the $^{14}CO_2$ produced from the potassium permanganate oxidation of excess methylmalonyl CoA is removed. Labelled succinate, resistant to the oxidation, is counted and MCM enzymatic activity is measured as μmol succinate formed per minute (10).

2.2.3 Intracellular Localization

MCM is most abundant in mammalian tissues with high concentrations found in kidney and liver (10). Studies utilizing specific enzyme markers to subcellular and submitochondrial fractions from human and rat liver localized MCM activity to the mitochondrial matrix (52). Intracellular enzyme distribution in rat liver identified

almost 97.5% of MCM in the mitochondria, and this compartmentalization was not affected by Cbl deficiency (52).

2.2.4 Requirement of Adenosylcobalamin (AdoCbl)

The MCM reaction requires AdoCbl, the coenzyme form of Cbl which is produced from dietary OH-Cbl. In 1958, AdoCbl was isolated, and was spectrophotometrically identified as the Cbl-containing cofactor required for the decomposition of glutamate in bacteria (50). Subsequent *in vitro* studies on liver homogenates (53) and liver mitochondria (51) from Cbl-deficient rats demonstrated an increase in MCM activity with the addition of AdoCbl, but not with CN-Cbl. Willard and Rosenberg (54) later established that endogenous holomutase activity in cultured human fibroblasts increased when incubated with OH-Cbl. They also deduced that the interaction between MCM, AdoCbl and methylmalonyl CoA was a result of independent, noncooperative active sites. Although the role of AdoCbl in the MCM reaction is obscure, it has been postulated that it is involved in the generation of free radicals of methylmalonyl CoA through oxidation and reduction of the cobalt atom by hydrogen transfers between substrate and coenzyme (10,48). This would facilitate the intramolecular rearrangement during isomerization. Such a molecular process was demonstrated recently by Murakami (55). An artificial enzyme simulating MCM catalytic function was capable of carbon-skeleton rearrangement of substrate in combination with hydrophobic Cbl attached to a synthetic lipid bilayer.

2.2.4.1 AdoCbl synthesis in bacteria

The synthesis of AdoCbl has been primarily studied in the bacteria *Clostridium tetanomorphum* (56). Three enzymatic steps are required for the conversion of OH-Cbl to AdoCbl. Upon entry into the mitochondrial matrix, Cbl^{+3} is reduced to Cbl^{+2} and subsequently to Cbl^{+1} by cob(III)alamin reductase (EC 1.6.99.8) and cob(II)alamin reductase (EC 1.6.99.9), respectively (57). This is followed by adenosylation with ATP by the enzyme 5'-adenosyltransferase (EC

2.5.1.17) to form AdoCbl (56) (see Fig.2). Adenosyltransferase activity in *Lactobacillus leichmannii* and *L. delbruekii* has been located to the ribosome, however, enzyme activity in *C. tetanomorphum* and *P. shermanii* has been associated with the 144,000g supernatant fraction (58). None of the reductases have been purified extensively, but it is known that both reduction steps are NADH-dependent and utilize FAD or FMN (57). Bacterial adenosyltransferase has been purified approximately 300 fold, and requires Cbl in a monovalent state and Mn^{+2} divalent cations for acitivity (56).

2.2.4.2 AdoCbl synthesis in mammals

AdoCbl synthesis was described in HeLa cells by Kerwar et al. (59). They showed that cell extracts were able to convert OH-Cbl to AdoCbl in the presence of ATP and a reducing system. Subsequently, Fenton and Rosenberg (60) demonstrated that intact rat liver mitochondria were also capable of synthesizing AdoCbl from OH-Cbl, requiring a source of ATP and reducing equivalents (NADH and glutamate). This implied that all three enzymatic steps for AdoCbl synthesis previously described in bacteria were also present in some form in mammalian mitochondria.

CHAPTER 3

INHERITED DISORDERS OF Cbl TRANSPORT AND METABOLISM

3.1 DEFECTIVE ABSORPTION AND TRANSPORT OF Cbl

3.1.1 R-binder Deficiency

R-binder (TCI) deficiency has been described in only a few patients and has no distinct phenotype. Although serum Cbl levels are low, these individuals do not exhibit clinical symptoms of Cbl deficiency (9). This is attributed to normal levels of the physiologically important TCII which can transport Cbl in serum (61).

3.1.2 Defective Intrinsic Factor

Patients with the inherited form of pernicious anemia exhibit low serum Cbl levels and megaloblastic anemia, but do not indicate any signs of gastric mucosal atrophy or autoantibodies as observed in patients with the acquired form (62) (see section 1.3.2). Cbl absorption is abnormal in these children, but can be restored by mixing Cbl with gastric juice as a source of normal IF (12). Symptoms of the inherited form manifest themselves in late infancy as this is believed to be the period at which Cbl transport switches from the pinocytic mechanism to the receptor-mediated form (4).

Functionally defective IF has been delineated into groups that include IF with reduced affinity for either Cbl or ileal receptor sites (63), no immunologically detectable IF (64), or IF with an increased susceptibility to proteolytic degradation (65).

3.1.3 Enterocyte Malabsorption (Imerslund-Grasbeck syndrome)

This syndrome results in selective Cbl malabsorption by enterocytes, and may encompass defects affecting Cbl-receptor formation, internalization of Cbl-receptor complex, and Cbl transfer to TCII (66). These patients exhibit megaloblastic anemia and low serum Cbl levels in early childhood, while some exhibit proteinuria as well (67). In contrast to IF deficiency, Cbl levels are not restored to normal after

mixing with gastric juice (9). The syndrome is characterized by normal TCII levels, normal IF function and secretion, absence of IF auto-antibodies, and normal gastrointestinal morphology (12). Recently, this malabsorption syndrome was described in a family of dogs (68). Although normal IF was present, there was an absence of the ileal IF-Cbl receptor complex, suggesting that the receptor was not expressed at the brush border.

3.1.4 TCII Deficiency

Patients with TCII deficiency generally display megaloblastic anemia, vomiting, failure to thrive, and pancytopenia, and eventually develop immunological and neurological disease (62). These patients are usually normal at birth, but symptoms manifest within the first two months of life. The transport of Cbl to cells is hindered by impaired TCII. However total circulating Cbl levels are normal. By testing the ability to bind Cbl in plasma, TCII has been demonstrated to be absent in affected individuals (9). The additional observation that Cbl absorption may also be affected in these patients, suggests that TCII synthesis is probably required for IF-mediated transport of Cbl (9).

This autosomal recessive disease may result from absent or defective TCII with no Cbl binding, and from physiologically inactive TCII with normal Cbl binding (4).

3.2 DISORDERS OF Cbl METABOLISM

3.2.1 Methylmalonic Aciduria (MMA)

MMA is a heterogeneous and rare autosomal recessive disease with an incident rate ranging from 1 in 50,000 to 1 in 29,000 (69,70). Normal human blood, urine and cerebral spinal fluid contain small amounts of methylmalonic acid; however, large amounts accumulate specifically in the blood and urine of affected children (9,28). Normal children and adults excrete less than 5 mg methylmalonate daily and have undetectable plasma levels. In contrast, children with MMA may excrete between 240 to 5700 mg methylmalonate daily and levels of 2.6 to 34 mg/dl (0.22 to

2.88mM) can be detected in their plasma (29). Characteristic laboratory findings indicate hyperammonemia, hypoglycemia, and elevated levels of ketones and methylmalonic acid in urine. Manifestations may range from benign to fulminant with persistent symptoms of ketoacidosis, lethargy, recurrent vomiting, dehydration, respiratory distress and muscular hypotonia (71,72).

MMA patients have demonstrated Cbl-responsive and unresponsive variants. Synthesis of AdoCbl is deficient only in fibroblasts from responsive patients (cbl class), whereas MCM synthesis is affected in unresponsive patients (mut class), thus indicating two forms of MMA (73,74). Clinical symptoms in the responsive form of the disease can be alleviated by Cbl administration and dietary restrictions (74), whereas children clinically unresponsive rely on dietary therapy. Carnitine deficiency may also arise due to the accumulation of unmetabolized organic acid esters (70). Therefore, limiting intake of amino acid precursors, and supplementation with sodium bicarbonate and carnitine is the mainstay of dietary treatment (69).

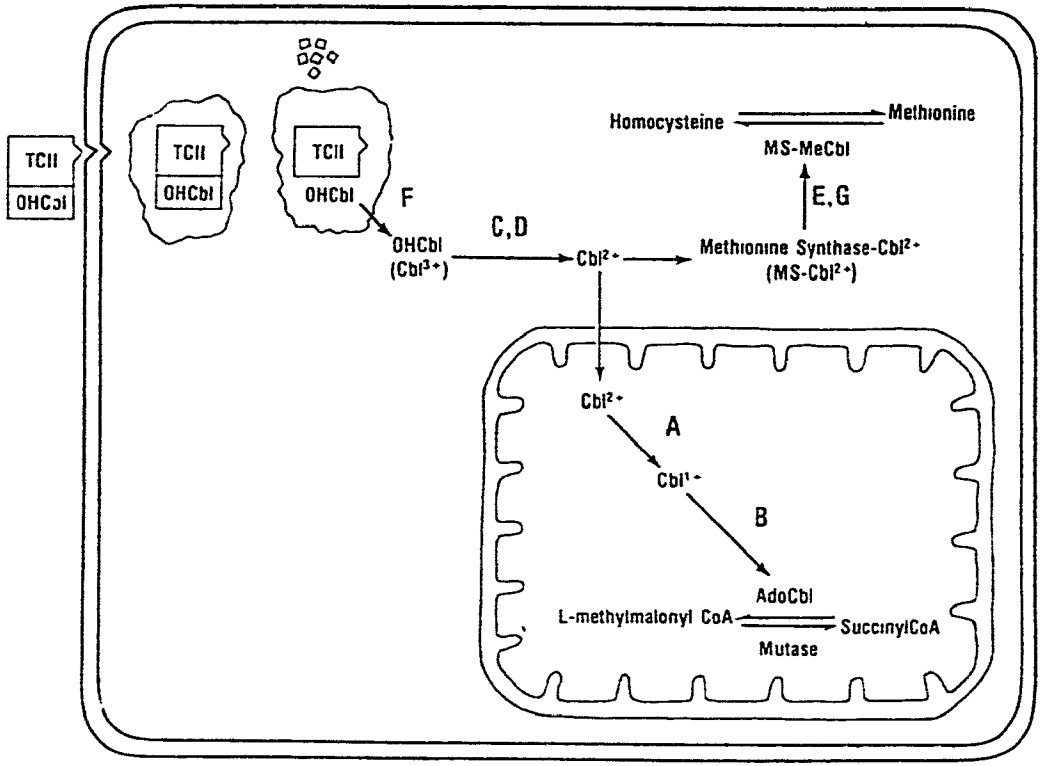
3.2.2 Somatic Cell Complementation Groups

Biochemical studies and somatic cell complementation of human cultured fibroblasts have delineated inborn errors of Cbl metabolism into independent genetic classes (28). These represent selective or combined deficiency of AdoCbl and MeCbl. Complementation analysis examines uptake of a labelled precursor by fused and unfused cells, co-incubated from two cell lines. Fused cell lines which stimulate incorporation complement each other and represent different complementation groups, whereas lack of stimulation assigns them the same complementation group (75).

A complementation test using Sendai virus-induced heterokaryons was developed by Gravel et al. (76). The ability of the heterokaryons to incorporate [^{14}C] propionate into trichloroacetic acid-precipitable material monitored the MCM route of Cbl metabolism. Likewise, incorporation of [^{14}C] MeTHF into acid-precipitable material analyzed the defects in the MS pathway (75). Presently, polyethylene glycol is used as a fusing agent (77). Both tests have defined seven distinct

FIG. 4 **INBORN ERRORS OF Cbl METABOLISM.** The letters A through G represent the different classes of Cbl mutations defined by complementation studies. Reprinted from Cooper and Rosenblatt (9).

Inborn Errors of Vitamin B_{12} (Cbl) Metabolism



complementation groups among patients with defective Cbl cellular metabolism. These groups are alphabetically designated cblA to cblG (4,9) (Fig.4), and presumably represent mutations at distinct genetic loci. An eighth complementation class, designated mut, constitutes defects of the MCM apoenzyme (73,76) and exhibits interallelic complementation (see 4.8.1).

3.2.3 cblA and cblB

The two classes of Cbl-responsive MMA have been defined as cblA and cblB (75). Patients exhibit MMA without homocystinuria, accumulate normal amounts of MeCbl, and present selective deficiency of AdoCbl synthesis (12).

Cell-free extracts from cblA patients were able to synthesize AdoCbl normally, but intact fibroblasts accumulated negligible amounts of AdoCbl (78). This discrepancy was due to the non-enzymatic reduction of cob(III)alamin to cob(I)alamin by the AdoCbl synthesis assay used for cell extracts. Although the biochemical abnormality in the cblA class is obscure, this evidence suggests a presumed defect in cob(III)alamin reductase or cob(II)alamin reductase (78).

Both intact fibroblasts and cell extracts from cblB patients were unable to synthesize AdoCbl from OH-[^{57}Co]Cbl. This implied normal Cbl reduction, but a presumable deficiency in cob(I)alamin:ATP-adenosyltransferase (79).

Almost 90% of cblA patients, and less than 40% of cblB patients, respond well to pharmacological doses of OH-Cbl. Intramuscular administration of AdoCbl to cblB patients have also failed to repress MMA symptoms (69,80).

Recently, Cooper and coworkers (81) have identified a patient who biochemically and clinically resembles other cblA patients. Fibroblasts complemented with cblB, and unexpectedly, with cblA cell lines, suggesting a genetically distinct abnormality. Inefficient entry of Cbl into the mitochondria, or decreased synthesis of cob(I)alamin have been postulated but not yet confirmed (81). Nevertheless, the identification of this new complementation group indicates the possibility of another genetic loci required for intracellular Cbl metabolism.

3.2.4 cblC and cblD

The cblC and cblD complementation classes represent patients exhibiting decreased AdoCbl and MeCbl synthesis, resulting in combined MMA and homocystinuria (75). Consequently, both propionate and MeTHF cellular uptake by cblC and cblD lines is low (75). The levels of accumulated metabolites are higher as compared to the levels accumulated in patients with Cbl deficiency or with Cbl transport defects (82). Serum Cbl and folate levels are within normal range (29).

Most cblC disease is the most common inherited disorder of Cbl metabolism, accounting for over 5O reported cases (82). CblC patients exhibit symptoms soon after birth, but onset may also occur in adolescence. Clinical symptoms include failure to thrive, lethargy, poor feeding, and hypomethioninaemia (9). Neurological manifestations are almost always prominent in cblC patients (29). This may be attributed to the MeCbl deficiency and the decreased activity of MS (see section 3.2.6). Some patients present hematologic abnormalities such as megaloblastic and macrocytic anemia (83), and ophthalmological deterioration is observed to be predominant in infants (82). Intramuscular treatment with OH-Cbl reverses the megaloblastic changes, and reduces the MMA and homocystinuria but does not fully eliminate them (9).

Transport of Cbl is normal in cblC cells as they are capable of internalizing TCII bound Cbl, and digesting the complex (84). Labelled-Cbl binding studies demonstrated that cblC fibroblasts were completely deficient in Cbl binding to MCM and MS (85). Further studies showed that cblC fibroblasts were unable to utilize CN-Cbl. This was assumed to be due to their inability to remove the cyanide group (85). The enzyme, ß-ligand transferase, is believed to be involved in the CN/GS axial group exchange (86). Recent work by Pezacka and Rosenblatt (87) have shown variability in decrease of specific activities of ß-ligand transferase and Cbl reductase in cblC cells. The latter enzyme reduces cob(III)alamin to cob(II)alamin after Cbl release from the lysosome, and has been suggested to be defective in cblC (85). However, due to the variable decrease in activity of both enzymes, Pezacka

suggests that cob(III)alamin reductase may not be the primary defect in cblC (87). There may be a defect in an earlier common metabolic step, however, as of yet, there is no evidence to support this (87). Thus, the specific defect is yet unknown.

The cblD class was first identified by complementation analysis of fibroblasts from two male siblings (77). A later age of onset and no apparent hemotologic abnormalities were apparent only with the original description of cblD, but subsequent variability of cblC age of onset has been observed (4). CblD cells show detectable, though decreased, binding ability of labelled Cbl to both apoenzymes (85). Thus, MS and MCM holoenzyme activity is somewhat maintained. CblD fibroblasts are unaffected by CN-Cbl in culture medium as they are capable exchanging the cyanide group for the hydroxyl group (85). Pezacka and Rosenblatt (87) have demonstrated that both ß-ligand transferase and Cbl reductase activities were 33% and 55% of controls, respectively. Interestingly, Byck and Rosenblatt (88) demonstrated the lack of metabolic cooperation between cblD and cblF cells when cocultivated in the absence of PEG. However, the cocultivation of cblC and cblF cells were able to partially increase propionate and MeTHF uptake. This further distinguished cblC and cblD defects. The nature of the cblD defect is obscure, but it has been suggested that this may be a "leaky" form of cblC (84), that it may interfere less severely within the same metabolic step (85), or that it may be affecting an unidentified step before or after the reduction of Cbl^{+3} to Cbl^{+2} (88).

3.2.5 cblF

Five patients have been described with cblF disease (89). The first reported case was an infant girl with developmental delay, Cbl-responsive MMA, but no indication of megaloblastic anemia or homocystinuria (90). The second infant presented with Cbl-responsive MMA and homocystinuria, and mild anemia (91). Subsequently, three other cblF patients were reported with similar findings (89). Onset of symptoms varied from infancy to late childhood. Incorporation of labelled propionate and MeTHF was low in cells from cblF patients, and consequently both holoenzyme activities were severely deficient (92). In contrast to cblC and cblD cells,

cblF cells demonstrated an increase in incorporation of labelled Cbl (93). Incorporation of [^{57}Co]CN-Cbl by cblF cells did not result in apoenzyme bound Cbl, but in almost 90% of unbound CN-Cbl (93). Subcellular fractions of cblF cell extracts were separated on Percoll gradients, and label was associated with lysosomes (90). Subsequently, EM autoradiography also demonstrated that free Cbl was abnormally accumulating in the lysosomes (94). However, TCII bound Cbl was shown to be normally internalized by cblF cells and the complex was also observed to dissociate normally (90). Further EM examinations did not indicate any abnormal lysosomal structure. Thus, the cblF defect is presumed to affect the release of free Cbl from the lysosome into the cytoplasm, thereby making it unavailable to the intracellular apoenzymes (95). Interestingly, cblF patients are unable to absorb orally administered Cbl, suggesting that internalization of IF-Cbl into the enterocyte may occur via the lysosome (89).

3.2.6 cblE and cblG

These inherited inborn errors of metabolism are characterized by defective methionine biosynthesis and accumulation of homocysteine in the urine. Serum Cbl and folate levels are normal (4). Patients usually manifest symptoms early in life, and most respond to OH-Cbl treatment. Most cases of cblE and cblG disorders exhibit pancytopenia, megaloblastic anemia, homocystinemia, hypomethioninemia, associated with poor feeding and vomiting, and homocystinuria without MMA (9,40). However, one case of cblE disease has been presented with transient MMA (96). Therapy for both diseases requires treatment with OH-Cbl, betaine and folates (75), however, many cblG patients do not respond well to therapy.

The cblE and cblG mutant classes are heterogeneous, and are characterized by functional MS deficiency due to decreased formation of MeCbl (95). MS and MeCbl deficiency may possibly lead to neurological manifestations. The development of myelopathy in those patients with decreased MS activity has suggested that the fall in AdoMet supply may interfere with essential methylation

reactions involved in myelination and neurotransmitter synthesis (62). Cbl treatment has promptly alleviated hypotonia and lethargy exhibited by MeCbl-deficient patients, but a longer period of treatment is necessary for slow, but incomplete, improvement in the psychomotor status (97).

Incorporation of labelled propionate is normal in both cblE and cblG cells, but MeTHF incorporation is approximately 10% of controls (4). CblE fibroblasts exhibit normal Cbl binding to MS apoenzyme. Intracellular levels of MeCbl are low and result in decreased methionine biosynthesis (39). However, cell extracts from cblE cells demonstrate normal specific MS activity under standard assay conditions, and decreased activity in suboptimal concentrations of reducing agents (39,98). Further studies with nitrous oxide (N_2O) have demonstrated inactivation of MS activity in controls and cblG cells, but no affect on activity in cblE cells (39). Christensen et al. (99) have demonstrated that the export of high levels of homocysteine from cblE cells is independent of methionine concentration or N_2O, and suggest that MS is inactive. Presumably, N_2O oxidizes the active MS bound cob(I)alamin to the inactive cob(III)alamin state. Thus, these studies suggest that the cblE defect may affect a MS-related reducing system (40,75). Cell extracts from a patient clinically and biochemically resembling the cblE phenotype was reported to have decreased MS activity even under standard assay conditions (100). This heterogeneity within the cblE class may imply yet another step in the Cbl reduction pathway.

CblG fibroblasts also exhibit normal Cbl binding to MS, however, enzyme activity is deficient even under optimal conditions (4). N_2O moderately inactivated MS in cblG cells and increased homocysteine export at low methionine concentration as observed in cystathionine ß-synthase deficient cells (99). This would suggest that there is a slow catalytic turnover of MS, and that the cblG defect is in the MS subunit itself. However, Hall et al. (101) studied a cblG cell line and demonstrated that Cbl bound normally to MS, but was unable to be methylated by AdoMet. This abnormality was overcome by supplying higher concentrations of AdoMet and was deduced to be associated to this methylating enzyme. Further

heterogeneity among the cblG class was demonstrated recently, as three of ten lines had low levels of Cbl accumulation and did not indicate binding of Cbl to MS (102). Although MS activity was almost undetectable, MeTHF uptake was higher as compared to other cblG lines. It was postulated that the defect in these three variants was due to the inability of MS to retain bound cob(I)alamin (102).

CHAPTER 4
MOLECULAR AND GENETIC CHARACTERIZATION OF METHYLMALONYL CoA MUTASE (MCM)

4.1 ISOLATION AND PURIFICATION OF MCM

Methylmalonyl CoA mutase has been purified to apparent homogeneity from the gram positive bacterium *Propionibacterium shermanii* (103), the intestinal worm *Ascaris lumbricoides* (104), sheep liver (44), human placenta (105), human liver (106), and very recently from *Streptomyces cinnamonensis* (107).

4.1.1 Isolation of Human MCM

Procedures involving column chromatography on DEAE-cellulose, Matex-Gel Blue A, hydroxylapatite, and Sephadex G-150 achieved an overall purification of 500 to 600 fold for MCM holoenzyme from human liver (106). Polyacrylamide sodium dodecyl sulfate gels exhibited a single protein band with apparent molecular weight of 77,500, while the native protein showed a molecular weight of approximately 150,000 by Sephadex G-150 chromatography. This suggested that the protein was composed of two identical subunits or homodimers. The ultraviolet absorption spectrum of pure MCM revealed two peaks around 520nm and 350nm, a characteristic of bound Adenosyl-Cbl, indicating that there is 1 mol of Adenosyl-Cbl bound to each enzyme subunit (106). These results were similar to those reported for MCM isolated from sheep liver and human placenta (105).

4.1.2 Isolation of Bacterial MCM

The native protein isolated from *Propionibacterium shermanii* revealed a single band of molecular weight 165,000 in polyacrylamide non-denaturing gels, but exhibited two bands of equal intensities in SDS/polyacrylamide gels with molecular weights of 79,000 and 67,000 (108). A 1:1 polypeptide chain ratio was constant in MCM samples, and suggested that the smaller subunit was not a proteolytic product of the larger subunit. The possibility that the bacterial mutase existed as a

heterodimer was further proven by sedimentation equilibrium studies showing the dissociation of two dissimilar subunits with increasing ionic strength (109).

4.2 CLONING OF MCM cDNA

In 1988, Ledley et al. (110) cloned the cDNA for human MCM by screening a human liver cDNA library in λgt11 with chicken antibodies raised against human placental MCM, and ^{125}I-labelled goat anti-chicken IgG. EcoRI fragments from each positive clone hybridized to total human liver RNA identified an mRNA species large enough to encode a protein of 77-78 kDa. A cDNA of corresponding length was able to increase MCM activity in transfected COS cells. In addition, several fibroblast cell lines deficient in MCM enzymatic activity had a specific decrease in hybridizable mRNA to the cDNA probe MCM26b as compared to hybridizable mRNA from control Cbl fibroblast cell lines (110). The full length human cDNA was cloned and sequenced, and the predicted peptide sequence corresponded to the tryptic fragments from purified human liver MCM (111).

4.3 MAPPING OF MCM GENE

A panel of human-Chinese hamster somatic-cell hybrids was constructed by Ledley et al. to study chromosome assignment of the MCM gene (112). Southern blots of DNA from hybrid cell lines cut with EcoRI or BamHI were probed with MCM26b. Concordance was observed between restriction fragments from human DNA and the presence of chromosome 6 from the five different corresponding hybrid panels. Regionalization of the MUT locus to 6p23-q12 was ascertained by identifying human EcoRI restriction fragments from DNA of cell lines containing portions of chromosome 6 (112). Further localization of the MUT locus to 6p12-21.2 was based on results of *in situ* hybridization (112).

Linkage between MUT and two other loci on the proximal arm of chromosome 6 confirmed the assignment of the MUT locus (113). Linkage was demonstrated with HLA (θ_{max}=0.28), and tight linkage was demonstrated with D6S4 at a recombination fraction of 0.01.

4.4 CHARACTERIZATION OF HUMAN MCM cDNA

The complete sequence of the MCM cDNA clone is comprised of 2798 base pairs, including a 28-base poly(A) tail. This codes for a protein of 750 amino acids and predicted molecular weight of 83 kDa (111). It contains a single, long open reading frame beginning with a CCAUG consensus sequence. Internal consensus CCAUG sequences are terminated early with stop signals. The cDNA sequence predicts a 32 amino acid mitochondrial leader sequence rich in arginine, serine, and leucine, and includes the amino-terminal methionine. The mature protein of 718 amino acids corresponds to the predicted molecular weight of 78 kDa (111).

4.5 STRUCTURE OF THE MUT LOCUS

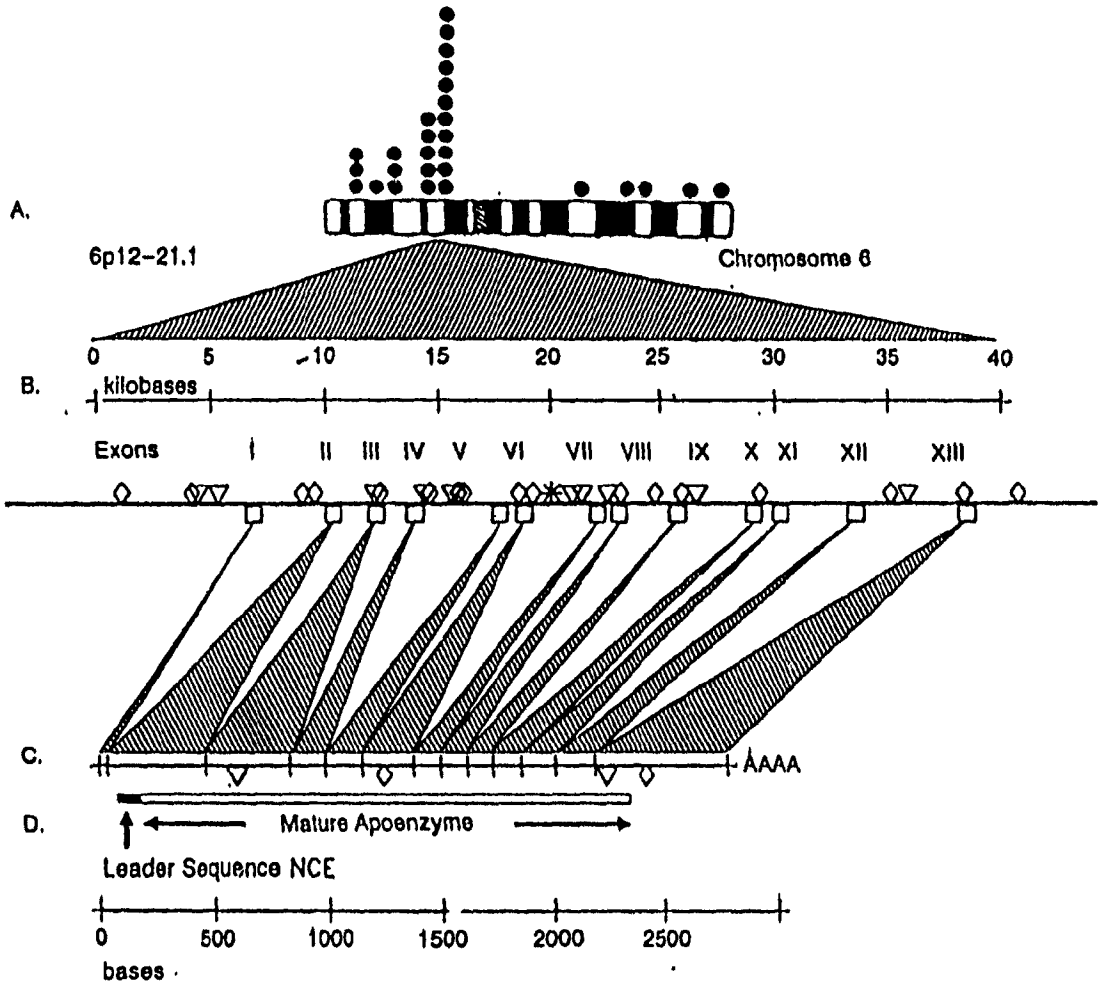
It was demonstrated that restriction fragments from somatic cell hybrids could independently be assigned to chromosome 6p12-21.1 (112), suggesting that a single locus encoded MCM. Various restriction digest analyses and southern blots identified a single MUT sequence (114). This indicated that there were no homologous genes or pseudogenes elsewhere in the genome, and that the locus contains a single copy of the MCM gene. Further analysis of the locus by cloning and sequencing clones from genomic libraries identified 13 exons spanning 40 kilobases (114). Consensus sequences for transcription, splicing, and polyadenylation are exhibited, and a GC rich region 5' to exon I expresses promoter function (114). There is no indication of an independent exon encoding the mitochondrial targeting sequence. Exon II contains the targeting sequence and the amino terminal of the mature MCM (114).

Molecular analysis of the MUT locus from fibroblast cell lines deficient in MCM activity identified no gross structural lesions. This suggests that most mutations involve point mutations, or small insertions and deletions (115).

4.6 PROCESSING OF THE MCM PRECURSOR

Initial experiments using a cell-free translation system, directed by rat liver RNA, and immunoprecipitation procedures, detected a precursor protein 3-4

FIG. 5 SCHEMATIC STRUCTURE OF MUT LOCUS AND MCM cDNA. ◊=EcoRI sites;▽=HindIII sites. Reprinted from Ledley (70).


A.
6p12-21.1
Chromosome 6
0
5
10
15
20
25
30
35
40
B.
kilobases
Exons
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
C.
AAAA
D.
Mature Apoenzyme
Leader Sequence NCE
0
500
1000
1500
2000
2500
bases

translationally processed *in vitro* by intact rat liver mitochondria, and the mature kDa larger than the mature MCM apoenzyme (116). The MCM precursor was post-MCM subunit of 78 kDa was localized only to the matrix subfraction (116). This indicates that the MCM precursor is concomitantly translocated and processed at the mitochondrial matrix. Presumably, the precursor protein, translated on cytoplasmic ribosomes, is actively transported into the mitochondria by means of a mitochondrial membrane receptor complex (117). The 32 amino acid target sequence is removed in the matrix by the mitochondrial processing peptidase. This enzyme recognizes and cleaves on the amino-terminal side of an octapeptide sequence (118). It is assumed that the octapeptide sequence functions as a structural prerequisite for cleavage at the junction between the target residues and the mature amino terminus (118). Inhibitors of energy metabolism prevented the accumulation of labelled MCM in the matrix, implying that transport and post-translational processing are tightly linked and energy-dependent (116,119).

It is presumed that during the evolution of endosymbiotic prokaryotes into modern mitochondria, genes for many proteins were translocated to the nuclear genome. Sequences were then acquired to enable transport of modern matrix proteins to the mitochondria (120). Based on the concurrence of the amino terminus sequences between human and *P. shermanii* MCM, Leadlay and Ledley (121) have suggested that the MCM target sequence evolved by extension of sequences at the 5' end of the reading frame. However, the emergence of target sequences by point mutations or rearrangements within the 5' untranslated region is also possible, and has been demonstrated under selective pressure in the yeast cytochrome oxidase subunit IV precursor (120).

4.7 MCM HOMOLOGY THROUGH EVOLUTION

4.7.1 Bacterial MCM

MCM from *Propionibacterium shermanii* has been crystallized (122) , and the structural genes coding for both polypeptides have been cloned and sequenced by Marsh et. al. (123). The Mut A gene is comprised of 1914 base pairs,

and encodes a 638 amino acid protein. This corresponds to the 67 kD small ß-subunit. The Mut B gene is comprised of 2184 base pairs, and encodes for a 728 amino acid large α-subunit of 79 kD. Both genes are observed to be closely linked and transcribed in the same direction as the last guanine nucleotide of the carboxyl-terminal of the Mut A gene is the first base of the GTG initiation codon of the Mut B gene (123). This suggests that both genes are plausibly transcribed as an operon. Both genes share 22% identity and close structural homology, reflecting the possibility of duplication of a common ancestral gene (123). Human MCM exhibits 22% homology to the Mut A gene and 60% homology to the Mut B gene (124).

Very recently, MCM has been cloned from the *Streptomyces cinnamonensis* actinomycete (107). Similar to *P.shermanii*, two polypeptides are encoded by two structural genes. The mutA gene contains 1848 bp, which encode a 616 a.a. protein of molecular weight 65kD. The mutB gene contains 2199 bp, which encodes a 733 a.a. protein of molecular weight 79kD. A potential 3bp streptomycete-ribosome binding site preceding the mutB gene, has been identified. Both genes of *S.cinnamonensis* share 29% identity. Comparison of MCM sequences from other organisms indicates that the mutA gene of *S.cinnamonensis* shares 27% and 43% identity to the Mut B and Mut A genes of *P.shermanii*, respectively, 27% identity to human MCM, and 25% identity to murine MCM. The mutB gene of *S.cinnamonensis* shares 72% and 27% identity to the Mut B and Mut A genes of *P.shermanii*, respectively, 63% identity to human MCM, and 61% identity to murine MCM (107). Although the bacterial MCM catalyses the reverse reaction, it is probable that the single gene in humans arose through divergent evolution. The importance of MCM structure and function may rely on the discrete regions of homology (121).

4.7.2 Murine MCM

The cDNA for murine MCM was cloned, sequenced and expressed in human fibroblasts in 1990 (124). The sequence contains 3245 nucleotide residues.

A single long open reading frame encodes a 748 amino acid protein of 82 kDa. The murine locus encoding MCM was localized to chromosome 17E-F by genetic linkage studies (125). Both mouse chromosome 17 and human chromosome 6 contain a syntenic group of genes which include MCM, glyoxalase, and HLA (125). Murine MCM exhibits 26% and 57% identity to Mut A and Mut B sequences from *P. shermanii*, respectively. However, 94% homology is observed between murine MCM and human MCM with divergence seen at the mitochondrial targeting sequence. Only 69% of residues are identical in this region (124). It is not unusual for homologous proteins to exhibit divergence at the mitochondrial targeting sequence. This is apparent in murine and human ornithine transcarbamoylases, which share an overall homology of 92% but only 69% identity is shared at the mitochondrial targeting sequence (124).

4.8 MCM APOENZYME DEFICIENCY (mut$^-$, mut^0)

The mut complementation class is characterized by mutations at the MUT locus, resulting in defects of the MCM apoenzyme (74,126). Thus, MMA without homocystinuria is characteristic of MCM apoenzyme deficiency. Mut and cbl forms of MMA are classically distinguished by somatic cell complementation (See section 3.2.2), and recently by DNA-mediated gene transfer (127). MCM cDNA transferred to mut cells restores enzyme activity as measured by [^{14}C]propionate uptake, while it does not reconstitute activity in cbl cells in the absence of cofactor. The results of one method correlate with the results of the other (127).

Mut MMA patients have been sub-classified into two groups designated mut^0 and mut$^-$. Pairwise crosses between mut^0 and mut$^-$ fibroblasts yielded non-complementing heterokaryons (128), and suggested that these are allelic mutations at the MUT locus. This was further supported by enzyme kinetic studies on fibroblast extracts which identified codominant expression of mut^0 and mut$^-$ alleles (126). Restriction fragment length polymorphisms have been identified from mut^0 and mut$^-$ lines with Hind III and Taq I enzymes, but no distinct relationship to mut phenotypes can be established due to small sample size (115).

Although clinical symptomology between cblA, cblB, and mut are similar, the mut class tends to be more severe and prognosis is worse (69). Mut patients usually become symptomatic in late infancy or childhood, and have a better outcome of survival than mut^0 patients. Shevell et al. (129) have reported clinical variability between mut$^-$ patients who exhibited normal to severe neurological dysfunction, with or without episodic acidosis. In contrast, mut^0 patients present symptoms early in neonatal life, with almost 90% of patients having onset before the end of the first month (29). These patients often die early in infancy, and those surviving suffer poor neurological outcomes (129). Hypotonia, microcephaly, and dystonia have been observed in surviving mut^0 patients, and neurodevelopmental delay has been identified in both mut$^-$ and mut^0 patients (129). The neurological dysfunctions in MMA have been attributed to the secondary effects of acidosis, ketosis, hyperammonemia, and episodic coma (62,70).

Mut$^-$ patients demonstrate residual enzyme activity in cell extracts when high concentrations of AdoCbl is added *in vitro* (128). Although abnormal K_m for cofactor have been previously described (130,131), further enzyme kinetic studies by Willard and Rosenberg (128) revealed that enzyme from mut$^-$ cells specifically displayed a 250 to 4000 fold lower affinity for AdoCbl than wild type MCM. Intact cells responded to medium containing excess OH-Cbl as measured by [^{14}C]propionate incorporation, however, mut$^-$ patients do not respond to Cbl therapy *in vivo* (29,128). Radioimmunoassay has detected cross reactive material (CRM) ranging from 20% to 100% of controls (132). Therefore, mut$^-$ mutations presumably represent a defective interaction between apoenzyme and cofactor.

Mut^0 patients have no detectable enzyme activity in cell extracts (<0.1% of controls) even in the presence of excess amounts of AdoCbl (128). Intact cells did not respond to OH-Cbl supplementation (128), and northern blot analysis revealed decreased levels of MCM mRNA in some mut^0 cell lines (115). Cross reactive material was not detected in some mut^0 cell lines, while others displayed ranges from 3% to 40% of controls (132). This led to immunoprecipitation studies assessing association between CRM and newly synthesized MCM from these patients

(119). Mut^0 cell lines that were CRM$^+$ had detectable newly synthesized MCM. Almost 50% of CRM$^-$ cell lines studied showed undetectable MCM, while others had MCM ranging from barely detectable to almost half that of controls. Other results from this study identified a CRM$^+$ mut^0 cell line which failed to accumulate mature MCM in the mitochondria due to faulty processing of the target sequence (119). This indicated that mut^0 mutations may result in either unstable or no protein synthesis, unstable mRNA, or post-translational defects.

4.8.1 Interallelic Complementation

Unusual complementation within the mut subclasses has been recently described (2). Cell-fusion complementation of a mut^0 cell line (WG 1130) was performed with cblA, cblB, mut^0, and mut$^-$ fibroblast cell lines. As expected, this cell line complemented with cblA and cblB cells, but also complemented three of nine mut^0 cell lines, and four of five mut$^-$ cell lines. Cloning and sequencing of MCM cDNA from WG 1130 characterized a homozygous G_{354}->A (R93H) substitution. Expression studies confirmed this change to be the pathogenic mutation. Compared to controls, there was no increase in [^{14}C]propionate uptake after transfection into mut^0 fibroblasts with low MCM mRNA (GM 1673). The recombinant MCM expression vector carrying this point mutation was also introduced into mut^0 and mut$^-$ cell lines which exhibited somatic cell complementation with WG 1130. Observation of complementation between the mut subclasses suggests that heterogeneity exists at the MUT locus due to interallelic complementation. Presently, WG 1130 complements five of thirteen mut^0 lines and six of eight mut$^-$ lines tested (Table 1).

4.8.2 Mutations at the MUT Locus

Figure 6 describes the location of previously identified mut mutations with respect to the MCM cDNA. A mutation in the leader sequence resulting in an amber termination signal (C_{128}->T, Q17STOP), was identified in the cDNA from a mut^0 cell line FB552 (133). This mutation prematurely terminated the transcription of the mitochondrial leader sequence. This resulted in a highly unstable protein

TABLE 1 COMPLEMENTATION PATTERNS OF mut CELL LINES WITH WG 1130.

TABLE 1 INTERALLELIC COMPLEMENTATION WITH WG 1130

COMPLEMENT WG 1130			DO NOT COMPLEMENT WG 1130	
mut°	mut⁻		mut°	mut⁻
WG 1517	WG 1511		WG 1510	WG 1599
WG 1601	WG 1562		WG 1512	WG 1618
WG 1609	WG 1610		WG 1602	
WG 1681	WG 1611		WG 1607	
WG 1713	WG 1613		WG 1608	
	WG 1738		WG 1612	
			WG 1727	
			WG 1864	

smaller than the normal precursor. The CRM^+ phenotype of this cell line reflected the initiation of translation downstream of the termination codon (133). Hence, the abnormal MCM was not cleaved or imported into the mitochondria, and this was the ultimate cause of the mut^0 MMA in this patient. Interestingly, the mutation was identified as a homozygous change in the cDNA; however, upon sequencing exon 2 of the genomic DNA, heterozygosity was observed at that position by the presence of the normal allele. This suggested that the heterologous allele was not represented in the mRNA.

Compound heterozygous mut^0 mutations were identified in the cDNA from the cell line WG 1607 (MAS) (134). A T_{389}->C transition resulted in a W105R change, and a C_{1206}->A transversion resulted in A378E change. Both mutations were expressed and confirmed pathogenicity. The mutations occurred at evolutionary preserved sites, and were believed to disrupt the secondary structure of the enzyme because a negatively charged amino acid replaced a hydrophobic one (134).

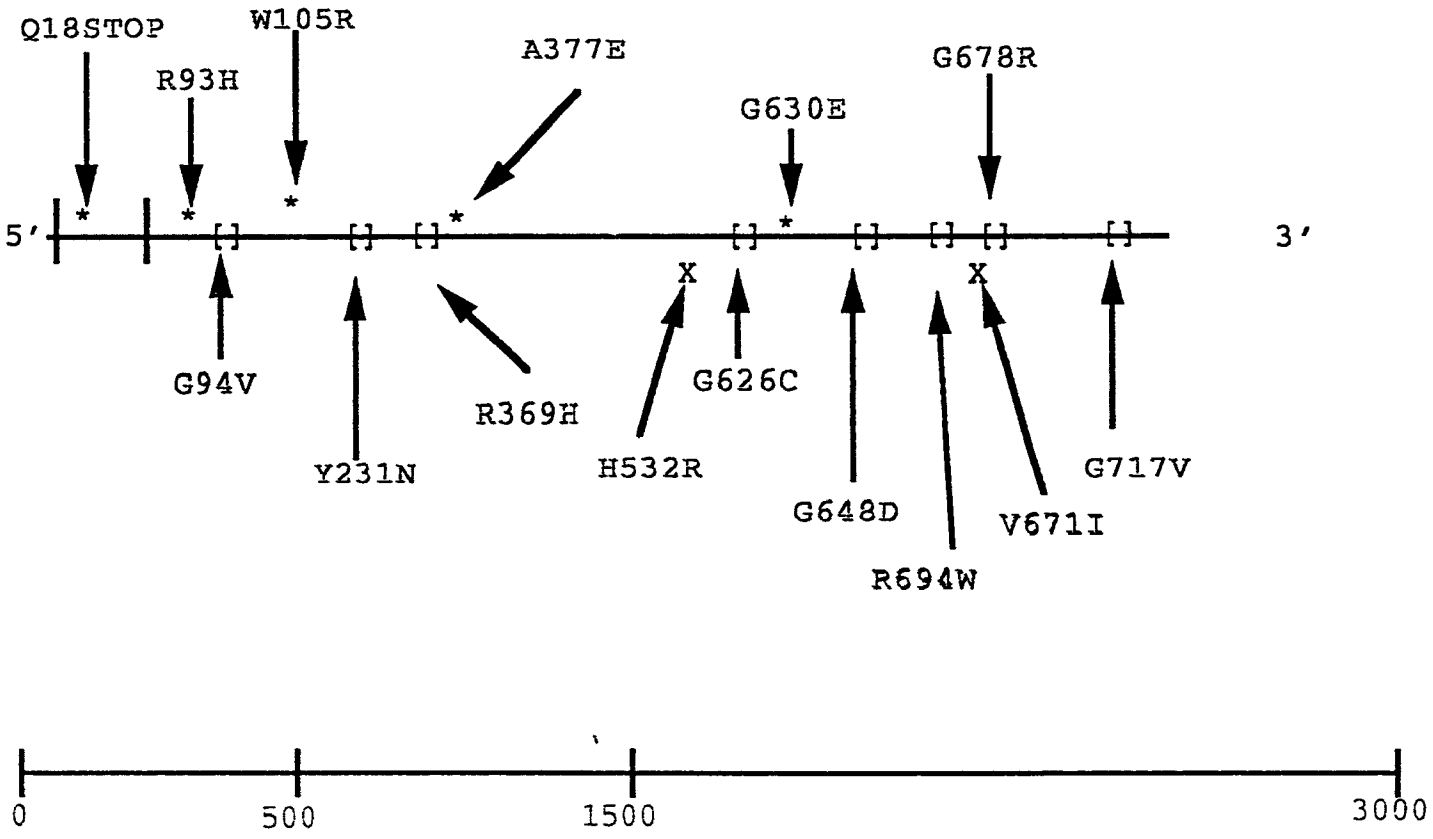
Recently, Crane et al. (1) identified a homozygous mutation in the cDNA of a mut^- cell line WG 1611, which complemented WG 1130. A G_{2226}->T transversion, resulting in G717V change, was determined to be the pathological mutation responsible for the mut^- phenotype, and was also able to complement the WG 1130 R93H mutation. This novel mutation occurred at a highly conserved MCM site, and was predicted to introduce an additional α-helix rotation, thereby disrupting protein folding (1). Enzyme kinetic studies demonstrated an elevated K_m, suggesting that the proposed molecular changes affected apoenzyme-cofactor binding (1). In addition, two other changes were identified as polymorphisms: A_{1671}->G (H532R), and G_{2087}->A (V671I). Subsequently, these three identical base pair changes were identified in two other mut^- cell lines (WG 1610, WG 1613) which also complemented WG 1130 (135). All three cell lines were derived from three-unrelated patients of African-American origin exhibiting similar disease phenotype (135). Interestingly, upon analysis of genomic DNA, only WG 1613 was identified to be heterozygous for the G717V mutation (135).

Other mutations listed in figure 6 have not been cited in the literature,

but have been conveyed through personal communication with Wayne Fenton. These mutations have been identified only at the level of the cDNA. The G94V, and the R369H changes are heterozygous. The H678R, and the Y231N changes are homozygous.

FIG. 6 **HUMAN MCM MUTATION.** Letters represent amino acid change. X= polymorphism; *= mut^{o}; []= mut^{-}.

MUTATIONS OF HUMAN MCM



key

X polymorphism	* mut(o)	[] mut(-)

CHAPTER 5
MATERIALS AND METHODS

5.1 PATIENTS

The 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 due to acidosis, and at 3 1/2 years of age had an approximate 6 months delay in his verbal and fine motor development.

Cultured cells from WG 1681 demonstrated a lack of stimulation of [^{14}C]propionate (New England Nuclear; Boston, MA) uptake in the presence of hydroxocobalamin. Thus, WG 1681 was defined to have a mut^0 phenotype (Table 2A).

WG 1130 is a well characterized mut^0 cell line (2) from a French Canadian Caucasian female who was diagnosed with MMA in the first week of life. She had recurrent vomiting, dehydration, metabolic episodes, and died at 5 months of age.

WG 1130 and WG 1681 have been observed to complement each other. An increase in labelled propionate incorporation was demonstrated by cells fused with PEG (S.T. Baker Chemical Co.; Phillipsburg, NJ) (Table 2B). The [^{14}C]propionate uptake and complementation analyses were performed by Nora Matiaszuk in Dr. Rosenblatt's laboratory at McGill University as described previously (2).

5.2 CELL CULTURE

Skin fibroblasts from control (MCH 24) and patients (WG 1130, WG 1681) were obtained from the Repository for Mutant Human Cells at the Montreal Children's Hospital (Montreal, Que.). All cell lines were determined to be free from mycoplasma contamination, and were grown as a monolayer in 175 cm^2 flasks (Falcon, Oxnard, CA). Cultures were maintained in 30 ml standard media consisting

TABLE 2A PROPIONATE UPTAKE FOR WG 1681.

TABLE 2B INTERALLELIC COMPLEMENTATION BETWEEN WG 1681 AND WG 1130. PEG= polyethylene glycol fusion (Both analyses were performed by Nora Matiaszuk).

TABLE 2A PROPIONATE UPTAKE OF WG 1681

CELL LINE	[^{14}C]PROPIONATE UPTAKE (nmole/mg protein/18h)	
	without OH-Cbl	with OH-Cbl
MCH 24 (Control)	9.6	9.0
WG 1681	1.1	0.85

TABLE 2B INTERALLELIC COMPLEMENTATION BETWEEN WG 1681 AND WG 1130

		FUSIONS WITH WG 1681	
		[^{14}C] PROPIONATE UPTAKE (nmoles/mg protein/18h)	
CELL LINE		without PEG	with PEG
WG 1681 (mut°)		0.69	0.53
WG 1607 (mut°)	*(1.7)	1.5	1.1
WG 1599 (mut⁻)	*(0.86)	0.86	0.89
WG 1588 (cblA)	*(1.5)	1.05	4.0
WG 1586 (cblB)	*(0.93)	0.79	3.6
WG 1130 (mut°)	*(0.56)	**0.74**	**2.4**

* denotes uptake values by cell line itself

of Eagles's minimum essential medium with Earle's salts, L-glutamine, and non-essential amino acids (Flow Laboratories, Mississuaga, Ont.), supplemented with 10% (v/v) fetal bovine serum (Gibco Laboratories, Grand Island, N.Y.). Cultures were kept at 37°C in an atmosphere of 5% CO_2, 95% O_2, and 86% humidity.

5.3 IDENTIFICATION OF MUTATIONS IN cDNA

5.3.1 RNA Extraction

The acid guanidinium thiocyanate-phenol-chloroform procedure (136) was used to isolate RNA from fibroblast cell monolayers. The cells were extracted and denatured at room temperature in a 4M guanidinium thiocyanate, 25mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1M 2-mercaptoethanol solution called solution D. Sequentially, 2M sodium acetate (pH 4), phenol saturated with water (1:1), and a chloroform:isoamyl alcohol (49:1) mixture were added to the lysate. After vigorous shaking and cooling on ice, the lysate was centrifuged at 23,281 x g for 20 min. at 4°C in a Beckman J2-21M centrifuge (Palo Alto, CA). The RNA-containing aqueous phase was precipitated for 1 hour at -20°C with 1 volume of cold isopropanol. RNA was sedimented according to the previous centrifugation parameters, and the pellet was resuspended in 1/10 volume of solution D. Another precipitation with 1 volume cold isopropanol (same conditions as above) was followed by centrifugation for 10 min. at 4°C in a Brinkmann 5415 Eppendorf centrifuge (Westbury, NY). The RNA pellet was washed once with cold 75% ethanol, air dried, and resuspended in 50μl DEP-treated water with 40U of RNasin inhibitor (Promega, Madison, WI). Concentration was determined by measuring absorbance at 260nm in a Beckman DU-64 spectrophotometer (Fullerton, CA.). Quality of RNA was determined by the 260nm/280nm absorbance ratio.

5.3.2 Reverse Transcription and Polymerase Chain Reaction

The RNA was amplified using a modified combined RT/PCR procedure of Wang et al. (137). Primers were synthesized based on the published cDNA sequence of MCM. The primer sequences used for amplification and sequencing are

shown in Table 3A and B. The positions of the primers with respect to the cDNA sequence, and the fragment size generated are shown in Figure 7A. The RNA was amplified separately in four overlapping fragments; fragment A being the most 3' and fragment D being the most 5'. Each 50μl reaction contained 0.5 to 1 μg RNA, 40U RNasin inhibitor, 1μM of each flanking primer, 200μM each dNTP (dATP, dGTP, dCTP, dTTP), 1X Perkin Eimer Cetus reaction buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% w/v gelatin), 2U Taq polymerase (Pharmacia), and 80U MMLV reverse transcriptase (BRL, Grand Island, NY). Samples were overlaid with 50μl of mineral oil and placed in a Diamed Coy thermal cyler (Mississuaga, Ont.). 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 minute, 60°C for 1.5 minutes, 72°C for 1.5 minutes, and 20 cycles of 94°C for 1 minute, 55°C for 1.5 minutes, 72°C for 1.5 minutes, followed by a final elongation at 72°C for 10 minutes.

The amplified fragments were then isolated from low melting agarose gel using a glassmilk method (GeneClean, Bio 101) in order to eliminate unamplified and degraded RNA.

5.3.3 Cloning

Each PCR fragment was ligated to the pCR1000 vector (Fig.8) provided by the Invitrogen TA Cloning Kit (San Diego, CA). This kit takes advantage of the terminal transferase activity of the Taq DNA polymerase which adds a single deoxyadenosine (A) nucleotide at the 3' end of the PCR product (138). These A "overhangs" enable the direct ligation of PCR products to the T-tailed vector. The ligation reaction was performed as directed by the kit, and incubated at 12°C for 10 hours followed by an enzyme inactivation at 65°C for 10 minutes.

Transformation of INVαF' bacterial cells (provided with the kit) was carried out as instructed. 100μl of transformed cells were plated on each of 3 plates containing 3.7% BHI, 1% agar (Difco Labs, Detroit, MI), 2.4% X-Gal, and

TABLE 3A SEQUENCES OF RT/PCR AND SEQUENCING PRIMERS.

TABLE 3B SEQUENCES OF INTERNAL SEQUENCING PRIMERS.

TABLE 3A RT/PCR AND cDNA SEQUENCING PRIMERS

PRIMER#	SEQUENCE	FRAGMENT
21*	5'TACTCTCTTCTTTGATCATAAC 3'	A
16	5'GAGAAATCACAGATGCCCT 3'	
37*	5'ATGAACCCTCTTGATAGCAGA 3'	B
35	5'TTTTGATGAAGCTTTGGGTTTGCC 3'	
30*	5'CCTCCCCAAGGATCAGCCAC 3'	C
23	5'AGTTTCCATGACTATGAAT 3'	
48*	5'AGAACTGGAATAACTGCTCC 3'	D
53	5'AGTCAGTTCTTATTTCTATTGGGTG 3'	

* denotes antisense primer

TABLE 3B INTERNAL SEQUENCING PRIMERS

PRIMER#	SEQUENCE	FRAGMENT
17*	5'CCTCCACACATGACAAG 3'	A
15*	5'AGGGCATCTGTGATTTCTC 3'	B
34	5'TTTCCTCCAGAACCATCCATGAA 3'	C
47*	5'GGCGGATGGTCCAGGGCCTA 3'	D

* denotes antisense primer

FIG. 7A **POSITION OF RT/PCR PRIMERS.** Numbers represent primers used.

FIG. 7B **POSITION OF cDNA SEQUENCING PRIMERS.** Flanking and internal primers used are shown.

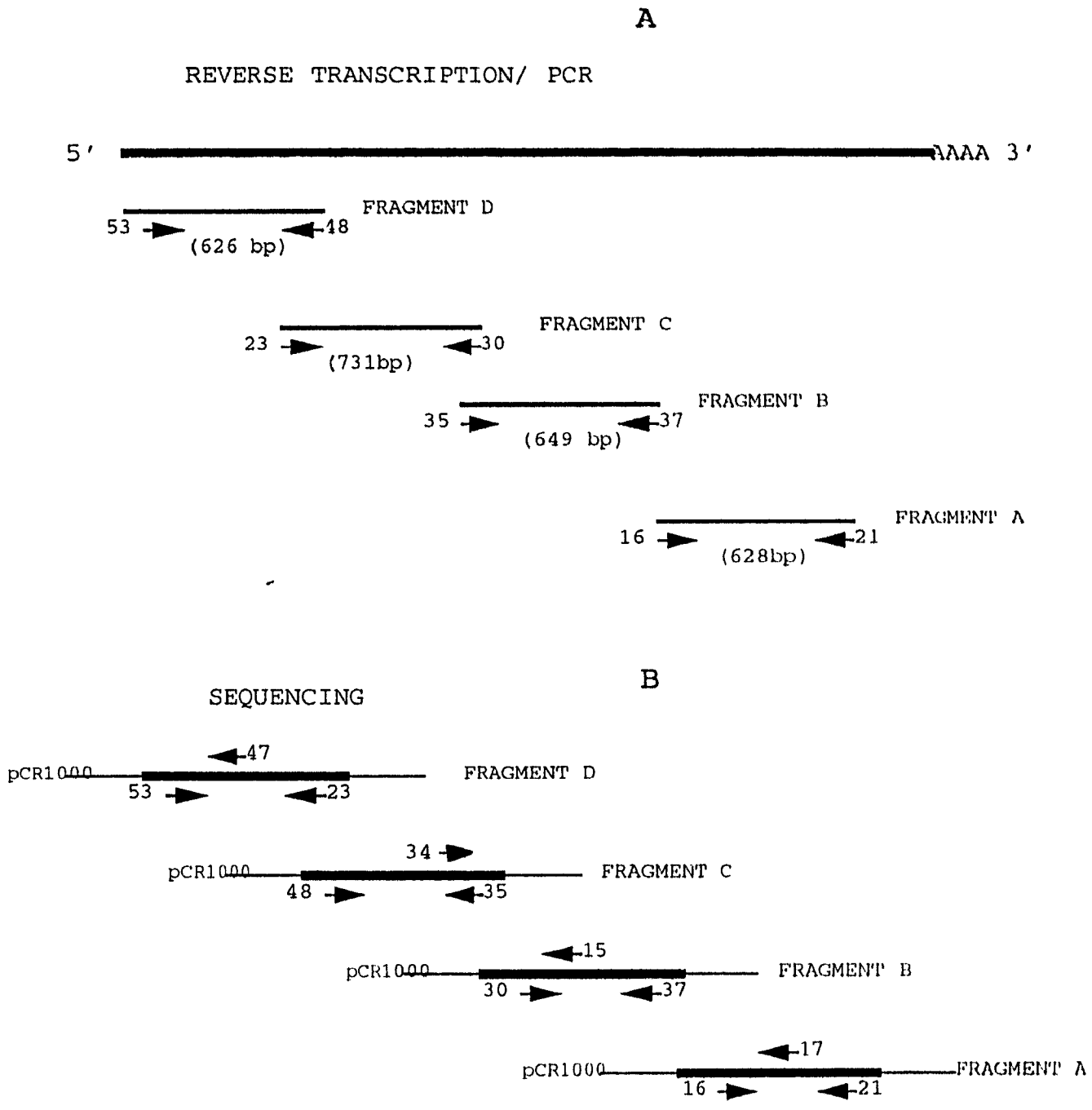
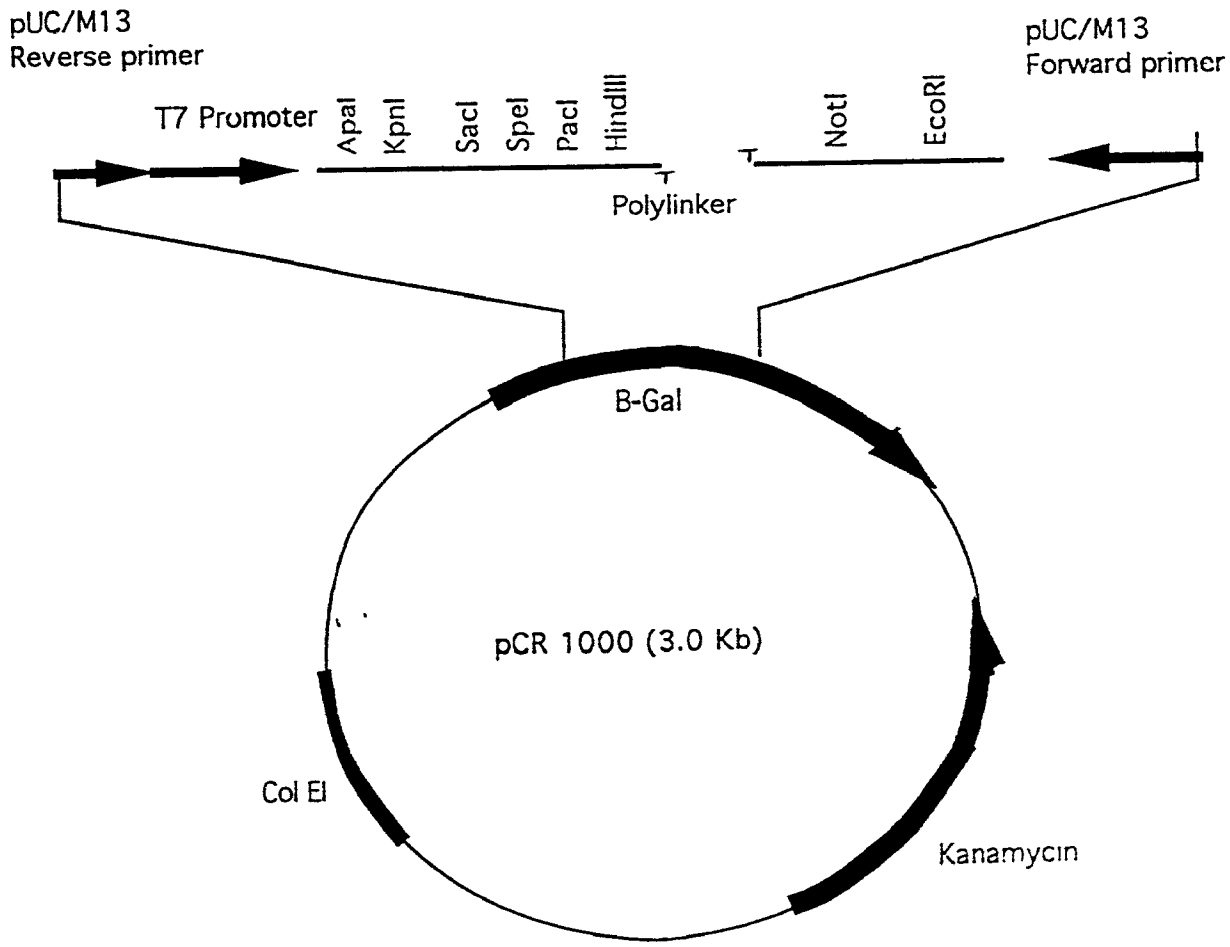

A
REVERSE TRANSCRIPTION/ PCR
5'
AAAA 3'
FRAGMENT D
53
48
(626 bp)
FRAGMENT C
23
30
(731bp)
FRAGMENT B
35
37
(649 bp)
FRAGMENT A
16
21
(628bp)
B
SEQUENCING
pCR1000
47
53
23
FRAGMENT D
pCR1000
34
48
35
FRAGMENT C
pCR1000
15
30
37
FRAGMENT B
pCR1000
17
16
21
FRAGMENT A

FIG. 8 **pCR 1000 CLONING VECTOR.** Provided by Invitrogen (San Diego, Ca.).

PCR CLONING VECTOR



kanamycin (50μg/ml). IPTG was not necessary because INVαF' is a lac⁻ bacterial strain. Plates were incubated overnight at 37°C.

5.3.4 Slot Lysis

A total of 24 white colonies for each fragment were separately grown in 0.5 ml of 3.7% BHI media (containing 50μg/ml kanamycin) by incubating overnight in a shaking incubator at 225 rpm and 37°C. A representative self-ligated vector (blue colony) was also grown. Slot lysis (Jansen, personal communication) of each clone allowed for a quick selection of those containing the proper ligated product. 10μl of each overnight culture were aliquoted into respective wells of a Falcon elisa plate (Becton Dickinson Co., Lincoln Park, NJ), and centrifuged in a micro-titre test plate swinging adaptor at 2500 rpm for 15 min. at 4°C in a Beckman TJ-6 bench top centrifuge (Palo Alto, CA). Each pellet was resuspended in 10μl of a protoplasting mix containing 260μl protoplasting buffer (0.03M Tris pH 8, 0.005M EDTA, 0.05M sucrose), freshly added lysozyme (0.05μg/μl) and RNase (0.096μg/ul). The resuspension was kept at 4°C for 45 min.. 2μl of lysis buffer (1X TAE, 2% SDS, 0.25g sucrose, 0.04% Bromophenol blue, and 3.1ml water) was applied to each 1mm thick well of a 20 X 25 cm 0.8% agarose gel (1X TAE, 0.5% SDS). To lyse the cells directly in the wells, 9μl of the refrigerated samples were added.

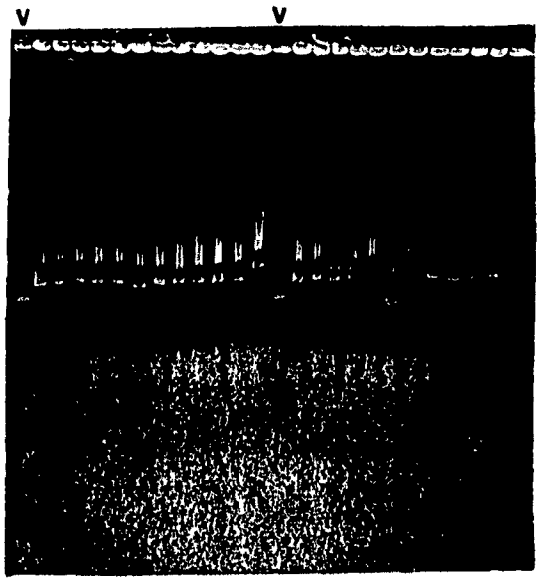
The samples were electrophoresed for 3 hours at 115 volts. The gel was stained for 45 min. in 1X TAE, 0.5mg/ml ethidium bromide, and then photographed under UV illumination (Fig.9).

5.3.5 Plasmid DNA Minipreparation

For each fragment, a total of 16 appropriate clones were chosen and separately grown in 3 ml of LB media containing 50μg/ml kanamycin by shaking overnight at 225 rpm at 37°C. Plasmid DNA mini-preparations were performed on 3ml of a single clone and 3mL of a pool of 15 independent clones by using the Promega Magic Mini-Preps DNA Purification System (Madison, WI) as instructed.

FIG. 9 **EXAMPLE OF SLOT LYSIS.** V=lane containing plasmid without insert. Remaining lanes contain separate clones of PCR fragment A.

Slot Lysis



plasmid with insert

plasmid alone

Each minipreparation was tested for quality by restriction digest of each fragment by an enzyme which cut within its sequence.

5.3.6 Double Stranded Sequencing

PCR and internal primer sequences used for sequencing are listed in Table 3A and 3B. The relative positions of PCR primers and internal sequencing primers for each amplified fragment are shown in Figure 7B. Each single and 15-combined plasmid DNA preparations for each fragment were subjected to double-stranded sequencing according to the procedure recommended by the Pharmacia T7 Sequencing Kit with slight modifications. An initial denaturation of 2μg of plasmid DNA was performed as directed, and the pellet was resuspended in 9μl of water. For each sequencing reaction, 3μl of a 1.2μM primer stock, and 10μCi of ^{35}SαdATP radiolabel (Amersham, Arlington, IL) were used. A 6% sequencing gel containing 7M urea, 1X TBE, 6% Bis Acrylamide (2:38) was prepared using wedged-spacers (0.25mm-0.75mm), and a shark-toothed comb. Samples were denatured at 83-85°C for 3 min., and 3μl were immediately applied to electrophores for 3 1/2 hours at 1300 volts in the Poker Face SE 1500 Sequencing apparatus (San Francisco, CA). The gel was dried for 1 hour at 80°C, sprayed with Krylon's Crystal Clear Acrylic Spray Coating (Borden Company Ltd., Willodale, Ont.), and exposed to X-ray film overnight.

Double stranded sequencing directly on PCR amplified exons 9,11, and 12 from genomic DNA of WG 1681 was also performed according to the instructions of the BRL dsDNA Cycle Sequencing System (Grand Island, NY). Genomic PCR primers (see section 5.4.2) were used as sequencing primers. Annealing temperatures for cycle sequencing were identical to those used for genomic PCR (see Table 4).

5.4 FAMILY STUDY

5.4.1 DNA Extraction

Confluent fibroblast monolayers from WG 1681 and WG 1130 were harvested from 175 cm^2 flasks by washing the cell surface twice with 1X PBS buffer.

Cells were released from the surface of the flasks by exposing to 0.25% trypsin for 10 min. at 37°C. Trypsinization was terminated by addition of normal culture media, and cells were then collected three times by centrifugation at 1500 rpm for 8 min. at room temperature in an IEC HN-S centrifuge (Needham, Mass.). Between each collection, the pellet was resuspended in 1X PBS buffer. The cell pellet was resuspended in 500μl PBS and underwent a final centrifugation at 14,000 rpm for 5 min. at 4°C. The pellet was then gently resuspended in 350μl solution A (1mM Tris pH 8, 1mM EDTA, 1mM NaCl). 350μl of solution B (solution A, 2% SDS), and proteinase K (0.25 mg/μl) were sequentially added. After a 3 hour incubation at 37°C, DNA was extracted once with equal volumes of Tris-phenol (Maniatis et al., 1989), once with phenol:chloroform, and once with TE:chloroform. DNA was precipitated by adding 1/25 volume of 5M NaCl and 2 volumes of cold absolute ethanol. The precipitate was dissolved in sterile water, and concentration was determined by measuring the absorbance at 260nm. Quality of DNA was determined by measuring the 260nm/280nm ratio.

DNA was also extracted from blood samples obtained from family members of the WG 1681 patient. Samples were centrifuged at 1900 rpm for 10 min. at 4°C in a Beckman TJ-6 centrifuge. The plasma was removed, and the nucleated cells were resuspended in 1X RSB (10mM Tris pH 7.6, 10mM KCl, 10mM $MgCl_2$), lysed with Nonidet P40 non-ionic detergent and centrifuged at 2500 rpm for 10 min. at 4°C. The pelleted cell nuclei were resuspended in 1X RSB and lysed with an SDS solution containing 1X RSB, 0.4M NaCl, 0.5% SDS and 2mM EDTA. DNA was extracted once with an equal volume of Tris-phenol and twice with an equal volume of chloroform. DNA was precipitated with 2 volumes of cold absolute ethanol. Sterile water was used to resuspend DNA, and the concentration was determined as described above.

TABLE 4 SEQUENCES OF PRIMERS USED FOR GENOMIC PCR AND DIRECT SEQUENCING.

TABLE 4 GENOMIC PCR PRIMERS

EXON	PRIMER#	SEQUENCE	ANNEAL TEMP.	#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'		

* denotes antisense primer
NOTE: primers were used in direct sequencing, and annealing temperatures were kept the same.

5.4.2 Genomic Polymerase Chain Reaction (PCR)

Primers were designed using OLIGO Primer Analysis Software (National BioSciences). Amplification of genomic DNA was performed using oligonucleotides specific for exons 9, 11, and 12. Each 50μl reaction contained 0.5μg DNA, 1μM of each 5' and 3' primers, 200μM each dNTP (dATP, dGTP, dCTP, dTTP), 1X Perkin Elmer Cetus reaction buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% w/v gelatin), and 2U Taq polymerase (Pharmacia) or 2U Amplitaq (Perkin Elmer Cetus). Samples were overlaid with 50μl 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 minute, annealing temperature for 1.5 minutes, and 72°C for 1.5 minutes. The primer sequences, temperatures used for annealing, and sizes of products are described in Table 4. Due to non-specific bands, amplification of exon 9 was performed according to a modification published by Yap and McGee (139). 10 cycles of amplification were performed as described above, followed by an additional 30 cycles using a denaturation temperature of 88°C for 1 minute.

Exon 2 was similarly amplified from WG 1130 and from a control DNA sample (normal half-sister of WG 1681).

5.4.2.1 Restriction digest analysis of Exon 2 from WG 1130

10μls of PCR amplified exon 2 from WG 1130 and from control was incubated overnight at room temperature with 7.2U of Bbr PI restriction enzyme (Boehringer Manneheim) and no buffer. In addition, 5μl of PCR amplified exon 2 was incubated for 1 hour at 37°C with 15U of PvuII restriction enzyme (Pharmacia) and 1X reaction buffer. Uncut and cut samples were analyzed on a 1% agarose gel.

5.4.3 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 in Table 5. 10μl of the PCR amplified

TABLE 5 SEQUENCES FOR ALLELE SPECIFIC OLIGONUCLEOTIDES.

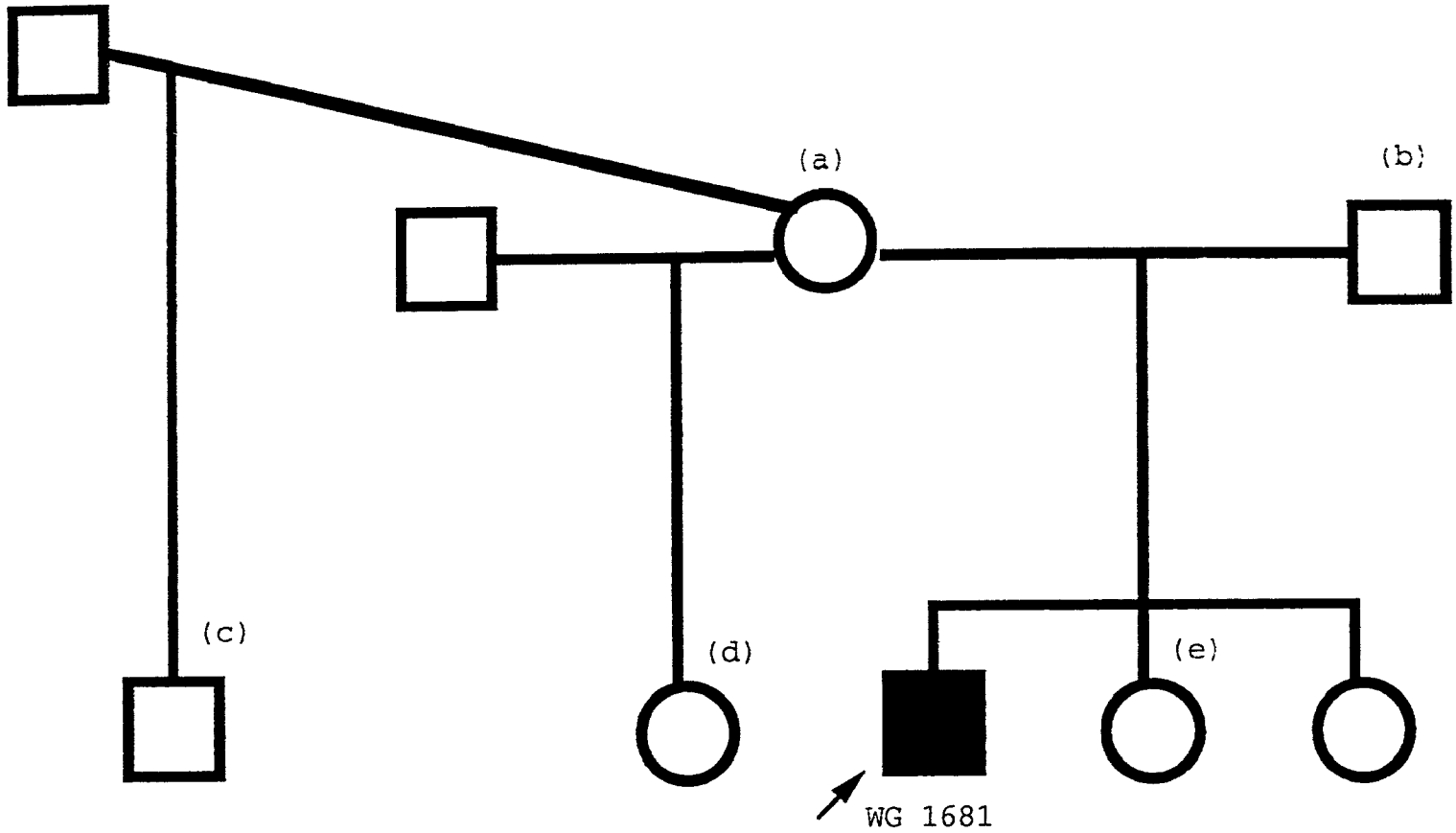
Numbers of ASO correspond to the specific change being studied.

TABLE 5 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

FIG. 10 **PEDIGREE OF WG 1681.** (a)=mother; (b)=father; (c)=half-brother; (d)=half-sister; (e)=sister; arrow=WG 1681 proband.


(a)
(b)
(c)
(d)
(e)
WG 1681

productsfrom exons 9, 11, and 12 were denatured by 100μl of a 0.4M NaOH, 25mM EDTA (pH 8) solution. The denatured DNA was blotted onto a wet nitrocellulose membrane by vacuum using the Schleicher and Schuell Minifold II Slot Blot System (Keene, NH) with slight modifications to the procedure described by the manufacturer. The nitrocellulose membrane was soaked in water and the wells were washed with 100μl of 20X SSPE (Maniatis). After transfer of samples, the membrane was baked for 1 1/2 hours at 80°C. 20μM of each oligonucleotide was end-labelled with 50μCi gamma-^{32}PATP, 9U of polynucleotide kinase (Pharmacia), and 1X Promega kinase buffer for 45 min. at 37°C. 5μl of end-labelled oligonucleotide was hybridized for 1 hour to the membrane pre-soaking in 5X SSPE, 0.5% SDS, and 5X Denhardt's (see Table 5 for hybridization temperatures). The membrane was washed twice in a 2X SSPE, 0.1% SDS solution for 5 min. each (see Table 5 for wash temperatures). The blot was placed between two intensifying screens and exposed to Kodak X-ray film for 2 hours at -70°C. The blots hybridized to the "normal" and V671I polymorphic ASO were exposed the identical way for 3 days.

5.4.4 PCR-Based Polymorphic Analysis

The gene encoding the human neurofilament-heavy chain subunit (NEFH) has been localized to chromosome 22q121-q131 (140). The polymorphism located at the 3' phosphorylated region of the NEFH gene (141) was amplified from genomic DNA of WG 1681 and family members. Primers were provided by Dr. Guy Rouleau. A standard 100μl PCR reaction consisted of 1x PCR buffer, 0.2mM dNTP's, 650ng of each primer, water, and 0.5μg of genomic DNA. After a hot start at 94°C for 8 minutes, 6.25U of Taq polymerase (Pharmacia) was added. Amplification consisted of 30 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 3 minutes. PCR products were digested for 2 hours at 37°C by 7μl of a PstI/PvuII and buffer mix. The mixture contained 1μl (15U) of each restriction enzyme (Pharmacia) and 5μl of 10X One-Phor-All buffer (Pharmacia). 25μl of each

digested product was loaded onto a 8% medium polyacrylamide gel (39:1), and electrophoresed in a BioRad unit. Electrophoresis was performed in 1X TBE for 24 hours at 100 volts, or until the xylene dye was near the bottom. The gel was stained for 20 minutes in an 0.5mg/ml ethidium bromide/1XTBE solution, and photographed under ultraviolet illumination.

Primer sequences: 544 5'gggatagaattcgtggtggagaagtctgagaa 3'
545 5'gggggagcctttgactttcacctcctggg 3'

5.5 EXPRESSION OF MUTATIONS

Expression vector and subcloning cassettes were provided by Dr. Fred Ledley at the Howard Hughes Institute. The expression vector, pCMV-hMCM, was constructed by inserting human MCM cDNA into the pNassCMV plasmid (142). This vector utilizes the cytomegalovirus (CMV) immediate early promoter, SV40 polyadenylation sequences and SV40 late viral protein splice donor and acceptor signals (Fig.11). 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 5Z+ plasmid (2) (Fig.12).

5.5.1 Subclone Constructs

Mutant subclones were constructed by combining mutant segments of the PCR sequencing-clones (section 5.3.3) with normal segments of either the 5' or 3' cassettes. Mutant and normal segments were extracted from PCR and subclone cassettes, respectively, by double restriction digests. Restriction enzymes were chosen so that respective cutting sites were represented once in the MCM cDNA sequence and were not found in the pGEM 5Z+ vector sequences. 18-20U of each enzyme was used as suggested by the manufacturers. Stratagene's 10X Universal Buffer was used at the required concentration suggested by the manufacturers for each enzyme used in the double digestion. Figure 13A lists the restriction enzymes used and the generated fragment sizes. Two different restriction enzymes could not be identified to extract the G703R change by itself. Therefore, the 703+P671 clone was digested

FIG. 11 **pCMV-hMCM EXPRESSION VECTOR.** SD= SV40 splice donor site; SA= SV40 splice acceptor site. (Figure provided by Ana M. Crane)

EXPRESSION VECTOR

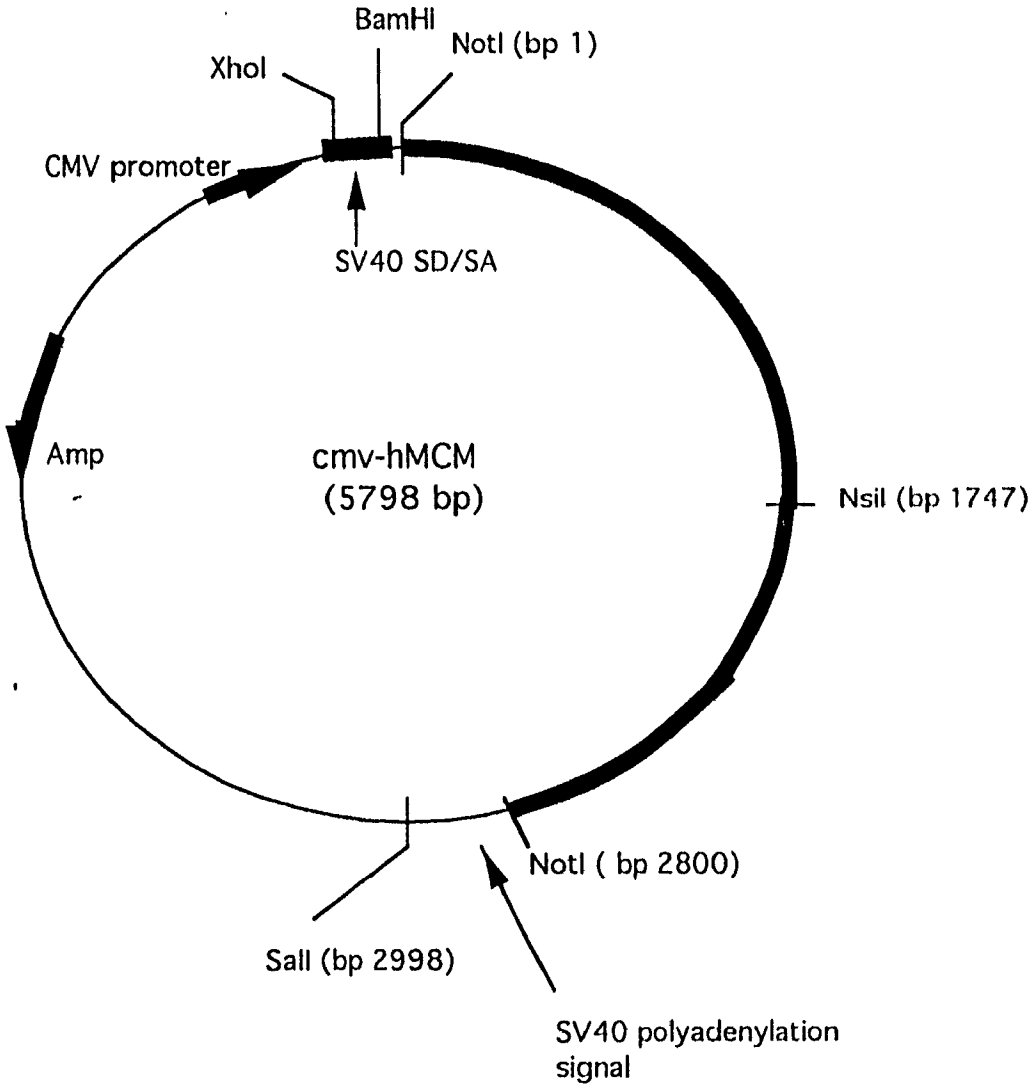
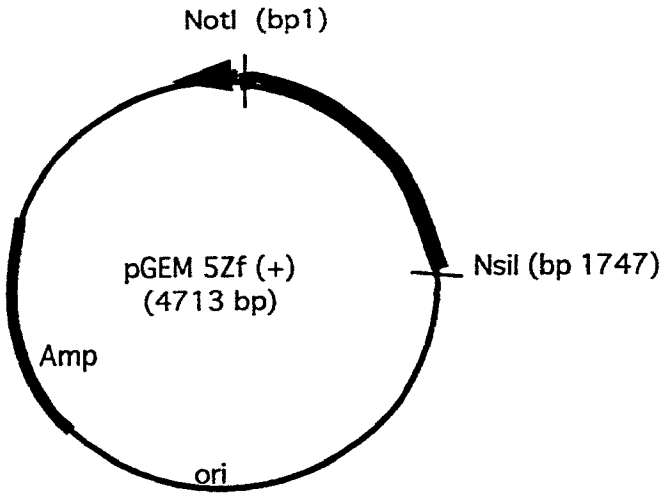
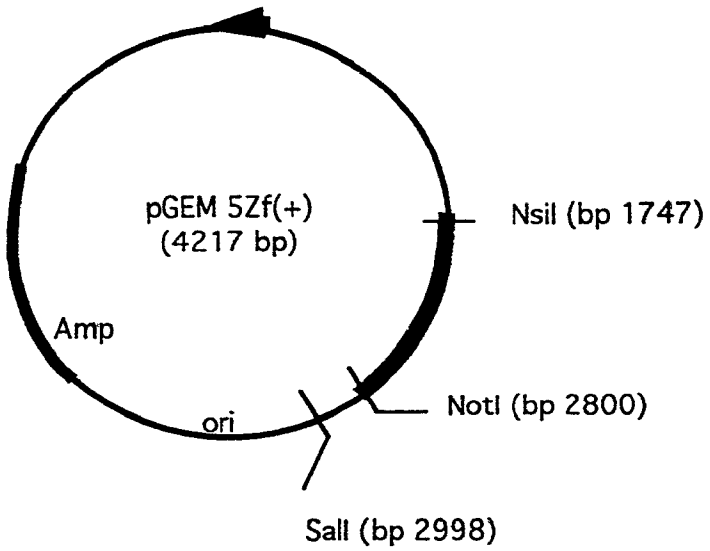


FIG. 12 **5' AND 3' pGEM 5Z+ SUBCLONING CASSETTES.** ori= origin of replication; amp= ampicillin resistance gene. (Figure provided by Ana M. Crane).

5' SUBCLONE CASSETTE



3' SUBCLONE CASSETTE



with StyI, which cut once in the cDNA sequence and once in the subclone vector. HindIII could be used to isolate the H532R polymorphism by partial digestion. Using the QIAEX extraction kit (QIAGEN, Chatsworth, Ca.), fragments of interest were isolated from 1% agarose gels as directed. Isolated fragments carrying each putative mutation singly or in combination with the V671I polymorphism were substituted for the equivalent normal segment in the subclone cassette by ligating for 10 hours at 14°C. A subclone carrying only the H532R polymorphism was also constructed. Ligation products were cloned in DH5α bacterial cells (Gibco BRL) as directed, however, agar plates did not contain X-Gal. Four colonies for each subclone were chosen and grown separately overnight in 3ml LB media containing 50μg/ml ampicillin. Plasmid DNA minipreparations were prepared as directed by Promega. Subclones were subjected to double stranded dideoxy sequencing as described in section 5.3.6 to confirm the absence of artifactual sequence changes.

5.5.2 Expression Constructs

SalI/NsiI fragments and/or NotI/NsiI fragments were substituted from mutant subclones into the normal expression vector by sequential multi-part ligation (Fig.13B). 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. These served not only as experimental controls, but also as controls for construction methodology. Transformation of DH5α bacterial cells, plasmid DNA minipreparations, and double-stranded sequencing were conducted as described above. All six expression constructs were individually grown overnight in 200ml of LB media containing 50μg/ml ampicillin. Plasmid extractions were performed using the Promega Magic Maxiprep Kit with slight modifications. The first two centrifugations were at 1879 x g for 10 min. and 20 min., respectively. Plasmid DNA was pelleted in 30ml corex tubes at 8816 x g for 15 min.. 1μl of each plasmid DNA elution was cut with BamHI restriction enzyme to check for plasmid quality. Each maxipreparation eluate was then precipitated overnight with 1/10 volume 3M

FIG. 13A CONSTRUCTION OF SUBCLONES. 1) G623R change alone; 2) G623R and V671I polymorphism; 3) G703R change alone; 4) G703R and V671I polymorphism; 5) H532R polymorphism alone.

A

SUBCLONE CONSTRUCTIONS

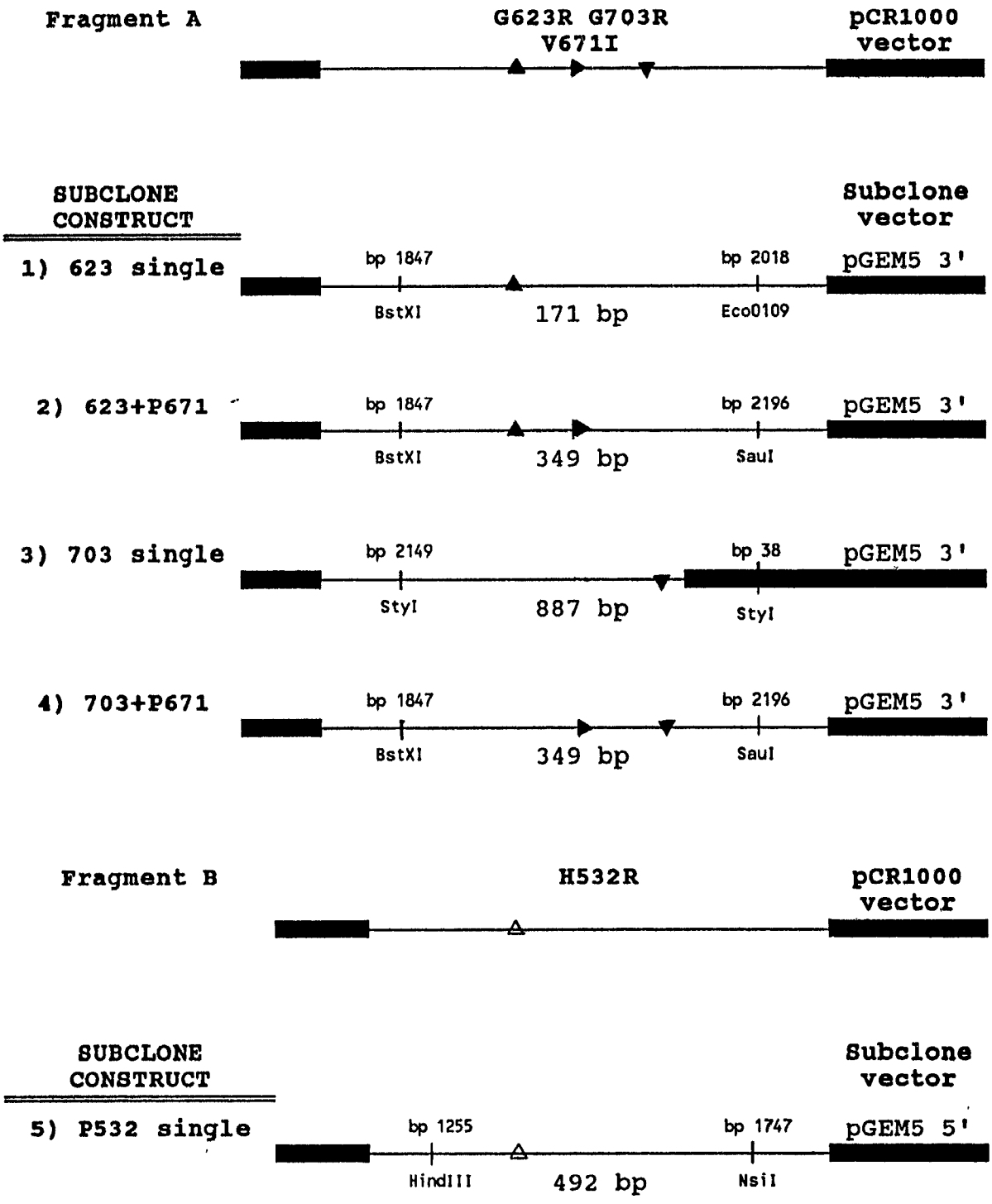
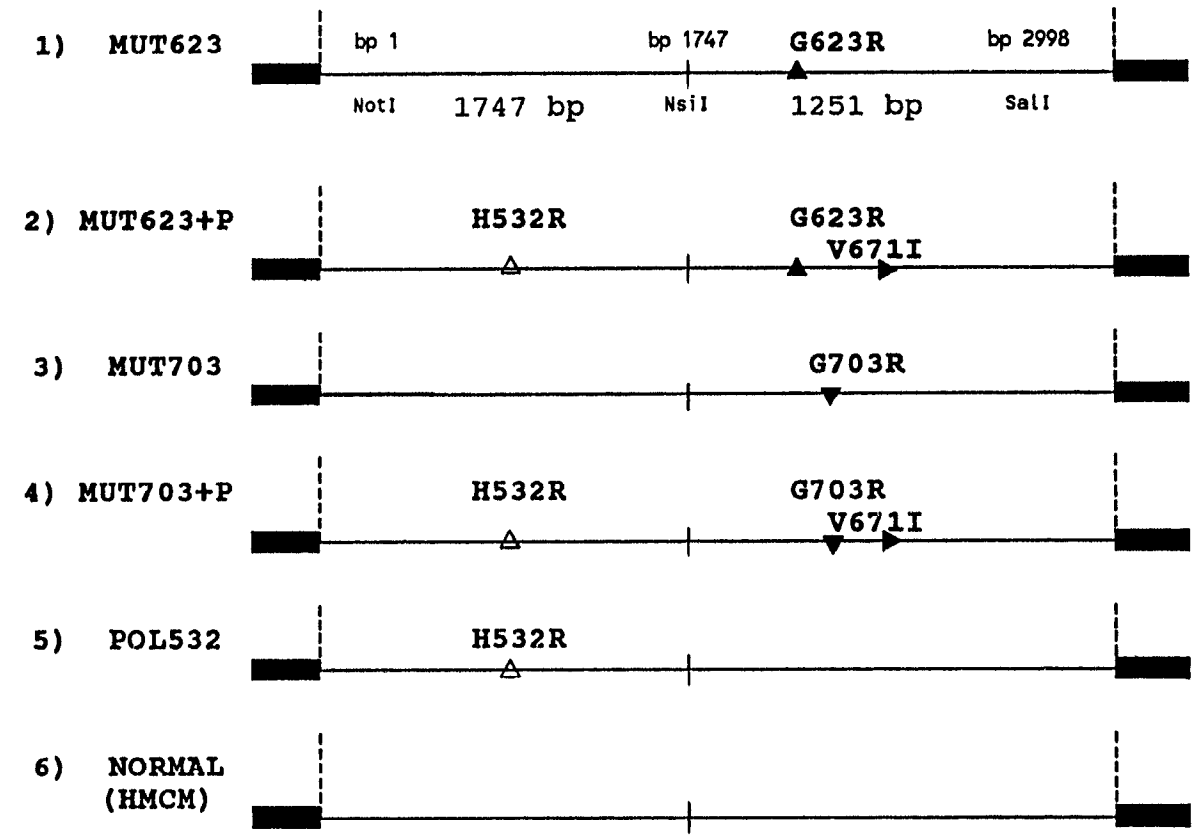


FIG. 13B CONSTRUCTION OF EXPRESSION CLONES. 1) G623R change alone; 2) G623R, and H532R and V671I polymorphisms; 3) G703R change alone; 4) G703R, and H532R and V671I polymorphisms; 5) H532R polymorphism alone; 6) normal MCM consensus sequence.

B

EXPRESSION CONSTRUCTS



NaAcetate pH 6 and 2 volumes absolute ethanol. Plasmid DNA was pelleted by centrifugation and washed twice with cold 70% ethanol. Resuspension of each expression plasmid DNA in 100μl sterile water resulted in 4-7μg/μl concentration as measured spectrophotometrically at 260nm.

5.5.3 Expression Studies

Expression constructs were sent to Dr. Fred Ledley's laboratory at the Howard Hughes Institute in Baylor, Texas. Expression studies were performed by Ana M. Crane as described before (1,2,134). 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 1.0μg/ml OH-Cbl. Results are expressed as the mean nanomoles of propionate per micromoles leucine incorporated in triplicate samples.

5.6 ANALYSIS OF INTERALLELIC COMPLEMENTATION

This experiment was also conducted by Ana M. Crane as described previously (1). Equal concentrations of plasmids carrying the G623R, G703R, H532R, G717V, G623R+ both polymorphisms, or G703R + both polymorphisms were electroporated into GM1673 mut^0 cells in combination with the R93H construct. [^{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.

CHAPTER 6
RESULTS

6.1 HOMOZYGOSITY OF R93H MUTATION IN WG 1130

The R93H mutation of WG 1130 has been identified in the cDNA from this cell line (2), but homozygosity had not been verified in the genomic DNA. This mutation eliminates a Bbr PI restriction site. PCR amplified exon 2 from genomic DNA of WG 1130 and from control genomic DNA generated a 485 bp fragment. Upon digestion with Bbr PI, two expected fragments of 368 bp and 117 bp were generated from the control, indicating the presence of a normal restriction site. However, this restriction site was absent in WG 1130 as indicated by an uncut 485 bp fragment (Fig.14A). This confirmed that the R93H mutation was a homozygous change in the WG 1130 cell line. Similar digestions with PvuII generated the expected 242 bp and 244 bp fragments from both control and WG 1130. Although the 1% agarose gel was unable to distinguish between the two similarly sized fragments, the distinction between cut and uncut product was clear (Fig 14B). This demonstrated that another restriction endonuclease site in exon 2 from WG 1130 was unaffected.

6.2 IDENTIFICATION OF MUTATIONS

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}->g (H532R) change was identified in fragment B, and three changes were identified in the most 3' fragment A: g_{2087}->a (V671I), g_{1913}->a (G623R), and g_{2185}->c (G703R). Sequencing the pooled clones authenticated any changes observed in the sequence of the single clone, minimizing errors due to PCR or cloning artifacts (134). When two bands comigrated in the pooled prep (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

FIG. 14 RESTRICTION DIGEST ANALYSIS OF EXON 2 OF WG 1130. (A) Results of Bbr PI digest. Lane 1= lambda digested with HindIII (marker); 2= undigested exon 2 of control; 3= exon 2 of control treated with Bbr PI; 4= undigested exon 2 of WG 1130; 5= exon 2 of WG 1130 treated with Bbr PI. (B) Results of Pvu II digest. Lane 1= lambda digested with HindIII (marker); 2= undigested exon 2 of control; 3= exon 2 of control treated with Pvu II; 4= undigested exon 2 of WG 1130; 5= exon 2 of WG 1130 treated with Pvu II.

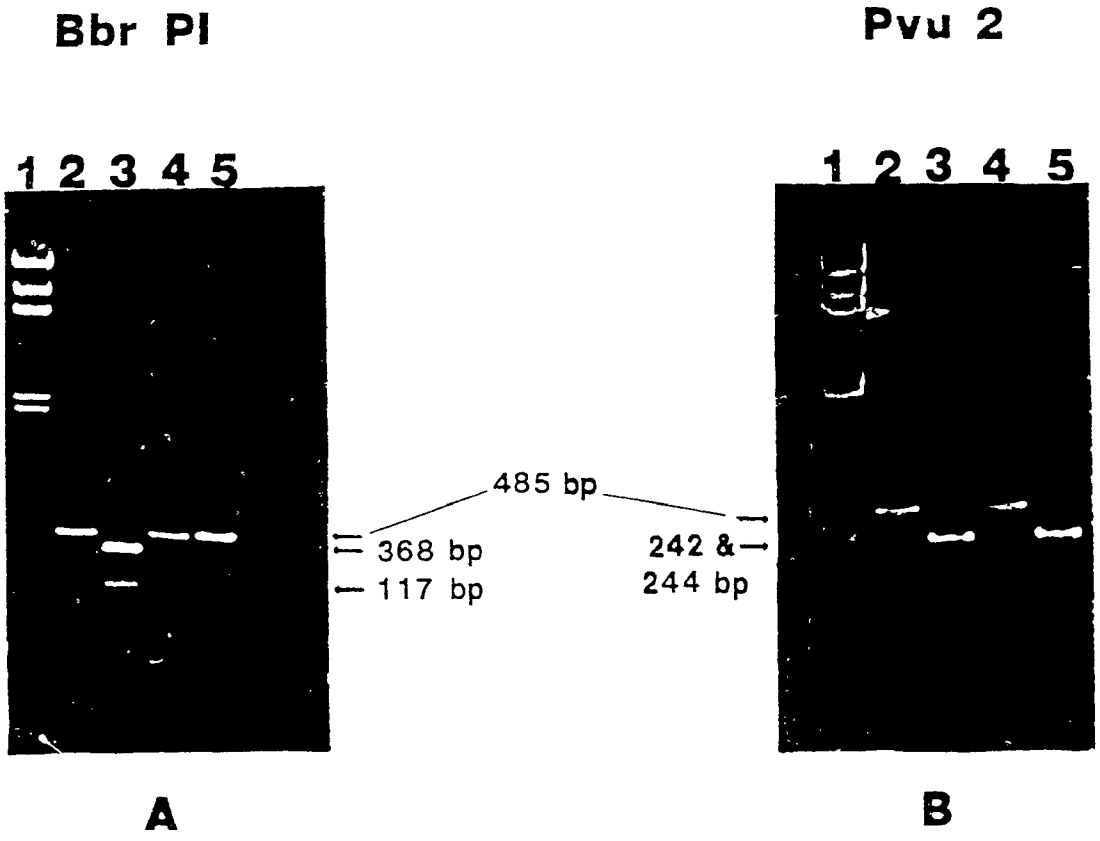

Bbr PI
Pvu 2
1 2 3 4 5
1 2 3 4 5
485 bp
368 bp
117 bp
242 &
244 bp
A
B

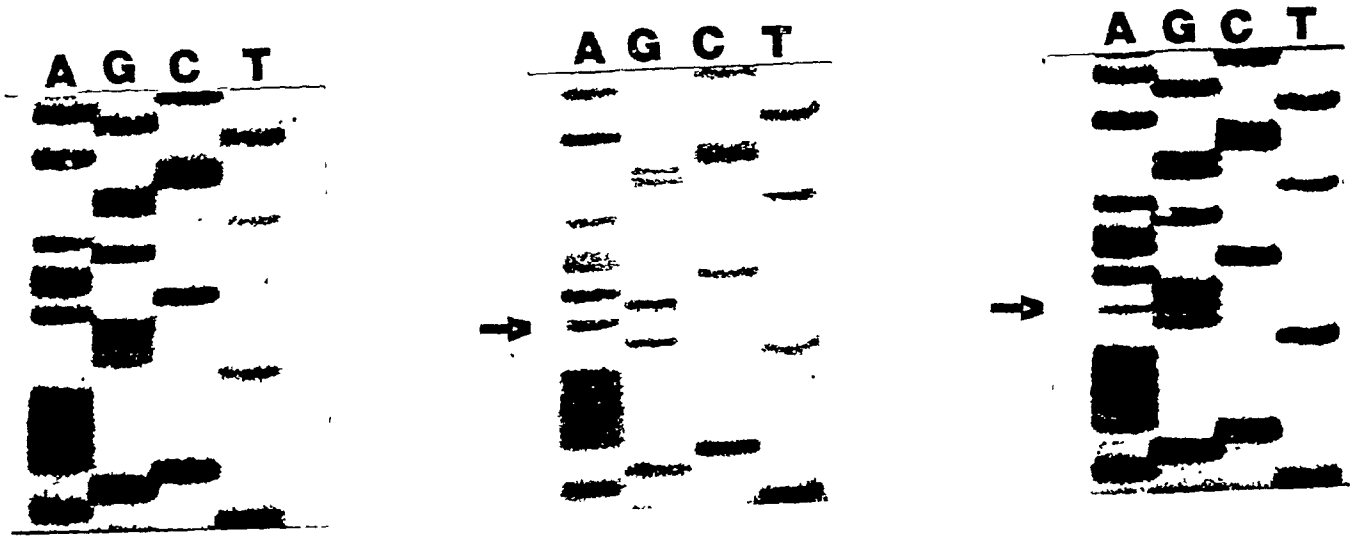
FIG. 15A cDNA SEQUENCING RESULTS FOR G623R CHANGE.

FIG. 15B cDNA SEQUENCING RESULTS FOR G703R CHANGE.

A

G623R

$g_{1943} \rightarrow a$



Control

Single

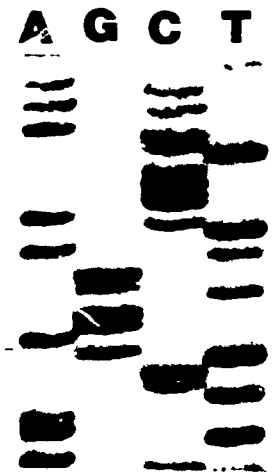
15 Pool

(Sense)

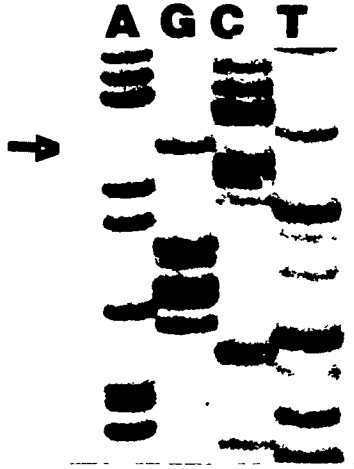
B

G 703 R

$g_{2183} \rightarrow c$

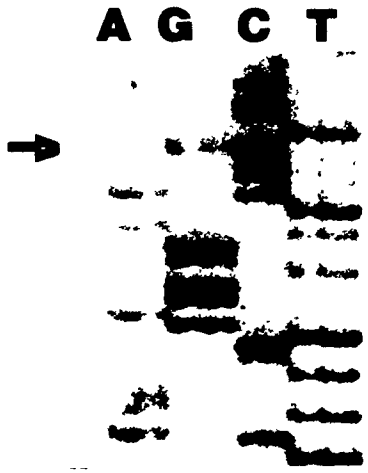


Control



Single

(Antisense)



15 Pool

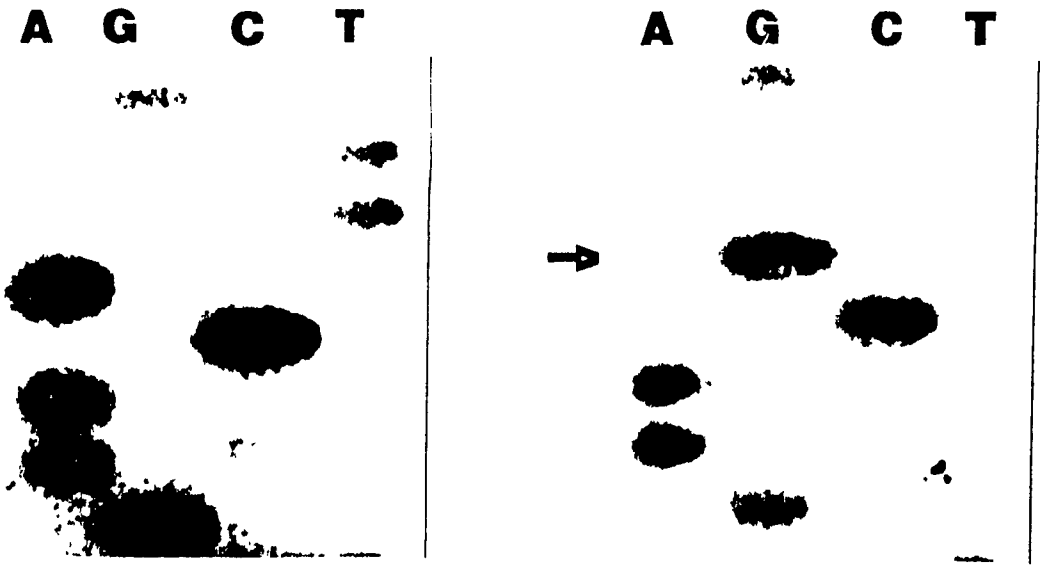
FIG. 16A DIRECT SEQUENCING RESULTS FOR H532R POLYMORPHISM.

FIG. 16B DIRECT SEQUENCING RESULTS FOR V671I POLYMORPHISM.

A

H 532 R Polymorphism

$^{a}_{1671} \rightarrow g$

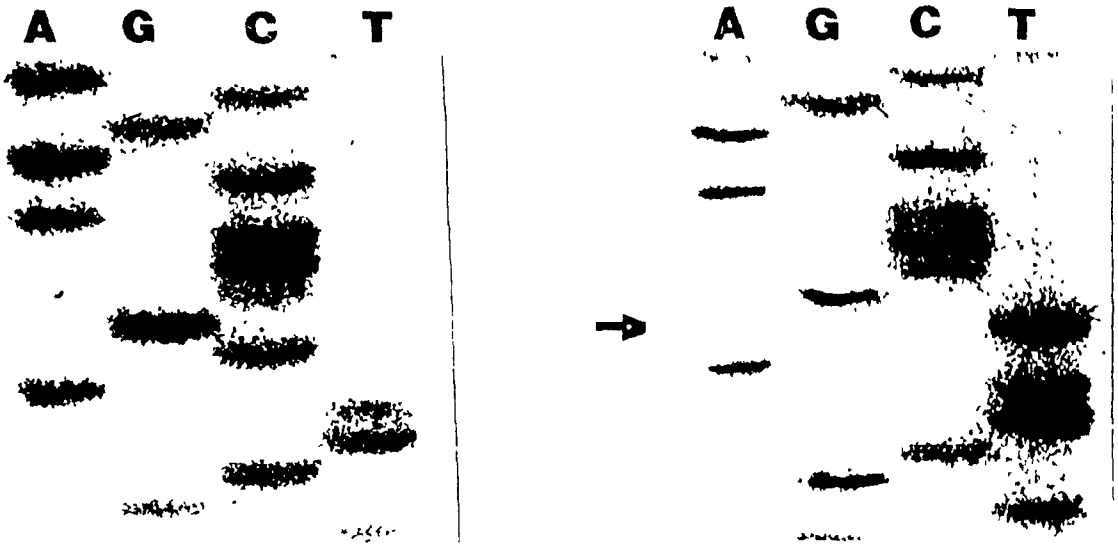


Control

Exon 9

V 6711 Polymorphism

$g_{2087} \rightarrow a$



Control

Exon XII

(Antisense)

substitutions, indicating homozygosity. These two changes have been previously identified as normal MCM polymorphisms (1) (see section 4.8.2). Putative heterozygosity was observed for the novel G623R and G703R changes. The pooled clones showed two equally intense co-migrating bands consistent with amino acids glycine and arginine at position 623 and 703 (Fig.15A and 15B, respectively).

The H532R, G623R, and both G703R and V671I changes are within exons 9, 11, and 12, respectively. The base pair changes observed in the cDNA were verified in genomic DNA by direct sequencing of PCR-amplified exons. Figures 16A and B show examples of direct sequencing results for the H532R and V671I polymorphisms, respectively.

6.3 FAMILY STUDY

The family members used in this study are identified in figure 10. PCR amplified exons 9, 11, and 12 from these individuals and from WG 1681 were analyzed by allele specific oligonucleotides (ASO) (Fig.17A). Haplotypes were then generated for each member as shown in figure 17B. Due to non-paternity (see section 6.4), the pedigree for the WG 1681 family was re-drawn. The new pedigree assumes that there exists a fourth mate for individual (a). WG 1681 showed heterozygosity for both G623R and G703R changes, and homozygosity for both H532R and V671I polymorphisms. This confirmed cDNA sequencing results. The mother was heterozygous for G703R, H532R, and V671I, and did not possess the G623R change. The father was homozygous for the H532R and heterozygous for the V671I polymorphisms. He also did not carry the G623R change. A clinically normal sister and half-brother were both heterozygous for G703R and V671I, and homozygous for H532R. A clinically normal half-sister did not possess any of the identified changes.

6.3.1 PCR-based Polymorphic Analysis

ASO analysis suggested that there may be a possible case of non-paternity. Polymorphic analysis was performed with all family members. Figure 18

FIG. 17A ALLELE SPECIFIC OLIGONUCLEOTIDE RESULTS. Letters correspond to individuals in fig. 10. N = normal allele; M = mutant allele; + = presence of polymorphism; - = absence of polymorphism.

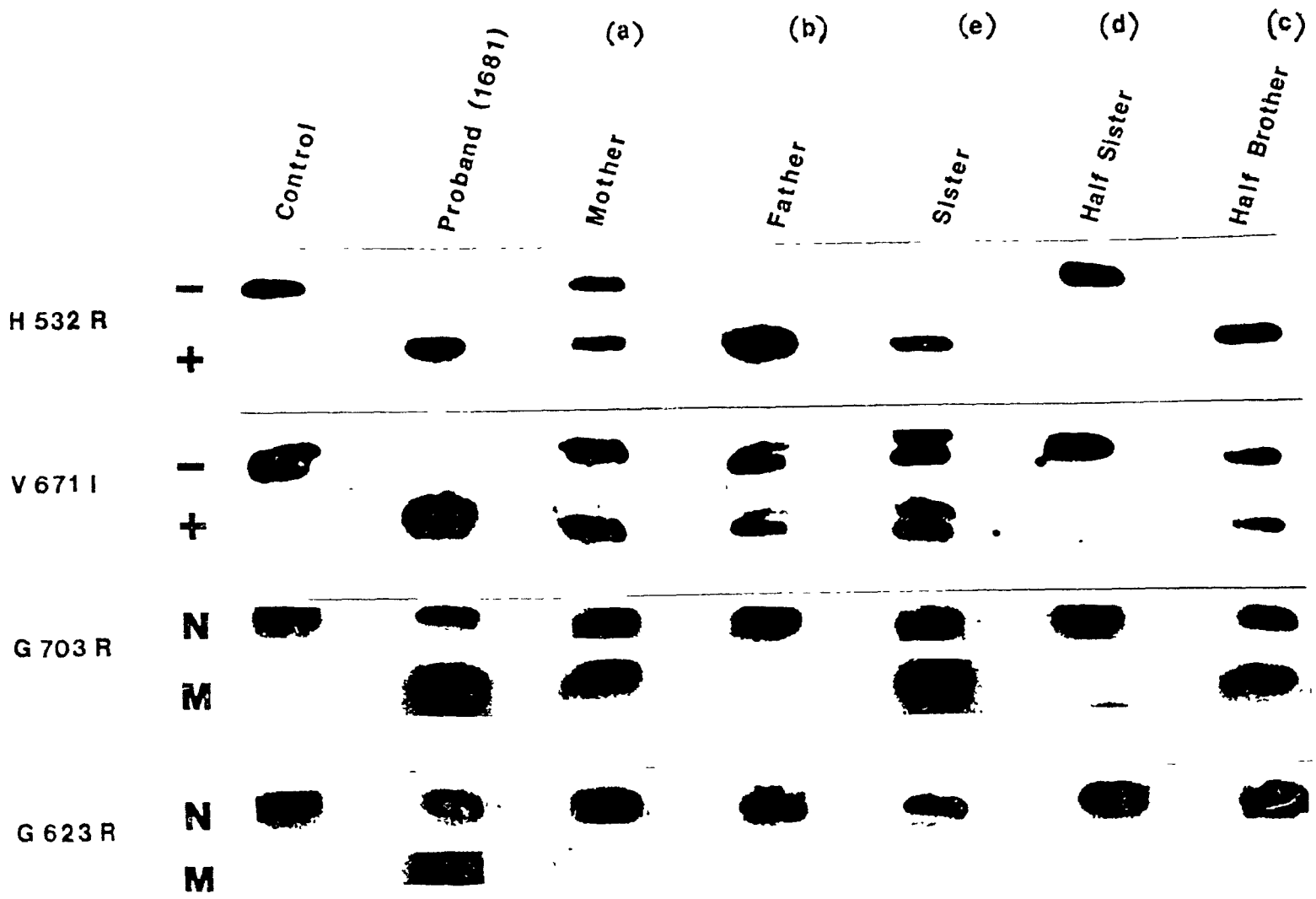

Control
Proband (1681)
(a)
Mother
(b)
Father
(e)
Sister
(d)
Half Sister
(c)
Half Brother
H 532 R
−
+
V 671 I
−
+
G 703 R
N
M
G 623 R
N
M

FIG. 17B HAPLOTYPE OF WG 1681 FAMILY. (a) mother (b) father of individual (e), (c)= half-brother; (d)= half-sister, arrow WG 1681 proband; (e)= sister M= mutant allele, N normal allele, + presence of polymorphism; -= absence of polymorphism, * carrier

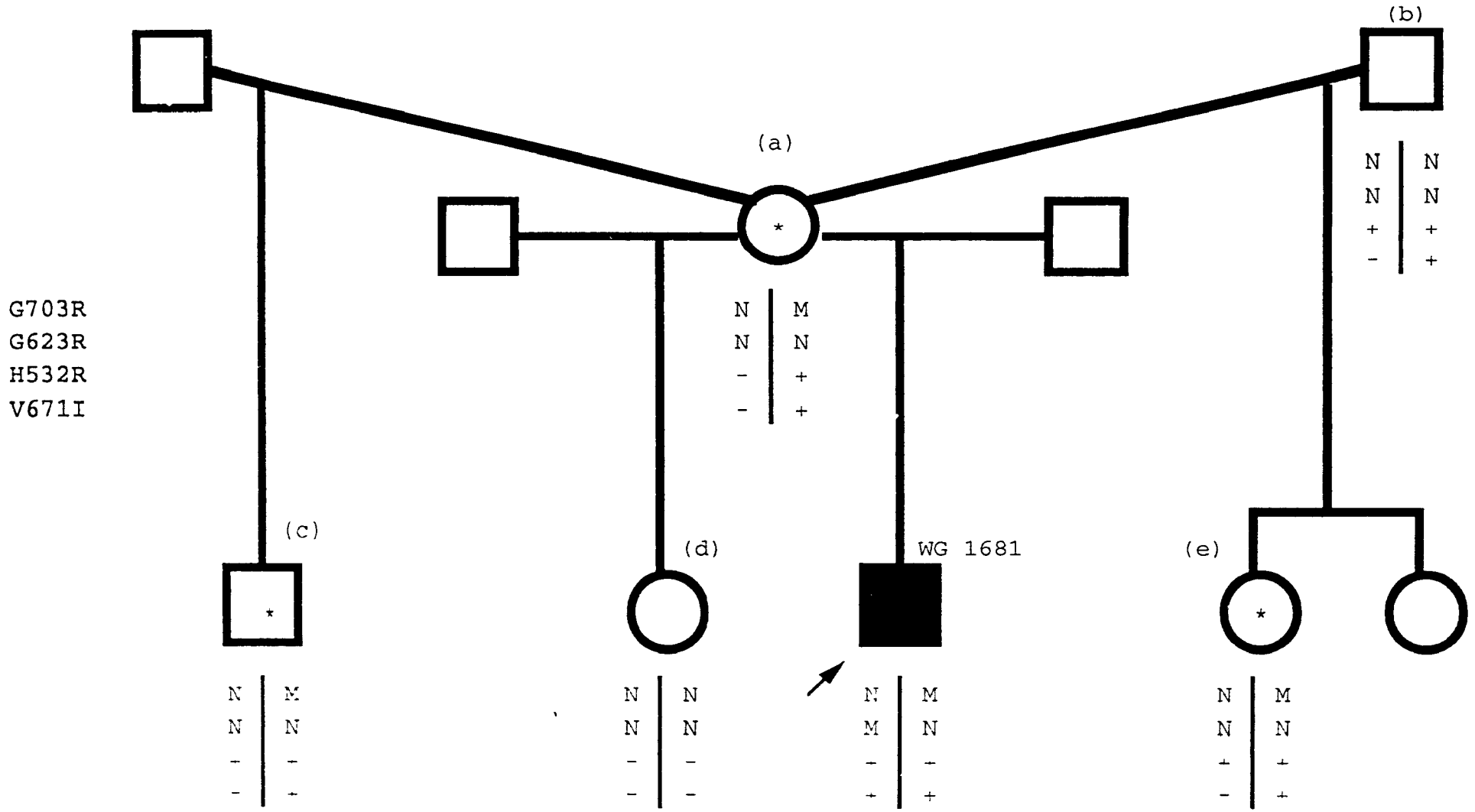
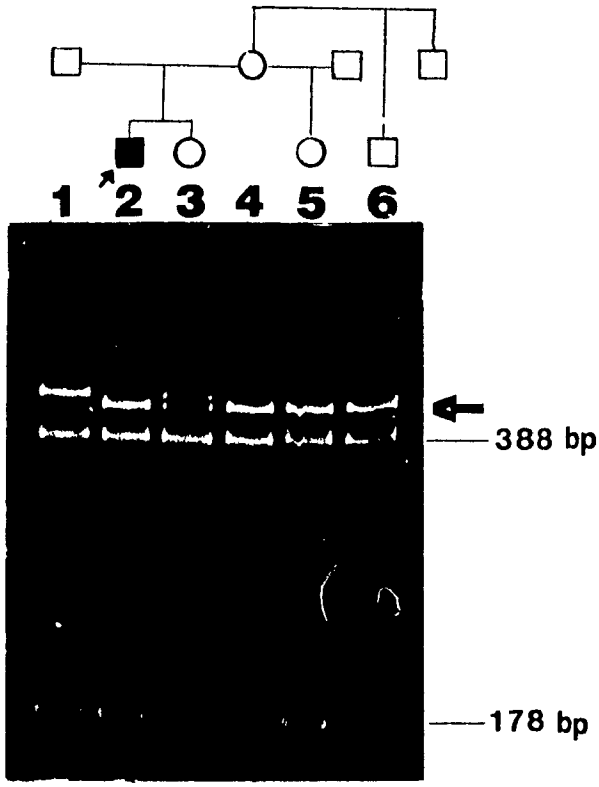

G703R
G623R
H532R
V671I
(a)
(b)
(c)
(d)
(e)
WG 1681
N M
N N
- +
- +
N N
N N
+ +
- +
N M
N N
- -
- +
N N
N N
- -
- -
N M
M N
- -
+ +
N M
N N
+ +
- +

FIG. 18 PCR-BASED POLYMORPHIC ANALYSIS. Lane 1= "father"; 2= WG 1681 proband; 3= sister; 4= mother; 5= half-sister; 6= half-brother.



genotype

1,1 2,2 1,2 2,2 2,2 2,2

shows the results of this analysis. The PCR product was 1.1 kb in size, but digestion with PstI and PvuII resulted in four fragments. The human neurofilament-heavy chain subunit gene polymorphism was observed in the largest of the four fragments (arrow). The other fragments of 388 bp, 178 bp, and 162 bp (off picture) are constant bands. The numbers 1 and 2 were assigned to the larger and smaller polymorphic alleles, respectively. Genotyping results are shown for each family member. WG 1681 (2), his mother (4), half-sister (5) and half-brother (6) were homozygous for the lower allele. The sister (3) was heterozygous, while the father (1) was homozygous for the larger allele. Thus, the blood sample obtained was not from the biological father of WG 1681.

6.4 EXPRESSION STUDIES

Expression of the G623R or G703R changes singly, and in combination with both H532R and V671I polymorphisms are shown in figure 19A. The GM1673 mut^0 cells, which express low MCM message, were transfected with these constructs, and [^{14}C]propionate incorporation was measured in triplicate. 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 considerably increased [^{14}C]propionate uptake. This indicated that the H532R substitution is a normal polymorphism. In comparison to these positive controls, the singly transfected G623R and G703R clones did not stimulate 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. Therefore, G623R and G703R are mutations which affect MCM function. An expression construct carrying the V671I polymorphism alone was not made due to difficulty in finding a restriction site. However, such a construct was previously made by Crane (1) from another cell line. Propionate uptake similarly increased upon transfection as observed in positive controls.

Expression studies in the presence and absence of 1μg/ml OH-Cbl were performed for the single clones in order to ascertain if the G623R and G703R mutations were responsible for the mut^0 phenotype exhibited by WG 1681 fibroblasts (Fig 19B). The G623R or G703R clones did not significantly stimulate (1 tail T-test, $p<0.05$) incorporation of labelled propionate in the presence of OH-Cbl. However, incorporation, after transfection of the construct carrying the normal consensus MCM, significantly increased in the presence of OH-Cbl ($p<0.05$). In addition, the co-transfection of the constructs carrying the G623R or the G703R mutations did not affect propionate uptake in the presence or absence of OH-Cbl.

6.5 INTERALLELIC COMPLEMENTATION

Somatic cell complementation analysis has demonstrated that WG 1681 is able to complement WG 1130. In order to ascertain which of the two alleles (G623R or G703R) is responsible for this phenomenon, each clone of WG 1681 was co-transfected with an R93H construct (2) of WG 1130. Results are shown in figure 20A. T-test analysis ($p<0.05$) indicated that the co-transfection of the construct carrying the H532R polymorphism with the R93H construct did not show a significant increase in propionate uptake when compared to uptake without R93H co-transfection. This indicated that the H532R polymorphism did not complement the R93H mutation, as expected. However, the co-transfections of the constructs carrying the G623R, or G703R, or G717V mutations with the R93H construct significantly stimulated ($p<0.05$) propionate incorporation. This indicated that G623R and G703R were independently capable of complementing the R93H mutation. Transfection of the R93H construct into WG 1681 fibroblasts resulted in a significant increase in labelled propionate incorporation, but the transfection of the G717V (mut$^-$ mutation characterized by Crane) construct (1) into WG 1681 fibroblasts did not stimulate significant incorporation (fig.20B). These results are consistent with the somatic cell complementation data. Interestingly, complementation at the somatic cell level (fig.20B) is less evident than at the molecular level (fig.20A). Co-transfection experiments are performed with equal concentrations of each clone

carrying the specific mutations so that presumably, equal subunit molar ratios exist. Thus, in co-transfection experiments, more of the stabilized dimers are formed between the two mutant subunits rather than in single transfections into fibroblasts where less dimers are presumably formed. This may be due to the unequal concentrations, and subsequent decreased molar ratios, of mutant subunits present during single transfections.

FIG. 19A EXPRESSION OF CONSTRUCTS. hMCM= normal clone with MCM consensus sequence; 623= clone carrying G623R change alone; 623+P= clone with G623R+V671I+H532R; 703= clone with G703R change alone; 703+P= clone with G703R+V671I+H532R; POL532= clone with H532R polymorphism alone; CMV= expression vector without insert; BLANK= non-electroporated cells; T-bars= standard error (Experiment performed by Ana Crane).

A

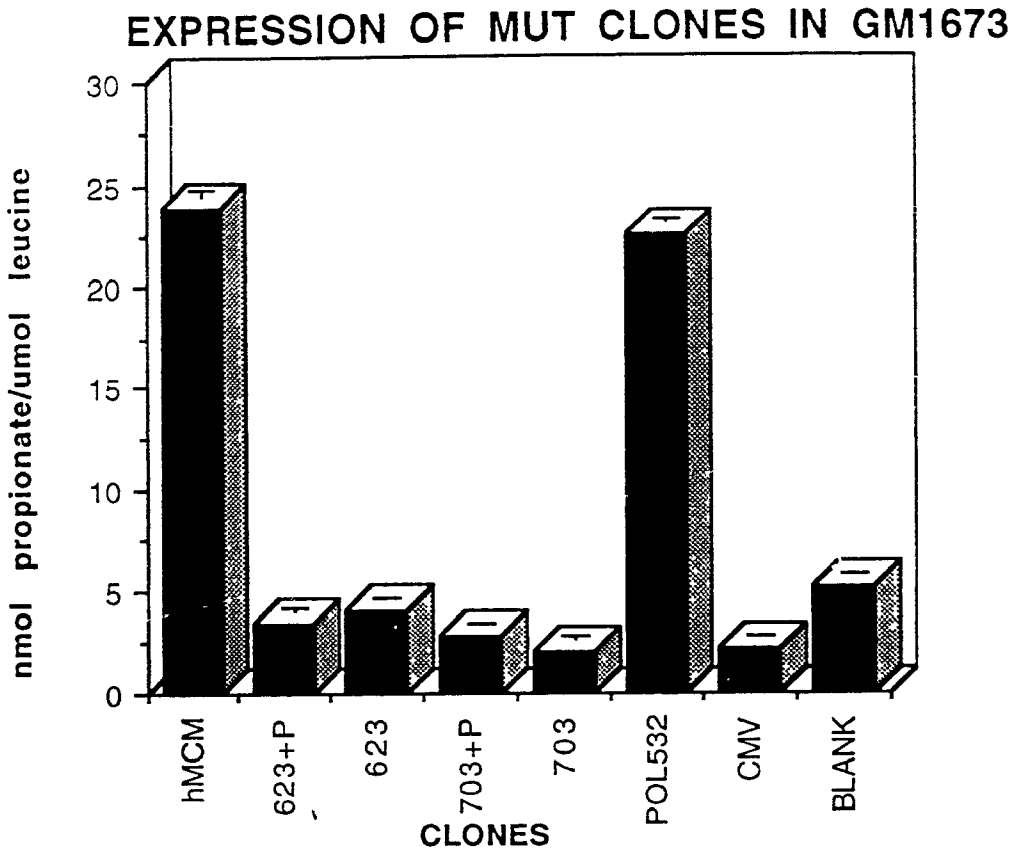

EXPRESSION OF MUT CLONES IN GM1673
nmol propionate/umol leucine
30
25
20
15
10
5
0
hMCM
623+P
623
703+P
703
POL532
CMV
BLANK
CLONES

FIG. 19b **EFFECT OF Cbl-Cbl ON EXPRESSION.** hMCM= normal consensus sequence; MUT623= clone with G623R change alone; MUT703= clone with G703R change alone; CMV= expression vector without insert; BLANK= non-electroporated cells; 623+703= co-transfection of MUT623 and MUT703 clones; T-bars= standard error. (Experiment performed by Ana Crane).

B

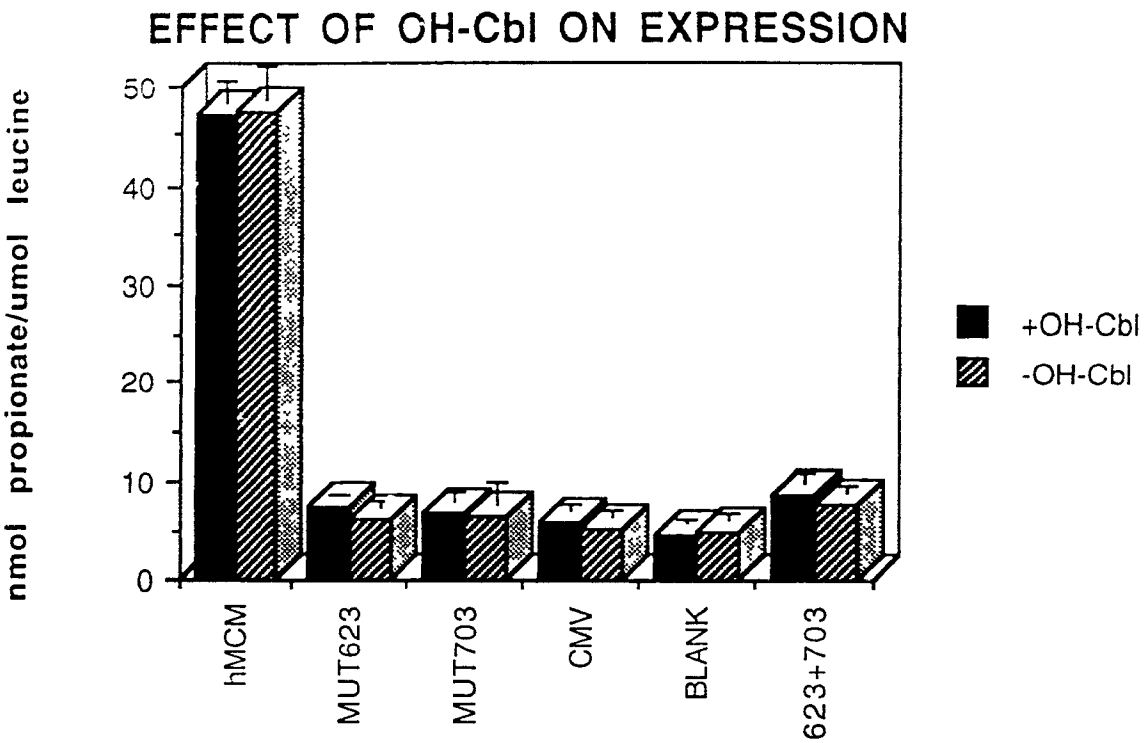
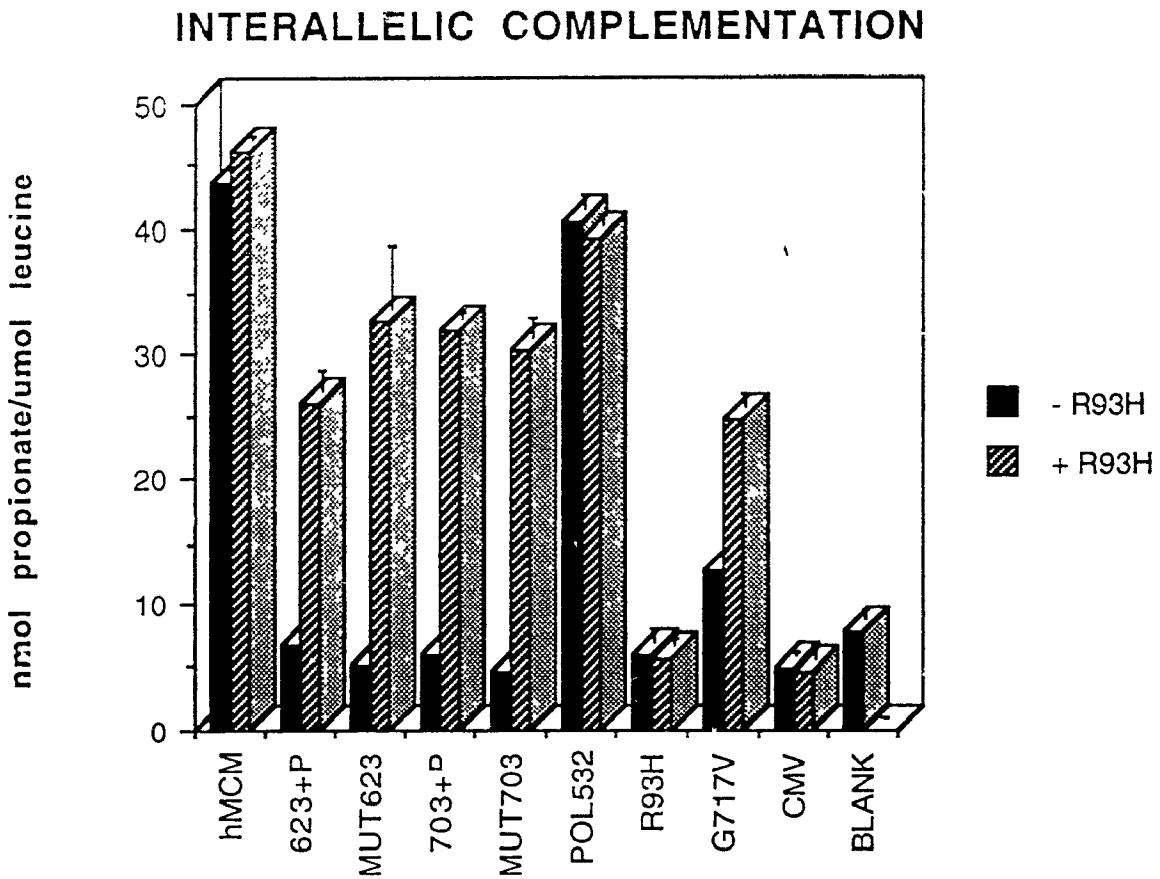

EFFECT OF OH-Cbl ON EXPRESSION
nmol propionate/umol leucine
50
40
30
20
10
0
hMCM
MUT623
MUT703
CMV
BLANK
623+703
+OH-Cbl
-OH-Cbl

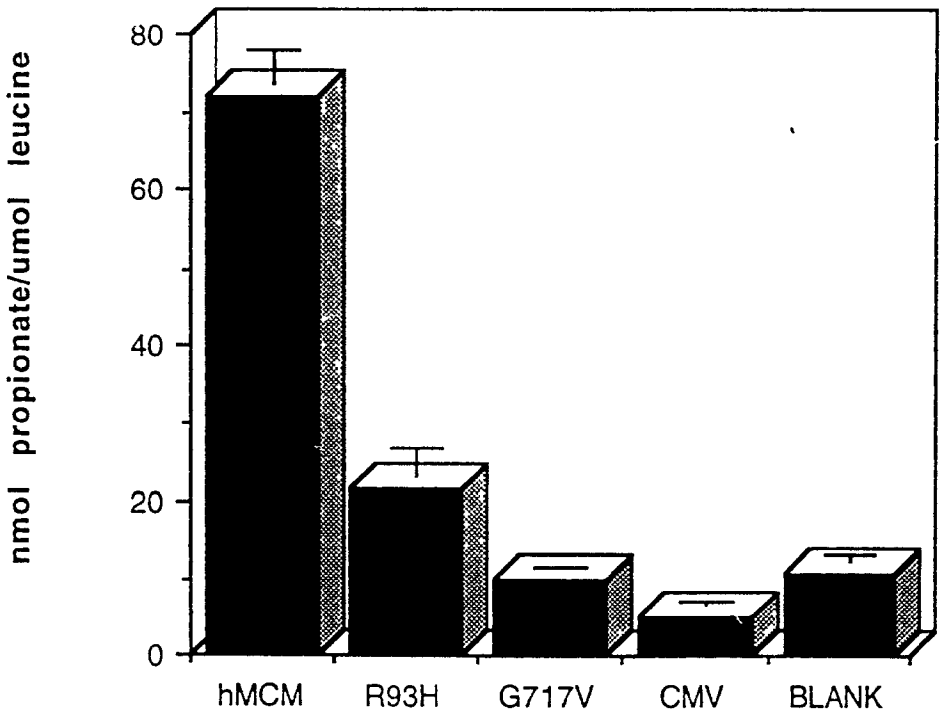
FIG. 20 INTERALLELIC COMPLEMENTATION ANALYSIS. (A) Co-transfection of each clone with R93H construct. hMCM= normal consensus sequence; 623+P= G623R change with both polymorphisms; MUT623= G623R change alone; 703+P= G703R change with both polymorphisms; MUT703= G703R change alone; POL532= H532R polymorphism; R93H= clone with WG 1130 mutation; G717V= clone with WG 1611 mutation; CMV= expression vector without insert; BLANK= non-electroporated cells; T-bars= standard error (B) Expression of clones in WG 1681 fibroblasts. (Experiments performed by Ana Crane).

A


INTERALLELIC COMPLEMENTATION
nmol propionate/umol leucine
50
40
30
20
10
0
hMCM
623+P
MUT623
703+P
MUT703
POL532
R93H
G717V
CMV
BLANK
- R93H
+ R93H

B

EXPRESSION IN WG 1681



CHAPTER 7
DISCUSSION

I have described two novel mutations involved in interallelic complementation at the MUT locus in fibroblasts from a single patient with MMA. The possible nature of the novel mut mutations in MMA, and their relevance to interallelic complementation have been examined.

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 which does not respond to OH-Cbl in medium. Sequencing of the MCM cDNA and genomic DNA from WG 1681 identified H532R and V671I as two homozygous variations, with respect to the consensus MCM cDNA sequence. These substitutions have been previously identified as polymorphisms in mut cell lines from three unrelated African-American patients (1,135). Although WG 1681 is a cell line obtained from an African-American male infant, we cannot presume that H532R and V671I are population-associated polymorphisms due to the small sample size. However, the H532R and V671I polymorphisms have been found in three other mut cell lines, WG 1511, WG 1599, and Yale 515 (personal communication by Wayne Fenton), with unknown ethnicity. The expression of my H532R construct in GM 1673 cells significantly increased [^{14}C]propionate incorporation. This indicates that the H532R substitution identified in WG 1681 is a normal polymorphism as identified previously. Although I was unable to construct a clone carrying the V671I substitution, previous expression studies by Crane (1) indicate that it is also a normal polymorphism. In addition, G703R and G623R were identified as two novel 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.

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. It should be noted that the mother in this study is common to WG 1681 and his half-siblings. Analysis using ASO identified the mother as the carrier of the G703R change; the G703R change was observed as well in a clinically normal sister and half brother of the proband. Haplotyping results summarized in figure 17B show that these (half-) siblings of WG 1681 also inherited at the H532R and V671I polymorphisms. This indicated that the G703R change, inherited from the common mother, segregated in cis with both polymorphisms. This observation was further supported by the haplotype of the clinically normal half-sister. She did not inherit the G703R change or the two polymorphisms from her mother.

Consequently, the ASO study confirmed the hypothesis that none of the (half-) siblings would carry the G623R change. Surprisingly, this amino acid substitution was also not identified in the father. At least two possible reasons for this outcome were suggested: 1) the G623R change was a *de novo* mutation, possibly arising in the paternal gonad; or 2) this was a case of non-paternity, eventhough the father carried both H532R and V671I polymorphisms. The latter hypothesis was tested by analyzing polymorphic fragments generated from PstI/PvuII restriction digests of the PCR amplified 3' region from the NEFH gene. Figure 18B shows that the father (1) and mother (4) have a 1,1 and 2,2 genotype, respectively, while the proband, WG 1681 (2), has a genotype of 2,2. This suggests that the blood sample obtained from individual (1) was probably not from the biological father. This would also explain why he did not carry the G623R change. However, the proband's sister (3) is heterozygous for the polymorphic alleles.

The ASO results supported the hypothesis that the G703R and G623R changes are inherited in trans, ie. each change is encoded separately within either subunit of the MCM homodimer of WG 1681. In order to demonstrate that these changes were in fact pathogenic mutations, expression studies were performed. Transfection of constructs with G703R or G623R singly, or in combination with both

polymorphisms, resulted in reduced incorporation of [^{14}C]propionate to 8.8-17% of that observed with transfection of 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. (1) 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 medium. These observations indicate that the novel G623R and G703R are mut^0 mutations.

Due to the undetectable enzyme activity in cell extracts, K_M studies of MCM from mut^0 lines have been difficult to perform. The mut^0 mutations have been proposed to involve defects at the catalytic or subunit dimerization sites of MCM (2). However, this does not eliminate the possibility that cofactor binding sites may also be affected by mut^0 mutations (128), perhaps to a greater extent than by the mut$^-$ mutations. Another possibility is that mut^0 mutations may affect protein folding or stability. This may be true for those mut^0 cell lines which are either CRM$^-$, have very low level of detectable CRM, or which have low levels of protein. The native three-dimensional structure of a polypeptide may also be defined by post-translational processing, attachment of cofactors and ligands, and intramolecular interactions (143). Therefore, amino acid alterations affecting critical sites or subunit organization may result in what has been defined as a mut^0 phenotype: the undetectabe enzyme activity in cell extracts, even in the presence of excess AdoCbl cofactor, and the lack of stimulation of propionate uptake in culture, in the presence of OH-Cbl.

Due to the unknown tertiary or quaternary structures of the MCM apoenzyme, one can only speculate as to the nature of the G623R and G703R mutations. The glycines at position 703 and 623 are conserved in human, and murine MCM sequences, and in *P.shermanii* MUTB sequences, suggesting that these sites may be consequential to MCM structure and function (fig 21). Prediction of secondary structure may provide possible explanations for the importance of these conserved sites. Based on the predictive model for secondary structure of proteins by Chou and Fasman (144), I analyzed the 623 and 703 amino acid regions of human MCM for

FIG. 21 HOMOLOGY OF G623 AND G703 SITES.

MCM SEQUENCES

	HOMOLOGY AT G623R SITE	HOMOLOGY AT G703R SITE
HUMAN	VAKM**G**QDGHD	VMCG**G**VIPPQ
MOUSE	VAKM**G**KDGHD	VMCG**G**VIPPQ
S.cinnamonensis	VAKM**G**KDGHD	IVVG**G**VIPPQ
S.erythraea	VAKM**G**QDGHD	ITVG**G**VIPPA
P.shermanii	LAKM**G**QDGHD	ITVG**G**VIPEQ
E.coli	IAKM**G**QDGHD	VVAG**G**VIPPQ

putative α-helical and ß-sheet formation. Glycine and proline are considered to be strong α-helix blockers. The glycine residue at position 623 may possibly be involved in the termination of an α-helix, which was predicted in this region. The substitution of a large, positively charged arginine at this position may permit the extension of the adjacent α-helix towards the C-terminus by three extra residues. If this predicted secondary change is correct, then possibly the association with other α-helices in the protein subunit may be hindered, resulting in improper folding and consequently in altered subunit structure. Alternatively, the extra helical rotation may itself disturb a defined catalytic, binding or dimerization determinant due to the large hydrophilic residue in place of a small uncharged amino acid. In contrast, the glycine at position 703 may be involved in ß-sheet formation. Charged residues rarely occur at the C-terminal of ß-strands (144), therefore, the presence of a positively-charged arginine may disrupt ß-strand interaction and thereby disrupt ß-sheet formation. Consequently, proper protein folding or important determinants may be altered. The decreased propionate incorporation in cells transfected by clones carrying the G623R and G703R mutations suggest that these mutations each result in defective subunits. Thus, the observed decreased uptake upon co-transfection of both mutations is consistent with the assumption that the association of the G623R and G703R subunits result in defective MCM apoenzyme. Although the precise mechanism for enzyme inactivation by these glycine to arginine substitutions awaits further knowledge of the tertiary structure of the MCM polypeptide, the functional effects of these amino acid changes on dimerization, cofactor binding and catalytic function can be tested by biochemical techniques using cells transfected with the expression constructs described in this thesis.

The most interesting aspect of the G623R and G703R mutations of WG 1681 is the correlation to the interallelic complementation observed at the somatic cell level. Table 6 shows the complementation pattern of those cell lines whose mutations have been identified. Interallelic (intragenic or intracistronic) complementation has been described most often in proteins with multiple subunits (145). This includes the heterotetrameric propionyl CoA carboxylase (PCC) (146), the homodimeric alkaline

phosphatase (147), the homotetrameric ß-galactosidase (148), the homotetrameric argininosuccinate lyase (149), the homohexameric glutamate dehydrogenase (150), and the heterodimeric tryptophan synthetase (145).

Interallelic complementation between mutations of the homodimeric MCM apoenzyme was first described by Raff et al. (2). As discussed in section 4.8.1, the mut^0 cell line WG 1130 has been observed to complement five of thirteen mut^0 lines and six of eight mut lines. Interestingly, Raff et al. (2) reported that complementation ability between mut cell lines and WG 1130 did not depend on mut or mut^0 phenotypes. Therefore, the observation that the mut^0 cell line WG 1681 complemented the mut^0 cell line WG 1130 was not of monumental surprise. Table 6 indicates that as of yet, WG 1681 has demonstrated complementation with WG 1130 and 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, co-transfection studies were performed. Results shown in figure 20A indicate that both G623R and G703R are separately capable of complementing the R93H mutation. A significant increase in propionate uptake was observed after co-transfection of different alleles. Co-transfection of each construct carrying the G623R or G703R mutation into GM 1673 (fig. 19B) indicated that the novel mutations of WG 1681 do not complement each other, as predicted. The R93H construct transfected into WG 1681 fibroblasts confirmed that the WG 1130 construct could complement the WG 1681 cell line.

Thus, interallelic 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 6).

Why do the G623R and G703R mut^0 mutations complement the R93H mut^0 mutation? It is instructive to examine theoretical models for the underlying

interallelic complementation. The first plausible model is referred to as homologous correction (151). It is assumed that the defective region in one monomer of a multimeric enzyme can be corrected with the normal homologous region of another monomer. In this case, active sites, cofactor binding sites, and subunit dimerization sites of multimeric proteins are the most likely regions involved in interallelic complementation (145). A variation of this model considers the case where an important site is the result of the association of half-sites from monomers (151). A second plausible hypothesis infers conformation-correction (152). Presumably, mutations not directly associated with detrimental sites may be involved in protein folding and stabilization. Therefore, subunit interaction may lead to important conformational changes that may be vital for function (151,153). Altered subunits, when formed as hybrids, may result in the proper conformation or stability of the protein (152). This was postulated by Coddington et al. (150), when mutants from *Neurospora crassa* demonstrated a "correction of conformation" when hybrid protein was formed. The third and generally accepted model postulates that interallelic complementation is a consequent of hybrid protein formation (152,153). The normal homomultimeric enzyme is the result of the association of two or more identical polypeptide chains (153). Therefore, restoration of function of the protein must involve the interaction of the differently altered polypeptide chains as a hybrid or heteromeric protein (152). This was observed in an experiment with *E.coli* mutants of alkaline phosphatase in which one subunit from each of various mutants was ^{35}S-labelled (154). Restoration of functional activity by different mutant subunits was observed in heterodimeric association.

As stated in section 4.8, mut^0 mutations may result in either unstable or no protein synthesis, unstable mRNA, or post-translational defects. If WG 1681 and WG 1130 present mut^0 phenotypes due to different mutational effects, as listed, then the formation of G623R + R93H and G703R + R93H heterodimers may result in the molecular stabilization of each defect (155). Protein and mRNA levels have not yet been studied in WG 1681. The R93H mutation of WG 1130 is near the

NH_2-terminal of the primary MCM sequence. Predictive analysis (144) of secondary structure does not indicate any specific conformation. Pulse-labelling studies with [^{35}S]methionine demonstrated no difference in MCM from WG 1130 when compared to other mut$^-$ cell lines (2). However, Western blot analysis with MCM-specific antibody has demonstrated a band intensity of 10% relative to the mut$^-$ cell line WG 1599 (normal levels of CRM and protein) (119). One can guess that this mutation may affect protein stability with respect to protein turnover, or with respect to subunit dimerization. The G623R and G703R mutations, as discussed in the previous pages, may affect protein stability, or important active, binding or dimerization sites. It is possible that the G623R monomer may stabilize the R93H monomer after dimerization, and the R93H monomer may contribute its normal determinant for G623R in a heterodimer. This hypothesis may also hold for the G703R and R93H heterodimers.

Alternatively, complementation between G623R, G703R and R93H may occur due to the alteration in the net surface charge of MCM. The substitution of a positively-charged arginine for glycine may change the net surface charge of each encoded subunit, thereby inactivating MCM in WG 1681. However, formation of heterodimers with R93H may restore the proper surface charge, thereby restoring enzyme activity. This form of interallelic complementation was observed between mutations of the glutamate dehydrogenase enzyme from *Neurospora crassa* (156). The am^{19} mutation in this homohexameric enzyme resulted in abnormal electrophoretic mobility due to a substitution of lysine for methionine. However, mobility was restored to normal upon formation of hybrids with an am^1 mutation which affected the enzyme active site. Enzyme activity was consequently activated, thus indicating that interallelic complementation between these mutants was due to a stabilization of net surface charge by hybrid formation (156).

The interallelic complementation pattern between WG 1130 and other mut$^-$ cell lines is quite puzzling. It has been proposed that the monomer from WG 1130 is capable of providing Cbl-binding determinants, in a heterodimer, to mut$^-$ monomers with defective Cbl-binding (2). This theory may be true for mut$^-$

TABLE 6 COMPLEMENTATION BETWEEN mut CELL LINES. + indicates complementation; - indicates no complementation; ND indicates complementation not done; htz= heterozygous mutation; hmz= homozygous mutation; htz, cDNA= heterozygosity obseved at cDNA level; hmz, cDNA= homozygosity observed at cDNA level.

TABLE 6

COMPLEMENTATION BETWEEN mut CELL LINES

	mut (o) WG 1130 R93H (hmz)	mut (-) WG 1511 H678R (hmz- cDNA)	mut (-) WG 1599 R369H (htz, cDNA)	mut (o) WG 1607 W105R, A377E (htz)	mut (-) WG 1610 G717V (hmz)	mut (-) WG 1611 G717V (htz)	mut (-) WG 1613 G717V (hmz)	mut (o) WG 1609 G630E	mut (-) WG 1618 UNKNOWN	mut (o) 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"

mutations such as G717V which complements R93H (1). This may also be conceivable for complementation observed between other mut° and mut⁻ cell lines such as WG 1681 and WG 1618, respectively. However, there exists a subset of mut° and mut⁻ alleles that complement neither WG 1130 nor each other. Although the mut° pattern of complementation has not been studied in much detail, it was suggested that only those mut° with decreased mRNA would not complement each other. However, this may be not the case. Ledley et al. (115) studied mRNA levels from certain mut° and mut⁻ cell lines. Of the 11 mut° cell lines studied, only WG 1606 (HAL), WG 1607 (MAS), WG 1608 (POU), WG 1609 (REG) and FB522 had normal levels of MCM mRNA. Of these lines, WG 1607 and WG 1608 do not complement WG 1130. Complementation for the other mut° lines have not been assessed. Thus, mut° lines with low mRNA showing complementation with WG 1130 have not been described yet.

Complementation between mut° and mut⁻ lines may relate to the position of the different mutations with respect to each other at the tertiary level, or it may relate to the specific amino acid substitution involved. Recently, studies on interallelic complementation between *E.coli* trp repressor mutations indicated that the specific amino acid alteration in the tryptophan-binding pocket of the dimeric protein determined whether or not two mutations complemented each other (155). A T44M mutation was able to complement G85R, G85W, and G85K mutations in the binding pocket, but was unable to complement a G85E mutation. The possibility that the presence of a positive charge or indole ring determined complementation between trp repressor mutations was suggested (155). Mut mutations identific in cell lines which exhibit interallelic complementation to WG 1130 do not seem o exhibit any specific pattern of charge or amino acid structure.

The relationship between amino acid position of a mutation, mut phenotype and interallelic complementation was analyzed. The positions of mut° and mut⁻ mutations at the primary amino acid level indicates putative clustering of mut⁻ mutations at the C-terminus and mut° mutations at the N-terminus. However, this observation is not absolute as certain mut mutations do not fall into the respective

clusters. Interestingly, a G94V change results in a mut phenotype (personal communication by Wayne Fenton) while the R93H mutation results in a mut^0 phenotype. The G623R, G703R, and G630E mut^0 mutations fall within the mut cluster at the C-terminus. Recently in our lab, a mut cell line, WG 1511 (H678R mutation), was shown to complement two other mut cell lines, WG 1611 and WG 1613 (both G717V mutation) (1). Therefore, neither mut^0/mut phenotypes nor interallelic complementation can be predicted by the relative primary amino acid position. Complementation is dependent not only on mutation position, but also on the specific amino acid substitution and the consequent effect on the tertiary structure.

Although little is known about MCM tertiary structure, the genetic study of interallelic complementation between mut mutations may offer some clues as to protein structure-function relationships. Interallelic complementation can be helpful in assigning functions to different portions of polypeptide chains, thereby defining specific domains (145). Using interallelic (intracistronic) complementation, Dobbelstein et al. (157) have recently localized a novel activity to the DNA-binding domain in SV40 T antigen. This novel activity was able to cooperate with retinoblastoma-suppressor protein and tumour suppressor p53. The ability of T antigen mutations and deletions to stimulate DNA synthesis in quiescent cells determined that at least three different functions of T antigen were involved in growth stimulation an cell transformation: Rb-binding, DNA-binding, and C-terminal p53 binding domains. A S189N mutation in the DNA-binding domain was able to complement a E107K mutation in the Rb-binding domain as well as to truncated peptides. The S189N mutation affected an unknown and novel activity which cooperated in growth stimulation (157). Therefore, if one examines the pattern of complementation between mut mutations, then possible structure-function relationships may be established. WG 1607 is a mut^0 cell line which does not complement WG 1130 or WG 1681. Its mutations were identified as compound heterozygous W105R and A378E (134). Thus, these mut^0 mutations are unable to complement the R93H, G623R, and G703R mutations. Possibly, the MCM region

between 93 and 358 is essential for protein stability or protein conformation. The W105R and A378E mutation may result in monomers less stable than the R93H monomers, resulting in failure to complement the G623R and G703R monomers.

Recently it has been shown in our lab that mut$^-$ cell lines carrying the G717V mutations (WG 1610, 1611, and 1613) are capable of complementing another mut$^-$ cell line WG 1618 (mutation unknown) (table 6). However, WG 1618 fails to complement WG 1130 in somatic cell experiments (table 1). Shapira et al. (158) have studied WG 1618 fibroblasts and have suggested that it is an atypical mut$^-$ line. Biochemical analysis has shown undetectable enzyme activity by *in vitro* assay of cellular extracts, however propionate incorporation in the presence of excess OH-Cbl is 2.5 fold higher as compared to other mut$^-$ cell lines. Therefore, the Cbl-binding site in WG 1618 may be indirectly affected, and could possibly be stabilized during heterodimer formation. Heterodimers formed between WG 1618 and those lines with the G717V mutation apparently restore proper Cbl-binding. This may suggest that the MCM region 717 may be a functional part of the Cbl-binding determinant. In contrast, no functional enzyme results from an interaction of R93H and WG 1618 (mutation unknown) dimers. If we presume that WG 1130 monomers are unstable, then WG 1618 monomers may not be able to restore proper Cbl-binding centres in a heterodimer. Therefore, both WG 1130 and WG 1618 monomers may remain unstable and fail to complement.

The G623R and G703R mutations of WG 1681 are novel mut^0 alleles which may be independently affecting α-helical or ß-sheet structures important in either subunit folding, dimerization, or MCM catalytic ability. 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. The observed complementation with WG 1130 (mut^0 R93H mutation) and WG 1618 (mut$^-$ mutation unknown) suggests that the heterodimers formed between mutant subunits are either able to compensate for an important site, or stabilize protein structure. The study of interallelic complementation promises to contribute a great

deal to our understanding of the structure and function of the MCM polypeptide, and ultimately to the diagnosis and treatment of MMA patients.

SUMMARY

This study has investigated mutations at the methylmalonyl CoA mutase locus from a mut^0 cell line, WG 1681. It has also investigated the contribution of the novel mutations to interallelic complementation. Two homozygous normal polymorphisms, H532R and V671I, were identified from WG 1681. In addition, two novel compound heterozygous mutations G623R and G703R, were identified. These novel mutations were demonstrated to be responsible for the mut^0 phenotype of the WG 1681 cell line. The G703R mutation was inherited in cis with both polymorphisms from the mother of the WG 1681 proband. Due to non-paternity, the origin of the G623R mutation is unknown. Both novel mutations were able to complement another mut^0 mutation, R93H, from WG 1130 at the molecular level, replicating the complementation pattern observed at the somatic cell level. These results indicate that heterogeneity at the MUT locus is more extensive than thought. Further studies of mut mutations demonstrating interallelic complementation will aid in the determination of the relationship between the structure of the MCM polypeptide and its function.

CLAIMS TO ORIGINALITY

1) This thesis describes the identification of two novel mutations from a mut^0 fibroblast cell line showing interallelic complementation in methylmalonic aciduria.

2) The novel mutations, G623R and G703R, are demonstrated to be presented in trans, and are responsible for the mut^0 phenotype.

3) The mut^0 cell line WG 1681 is capable of complementing the mut^0 cell line WG 1130, and this phenomenon was replicated at the molecular level. The novel G623R and G703R mutations of WG 1681 were independently capable of demonstrating interallelic complementation with the previously described R93H mutation of WG 1130.

PUBLICATIONS

ABSTRACTS AND ACKNOWLEDGEMENT

1) Qureshi A.A., Matiaszuk N.V., and Rosenblatt D.S.: Two mut fibroblasts lines showing intragenic complementation in Methylmalonic aciduria (MMA). Am J Hum Genet 51:A355 1992.

2) Qureshi A.A, Matiaszuk N.V., Rezvani I., and Rosenblatt D.S.: A family study of two novel mutations from a mut^0 fibroblast showing intragenic complementation in Methylmalonic aciduria (MMA). To be presented in poster session at the Annual Meeting of the Canadian Society for Clinical Investigation in Vancouver, B.C. Sept. 9-13, 1993. (CSCI Student Award winning presentation).

3) Qureshi A.A., Crane A.M., Rezvani I., Ledley F.D., and Rosenblatt D.S.: Identification and expression of two novel mutations from a mut^0 fibroblast showing intragenic complementation in Methylmalonic aciduria (MMA). Submitted to the American Society of Human Genetics.

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