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ARTICLE

Defects in human methionine synthase in cblG patients

Sumedha Gulati[1], Priscilla Baker[2], Yunan N. Li[3], Brian Fowler[4], Warren Kruger[3,*], Lawrence C. Brody[2,*] and Ruma Banerjee[1,*]

[1]Biochemistry Department, University of Nebraska, Lincoln, NE 68588-0664, USA, [2]Laboratory of Gene Transfer, National Center for Human Genome Research, NIH, Bethesda, MD 20892-4442, USA, [3]Division of Population Science, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA, 19046, USA and [4]Universitäts-Kinderklinik, Postfach, CH-4005 Basel, Switzerland

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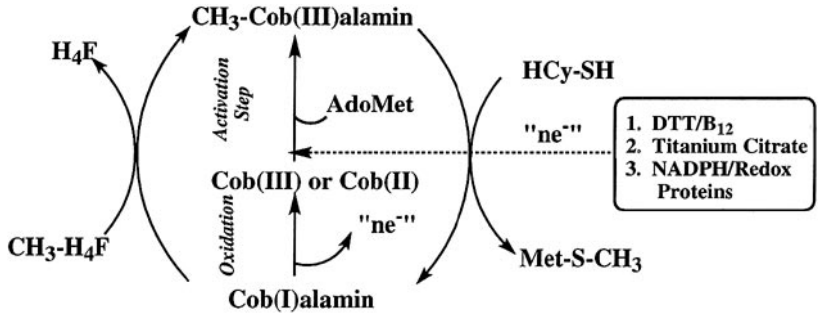
Inborn errors resulting in isolated functional methionine synthase deficiency fall into two complementation groups, cblG and cblE. Using biochemical approaches we demonstrate that one cblG patient has greatly reduced levels of methionine synthase while in another, the enzyme is specifically impaired in the reductive activation cycle. The biochemical data suggested that low levels of methionine synthase activity in the first patient may result from mutations in the catalytic domains of the enzyme, reduced transcription, or generation of unstable message or protein. Using Northern analysis, we demonstrate that the molecular basis for the biochemical phenotype in this patient is associated with greatly diminished steady-state levels of methionine synthase mRNA. The biochemical data on the second patient cell line implicated mutations specific to reductive activation, a function that is housed in the C-terminal AdoMet-binding domain and the intermediate B_{12}-binding domain, in the highly homologous bacterial enzyme. We have detected two mutations in a compound heterozygous state, one that results in conversion of a conserved proline (1173) to a leucine residue and the other a deletion of an isoleucine residue (881). The crystal structure of the C-terminal domain of the *Escherichia coli* MS predicts that the Pro to Leu mutation could disrupt activation since it is embedded in a sequence that makes direct contacts with the bound AdoMet. Deletion of isoleucine in the B_{12}-binding domain would result in shortening of a β-sheet. Our data provide the first evidence for mutations in the methionine synthase gene being culpable for the cblG phenotype. In addition, they suggest directly that mutations in methionine synthase can lead to elevated homocysteine, implicated both in neural tube defects and in cardiovascular diseases.

INTRODUCTION

Cobalamin is an organometallic cofactor required by only two known enzymes in mammals (1,2). Intracellular cobalamin metabolism is complex, compartmentalized, and requires the interplay of a variety of gene products. In the mitochondrion, the processing pathway leads to adenosylcobalamin (AdoCbl), whereas the cytoplasmic route leads to methylcobalamin (MeCbl). In both compartments, the component steps have been characterized largely at the genetic level by complementation analyses (3). In cblC, D and F groups both AdoCbl and MeCbl are defective. However, in cblE and cblG, MeCbl is specifically lowered, and this results from an apparent functional deficiency of methionine synthase (4,5). Both clinically and biochemically, cblG and E patients have similar phenotypes, displaying megaloblastic anemia, homocystinuria, and hypomethioninemia, together indicative of a block in the biosynthetic pathway unique to MeCbl formation. Based on biochemical studies, it has been postulated that cblG patients may have mutations in MS whereas the cblE patients have defects in one of the two redox proteins that activate MS (4). However, direct evidence from mutational analysis of the respective genes has been lacking.

The cblG phenotype is inherited as an autosomal recessive disorder (5). We have employed a combination of biochemical and molecular approaches to characterize two cblG cell lines. The first, cblG 79/96, is from a patient with severe neurological dysfunction and homocystinuria but no megaloblastic anemia (6), while the second, cblG WG1892, is from a patient with mental retardation, macrocytic anemia, and homocystinuria. Both presented with their symptoms within the first four months of life.

*To whom correspondence should be addressed



Scheme 1. Postulated reaction mechanism of methionine synthase. The different reductants that are employed in the *in vitro* assay systems are indicated.

Heterogeneity in cblG patients is displayed at the biochemical level (7) and has been categorized broadly into two subgroups. In the first subclass, cblGs are indistinguishable from control cell lines in the standard MS activity assays. In the second cblG subclass, the catalytic activity of MS is markedly reduced (Gulati, Rosenblatt and Banerjee, manuscript in preparation). We have measured MS activity in fibroblast cell lines using a variety of reductants and AdoMet, required for reductive methylation of oxidized and inactive enzyme (Scheme 1). When artificial reductants (such as titanium citrate or DTT) employed in the standard assay are replaced by the physiological reducing agent, NADPH, activity of MS is ~50% in cblG WG1892 compared with that in a control cell line. This information led us to suspect that cblG WG1892, which is impaired in reductive activation but not in catalysis, may have mutations in the activation domain located in the carboxy-terminal half of the protein. We have recently cloned, sequenced and mapped the human cDNA encoding MS (8). Using SSCP analysis and sequencing, we have detected a single base pair change resulting in a Pro1173 to Leu mutation, and a 3 bp deletion resulting in loss of Ile881 in the activation domain of MS of patient WG1892. In addition, we demonstrate that the low catalytic activity observed in the cblG 79/96 patient is due to greatly reduced steady-state levels of MS mRNA. Our data provide evidence of the MS gene being directly involved in the pathogenesis of the cblG phenotype.

Homocystinuria is a common clinical characteristic presented by cblG patients. Severe homocystinuria has been previously shown to result from mutations in both cystathionine β-synthase and methylenetetrahydrofolate reductase, two enzymes involved either directly or peripherally, in homocysteine metabolism (9,10). Our study presents the first evidence that MS, an enzyme central to this metabolic circuit, is an additional genetic target for defects leading to homocystinuria.

RESULTS

Biochemical characterization of cblG cell lines

Two cblG cell lines, WG1892 and 79/96, have been characterized biochemically. MS in the cblG WG1892 cell line is competent in catalysis showing an almost two-fold higher specific activity versus a control fibroblast cell line (Table 1). However, an impairment in the reductive activation cycle is unmasked when the physiological NADPH-driven reducing system is employed to activate MS, with approximately 50% of the activity seen in the patient versus a control cell line. In contrast to cblG WG1892, cblG 79/96 exhibits less than 1% of the activity seen with control cell lines under all conditions employed, including high concentrations of reducing agents (data not shown).

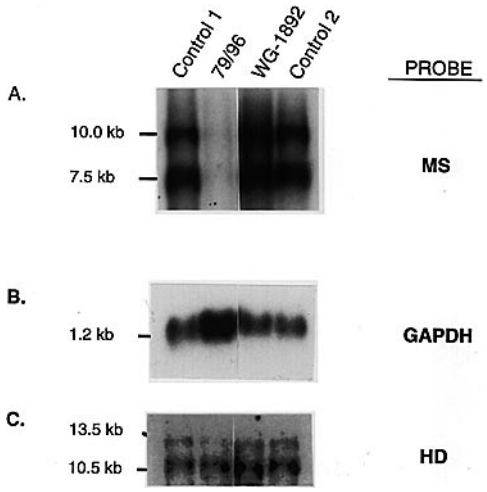


Figure 1. Northern blots of cblG and control cell lines probed with either MS, GAPDH or Huntington's disease cDNA fragment. Lanes 1 and 4 represent control fibroblast cell lines, the other two are from cblG patient cell lines: 79/96 (lane 2), WG1892 (lane 3). The autoradiogram with the MS probe was developed after a 3-week exposure to maximize signal in lane 2.

Northern analysis of methionine synthase mRNA in cblG cell lines

Steady-state levels of MS in cblG and control cell lines were compared by Northern analysis. As shown in Figure 1, MS mRNA levels in cblG WG1892 (lane 3) were comparable to, and perhaps even slightly higher than in the controls (lanes 1 and 4), but barely detectable in cblG 79/96 (lane 2) even after prolonged exposure. In contrast, the GAPDH and Huntington's disease gene mRNA levels were comparable in all cell lines, ruling out lack of mRNA integrity as an explanation for the extremely low level of MS-specific RNA in the cblG 79/96 patient cell line.

Table 1. Methionine synthase activity in control and patient cell lines

Cell line	Specific activity*		Specific activity	% Holo-enzyme
	TiC/-B_{12}	TiC/+B_{12}	NADPH/-B_{12}	
Control	157 ± 6	224 ± 1	35 ± 2	70
cblG 1892	369 ± 29	446 ± 31	17 ± 2	83

*Specific activity is expressed as pmol methionine/min/mg protein at 37°C. Assays were performed in duplicate.

Table 2. Description of PCR primers employed in this study

Primer	Sequence
P1-S*	5′-ATGAGGATGATGGTGACGACTACAGC-3′
P2-A	5′-AAATCTTCCCCACAGCAAAATA-3′
P3-S	5′-ACATGTCACCCGCGCTCCAAGACCTGTC-3′
P4-A	5′-TCGACAGGGCCATTTCTCCACTCATC-3′
P5-S	5′-TCCGAGGAATGGAAGCCATTCGAGAAGC-3′
P6-A	5′-AGTTAGTCTGTATCATATCCCAAAATGG-3′
P7-S	5′-CCGAGAACTGTGGGCCTACTGTG-3′
P8-A	5′-GGTCGGGCTGGCTGGGGTA-3′
P9-S	5′-GGAGGAGCAACCACTTCAAAAAC-3′
P10-A	5′-AGCTTCTGCAGGTCATAGTCTTCAA-3′
P11-S	5′-AGCAACCACTTCAAAAACCCACAC-3′
P12-A	5′-CCACCACATTCTTGGACGCG-3′
P13-A	5′-CGCGTCCAGGACATGGACA-3′

*S and A refer to sense and antisense respectively.

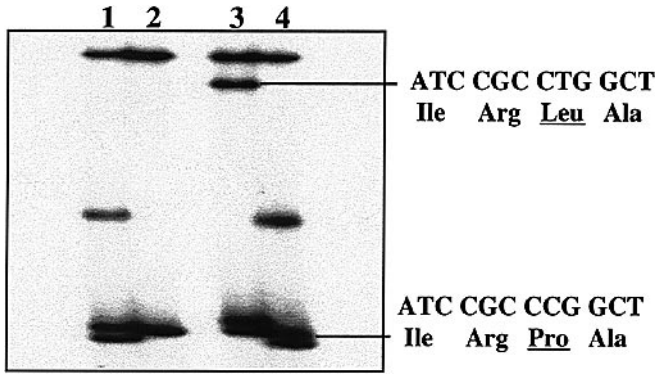


Figure 2. SSCP analysis of control and cblG cell lines. Lanes 1 and 2 represent control cell lines, lanes 3 (cblG WG1892) and 4 (cblG WG1765) represent patient cell lines.

SSCP and nucleotide sequencing-based detection of mutations and polymorphisms in the methionine synthase cDNA

We have employed two complementary methods to detect mutations in cblG WG1892. In the first approach, SSCP analysis was used to detect single base changes in the MS cDNA. Seventeen overlapping primer pairs spanning the MS ORF were employed covering 100% of the coding sequence. In the second approach, MS cDNA from cblG WG1892 was amplified in two fragments spanning the 5′- and 3′-halves respectively by RT–PCR (see Table 2 and Materials and Methods for primer descriptions). The resulting products were subcloned, and the insert containing the 3′-half of the gene was sequenced.

Several variant conformational polymorphisms were observed in one of the amplicons shown in Figure 2. One of the control cell lines (lane 2) is homozygous, whereas the other three [representing one control (lane 1) and two cblG patients, cblG WG1892 (lane 3) and cblG WG1765 (lane 4)] are heterozygous. Anomalously migrating bands were excised from the gel and sequenced. Two of the mutations are CpG changes, consistent with methylation-derived transitions. The heterozygosity in the control and one of the cblG cell lines (WG1765), which exhibit identical banding patterns, is due to two silent mutations: 3862C to T and 3778C to A (nucleotide numbering is based on the DNA sequence in reference 8 that contains 286 bp upstream of the start codon). Both these changes occur in the wobble position of the respective codons. The anomalously migrating pair of bands in cblG WG1892 is due to a 3804C to T transition, which results in the codon for Pro1173 being changed to Leu. The complete cDNA has been analyzed by the SSCP method, and only the Pro→Leu mutation described here has been detected in this patient. A third likely polymorphism was detected by SSCP analysis at the 5′ end of the cDNA (data not shown). This resulted from a G→A transition at nucleotide 468, and resulted in Arg61 changing to Lys.

The 3804C to T transition destroyed an *Msp*I restriction site and permitted convenient confirmation of the Pro1173 to Leu mutation by RT–PCR. The uncut PCR product (Fig. 3, lanes 1–3) is 359 bp long. In control DNA, this fragment has two *Msp*I sites which results in cleavage into three bands (183, 124, and 52 bp in length) which are observed (lanes 4–5) following restriction digestion. In the patient, who is heterozygous for the mutation, two different sets of digestion products are expected. The normal allele produces the same three fragments as the controls, whereas the mutant allele, missing one of the *Msp*I sites, generates two fragments that are 307 and 52 bp long. Hence, four bands are observed upon digestion (lane 6). In the last lane, one of the bands from the SSCP gel (Fig. 3, lane 3), carrying the C to T mutation in a homozygous state, was excised, amplified and subjected to resriction digestion. Only two bands were observed, as predicted.

A similar approach was employed to examine the prevalence of this mutation in the genomic DNA from 160 control DNA samples. Since the PCR product generated by the previously described primer pair spans an intron (Baker and Brody, unpublished data), a second set of primers, P7-S and P8-A (Table 2), were constructed to amplify a region of 183 bases spanning the mutation, and avoiding the intron. The 3804C to T transition mutation was not detected in any of the controls.

Eight clones containing the 3′ half of the MS gene (between nucleotides 2113 and 4086) from cblG WG1892 were analyzed. An 800 bp PCR product spanning the 3804C to T transition mutation was amplified from each of these clones and subjected to MspI

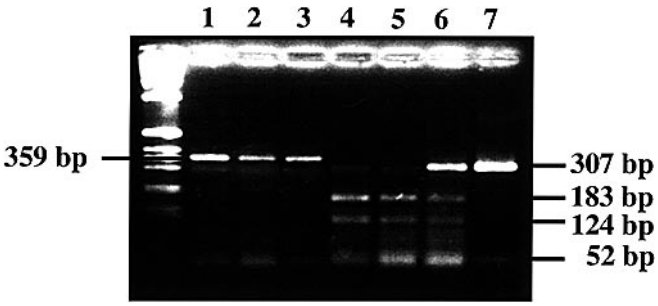


Figure 3. Confirmation of C to T mutation in cblG WG1892 by restriction digestion with *Msp*I. PCR products were amplified as described in Materials and Methods and separated on a 4% agarose gel. The lane at the far left has molecular weight standards. Lanes 1–3 have uncut DNA from controls (lanes 1 and 2) and patient (lane 3) cell lines. *Msp*I digested DNA was separated in lanes 4–7, with DNA from controls (lanes 4, 5) and patient (lane 6). Lane 7 has homozygous DNA from the SSCP gel (Fig. 2, lane 3, band 2 from top) which was excised, reamplified and digested with *Msp*I.

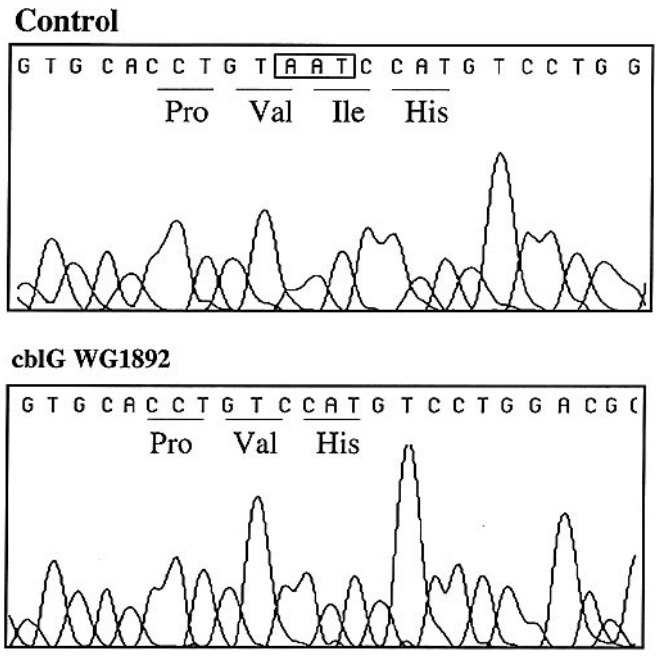


Figure 4. Nucleotide sequence analysis showing the 3 bp deletion (of AAT) in cblG WG1892.

digestion. Of these, three did not harbor the 3804C to T transition mutation. DNA from these three clones were subjected to double stranded nucleotide sequencing. A 3 bp deletion (Δ2926A–2928T) was detected (Fig. 4). This mutation resulted in an in-frame deletion of Ile881 from the human sequence. In addition, this experiment confirmed that the ΔIle881 and Pro1173→Leu mutations are on different chromosomes. The Ile deletion mutation was confirmed using a mutation-specific PCR assay (described in Materials and Methods). In addition, it was not detected in the 12 controls that were screened (data not shown).

Modelling of the Pro1173→Leu and ΔIle881 mutations in the activation domains of MS

Pro1173 (in the human sequence) is conserved in the human, *C.elegans* and *E.coli* methionine synthases (8). Recently, the crystal structure of the AdoMet binding domain of the *E.coli* MS has been determined (11). Two residues, arginine and alanine, that immediately flank the mutated proline make direct contacts to AdoMet. The β carbon of Pro1135 (corresponding to Pro1173 in the human sequence) is in Van der Waal's contact with C2 of the adenine ring of AdoMet (Fig. 5A). Replacement of a rigid proline by a flexible leucine in this position at the active site is expected to be detrimental.

Ile881 in the human sequence is replaced by a valine in both the *E.coli* and *C.elegans* sequences. It lies within a region of the protein where the primary sequence is not well conserved across organisms (8). The crystal structure of the cobalamin binding domain of the *E.coli* MS (12) reveals that the homologous Val855 lies within a β-sheet (IIβ5), which contributes to the protein wall surrounding the nucleotide tail, dimethylbenzimidazole (Fig. 5B). Truncation of the β-sheet resulting from deletion of the corresponding Val855 could lead to structural perturbations in this region. It is not obvious from the structure why such an alteration would specifically impair reductive activation. Alternatively, it is possible that this deletion leads to inactive enzyme, and that the biochemical phenotype of cblG WG1892 is due to the Pro1173 to Leu mutation.

DISCUSSION

A number of lines of evidence have pointed to impairments in MS as the basic defect in cblG patients. MS catalyzes a transmethylation reaction in which successive methyl group transfers from CH_3-H_4folate to cob(I)alamin to homocysteine lead to the products, H_4folate and methionine (Scheme 1). Due to the superreactivity of the intermediate, cob(I)alamin, oxidation competes with remethylation, and over time, the enzyme accrues in an oxidized and inactive state. For reentry into the catalytic cycle, a source of reducing equivalents and AdoMet, as a methyl group donor, are required. Under *in vitro* assay conditions, the requirement for reducing equivalents is met by DTT/OHCbl or titanium citrate. The physiological reducing system can utilize NADPH as the ultimate source of electrons, and with the participation of two redox proteins, can activate MS (13). This activity can be exploited under *in vitro* assay conditions using cell extracts, where MS and the two redox-active proteins are all present (13).

We have employed a combination of these assay methods to localize defects that are specific to the reductive activation cycle. MS in one of the cell lines, cblG WG1892, has such a biochemical phenotype, being impaired in reductive activation but robust in catalysis (Table 1). These data suggested that, at the molecular level, mutations might be present in the 3′-half of the gene encoding the B_{12}- and AdoMet-binding domains involved in reductive activation. CblG 79/96 on the other hand displays very low levels of MS activity. In this cell line, one or more mutations affecting catalysis, transcription, or stability of either the message or the protein could explain the reduced enzyme activity.

Northern analysis revealed a greatly diminished level of MS mRNA in cblG 79/96 accounting for its biochemical phenotype. In contrast, the level of MS mRNA in cblG WG1892 is comparable to that of control cell lines (Fig. 1). In this patient, two mutations were found in *trans* in the activation domains of the

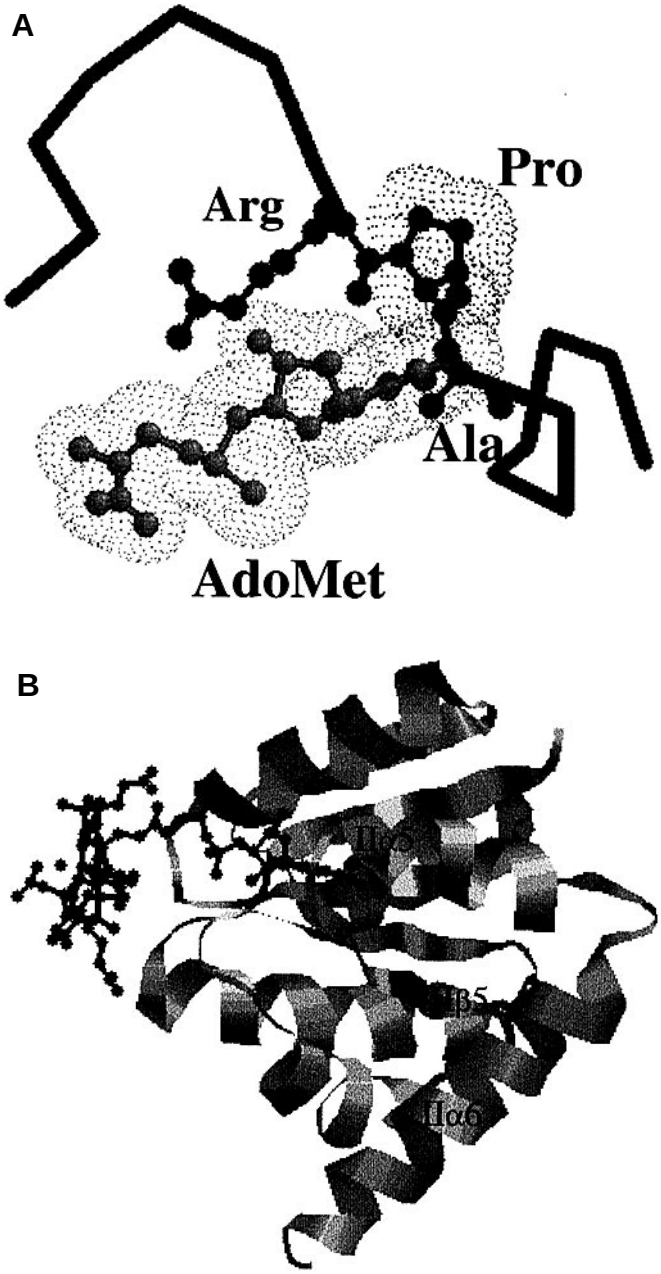


Figure 5. Location of the analogous mutated residues in the three dimensional structures of *E.coli* MS domains. (**A**) Active site structure of the AdoMet-binding domain of *E.coli* MS showing Van der Waal's contacts between Pro1135 and the bound AdoMet. The flanking and conserved arginine and alanine residues are also indicated. (**B**) Cobalamin-binding domain of MS showing interactions between the protein and the lower face of the cofactor. Location of Val855, corresponding to the deleted Ile881 in the human sequence, is indicated in black in the middle of the β sheet, IIβ5. The α helices downstream of the cofactor (IIα5 and IIα6) are labeled, and bound MeCbl is indicated by the ball and stick display.

enzyme, consistent with the biochemical data. A single base transition mutation resulting in a Pro1173 to Leu change in the AdoMet-binding domain was detected by SSCP analysis. The second mutation, a 3 bp deletion resulting in loss of Ile881 in the B_{12}-binding domain was detected by nucleotide sequence analysis. The primers (P9-S and P10-A) spanning this region resulted in a 331 nucleotide-long amplicon. It is likely that the relatively long length of the PCR product precluded detection of this mutation by SSCP analysis.

Based on the high degree of homology between the human and *E.coli* methionine synthases [55% identical (8)], we predict that the modular organization of the two enzymes will be similar. The *E.coli* enzyme has been shown to be a three-domain protein, with the substrates, cobalamin, and AdoMet binding to the N-terminal, middle, and C-terminal domains respectively (14,15). The crystal structures of the cobalamin (12) and AdoMet (11) binding domains of the bacterial protein have been determined. While the proline residue is conserved in the *E.coli, C.elegans* and human MS sequences, Ile881 in the human sequence is substituted by valines in the other two.

The homologous proline residue in the bacterial MS is in Van der Waal's contact with the bound AdoMet (11). Its role, along with two proximal prolines (two and five residues downstream), is postulated to be to stiffen the loop thereby fixing the orientation of the backbone (11). The flanking and conserved arginine and alanine residues make direct docking interactions that have been identified as being important for AdoMet binding. The three dimensional structure of this domain provides a structural rationale for the detrimental effect of this mutation and provocative evidence for the involvement of the AdoMet-binding domain in electron transfer from the proximal redox partner to MS.

Isoleucine at position 881 in the human sequence corresponds to Val855 in the *E.coli* sequence and resides in the cobalamin-binding domain. The three dimensional structure of this domain (12) reveals that Val855 is in the middle of a six residue long β-sheet (IIβ5), which together with the adjoining α helix (IIα5) forms one of the walls around the buried dimethylbenzimidazole moiety of the bound cobalamin (Fig. 5B). Helix, IIα5, then leads into helix IIα6, which connects to the AdoMet binding domain. Deletion of the corresponding Val855 would lead to contraction of the β-sheet potentially creating structural deformations in the protein wall cupping the cofactor tail. This could either impair cofactor binding or affect domain rearrangement that is expected to occur as the protein switches from the catalytic to the activation mode. It is interesting that a mutation in this region that is distal to the corrin ring is associated with a biochemical phenotype in which reductive activation is specifically impaired. It is possible that the ΔIle881 mutation results in inactive enzyme, and that the biochemical phenotype of the cblG WG1892 patient cell line stems entirely from the Pro1173 to Leu mutation. Characterization of the recombinant mutant human enzymes in the future will permit direct evaluation of this issue.

Mutations in methylenetetrahydrofolate reductase and cystathionine β-synthase, both involved in homocysteine metabolism, have been described (9,10). These mutations result in homocystinuria in homozygous affected patients. Similarly, dysfunction of MS has been implicated in homocystinuria inherited as an autosomal recessive inborn error of metabolism. Our results now furnish direct evidence for the complicity of MS in homocystinuria.

MATERIALS AND METHODS

Cell culture

Fibroblast cell line cblG WG1892 was purchased from the Repository for Mutant Human Cells, Montreal Children's

Hospital. Control cell lines and cblG 79/76 were available in our laboratories. The control DNAs were a subset of those described previously (16). Cultures were maintained in Eagle's minimum essential medium plus nonesssential amino acids (Sigma), and supplemented with 10% (v/v) fetal bovine serum (Calbiochem). Cells were grown to confluency on ten 150 mm petri dishes, and harvested using a cell scraper. The cells were pelleted at 1200 r.p.m. for 15 min, washed 2 times with phosphate buffered saline and frozen at –80°C.

Methionine synthase assay

Approximately 10^8 cells were resuspended in 1 ml of 100 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose. The cell membranes were disrupted by sonication on ice using the miniprobe (power output setting of 4) and the following cycle: 20 s pulse, 1 min pause, repeated five times. The homogenate was centrifuged at 4°C, at 12 000 × *g* for 60 min. The resulting supernatant was aliquoted and stored at –80°C. The *in vitro* anaerobic assays were performed essentially as described previously (17) at 37°C for 75 min. When the physiological activation system was employed to measure MS activity, 1 mM concentration of NADPH was used.

Northern blots

RNA was isolated from approximately 10^6 cells from control and patient cell lines using RNAStat-60, following the procedure recommended by the vendor (Tel-Test 'B' Inc, Texas). The RNA was quantitated spectrophotometrically, and ~10 µg RNA/lane was loaded on a 1% formaldehyde agarose gel. The RNA was transferred from the gel to a Hybond N+ membrane (Amersham). A 2.2 kb *Bgl*I fragment from the coding region of the human MS was end-labeled with [^{32}P] γ-dCTP (3000 Ci/mol) and 3 ml (containing ~10^6 counts/ml) of Church and Gilbert hybridization solution and used to probe the northern blot. Hybridization was conducted overnight at 65°C, and the membrane was washed three times with 2× SSC/10% sodium dodecyl sulfate at the same temperature. For hybridization to control probes the membrane was later stripped by boiling in 0.1× SSC, 0.1% SDS for 10 min, and reprobed with human GAPDH cDNA and a 670 bp fragment from Huntington's disease gene cDNA.

SSCP analysis

RNA isolated as described above was employed as template for first strand cDNA synthesis. The reaction mixture containing 1 µg of RNA in DEPC-treated water (total volume 13 µl) and 1 µl of 1 µg/µl of oligo dt (from Invitrogen) was mixed and heated at 65°C for 10 min, followed by incubation on ice for 2 min. Then the following solutions were added: 2 µl of 10× synthesis buffer (Gibco BRL), 2 µl of 0.1 M DTT, and 1 µl of 10 mM dNTP (Amersham). The sample was mixed by vortexing, spun down and incubated at 42°C for 2 min before 1 µl of Reverse Transcriptase (BRL) was added. Incubation was continued at 42°C for 30 min, the sample was centrifuged and placed on ice. RNaseH (1 µl) was added and the mixture was incubated for 10 min at 42°C. The sample was cooled on ice, then stored at –20°C.

First strand material was amplified for SSCP. Seventeen primer pairs were employed to completely span the open reading frame. The sequences of these primers are available from the authors on request. Primers P1-S and P2-A generated an amplicon harboring the C3804→T mutation. Radiolabeled dCTP (α-dCTP, 3000 Ci/mol) was added to the PCR mixture. The following temperature cycling conditions were employed using the touch down program: 95°C for 2 min followed by 94°C for 30 s, annealing/extension at 67°C for 40 s, with the annealing temperature decreasing by 1.1°C/cycle for 12 cycles. The next 35 cycles used the following conditions: 94°C for 30 s, 54°C for 30 s, 72°C for 30 s.

PCR products were diluted ten-fold with loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue) prior to use. The heat denatured PCR products (95°C for 5 min followed by incubation on ice for 3 min) were separated on a 0.5× MDE (FMC) sequencing gel at ambient temperature. Bands of interest were excised from the sequencing gel and reamplified using the conditions described above. The PCR products were purified using the Wizard PCR purification system (Promega) and subjected to fluorescent nucleotide sequencing on an ABI 377 Automated Sequencer.

Mutation identification by nucleotide sequence analysis

RT–PCR was carried out using Advantage Klen Taq DNA Polymerase (Clontech Inc.) from first strand cDNAs of two controls, HS-68 and MRC-5, and two clbG cell lines, WG1892 and 79/96. The amplifications produced ~2 kb DNA fragments that spanned the 5′ half and 3′ halves of the gene respectively, and overlapped by 265 bp. The primer sets used for these two PCR reactions were P3-S/P4-A and P5-S/P6-A respectively. Since the enzymatic assay data suggested that mutations in cblG WG1892 are likely to be in the C-terminal reductive activation domains, clones containing the 3′ half of the gene were further studied. Semi-nested PCR was performed using 1 µl of 100-fold diluted PCR product containing the 3′ half of the gene by using 3NGSP1/P6-A [Table 2 and Table in reference (8) for primer description] and the amplified DNA fragments were cloned into the T/A cloning vector, pCR2.1 (Invitrogen Inc.). Eight clones were then used as templates for PCR amplification of a 800 bp fragment (using primers P15F/ P6-A, Table 2; ref. 8) containing the C3804→T transition mutation (Pro1173→Leu) previously detected by SSCP in a heterozygotic state. To distinguish the known allele from the unknown allele the 800 bp fragments were digested with *Msp*I and separated on a 2% agarose gel. Three clones that did not have the C3804→T mutation were completely sequenced on both strands using the automated sequencing facility at Fox Chase Cancer Center.

Confirmation of the Ile deletion mutation

To confirm Ile881 deletion in genomic DNA and to examine its prevalence in control samples, a mutation specific primer assay system was utilized. Two primers flanking the mutation site, P-11S and P12-S (Table 2) were synthesized. A third primer, specific for the mutant allele, P13-A, was also synthesized. This primer is designed to be complementary to the mutant sequence and will contain a mismatched 3′ terminus when annealed to wild type DNA. Genomic DNA was PCR amplified in a reaction containing all three primers. Reaction conditions were as previously described except that 5-fold less of the flanking mutation specific primer, P13-A was added. The outside primer pair amplifies a 95 bp fragment in all samples. If the mutant sequence is present an additional fragment of 79 bp is observed. PCR products were resolved on 10% acrylamide minigels in 0.5×

TBE buffer. The control cell lines employed in this study have been described previously (16).

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ABBREVIATIONS

AdoCbl: adenosylcobalamin; MeCbl: methylcobalamin; OHCbl: hydroxocobalamin; MS: methionine synthase; SSCP: single strand conformational polymorphism; AdoMet: S-adenosylmethionine; GAPDH: gylceraldehye 3′ phosphate dehydrogenase; DTT: dithiothreitol; DEPC: diethylpyrocarbonate.

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NOTE ADDED IN PROOF

The Ile881 deletion mutation in WG1892 has been found independently by Dr R.Rozen, Montreal Children's Hospital (personal communication; *Hum. Mol. Genet.* **5,** 1867–1874).