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ARTICLE

Human methionine synthase: cDNA cloning and identification of mutations in patients of the *cblG* complementation group of folate/cobalamin disorders

D. Leclerc, E. Campeau, P. Goyette, C. E. Adjalla, B. Christensen, M. Ross, P. Eydoux, D. S. Rosenblatt, R. Rozen and R. A. Gravel*

MRC Group in Medical Genetics, Departments of Pediatrics, Human Genetics, Biology, Pathology and Medicine, McGill University, Montreal, Quebec, H3Z 2Z3, Canada

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Methionine synthase catalyzes the remethylation of homocysteine to methionine in a methylcobalamin-dependent reaction. We used specific regions of homology within the methionine synthase sequences of several lower organisms to clone a human methionine synthase cDNA by a combination of RT–PCR and inverse PCR. The enzyme is 1265 amino acids in length and contains the seven residue structure-based sequence fingerprint identified for cobalamin-containing enzymes. The gene was localized to chromosome 1q43 by the FISH technique. We have identified one missense mutation and a 3 bp deletion in patients of the *cblG* complementation group of inherited homocysteine/folate disorders by SSCP and sequence analysis, as well as an amino acid substitution present in high frequency in the general population. We discuss the possibility that a mild deficiency of methionine synthase activity could be associated with mild hyperhomocysteinemia, a risk factor for cardiovascular disease and possibly neural tube defects.

INTRODUCTION

Methionine synthase (EC 2.1.1.13, 5-methyltetrahydrofolate-homocysteine methyltransferase) catalyzes the remethylation of homocysteine to methionine in a reaction in which methylcobalamin serves as an intermediate methyl carrier. This occurs by transfer of the methyl group of 5-methyltetrahydrofolate to the enzyme-bound cob(I)alamin to form methylcobalamin with subsequent transfer of the methyl group to homocysteine to form methionine. Over time, cob(I)alamin may become oxidized to cob(II)alamin rendering the enzyme inactive. Regeneration of the functional enzyme occurs through the methionine synthase-mediated methylation of the cob(II)alamin in which S-adenosylmethionine is utilized as methyl donor. In *Escherichia coli*, two flavodoxins have been implicated in the reductive activation of methionine synthase (1). A methionine synthase-linked reducing system has yet to be identified in mammalian cells.

Deficiency of methionine synthase activity results in hyperhomocysteinemia, homocystinuria and megaloblastic anemia without methylmalonic aciduria (2,3). Two classes of methionine synthase-associated genetic diseases have been proposed based on complementation experiments between patient fibroblast cell lines (4). One complementation group, *cblE*, has been postulated to be due to deficiency of the reducing system required for methionine synthase (5). Cells from patients in the *cblE* group fail to incorporate ^{14}C-methyltetrahydrofolate into methionine in whole cells but have significant methionine synthase activity in cell extracts in the presence of a potent reducing agent. The *cblG* group is thought to be due to defects of the methionine synthase apoenzyme. Mutant cells from this group show deficient methionine synthase activity in both whole cells and cell extracts (4,6). Moreover, some *cblG* patients show defective binding of cobalamin to methionine synthase in cells incubated with radiolabelled cyanocobalamin (7).

The gene encoding methionine synthase has been cloned from several lower organisms, but not from mammals (Fig. 1). The cobalamin-dependent methionine synthase of *E.coli* has been crystallized and the structure of its active site determined (8,9). We used specific regions of homology within the methionine synthase sequences, including a portion of the cobalamin binding site determined from the *E.coli* enzyme, to design degenerate oligonucleotides for RT–PCR-dependent cloning of human methionine synthase. We confirm the identification of the cDNA sequences for human methionine synthase by the high degree of homology to the enzymes in other species and the identification of mutations in patients from the *cblG* complementation group.

*To whom correspondence should be addressed at: McGill University-Montreal Children's Hospital Research Institute, 4060 Ste-Catherine St. W., Montreal, Quebec, H3Z 2Z3, Canada

Table 1. Oligonucleotides used for cDNA cloning, chromosome mapping and mutation detection

Oligonucleotides[a]	Sequence	Location[b]
D1729	5′-GAYGGNGCNATGGGNACNATGATHCA	100–125
D1730	5′-GCNACNGTNAARGGNGAYGTNCAYGAYAT	2332–2360
D1731	5′-RTTYTTNCCDATRTCRTGNACRTCNCCYTT	2370–2341
D1733	5′-RTGNAGRTAYTCNGCRAANGCYTCNGC	3426–3400
D1754	5′-ATRTGRTCNGGNGTNGTNCCRCARCANCCNCC	992–961
D1755	5′-GGNGGNTGYTGYGGNACNACNCCNGAYCAYAT	961–992
M1806A	5′-GTCTGTGTCATAGCCCAGAATGGG	3795–3772
M1806B	5′-TCAGTCTGTGTCATAGCCCAGAAT	3798–3775
305A	5′-GAACTAGAAGACAGAAATTCTCTA	(intronic)
407A	5′-TTCCGAGGTCAGGAATTTAAAGATCA	151–176
407B	5′-GTGTTCTTCGTTTAGCTTCTCCCG	150–127
407D	5′-CCCCAGCCAGCAAGTATTCCTTAT	268–245
1107A	5′-CTAGGTTGTATTTCCTTGAGGATC	3856–3833
1406D	5′-GGAGCTGGAAAAATGTTTCTACCTC	2170–2194
1406E	5′-ACAGGAGGGAAGAAAGTCATTCAG	1963–1986
1706A	5′-CCTTCAATTATATTGAGAGGTCGGG	2129–2105
1707A	5′-CAACCCGAAGGTCTGAAGAAAACC	28–51
1707B	5′-CCCGCGCTCCAAGACCTGTCG	7–27
1707C	5′-CGACAGGTCTTGGAGCGCGGG	27–7
1758	5′-GGAGTCATGACTCCTAAATCAATAACTC	2432–2405
1760	5′-GACGACTACAGCAGCATCATGGT	3355–3377
1766	5′-AAAAATCATTTCATCCAGGGAA	2526–2505
1772	5′-ATAGGCAAGAACATAGTTGGAGTAGT	2359–2384
1773	5′-TTTCATCTAACAGCTGGGAACACAC	2698–2674
1774	5′-TGCCTCTCAGACTTCATCGCTCCC	3241–3264
1780	5′-TGCAGCCTGGGGCACAGCAGC	3168–3148
1782	5′-ATGGATTGGCTGTCTGAACCTCAC	2824–2847
1796	5′-CATGGAAGAATATGAAGATATTAGAC	2727–2752
1803	5′-ACCATCATCCTCATAGGCCTTGCT	3354–3331
1806C	5′-CAGACCTGCGAAGGTTGCGGTAC	3482–3504
1806F	5′-GAAGTGGTTGCTCCTCCAATCAAC	2591–2568
1808	5′-GAGCAGCTTTCAGTATCTTATCACAT	2458–2433
1827	5′-ACAAGTTGTGTTCCTCCATTCCAGT	1657–1633
1828	5′-AGAGCGCTGTAATGTTGCAGGATCA	1125–1149
1907B	5′-TGTTTTTCAATGCCCTTCACAAGGG	2057–2033
1907C	5′-TAAAAAGTATGGAGCTGCTATGGTG	1464–1488
2606A	5′-GACCAGACAGTAACATATGTCCTTC	1078–1054
2606B	5′-ACATTACAGCGCTCTCCAATGTTAAC	1139–1114
2706A	5′-TGAGGTTGAGAAATGGCTTGGACC	3750–3773
2706B	5′-GCCACAGATATGTTCTTCCTCAATG	3749–3725
3107A	5′-TGTGGAGAGCACGTCTTCTCTGCC	–55 – –32

[a]Numbers with the prefix ‘D’ refer to oligonucleotides with degenerate bases shown as N (any base), H (A, C, or T), D (A, G, or T), Y (T or C), or R (A or G); those with the prefix ‘M’ refer to mouse sequences (see Fig. 3).
[b]From the first methionine codon, see Fig. 3.

RESULTS

Cloning human methionine synthase cDNA

An initial survey of the NCBI databases yielded several sequences corresponding to methionine synthase from different organisms. Comparison of these sequences generated four very conserved regions identified as Boxes 1–4 in Figure 1. Degenerate oligonucleotides were synthesized corresponding to these conserved sequences. These were used as primers for RT–PCR with human and mouse mRNA. These experiments yielded PCR products which were subcloned, sequenced and aligned as shown in Figure 2. In subsequent experiments, oligonucleotide primers were specified from the non-degenerate internal sequences of the subclones and additional PCR products encompassing the conserved boxes were obtained. In later experiments, additional sequences were obtained by inverse PCR (iPCR in Fig. 2) to obtain upstream or downstream sequences from those already determined. At the 3′ end, a mouse sequence was obtained from the dbEST database (Accession Number W33307). This sequence was used as the source of primers for additional PCR experiments. Throughout the experiments, the sequences of the PCR products were considered provisionally authentic if they were homologous to the methionine synthase sequences obtained from the databases. The sequences were taken as error free by comparison of the sequences of at least two, and usually three, independent PCR reactions. Sequences were linked into a common sequence if RT–PCRs bridging independently isolated sequences were successful. Through this approach, the complete coding sequence was determined through exclusive use of PCR reactions.

The coding sequence of human methionine synthase contains 3795 bp encoding a polypeptide of 1265 amino acids in length (Fig. 3), exceeding the length of published methionine synthases by 11–29 residues. The putative initiation codon is in a sequence of good context for the initiation of translation in eukaryotic cells [GACAACATGT, underlined nucleotides matching Kozak consensus, (10)]. The predicted Mr of methionine synthase is 141 000, comparing favorably with the published size of 151 000 based on SDS-polyacrylamide electrophoresis of the pig enzyme (11). It shares 58% identity with the *E.coli* and 65% identity with the *Caenorhabditis elegans* enzyme.

Chromosomal location

Using FISH, the gene encoding methionine synthase was mapped to chromosome band 1q43, close to the telomeric region of the long arm (Fig. 4). A total of 50 cells with at least one signal were observed. A signal was seen on one chromatid in 26 cells, on two chromatids in 14 cells, on three chromatids in seven cells, and on four chromatids in three cells. These results confirm the previous assignment of the gene to chromosome 1 by Mellman *et al.* (12), who used cobalamin binding as a marker for the enzyme in human-hamster hybrids.

Mutations in the *cblG* complementation group

Patients with deficiency of methionine synthase activity have been grouped into the *cblG* complementation group in cell fusion experiments (4). Fibroblast cultures from patients assigned to *cblG* were examined by RT–PCR based SSCP analysis. Three variants were identified by sequencing PCR fragments showing band shifts by SSCP (Fig. 5). In each case, the change was confirmed by an independent diagnostic test on genomic DNA or a separate preparation of cDNA from patient fibroblasts. One of the mutations, 2756A→G (D919G), was confirmed by a diagnostic test that monitored the presence of a *Hae*III site created by the mutation (Fig. 5A). Using this test, it was identified as a polymorphism since it was seen in eight of 52 control alleles (15%). In two other cases, candidate deleterious mutations were identified. One is a 3 bp deletion, bp 2640–2642, that results in the deletion of an isoleucine codon (ΔIle881). It was confirmed by heteroduplex analysis of cDNA generated by RT–PCR (Fig. 5B). The second is a point mutation, 2758C→G. It results in the amino acid substitution H920D. It was confirmed in genomic DNA by the loss of a *Sau*96I site (Fig. 5C). The latter two mutations were heterozygous in the patient cell lines. Their second mutation has not been identified. The candidate deleterious mutations were not seen in a panel of 68 or 52 control alleles, respectively.

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BOX 1:
                    ** ***  *
     Ec      (20) DGGMGTMIQ
     Ss      (20) DGAMGTNLQ
     Ml2      (5) DGAMGTQLQ
     Hi      (20) DGAMGTMIQ
     Ce      (22) DGAMGTMIQ
     Hs      (34) DGGMGTMIQ

BOX 2:
                    ************
     Ec    (752) ATVKGDVHDIGKN
     Ss    (729) ATVKGDVHDIGKN
     Ml2   (726) ATVKGDVHDIGKN
     Hi    (142) ATVKGDVHDIGKN
     Ce    (766) ATVKGDVHDIGKN
     Hs    (778) ATVKGDVHDIGKN

BOX 3:
                      ** **  *
     Ec   (1095) LAEAFAEYLH
     Ss   (1085) MAEALAEWTH
     Ml1    (56) LTEALAEYWH
     Hi    (490) LAEAMAEYLH
     Ce   (1084) LAEAYAEYLH
     Hs   (1133) LAEAFAEELH

BOX 4:
                    *****  * **
     Ec      (262) GGCCGTTPQHI
     Ss      (243) GGCCGTRPDHI
     Ml2     (226) GGCCGTTPDHI
     Ce      (264) GGCCGTTPDHI
     Hs      (321) GGCCGSTPDHI
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Figure 1. Four homologous regions among methionine synthases. Boxes 1 to 4 were used to design degenerate oligonucleotides for the initial cloning experiments. Ec: *Escherichia coli*, accession number J04975; Ss: *Synechocystis sp.*, accession number D64002; Ml1 and Ml2: *Mycobacterium leprae*, accession number U000175 (9); Hi: *Haemophilus influenzae*, accession number U32730; Ce: *Caenorhabditis elegans*, accession number Z46828; Hs: *Homo sapiens*, this work. Identical residues are indicated by a star above the alignment. Amino acid position for each protein is shown at left.

DISCUSSION

We conclude that the cDNA that we have identified corresponds to human methionine synthase on the basis of homology to known methionine synthases and by the identification of mutations in patients with a deficiency of enzyme activity. The most striking sequence conservation was found in four boxes of 9–13 amino acids. Box 2 has been proposed to correspond to part of the cobalamin binding domain (9). It contains 13 consecutive residues that are identical in all known methionine synthases. Three amino acids within box 2 and four others C-terminal to it

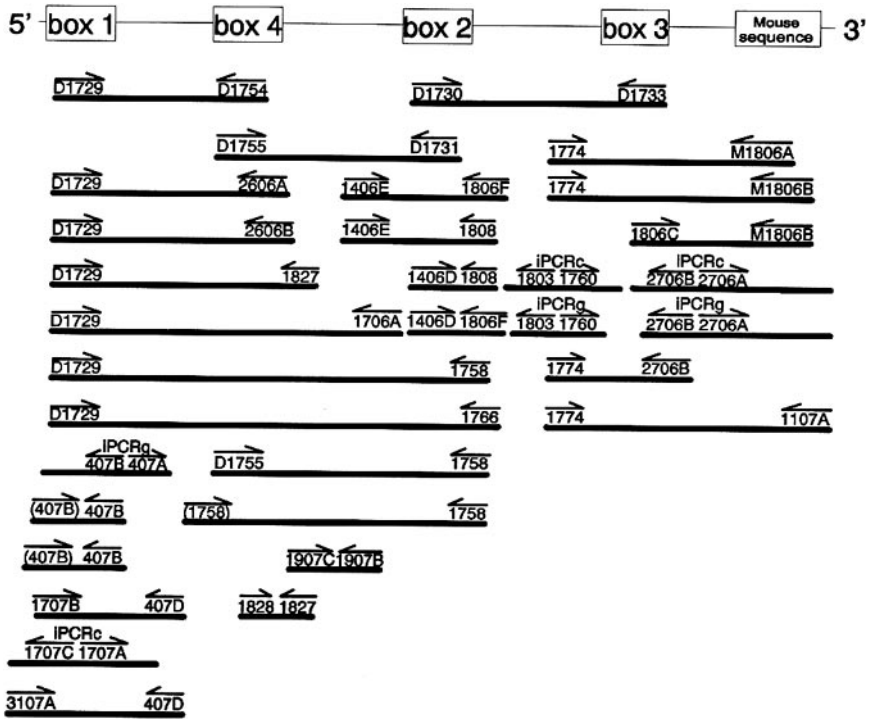

Figure 2. Overlapping PCR fragments generated to clone human methionine synthase. Oligonucleotides are described in Table 1. Primers in parenthesis designate mispriming outcomes that generated valid internal sequence. iPCRc: inverse PCR on cDNA, iPCRg: inverse PCR on genomic DNA.

correspond to residues proposed by Drennan *et al.* (9) as a structure-based sequence fingerprint for cobalamin binding. They appear to interact with the lower face of the corrin ring and dimethylbenzimidazole tail of cobalamin, determined from the crystal structure of the *E.coli* enzyme at 3 Å resolution (9). All seven residues are identical in the human sequence (Fig. 6).

A survey of the NCBI databases for homology to the human methionine synthase using BLASTP yielded the various methionine synthases listed in Figure 1, as well as the glutamate mutase (S41332, Q05488) and methylmalonyl-CoA mutase (P11653) (adenosyl-cobalamin dependent mutases) used to deduce the sequence fingerprint for cobalamin binding (9). Homology was also found with the cobalamin binding region of the corrinoid:coenzyme M methyltransferase of *Methanosarcina barkeri* (U36337), the 5-methyltetrahydrofolate corrinoid/iron sulfur protein methyltransferase of *Clostridium thermoaceticum* (L34780) and the B12-dependent 2-methyleneglutarate mutase of *Clostridium barkeri* (S43552, S43237). Further, homology was found with the N-terminal sequence of the recently identified putative methionine synthase of *Agrobacterium tumefaciens* (U48718; partial N-terminal sequence is given, up to region of box 4). Significantly, homology with the B12-binding site domain was also found in the Hg resistance protein of *Myxococcus xanthus* (Z21955). This protein has not been described as having a cobalamin prosthetic group.

The two mutations we have identified as candidates for causing *cblG* disease are located in the vicinity of the cobalamin binding domain by comparison with *E.coli* methionine synthase (Fig. 6). Ile881 corresponds by sequence alignment to Val855 in the *E.coli* enzyme. Val855 is within a β sheet strand that is part of an α/β domain that is a variant of the Rossmann nucleotide binding fold. The H920D substitution is found in a region which, in the *E.coli* enzyme, is in an α helix at the C-terminal end of the α/β domain. It is interesting that the polymorphism we have identified is at the adjacent residue (D919G). The functional significance, if any, of the polymorphism as well as that of the candidate deleterious mutations will have to be examined in expression experiments to confirm their impact on the protein.

Through the cloning of a cDNA for human methionine synthase, it will now be possible to determine the properties of the human enzyme and to complete the characterization of mutations in patients with severe synthase deficiency. Further, the identification of mild hyperhomocysteinemia as a risk factor for cardiovascular disease (13) and the reports of hyperhomocysteinemia in families with neural tube defects (14,15) have generated an increased interest in the genes involved in homocysteine metabolism. A recently-identified mutation in methylenetetrahydrofolate reductase, the enzyme that synthesizes the 5-methyltetrahydrofolate substrate for the methionine synthase reaction, results in mild hyperhomocysteinemia (16). Evidence is accumulating that this mutation, present in 35–40% of alleles, is a risk factor in both cardiovascular disease and neural tube defects (reviewed in ref. 17). Genetic variants of methionine synthase might similarly lead to mild hyperhomocysteinemia with consequent impact on these two multifactorial disorders.

MATERIALS AND METHODS

Cell lines

The skin fibroblast lines are from patients with methionine synthase deficiency. They were assigned to the *cblG* complementation group in cell fusion experiments assayed by ^{14}C-methyltetrahydrofolate incorporation into cellular macromolecules

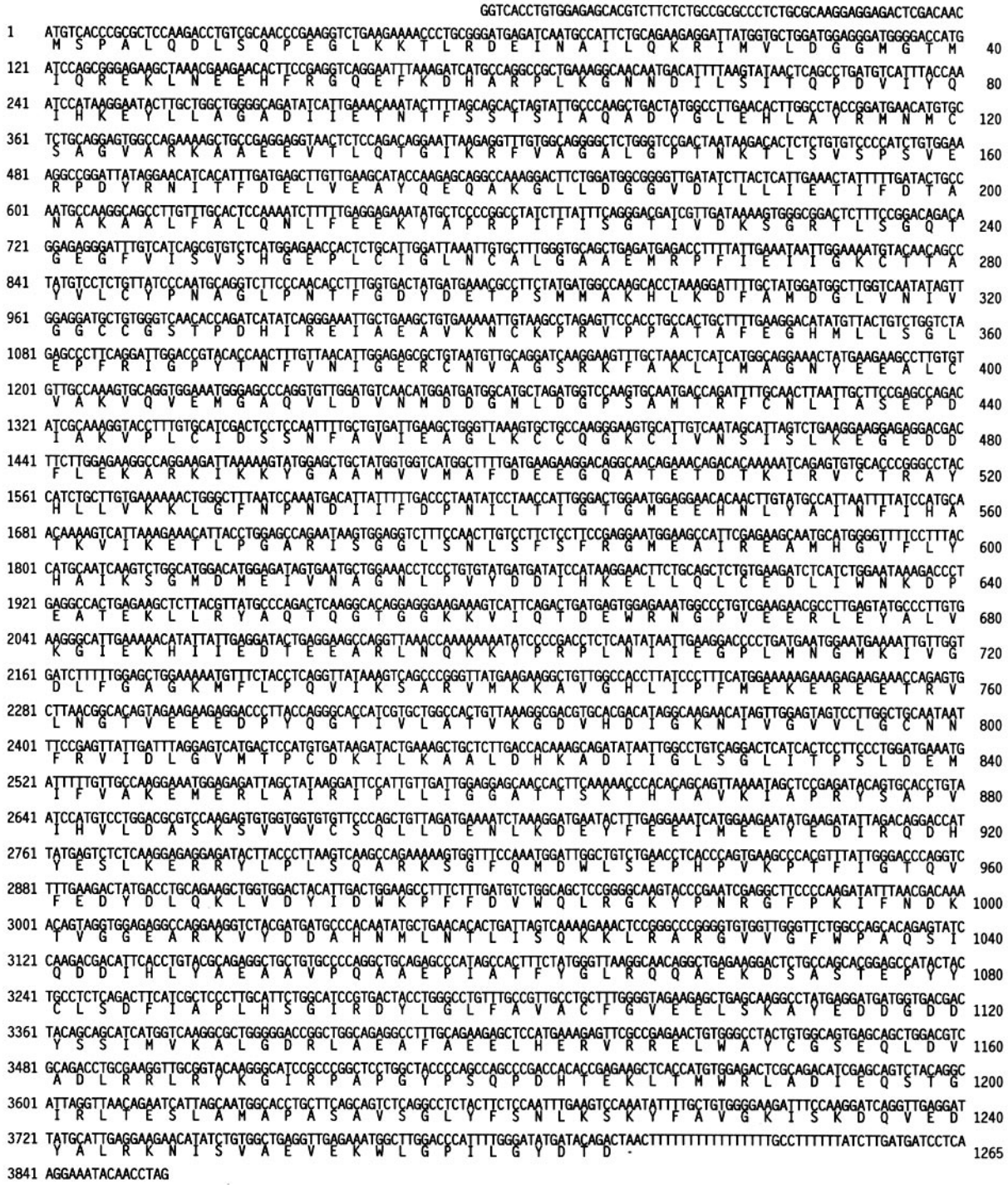

Figure 3. Nucleotide and deduced amino acid sequence of human methionine synthase. The nucleotide residues are numbered on the left margin, while the amino acid residues are numbered on the right margin. The sequence reported in this paper has been deposited in the GenBank database, accession no. U71285.

(4). Control fibroblasts were from other laboratory stocks or the Montreal Children's Hospital Cell Repository for Mutant Human Cell Strains. Of the patients for which non-polymorphic mutations were found, WG 1892, a Caucasian male, was diagnosed at the age of 4 years with developmental delay, tremors, gait instability, megaloblastic anemia and homocystinuria; and WG2290, also a Caucasian male, was diagnosed at age 3 months with failure to thrive, severe eczema, megaloblastic anemia and surprisingly both homocystinuria and methylmalonic aciduria.

Materials

The T/A cloning kit was from Invitrogen. The Geneclean III kit was obtained from Bio 101 Inc. and the Wizard Mini-Preps were

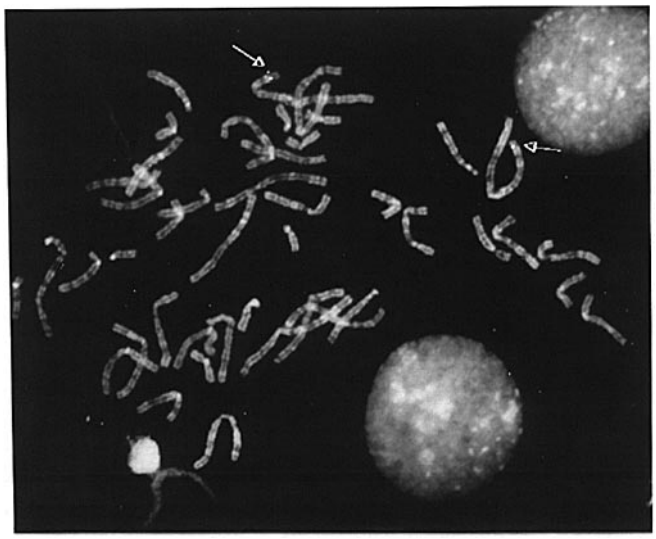

Figure 4. Mapping of the human methionine synthase gene using FISH. Signals are clearly visible at 1q43 (arrows).

from Promega. The random-primed DNA labelling kit was from Boehringer-Mannheim. Taq polymerase, Superscript II reverse transcriptase, AMV reverse transcriptase, Trizol reagent, DNAzol reagent, T4 DNA ligase, and restriction enzymes were purchased from Gibco BRL. The Sequenase kit for manual sequencing was from United States Biochemicals. The α-[^{35}S]dATP (12.5 Ci/mole) was from Dupont or ICN. The oligonucleotide primers were synthesized by R. Clarizio of the Montreal Children's Hospital Research Institute Oligonucleotide Synthesis Facility or the Sheldon Biotechnology Centre, McGill University.

Homology matches

Comparisons were made between the published *E.coli* cobalamin-dependent methionine synthase sequence and sequences in the NCBI databases (dbEST and GenBank) using the BLAST programs.

PCR cloning and DNA sequencing

DNA was prepared from fibroblast pellets by the method of Hoar *et al.* (18). Total cellular RNA was isolated by the method of Chirgwin *et al.* (19) and reverse-transcribed using oligo-dT$_{15}$ as primer. PCR was conducted using degenerate oligonucleotides as primers, paired so as to link the sequences of different homology boxes. The PCRs were conducted as described previously (20) except that the temperature of incubation was modified to accommodate the use of reduced temperatures in the annealing step or by step-down PCR (21). In some experiments, inverse PCR was used to determine sequence upstream or downstream of known sequence (22). In these instances, genomic DNA or cDNA prepared by reverse transcription of RNA was digested with different four base restriction endonucleases, ligated with T4 DNA ligase, and amplified by PCR using adjacent oligonucleotides priming in opposite directions. Templates for inverse PCR at the cDNA level were generated with 12.5 µg RNA reversed transcribed using AMV-RT. Second strand synthesis was carried out using the random-primed DNA labelling kit adding 1 µl of each dNTP. Samples were incubated for 30 min at 37°C. Template was then treated as genomic DNA for digestion and ligation. Inverse PCR was used to obtain the 5′ and 3′ ends of the cDNA and to define an intron sequence adjacent to a splice junction for the design of a mutation diagnostic test. The PCR products were purified with Geneclean and were subcloned in the pCR2.1 vector and transformed into *E.coli* as per the supplier's protocol (TA Cloning Kit). The candidate clones were sequenced manually or by the DNA Core Facility of the Canadian Genetic Diseases Network or the McGill University Sheldon Biotechnology Centre.

Mutation analysis

Genomic DNA and RNA were isolated from control or patient fibroblast pellets using the DNAzol or Trizol reagents, respectively, as per the manufacturer. The cDNA template for PCR was prepared by reverse transcription of 3–5 µg total RNA in reactions containing 400 U of Superscript II reverse transcriptase and 100 ng random hexamers in a total reaction volume of 20 µl. SSCP analysis was performed as described previously (20) in reactions containing 4 µl of template, 1 µl of each dTTP, dCTP,

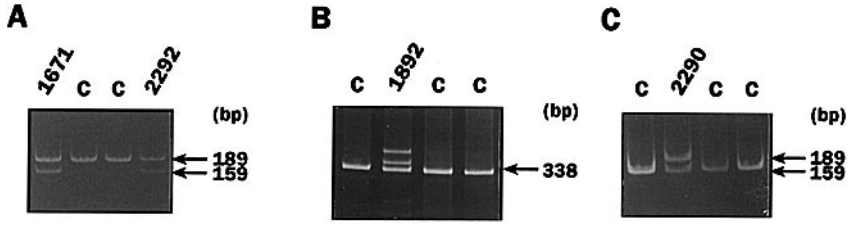

Figure 5. Diagnostic tests for mutations in the methionine synthase gene. Numbers above the gel lanes correspond to patients cell lines whereas the letter 'c' identifies wild-type controls. (**A**) *Hae*III restriction analysis of genomic DNA PCR products using primers #1796 and #305A. The 2756A→G change creates a *Hae*III site. Expected fragments, 2756A allele: 189 bp, 2756G allele: 159 and 30 bp (the 30 bp fragment was run off the gel). (**B**) Heteroduplex analysis of PCR products amplified from RT reactions of patient 1892 and three controls. RT–PCR was done with primers #1772 and #1773. Expected PCR product: 338 bp, heteroduplexes can be seen above this band in patient 1892 (heterozygous for Δ2640–2642). (**C**) *Sau*96I restriction analysis of genomic DNA PCR products. PCR was done as in (A). The 2758C→G mutation abolishes a *Sau*96I restriction endonuclease site in patient 2290. Expected fragments, control allele: 159, 30 bp, mutant allele: 189 bp (the 30 bp fragment has been run off the gel).

Figure 6. Amino acid sequence comparison among methionine synthases in the Box 2 region. Identical residues are indicated by a star above the alignment. Dots show partially conserved residues, for which at least 6/7 identical or similar residues can be aligned [A,G,S,T; D,E,N,Q; V,L,I,M; K,R; and F,W,Y (25)]. Mutations identified in this work are shown below the alignment. For abbreviations, see Figure 1; Mm: *Mus musculus*. The seven amino acids conserved in cobalamin-binding proteins (9) are underlined.

dGTP (0.625 mM), 0.5 µl of dATP (0.625 mM), 1 µl α-[^{35}S]-dATP (12.5 Ci/mol). The radiolabelled PCR products mixed with sequencing stop solution were heat denatured and quick chilled on ice prior to loading (20). As well, an aliquot of each sample was run without prior heating to identify the duplex product. The fragments were subjected to electrophoresis in a 6% acrylamide/10% glycerol gel in 1× TBE for 18 h at 8 Watts at room temperature. The gel was dried and exposed to Biomax film (Kodak). Fragments that displayed band shifts were sequenced directly.

Two mutations were confirmed directly in PCR amplification products from genomic DNA and one mutation was confirmed in reversed transcribed mRNA. The PCR reactions for mutation confirmation were performed using 4 µl of cDNA template or 500 ng genomic DNA, 500 ng of specific primers, 2.5 U *Taq* polymerase and 1.5 mM $MgCl_2$ in a 50 µl volume. Heteroduplex analysis was accomplished by preheating PCR products to 95°C for 5 min and subjecting the samples to electrophoresis in a 9% polyacrylamide gel (20). Other diagnostic assays were accomplished by digesting a 15 µl sample of the PCR products with the indicated restriction endonuclease prior to electrophoresis.

Chromosomal localization

Human metaphase spreads were obtained from short-term cultures of phytohemaglutinin-stimulated peripheral blood lymphocytes. The cells were synchronized with thymidine and treated with BrdU during the late S-phase before harvesting for simultaneous observation of the hybridized sites and chromosome banding. The protocol for FISH was essentially as described previously (23,24). Briefly, a 5 kb DNA fragment of the methionine synthase genomic DNA (generated by PCR using oligonucleotides #1782 and #1780) was labelled by nick translation with biotin-16-dUTP (Boehringer-Mannheim), ethanol precipitated and dissolved in hybridization buffer at a final concentration of 8 ng/µl. The slides were denatured in 70% formamide, 2× SSC at 70°C for 2 min. The biotinylated probe was denatured in the hybridization buffer at 95°C for 10 min, quickly cooled on ice, then applied on slides. Post-washing was done by rinsing in 50% formamide, 2× SSC at 37°C. The slides were incubated with rabbit antibiotin antibody (Enzo Biochemicals), biotinylated goat anti-rabbit antibodies (BRL) and streptavidin-FITC. They were stained with propidium iodide and mounted in p-phenylenediamine, pH 11. Cells were observed under the microscope (Zeiss), then captured through a CCD camera and processed using a FISH software (Applied Imaging).

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