

Defective Cystathionine β -Synthase Regulation by S-Adenosylmethionine in a Partially Pyridoxine Responsive Homocystinuria Patient

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Abstract

We determined the molecular basis of cystathionine β -synthase (CBS) deficiency in a partially pyridoxine-responsive homocystinuria patient. Direct sequencing of the entire CBS cDNA revealed the presence of a homozygous G₁₃₃₀A transition. This mutation causes an amino acid change from aspartic acid to asparagine (D444N) in the regulatory domain of the protein and abolishes a TaqI restriction site at DNA level. Despite the homozygous mutation, CBS activities in extracts of cultured fibroblasts of this patient were not in the homozygous but in the heterozygous range. Furthermore, we observed no stimulation of CBS activity by S-adenosylmethionine, contrary to a threefold stimulation in control fibroblast extract. The mutation was introduced in an *E. coli* expression system and CBS activities were measured after addition of different S-adenosylmethionine concentrations (0–200 μ M). Again, we observed a defective stimulation of CBS activity by S-adenosylmethionine in the mutated construct, whereas the normal construct showed a threefold stimulation in activity. These data suggest that this D444N mutation interferes in S-adenosylmethionine regulation of CBS. Furthermore, it indicates the importance of S-adenosylmethionine regulation of the transsulfuration pathway in homocysteine homeostasis in humans. (*J. Clin. Invest.* 1996. 98:285–289.) Key words: homocysteine • cystathionine β -synthase deficiency • genetic mutation • in vitro expression • methionine

Introduction

Homocystinuria due to cystathionine β -synthase (CBS,¹ L-serine hydro-lyase [adding homocysteine], EC 4.2.1.22) defi-

ciency is an inborn error of methionine metabolism. CBS-deficiency is inherited as an autosomal recessive trait and is clinically characterized by premature arteriosclerosis and thrombosis, ectopia lentis, skeletal abnormalities, and mental retardation (1, 2).

In a large international survey of 629 homocystinuria patients, Mudd et al. (3) showed that about 50% of these patients were considered pyridoxine responsive, i.e., a large decrease in homocysteine concentrations was obtained upon administration of pharmacological doses of pyridoxine (vitamin B₆), the precursor of the co-factor for CBS.

CBS catalyzes the condensation of homocysteine and serine to cystathionine, an irreversible step in the transsulfuration pathway. This catalytic active enzyme consists of four identical subunits. The CBS gene has been mapped to the subtelomeric segment of chromosome 21: 21q22.3 (4) and encodes a subunit of 63 kD.

Several mutations have been reported in the human CBS cDNA (5–7), of which most were screened for pathogenicity in a bacterial or yeast expression system (8, 9). The mutations described so far, are located in the catalytic domain of the protein (amino acids 1 to 418; J.P. Kraus, unpublished results), with some clusters of mutations in exon 3 and 8 (10). No mutations have been reported in the regulatory domain of this protein.

S-Adenosylmethionine (AdoMet), an intermediate in the conversion of methionine to homocysteine, is the methyl donor in various transmethylation reactions including nucleic acids, neurotransmitters, phospholipids, and hormones. It is an important regulator of homocysteine/methionine metabolism (11, 12), by stimulating CBS activity about threefold (13) and decreasing the formation of 5-methylenetetrahydrofolate by inhibition of methylenetetrahydrofolate reductase (MTHFR). The result of elevated AdoMet levels is a decreased remethylation of homocysteine to methionine and an enhanced clearance of homocysteine via the transsulfuration pathway.

In this report, we describe a homocystinuria patient with severely elevated homocysteine concentrations, but with CBS activities in cultured fibroblasts in the heterozygous range. DNA analysis revealed a novel mutation in the regulatory domain of the CBS protein. We were able to correlate this mutation to a defective regulation of the CBS protein by AdoMet in both fibroblasts and an *E. coli* expression system, which provides evidence for the important regulatory function of AdoMet in homocysteine metabolism.

Methods

Patient. The patient is a woman, now 20 y of age, who had been admitted to the hospital at the age of nine years because of psychomotoric retardation and marfanoid features such as excessive height,

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1. Abbreviations used in this paper: AdoMet, S-adenosylmethionine; CBS, cystathionine β -synthase; MTHFR, methylenetetrahydrofolate reductase.

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dolichostenomelia, and arachnodactyly. The diagnosis homozygous homocystinuria has been made then, and since she is treated with a combination of pyridoxine (vitamin B6, 500–750 mg daily), folic acid (5 mg daily), and betaine (6 grams daily). At present time, i.e., 11 y after diagnosing and start of therapy, she is in a very good physical condition and her intellectual development has reached an average level. Her length is 182 cm and weight 75 kg. She has not any physical complaint. Ectopia lentis, osteoporosis, and vascular complications did not occur until now.

Biochemical analysis. Determination of homocystine, homocysteine-cysteine mixed disulphide and methionine in serum of the patient was performed as described earlier by us (14). The amount of nonprotein-bound homocysteine was calculated as twice the concentration of homocystine plus the concentration of the homocysteine-cysteine mixed disulphide. Total plasma homocysteine concentrations, consisting of the total amount of protein and nonprotein-bound homocysteine, of a sister were determined according to Te Poele-Pothoff et al. (15).

CBS activities in extracts of cultured skin fibroblasts were determined in the absence and presence of 1 mM pyridoxal 5'-phosphate (PLP) or different concentrations of AdoMet, essentially according to Engbersen et al. (16). Activities are expressed in nmol cystathionine formed per mg protein per hour at 37°C.

Methylenetetrahydrofolate reductase (MTHFR) activity was measured in isolated lymphocytes (16) in the absence and presence of 75 or 400 μ M AdoMet, and in cultured fibroblasts (17). The specific MTHFR activity is expressed in nmol formaldehyde formed per mg protein per hour. Protein concentrations were determined as described by Lowry et al. (18). Folate and vitamin B12 concentrations were determined in heparinized plasma by routine hospital assays.

Mutation analysis. Genomic DNA was isolated from peripheral blood leukocytes according to a protocol by Miller et al. (19). Total RNA was extracted from cultured fibroblasts (20) and stored as an ethanol precipitate at -80°C . A 5 μ g sample of RNA was reverse transcribed to cDNA in 1 h at 42°C with 200 units of Superscript II reverse transcriptase (Life Technologies, Breda, The Netherlands), using oligo(dT) and random hexamer primers. 1 μ l of this first-strand cDNA was subjected to PCR amplification. The oligonucleotides used were described elsewhere (7). These amplifications were carried out in a total volume of 100 μ l, containing 100 ng forward and reverse primer, 200 μ M each dNTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin and 1 unit *Taq* polymerase (Life Technologies, Breda, The Netherlands). Amplification parameters were as follows: 35 cycles of $92^{\circ}\text{C}/60$ s (denaturation), 52 – $58^{\circ}\text{C}/60$ s (annealing) and $72^{\circ}\text{C}/90$ s (extension). The cycles were preceded by an initial denaturation step of 5 min at 95°C and were followed by a final extension of 7 min at 72°C .

By PCR, we generated three overlapping fragments which covered the entire coding region. Direct sequencing of these products was performed using the Taq Dye Deoxy™ Terminator Cycle Sequencing Kit according to the manufacturer's recommendations (Applied Biosystems, Forster City, CA). The dye-labeled sequencing products were purified by phenol/chloroform extraction and ethanol precipitation at room temperature. The pellets were washed in 70% ethanol, solubilized in loading buffer (96% deionized formamide, 20 mM EDTA, pH 8.0, dextran blue) and electrophoresed in a 6% polyacrylamide/8 M urea sequencing gel on a ABI 373A DNA sequencer. All PCR fragments were sequenced on both strands.

Genomic DNA amplification. To confirm the mutation at the genomic DNA level, a fragment consisting of exon 11, intron 11 and exon 12 was amplified in the same PCR buffer as described above, using forward primer 5'-GACCAAGTTCCTGAGCGACAGGTG-3' (cDNA 1146–1169) and reverse primer 5'-CCCGCCTCATCCAC-CACGG-3' (cDNA 1358–1340). Amplification conditions were as follows: 5 min initial denaturation at 95°C , 35 cycles of $92^{\circ}\text{C}/60$ s, $59^{\circ}\text{C}/60$ s and $72^{\circ}\text{C}/45$ s, followed by a final extension of 7 min at 72°C . TaqI restriction enzyme analysis was performed according to the recommendations of the manufacturer (Life Technologies,

Breda, The Netherlands). Restriction fragments were resolved in a 15% polyacrylamide gel containing 5% glycerol. Ethidium bromide staining was used to visualize the restriction fragments.

Cloning and expression of the mutation. The cDNA fragment containing the mutation was amplified by PCR and subcloned into an expression cartridge derived from pHCS3, using BstXI and KpnI, as described extensively (8, 21). *E. coli* JM109 cells were transformed and recombinants were selected by PCR and restriction enzyme analysis. The integrity of the mutated clone was established by sequencing analysis. Expression of the CBS cDNA was induced by IPTG addition (21) and the CBS enzyme assay (16) was performed without addition of BSA to the incubation mixture. Cystathionine (Sigma, Amsterdam, The Netherlands) was added to a final concentration of 2 mM.

Results

Biochemical analysis. Hyperhomocysteinemia and hypermethioninemia were observed in our patient when compared to controls (Table I). Daily administration of 500 mg pyridoxine for six weeks resulted in only a minor reduction of homocysteine levels of $\sim 30\%$. Additional administration of folic acid (5 mg daily, for 6 wk) and later on also of betaine (6 grams daily, for 6 wk) resulted in a marked decrease in homocysteine, however homocysteine was still elevated compared to controls (Table I). Therapy left methionine concentrations virtually unchanged. In her sister, total plasma homocysteine concentration (protein plus nonprotein-bound) was elevated in fasting state (26 μ mol/liter; reference values: 6–15 μ mol/liter) and after a methionine-loading test (72 μ mol/liter; reference values: 18–51 μ mol/liter).

Folate concentration prior to treatment was 10 nmol/liter (control values: 5.5–40 nmol/liter) and vitamin B12 concentration was 180 pmol/liter (control values: 150–380 pmol/liter).

Specific MTHFR activity was normal in isolated lymphocytes of our patient (12.6, reference values: 9.1–23.9 nmol CH₂O/mg protein.h; $n = 18$) as well as in cultured fibroblasts (6.2, reference values: 4.9–10.0 nmol CH₂O/mg protein.h; $n = 6$). In isolated lymphocytes, we found the same inhibition by AdoMet as observed in control lymphocytes (data not shown). She was found to be heterozygous for the previously described 677 C→T transition in the MTHFR gene (22).

Extracts of cultured fibroblasts were assayed for CBS activity in the presence and absence of 1 mM PLP, as shown in Ta-

Table I. Fasting Serum Levels of Nonprotein-bound Homocysteine and Methionine at Base-line and During Therapy, at the Patient's Age of Nine Years

| | Base-line | Therapy | | |
|-----------------------------|----------------------------|------------------------------|------------------------------|------------------------------|
| | | Pyridoxine (500 mg daily) | + Folic acid (5 mg daily) | + Betaine (6 grams daily) |
| Patient | HCy*: 178 Met: 83 | HCy: 121 Met: 79 | HCy: 62 Met: 80 | HCy: 18 Met: 69 |
| Controls (premenopausal) | HCy: 0.2–2.6 Met: 16–38 | | | |

*HCy refers to the amount of nonprotein-bound homocysteine (in μ mol/liter), and is calculated as twice the concentration of homocystine plus the concentration of homocysteine-cysteine mixed disulphide. *Met*, methionine.

Table II. CBS Activities in Extracts of Cultured Fibroblasts

| | -PLP | +PLP |
|---------------------------------|----------|----------|
| | | 1 mM |
| Controls (n = 12) | 2.3–18.2 | 4.0–22.3 |
| Obligate heterozygotes (n = 14) | 0.17–2.4 | 0.39–5.4 |
| Homozygotes (n = 14) | 0–0.19 | 0–1.6 |
| Patient | 1.7 | 2.6 |

Activities are expressed in nmol cystathionine formed per mg protein per hour. PLP, pyridoxal 5'-phosphate.

ble II. Both measurements show CBS activities in the heterozygous range which is in contrast to the severely elevated homocysteine levels observed in this patient. DNA analysis of the CBS cDNA was performed to explore the genetic basis underlying this contradiction.

DNA analysis. We synthesized three sets of oligonucleotides which enabled us to amplify overlapping fragments covering the entire CBS cDNA. The PCR fragments generated were all of the expected size, suggesting the existence of point mutations rather than small deletions, insertions or splice error mutations in the cDNA of this patient.

Direct sequencing of the entire coding region revealed a novel homozygous G₁₃₃₀A missense mutation. No other sequence aberrations could be detected in the CBS cDNA. This mutation is expected to cause an amino acid change from aspartic acid (GAC) to asparagine (AAC) at position 444 of the mature protein (D444N), i.e., a negatively charged amino acid is replaced with a neutral one. The mutation abolished a TaqI restriction site which was used to confirm the mutation at the genomic DNA level. Screening of both parents and an unaffected sister revealed their heterozygous state for this transition (Fig. 1). We were unable to detect this mutation among 14 other Dutch homozygotes for CBS deficiency, indicating that this transition is rare among Dutch homocystinurics. The D444N substitution was not observed in 80 control chromosomes.

In vitro expression and AdoMet regulation. The pathogenic nature of the G₁₃₃₀A transition was investigated both in cultured fibroblasts and in an *E. coli* expression system. CBS assays in extracts of cultured fibroblasts were performed after addition of different AdoMet concentrations to the incubation mixture. As shown in Fig. 2, AdoMet stimulates CBS activity about threefold in control fibroblasts, contrary to virtually no stimulation observed in fibroblast extracts of the patient.

We cloned the cDNA fragment, containing the G₁₃₃₀A transition, into an expression cartridge to restore the CBS cDNA in plasmid pHCS3. The presence of the mutation and the absence of artifacts introduced by PCR were confirmed by sequence analysis of the cloned fragment and its cloning sites (data not shown). Cell lysates were analyzed for CBS activity in the presence of different concentrations of AdoMet (0–200 μM; Fig. 3). This figure clearly demonstrates that CBS activity in the mutant is indistinguishable from the control when measured in the absence of AdoMet. AdoMet, however, is unable to stimulate the CBS protein with the D444N substitution to the same extent as the CBS protein translated from the control construct, which is stimulated threefold.

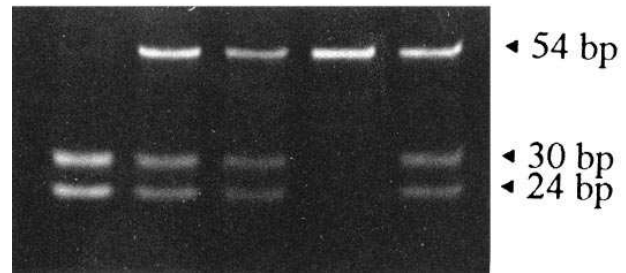
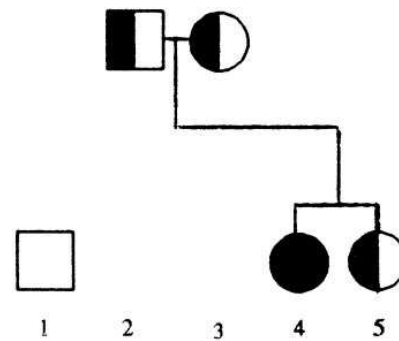


Figure 1. Pedigree of the family. A genomic DNA fragment was amplified by PCR, screened for the mutation by TaqI restriction enzyme analysis and subsequently electrophoresed in a 15% polyacrylamide gel. The mutated allele yields one fragment of 54 bp, whereas the wild-type allele yields two fragments of 30 and 24 bp, respectively. A larger fragment of ~500 bp, present in all samples, was cut off the photo for clarity. Lane 1: healthy male control; lane 2: father; lane 3: mother; lane 4: patient; lane 5: sister.

Discussion

Severe hyperhomocysteinemia, as observed in our patient, may be caused by a homozygous deficiency in CBS or MTHFR, two regulating enzymes in homocysteine metabolism. In this study, we detected a novel G₁₃₃₀A (D444N) missense mutation in the regulatory domain of the CBS protein,

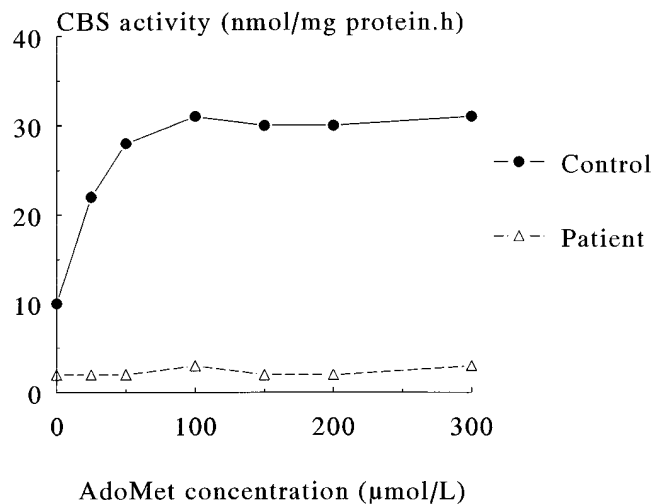


Figure 2. CBS activities in extracts of cultured fibroblasts of a healthy control and the patient in the presence of different concentrations of S-adenosylmethionine. Each point represents the mean of two independent experiments.

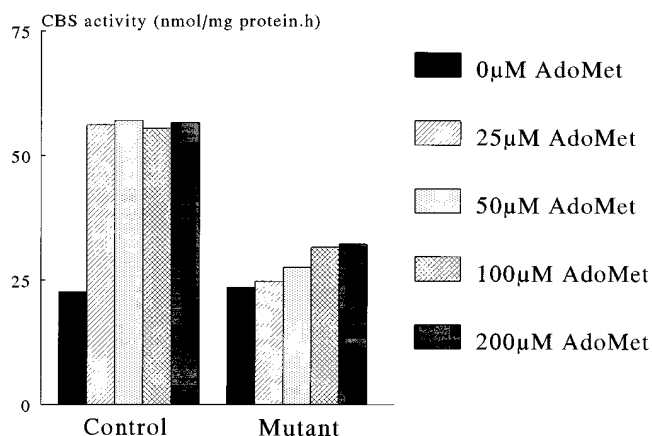


Figure 3. Regulation of CBS activity by S-adenosylmethionine in cell lysates of the *E. coli* expression system. Control denotes expression plasmid pHCS3 harboring the wild-type CBS cDNA sequence. Mutant denotes the mutated plasmid pHCS3 containing the G₁₃₃₀A transition. Each bar represents the mean of two independent experiments.

which is associated with a defective regulation of the CBS protein by AdoMet.

Until now, about 18 mutations have been described in the CBS gene in patients with homocystinuria due to CBS deficiency (5–7). Some mutations seemed to be rather unique to one family, while others, especially the I278T (21) and G307S (23), occur more frequently. All mutations described so far, are located in the NH₂-terminal domain and are thought to interfere in the catalytic activity of the enzyme.

In our patient, we detected an apparent mild D444N mutation in the regulatory domain of the CBS protein, implying a mild CBS deficiency. However, the clinical manifestations and the severe hyperhomocysteinemic status of the patient and the moderate hyperhomocysteinemia of the heterozygous sister, indicate that this mutation may interfere in a very important regulation of CBS activity.

In extracts of cultured fibroblasts, we observed an exceptionally high residual CBS activity for a homocystinuria patient compared to 14 other diagnosed CBS deficient patients (Table II). Homocystinuria patients described so far, exhibited dramatically decreased CBS activities in cultured fibroblasts. However, Uhlendorf, Bittles and their co-workers (24, 25), described each a homocystinuria patient with CBS activities in the heterozygous or low-normal range. They ascribed these findings to either a very mild homozygous CBS deficiency, or to a heterozygous deficiency with hepatic CBS activities unusually low for heterozygotes, which could lead to homocystinuria. According to our data, those patients may be homozygotes for CBS deficiency defective in CBS regulation.

Expression of the D444N mutation was performed in an *E. coli* expression system (8, 21). CBS enzyme expressed in *E. coli* is indistinguishable from human CBS enzyme present in cultured fibroblasts. It is able to bind PLP, heme and AdoMet and exhibits catalytic activity (21, 26). Activities obtained when expressing the mutant construct were equal to those observed in the control construct in the absence of AdoMet. Thus, the mutation has no pathogenic nature in the absence of AdoMet in our in vitro expression system. Addition of AdoMet to the incubation mixture reveals the pathogenic

nature of the mutation: it interferes in the regulation of the CBS protein by AdoMet.

The *E. coli* expression with our mutant CBS construct showed a normal basal activity, contrary to fibroblasts, in which we observed a reduced basal activity. However, the effect of a mutation on protein activity in cultured fibroblasts cannot be compared directly with its effect in *E. coli*. It is known that mutant polypeptides are degraded more rapidly in cultured fibroblasts than in *E. coli*, which has also been demonstrated for mutant CBS subunits (21). The discrepancy observed may depend on differences in protein catabolism between both systems, heme status (important in CBS function) and the concentration of stabilizing (co)-factors.

Although our experiments in the *E. coli* expression system show that this D444N mutation is causing a regulatory defect, we were unable to exclude a possible influence of the mutation on CBS activity in liver, the main site of homocysteine catabolism. The mutation may also have an effect on protein synthesis and/or turnover of CBS in liver affecting hepatic CBS activity and plasma homocysteine concentration in this patient. For ethical reasons we were unable to obtain a liver specimen to address this issue.

AdoMet is an allosteric regulator of the homocysteine flux through either the transsulfuration or remethylation pathway. Low levels of AdoMet favor the conservation of the homocysteine-skeleton of methionine, whereas high levels of AdoMet stimulate the irreversible conversion of homocysteine to cystathionine in the transsulfuration pathway (12). The disruption of this regulation of homocysteine metabolism may explain the severe hyperhomocysteinemia observed in our patient. Due to the homozygous G₁₃₃₀A transition in the CBS gene, AdoMet is unable to stimulate CBS, so a surplus of homocysteine will not be degraded via the transsulfuration pathway. Furthermore, the elevated AdoMet levels will inhibit MTHFR, resulting in an additional accumulation of homocysteine.

MTHFR deficiency was excluded as a possible contributor to hyperhomocysteinemia. Specific MTHFR activity in isolated lymphocytes as well as in cultured fibroblasts of our patient was well within the reference range, excluding a major defect in MTHFR. Furthermore, regulation of MTHFR by AdoMet was found to be normal. The patient is heterozygous for the previously described 677C→T transition in the MTHFR gene. The prevalence of this heterozygous state in the Dutch population is ~40% (27, 28). In homozygous state, this mutation is associated with elevated homocysteine concentrations (22, 27, 28), especially in circumstances of low (normal) folate (29). Plasma homocysteine concentrations in heterozygotes however, do not differ significantly from those observed in wild-type individuals (22, 27–29). An interaction between lowered folate and plasma homocysteine has only been described in individuals with the homozygous mutant genotype and not in heterozygous individuals (29).

Comparison of the human CBS to the rat enzyme and several homologous cysteine synthases from plants and bacteria (5), indicates an extensive homology in the NH₂-terminal part of these proteins. This NH₂-terminal domain, encoded by exon 1 to 12, is believed to bind both substrates (homocysteine and serine), heme, and its cofactor PLP. The COOH-terminal domain of the CBS protein is only conserved in rat CBS (30) and partly in yeast CBS (9), in which also the aspartic acid-444 residue is conserved, but is absent in O-acetylserine (thiol)-lyase (*cysK*) from *E. coli* (31). However, this latter enzyme, which

shows 52% sequence similarity to human CBS, catalyzes the formation of cysteine from *O*-acetyl-L-serine and inorganic sulfide and not the homocysteine conversion into cystathionine in this organism. Although the enzymatic function is different, both enzymes, CBS, and *cysK*, condense serine and a sulfur containing compound to either cystathionine or cysteine.

Pathogenicity of the G₁₃₃₀A mutation was ascertained by four different methods. First, we found no other mutations in the coding region of the *CBS* gene, which could be responsible for the severely elevated homocysteine and methionine levels. Second, the mutation was absent in 80 control alleles, indicating that it is not a benign polymorphism. Third, the wild-type aspartic acid-444 residue is conserved in the evolution, which is evidence for functional relevance of this amino acid in the biological function of the protein. Fourth, we observed a defective regulation of CBS by AdoMet in extracts of cultured fibroblasts of this patient and in a prokaryotic expression system in which we introduced this mutation.

To our knowledge, this is the first report of a mutation in the regulatory domain of the CBS protein. We have provided substantial evidence that the D444N substitution is a pathogenic mutation and interferes in the normal regulation of the CBS protein by AdoMet, although a possible effect on CBS synthesis and turnover in liver could not be excluded. From a diagnostic point of view, we urge others to include an AdoMet-dependent enzyme assay in the diagnosis of homocystinuria, because similar patients could be missed in the regular screening methods, which could lead to a false conclusion regarding the origin of homocystinuria in such patients.

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