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Characterisation of Five Missense Mutations in the Cystathionine Beta-Synthase Gene from Three Patients with B₆-Nonresponsive Homocystinuria

Abstract

Homocystinuria, due to a deficiency of the enzyme cystathionine β-synthase (CBS), is an inborn error of sulphur-amino acid metabolism. This is an autosomal recessive disease which results in hyperhomocysteinaemia and a wide range of clinical features, including optic lens dislocation, mental retardation, skeletal abnormalities and premature thrombotic events. We report the identification of 5 missense mutations in the protein-coding region of the CBS gene from 3 patients with pyridoxine-nonresponsive homocystinuria. Reverse-transcription PCR was used to amplify CBS cDNA from each patient and the coding region was analysed by direct sequencing. The mutations detected included 3 novel (1058C→T, 992C→A and 1316G→A) and 2 previously identified (430G→A and 833C→T) base alterations in the CBS cDNA. Each of these mutations predicts a single amino acid substitution in the CBS polypeptide. Appropriate cassettes of patient CBS cDNA, containing each of the above defined mutations, were used to replace the corresponding cassettes of normal CBS cDNA sequence within the bacterial expression vector pT7-7. These recombinant mutant and normal CBS constructs were expressed in *Escherichia coli* cells and the catalytic activities of the mutant proteins were compared with normal. All of the mutant proteins exhibited decreased catalytic activity in vitro, which confirmed the association between the individual mutation and CBS dysfunction in each patient.

Key Words

Homocystinuria
Cystathionine β-synthase
Mutation analysis
Cystathionine β-synthase genetic variants
B₆-nonresponsive homocystinuria

Introduction

Cystathionine β-synthase (CBS; EC 4.2.1.22) is an enzyme of the transsulphuration pathway that catalyses the condensation of homocysteine with serine to form cystathionine. CBS enzyme deficiency leads to an abnormal accumulation of homocysteine in the blood (hyperhomo-

cysteinaemia) and excretion of large amounts of homocystine into the urine (homocystinuria). Homocystinuria due to CBS deficiency is an autosomal recessive disease characterised by mental retardation, dislocation of the optic lens, thinning and lengthening of the long bones, and thrombotic tendencies [1, 2]. The incidence of homozygous CBS deficiency is approximately 1:344,000 world-

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Table 1. Mutations detected in the CBS cDNA and confirmed in genomic DNA of 3 patients with homocystinuria nonresponsive to vitamin B₆

Patient	CBS mutation and genotype	Predicted amino acid change
1	833T→C (heterozygous) ¹	I278T ¹
	1058C→T (heterozygous) ¹	T353M ¹
	699C→T (heterozygous)	Y233Y
	1080T→C (homozygous)	A360A
2	992C→A (heterozygous) ¹	A331E ¹
3	430G→A (heterozygous) ¹	E144K ¹
	1316G→A (heterozygous) ¹	R439Q ¹
	699C→T	Y233Y

¹ Predicted pathological mutations in the CBS protein. Synonymous mutations are also shown.

wide, although higher in Ireland (1:58,000) [1]. The human CBS enzyme is a homotetramer of 63-kDa subunits, which binds its 2 substrates, homocysteine and serine, and 3 additional ligands: pyridoxal 5'-phosphate, S-adenosylmethionine and haem [3, 4]. The CBS gene maps to human chromosome 21 at position q22.3 [5, 6]. This gene contains a protein-coding region of 1653 nucleotides which is translated into the CBS subunit of 551 amino acids. Since the isolation and characterisation of the human CBS cDNA sequence in 1993 [3], interest in the detection of CBS mutations in patients with homocystinuria has expanded [6–15]. To date, 26 pathogenetic mutations within the CBS gene have been isolated, and some of these have been assayed for catalytic activity in vitro [16, 17]. In this communication, we report the identification and expression of 3 novel mutations and 2 previously identified mutations within the protein-coding region of the CBS gene from 3 patients with pyridoxine-nonresponsive homocystinuria.

Results

Detection of CBS Mutations from Patients

Direct-sequence analysis of PCR-amplified CBS cDNA was employed to identify sequence mutations in the CBS gene from 3 patients with pyridoxine-nonresponsive homocystinuria. These mutations are summarised in table 1 together with the predicted single amino acid substitutions in the CBS polypeptide.

Patient 1. Patient 1 is a 50-year-old male and was first diagnosed with homocystinuria at the age of 29 years. He has dislocated optic lenses, mild mental retardation and osteoporosis. His current treatments include betaine, folic acid, B₁₂ and pyridoxine. Clinical information obtained on patient 1 documented that following 5 years of treatment with pyridoxine (100 mg/day) plus folic acid (5 mg/day), his concentration of serum homocysteine (non-protein-bound) was 100 µmol/l and his methionine concentration was 93 µmol/l. Since his homocysteine did not fall to below 20 µmol/l on this treatment, he was regarded as pyridoxine-nonresponsive [Wilcken, pers. commun.]. After the inclusion of betaine (3 g twice daily) to his regimen, his homocysteine concentration dropped immediately to 18 µmol/l and remained generally below 50 µmol/l for years. This patient is a compound heterozygote for a previously described 833T→C mutation in exon 8 [7, 14] and a novel 1058C→T mutation in exon 10 of CBS cDNA (data not shown). These mutations are predicted to change an isoleucine to threonine at position 278 (I278T), and a threonine to methionine at position 353 (T353M) in the CBS polypeptide. In addition, a heterozygous 699C→T synonymous mutation and a homozygous 1080T→C synonymous mutation were detected in the CBS cDNA of this patient (data not shown). Because the cell line from this patient was lost during culture manipulations and further samples from this patient or his parents were not obtainable, confirmation of the above mutations from genomic DNA was not possible.

Patient 2. Patient 2 is a 6-year-old female, first diagnosed with homocystinuria at the age of 2 years, with delayed development, osteoporosis and osteogenesis imperfecta type IV. She is currently on treatment with betaine, folic acid and aspirin. Serum concentrations of homocysteine were not determined, but her level of urinary homocysteine before and after treatment were 42 and 14 µmol/l, respectively. Direct-sequence analysis revealed an apparent homozygous novel 992C→A mutation in exon 9 of the CBS cDNA (data not shown). This mutation is predicted to change alanine to glutamic acid at position 331 (A331E) in the CBS polypeptide. However, both direct-sequence analysis (data not shown) and *Hinf*I restriction endonuclease analysis (fig. 1) of a portion of exon 9 amplified from genomic DNA from patient 2 identified the 992C→A mutation as heterozygous.

The mother of patient 2 was also identified as heterozygous for the 992C→A mutation, whereas the father was normal (fig. 1), indicating that the 992C→A mutation is of maternal origin. No other alterations were detected in the 1653-bp CBS coding region of this patient. These

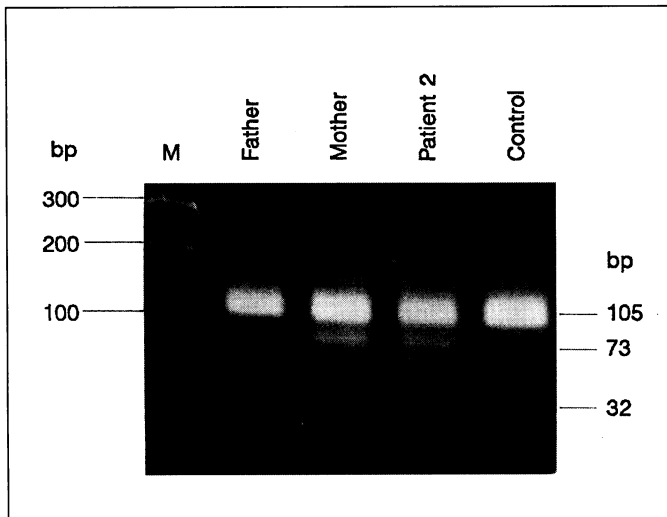


Fig. 1. Confirmation by restriction enzyme analysis of the 992C→A mutation in patient 2 and family members. A portion of exon 9 of the CBS gene amplified by PCR from genomic DNA and subjected to digestion with *HinfI* as described in Materials and Methods. Lane M is a 100-bp ladder of molecular-weight markers. The sizes of the fragments are indicated on the right.

results suggest that the paternally derived allele is not expressed. The reason(s) for the lack of expression of this allele are unknown. One possibility would be a mutation(s) in the 5' region of the CBS gene that is important for expression. Other possibilities include mutations downstream of the coding sequence that may affect the stability of the CBS transcript. It is difficult to screen these regions since, as yet, they have not been fully characterised for the CBS gene. Reduced amounts of mRNA have been found as a result of point mutations in other genes [see ref. 18 and references within]. In the present study, levels of CBS mRNA were not assessed; however, if mRNA derived from one allele were in low concentration it could lead to the preferential amplification in a PCR of cDNA from the second allele. A mutation detected on this second allele would appear as apparently homozygous. It is also possible that a mutation causing a break in the reading frame on one allele could lead to reduced level of transcripts from this allele, resulting in apparent homozygosity at the level of cDNA. Conversely, a point mutation appearing homozygous at the level of both cDNA and genomic DNA could be caused by a heterozygous mutation in a region of the gene on one chromosome that is deleted on the other homologue.

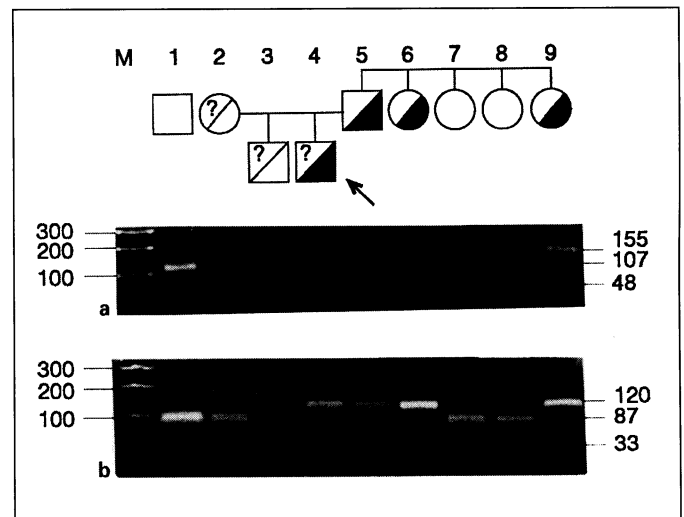


Fig. 2. Confirmation by restriction enzyme analysis of the 430G→A and the 1316G→A mutations in patient 3 and family members. Appropriate fragments of the CBS gene were amplified by PCR from samples of genomic DNA and subjected to digestion with either *TaqI* (a) or *HpaII* (b) as described in Materials and Methods. The arrow denotes the proband. Lane M is a 100-bp ladder of molecular-weight markers. The sizes of the fragments are indicated on the right.

Patient 3. Patient 3 is a 14-year-old male who was first diagnosed with homocystinuria at the age of 9 years. He has dislocated optic lenses, delayed development, osteoporosis and juvenile arthritis. He is on a treatment of betaine, folic acid and low-dose aspirin. His serum concentration of homocysteine (total) was 252 $\mu\text{mol/l}$ before treatment and is 34 $\mu\text{mol/l}$ after treatment. Direct-sequence analysis of CBS cDNA revealed an apparent homozygous previously reported 430G→A mutation in exon 3 [14] and an apparent homozygous novel 1316G→A mutation in exon 12 of this patient (data not shown). These mutations are predicted to change glutamic acid to lysine at position 144 (E144K), and arginine to glutamine at position 439 (R439Q) in the CBS polypeptide. An apparently homozygous synonymous mutation (699C→T) was also detected in the CBS cDNA from patient 3 (data not shown). Subsequent confirmation of the 430G→A and 1316G→A mutations in genomic DNA from patient 3 showed both mutations to be clearly heterozygous (fig. 2). To trace the origin of the mutations that were initially identified in the CBS cDNA of patient 3, the parents and other family members were analysed (fig. 2). The 430G→A and 1316G→A mutations were both demonstrated in patient 3 and in his father, and in 2 paternal aunts (fig. 2, lanes 4–6 and 9). This result demon-

Fig. 3. Western blot analysis of wild-type and mutant human CBS proteins expressed in *E. coli*. Cell lysate (equivalent to 1 µg of total protein) from clones harbouring the amino acid substitution indicated were subjected to SDS gel electrophoresis and Western blotting as described in Materials and Methods. The migration of prestained molecular-weight marker proteins is indicated on the left.

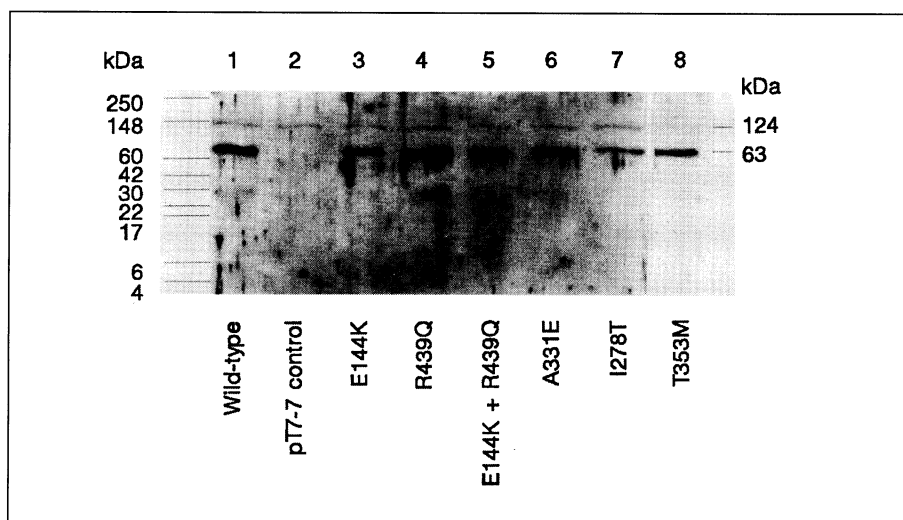


Table 2. Enzymic activity of recombinant human CBS proteins

Amino acid substitution	CBS-specific activity nmol cystathionine/h/mg protein		
	mean	range	mean wild-type level, %
I278T	2	0.4–3.7	0.7
T353M	0.9	0.3–1.5	0.3
A331E	0.9	0.8–1.1	0.3
E144K	0.9	0–1.8	0.3
R439Q	90	84–97	31
E144K + R439Q	1.6	0.7–2.5	0.5
Wild-type	288	276–309	100

The vector pT7-7 containing wild-type or mutant CBS cDNA sequence was used to transform *E. coli* (BL21/DE3) cells. Expression of recombinant proteins was induced with IPTG and cell lysates were prepared. CBS activity was assayed with [¹⁴C]serine as described in Materials and Methods.

strates that both mutations are linked in cis on the same CBS allele. The maternal CBS mutation in patient 3 has yet to be identified. Similar reasons to those stated above for patient 2 may be responsible for the failure to detect a maternally derived allele in patient 3.

Expression of CBS Mutants

CBS Assays. We expressed mutant CBS proteins containing the individual substitutions I278T, T353M, A331E, E144K, and R439Q, as well as the substitutions E144K and R439Q in cis, in *Escherichia coli* (BL21/DE3)

cells. Table 2 summarizes the specific activities of wild-type and mutant CBS proteins, with the exception of R439Q, were associated with negligible catalytic function (table 2).

Western Blots. Western blot analysis was performed on *E. coli* extracts containing the expressed CBS recombinant proteins. The results of this analysis revealed a band corresponding to a 63-kDa CBS protein in each mutant and wild-type sample (fig. 3). A band corresponding to 63 kDa was not observed in the lane containing the pT7-7 negative control (fig. 3, lane 2). This result is consistent with the 63-kDa band being derived from the expressed human CBS proteins and confirms that the wild-type CBS protein and all of the mutant CBS proteins are expressed in the *E. coli* cells.

Discussion

Using direct-sequence analysis of PCR-amplified cDNA we identified 3 novel mutations and 2 previously reported mutations in the CBS gene of 3 patients with homocystinuria. Each of the mutations identified is predicted to result in a single amino acid substitution in the CBS polypeptide (table 1). In 2 patients, direct-sequence analysis of PCR-amplified CBS cDNA indicated apparent homozygous mutations. Subsequent testing to confirm these mutations in genomic DNA samples revealed the mutations to be heterogeneous. The most likely explanation for the direct sequencing results is that only mRNA from one allele has been amplified. This can occur if the other allele is not transcribed or its mRNA is present

in limiting amounts due to an inherent instability of the primary message. Results from patient 3 offer support for this explanation, since analysis of cDNA indicated 3 apparently homozygous mutations and at least 2 of these mutations were shown to be linked in cis on the same allele (fig. 2). Patients 2 and 3 both have classical homocystinuria and must clearly be either homozygous or compound heterozygotes for 2 defective CBS alleles. Thus a second abnormal CBS allele must exist. In both of these patients, the second allele is not apparent from direct-sequencing analysis, suggesting reduced transcription or instability of the message. Therefore the results of the present study add a cautionary note for mutation screening using cDNA and emphasise the importance of confirming mutational analysis of cDNA by other means.

The expression studies of recombinant protein revealed that all the mutant alleles, with the exception of R439Q, have negligible catalytic activity (table 2) but retain some immunoreactivity and are apparently stable in *E. coli* lysates (fig. 3). Both the E144K and R439Q mutations detected in patient 3 were in cis. The E144K mutation, when expressed as recombinant protein, had <1% activity of the wild type while the R439Q mutation had 31% activity. When expressed in cis the double mutant exhibited <2% activity (table 2). Patient 3 also had a Y233Y synonymous mutation which, by cDNA analysis, was apparently homozygous. This mutation was presumably also in cis with the other 2 mutations. However, this was not substantial further. Others have reported multiple mutations in cis in CBS alleles of patients with homocystinuria. Marble et al. [10] reported a patient who was B₆-nonresponsive with homozygous mutations of R125Q, E131D and a synonymous P145P mutation. All 3 mutations were in cis. Each of the pathological mutations, when expressed as recombinant protein, had <2% activity of the wild-type CBS protein. Also de Franchis et al. [11] reported an allele from 3 siblings with homocystinuria, a P78R which had approximately 50% activity and a K112N which also had approximately 50% activity. However, expression of the combined P78R and K112N allele gave no activity. Gene conversion or nonhomologous recombination events have been suggested as a possible explanation for the occurrence of multiple mutations in cis [10]. However, these authors were unable to identify any putative source sequence for these events.

All 3 patients in the present study were reported by their physicians as being pyridoxine-nonresponsive. The I278T allele, found in patient 1, has not been detected so far in pyridoxine-nonresponsive patients but has been reported in a number of pyridoxine-responsive patients

[14]. Thus it is likely that the phenotype conferred by the I278T mutation is modified by the additional T353M allele in patient 1. Further study will be required to substantiate this hypothesis. Likewise patient 3, with an E144K mutation in cis with an R439Q mutation, would appear to differ in response to pyridoxine from a report of the E144K allele in 2 pyridoxine-responsive patients [14].

The present study takes the total number of unique pathogenetic mutations, so far reported, in the CBS gene to 29, indicating a heterogeneous group of alleles causing CBS deficiency and leading to homocystinuria. Some alleles appear to be prevalent in patients of certain ethnic origin [16, 19, 20].

This observed heterogeneity makes it difficult to screen for new mutations in the CBS gene of homocystinuric patients and their family members (potential heterozygotes). Mutation detection methods need to cover at least the entire coding sequence. This limitation applies to a number of diseases resulting from single gene defects. Screening methods such as single-strand conformation polymorphisms [21], which exhibit best results with small fragments of DNA (300–400 bp), have to be applied to multiple sections of coding sequence or replaced by other methods such as mismatch cleavage analysis [22, 23] or methods using mismatch binding proteins (mutS) [24], which permit longer stretches of DNA (1–1.6 kb) to be analysed. These methods do not allow precise information on the nature of the mismatched base(s) and since direct-sequence analysis is becoming technically less arduous and more routine, the latter technology seems likely to be a preferable screening option.

Materials and Methods

Patients

The 3 patients had been diagnosed and treatments initiated by different clinicians within Australia. Each patient had been assessed and described by the respective physician as having pyridoxine-nonresponsive homocystinuria. An in vitro culture of fibroblast cells from patient 1 was available, and lymphoblast cultures were established from blood samples from patients 2 and 3, following informed consent. In vitro cultures of these cells were grown as previously described [25, 26] in RPMI medium in a humidified 37°C, 5% CO₂ incubator.

DNA and RNA Preparation

Cultured cells, derived from patients and controls, were harvested and the DNA was isolated according to Miller et al. [27]. Total RNA was extracted from the cells by the procedure described by Chirgwin et al. [28]. Poly(A)⁺-RNA was isolated from total RNA by magnetic bead separation technology using Dynabeads oligo(dT)₂₅ as

described by the manufacturer (Dynal). For the confirmation of the detected mutations in family members, DNA was extracted from blood samples obtained by venipuncture.

PCR Amplification of CBS cDNA

Poly(A)⁺-RNA (5 µg) was reverse-transcribed with Moloney murine virus reverse transcriptase (200 U; BRL) with the conditions recommended by the manufacturer and using oligo(dT)₂₅ as a primer. The 30-µl reaction was incubated at 25 °C for 5 min followed by 42 °C for 1 h. A nested PCR strategy was employed to amplify the entire protein-coding region of the CBS cDNA. An aliquot (2 µl) of the reverse-transcriptase reaction was used as a template for an initial PCR with the sense primer (nt -94 to -76; 5'-GGAACACCAGGATCCCATG-3') and the antisense primer (nt 1817 to 1800; 5'-TGGAGGATGCTGAGCCACG-3'). The nt refers to the nucleotide position in the CBS cDNA sequence, with the A of the ATG initiation codon being +1 [3]. PCR was performed in a total reaction of 18 µl using 10 mM Tris-HCl (pH 8.6), 50 mM KCl, 2 mM MgCl₂, 0.1 mg/ml gelatin, 5% DMSO, 155 µM of each dNTP with 0.5 U *Taq* polymerase (BTQ-1, Bresatec). Thermal cycling was performed using a capillary thermal cycler (Corbett Research) with the following cycling conditions: 3 min at 95 °C, 30 s at 60 °C and 1 min at 72 °C for 1 cycle, then 15 s at 95 °C, 15 s at 60 °C and 1 min at 72 °C for 4 cycles, then 5 s at 95 °C, 5 s at 60 °C and 1 min at 72 °C for 30 cycles. An aliquot (1 µl) of the first PCR was then used as a template with identical reaction and cycling conditions with the internally nested sense primer (nt -31 to -12; 5'-GAACTGTCAGCACCATCTGT-3') and the nested antisense primer (nt 1735 to 1718; 5'-CATTGCCTGTGTTTCATCC-3'). In each PCR, 1 of the nested primers was biotinylated at the 5' terminus, which facilitated the isolation of single-stranded DNA using streptavidin-coated magnetic beads (Dynal). For amplification of portions of CBS exons 3, 9 and 12 from genomic DNA, 200 ng of DNA was used as a template in a reaction volume of 100 µl containing 67 mM Tris-HCl (pH 8.6), 6.7 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 5 mM β-mercaptoethanol, 6.8 µM Na₂EDTA, 310 µM each dNTP with 2.0 U *Taq* polymerase. The sense and antisense oligonucleotide primers used were: for exon 3, sense (nt 322 to 341; 5'-AAGTGTGAGTCTCTTCAACGC-3') and antisense (nt +9 to +25 of the 5' end of intron 3; 5'-ccaccctctggcctg-3'); for exon 9, sense (nt -36 to -14 of the 3' end of intron 8; 5'-tccacgctgacggctgtggtg-3') and antisense (nt 1023 to 993; 5'-TTGCGCGATCAGCATGCGG-GCAAAGGTGGAC-3'); the mismatch G in this antisense primer creates a *Hinf*I site with the 992C→A transversion found in patient 2; see Restriction Enzyme Analysis), and for exon 12, sense (nt 1228 to 1245; 5'-TGGCACCTCCGTGTTTCCAG-3') and antisense (nt 1347 to 1330; 5'-CACCACGGGCGCCTGGTC-3').

DNA Sequencing

Single-stranded DNA for sequencing was isolated from PCR-amplified fragments of CBS cDNA using magnetic bead technology (Dynabeads M-280 streptavidin, Dynal). The entire coding region of the CBS cDNA from each patient was manually sequenced in both directions at least twice from different batches of RNA. Direct sequencing was performed using the chain termination procedures [29] with Sequenase V2 (USB).

Restriction Enzyme Analysis

To confirm the detected mutations, segments of patient CBS genomic DNA which contained the individual mutations were amplified by PCR. These segments were digested with the appropriate

restriction endonuclease (New England Biolabs) under conditions recommended by the manufacturer and electrophoresed on a 2.5% agarose gel in 1 × TBE at 80 mA for 1 h. The DNA bands were stained with ethidium bromide and visualised under ultraviolet light.

The 992C→A mutation does not alter a restriction endonuclease site. To overcome this problem, a mismatched G was introduced near the 3' terminus of the antisense primer (see above) used to amplify a portion of exon 9. This primer flanks the 992C→A mutation and creates a *Hinf*I site in the sequence containing the 992C→A mutation. PCR fragments of 105 bp were amplified from genomic DNA from patient 2 and her parents with sense primer and the antisense primer containing the G mismatch. Carriers of the 992C→A mutation will have 2 fragments of 73 and 32 bp following digestion with *Hinf*I, whereas the wild-type PCR product will remain uncleaved.

The 430G→A and 1316G→A mutations detected in patient 3 were verified by restriction enzyme analysis with *Taq*I and *Hap*II, respectively. Individuals who carry the 430G→A mutation will have the 155-bp PCR fragment refractile to digestion with *Taq*I, whereas the wild-type allele yields digestion products of 107 and 48 bp. Carriers of the 1316G→A mutation will have a PCR fragment that is resistant to digestion, whereas the wild-type allele produces 87- and 33-bp pieces.

Expression Constructs

To construct a wild-type human CBS cDNA, we amplified a 1719-bp fragment from a control cell line which encompassed the entire coding region of the CBS cDNA. The oligonucleotide primers used in this PCR were sense (nt 1 to 16; 5'-ggaacatATGCCCTTCTGAGACCC-3') and antisense (nt 1706 to 1688; 5'-cccaagCTTCTCTCTCAATATTTCC-3'). The lower-case letters in these primer sequences represent artificial *Nde*I and *Hind*III sites, respectively. The amplified product was purified from a 1% agarose gel and cloned into the bacterial expression plasmid pT7-7 [30] following digestion with *Nde*I and *Hind*III. The entire sequence of the cloned CBS was checked by sequence analysis. To construct mutant CBS sequences, segments of the patient CBS cDNA which contain the individual mutations were amplified by PCR and used to replace the corresponding segment of normal CBS cDNA in the recombinant pT7-7/CBS plasmid. All mutant CBS constructs were checked by sequence analysis. Recombinant CBS protein was expressed in *E. coli* (strain BL21/DE3) following induction with 0.4 mM IPTG for 2 h.

CBS Assays

CBS assays were performed essentially as previously described using the *E. coli* lysates which contain the expressed CBS protein [7]. The amount of [¹⁴C]cystathionine formed from [¹⁴S]serine was determined by scintillation counting following paper chromatography. All of the expression experiments were assayed in duplicate, which agreed within ±5%. Only the mean specific activity of CBS from these duplicate assays is shown in table 2. A negative control, consisting of the parent pT7-7 expression plasmid minus the CBS cDNA insert, gave <1% of the counts found in the wild-type CBS in all assays. This background count was subtracted from the counts in the wild-type and mutant CBS samples.

Western Blot Analysis

Aliquots (5 µl) of protein samples (corresponding to 1 µg protein) were subjected to SDS/PAGE for 10 min at 50 mA then for 1 h at 100

mA [31]. Following transfer to a nitrocellulose membrane at 100 mA for 2.5 h in 39 mM glycine, 48 mM Tris-HCl (pH 8.3), 0.037% SDS and 20% methanol, the blots were incubated with rabbit antihuman CBS and then with goat antirabbit IgG peroxidase conjugate (Cap-pel). Immunological detection of CBS protein was performed using the Enhanced Chemiluminescence kit as described by the manufacturer (Amersham), and exposure to film for 20 h.

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