

Detection of a novel deletion in the cystathionine β -synthase (CBS) gene using an improved genomic DNA based method

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Abstract We elucidated the intron-exon boundaries of the 15 coding exons of the human cystathionine β -synthase (CBS) gene in order to establish an improved method based on PCR and direct sequencing for detection of CBS mutations. Using this method we identified the pathogenic mutations in two Danish siblings with CBS deficiency. Patients were compound heterozygotes: we detected the 833T $\rightarrow$ C mutation and a novel 22 bp deletion of exon 4 (493–514del) that introduces a frameshift and a stop codon immediately after the deletion. The deletion resulted in no detectable mRNA from this allele, as assessed by sequencing of cDNA. The established method represents an improvement of the existing method based on sequencing of cDNA because it permits the detection of mutations within the entire coding region of the CBS gene from a peripheral blood sample, including splice mutations and mutations resulting in the lack or a reduced amount of transcript.

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1. Introduction

Homocystinuria due to cystathionine β -synthase deficiency (CBS; EC 4.2.1.22) is the most common inborn error of sulphur amino acid metabolism. It is an autosomal recessive disease with an estimated incidence of approximately 1:200 000. The clinical manifestations include premature atherosclerosis and early thromboembolism, mental retardation, psychotic behaviour and seizures, optic lens dislocation, osteoporosis and skeletal abnormalities. Vascular complications comprise the major cause of death [1].

The human CBS gene maps to chromosome 21q22.3 [2] and encodes a protein of 551 amino acids [3]. The CBS enzyme is a cytosolic homotetramer of 63 kDa subunits [4] that catalyses the condensation of homocysteine with serine, forming cystathionine. The enzyme requires pyridoxal 5'-phosphate (PLP,

the active form of vitamin B₆) as co-factor, and patients are classified as pyridoxine-responsive or non-responsive according to the homocysteine lowering effect of pyridoxine treatment. Usually, pyridoxine-responsive patients tend to have a milder clinical phenotype than do pyridoxine non-responsive patients. However, the severity of the disease varies strongly and can vary from normal to severe in patients with no measurable CBS activity although many of these patients are pyridoxine-responsive. Furthermore, variation in phenotypes between patients with the same genotype is observed [5], demonstrating the lack of a well-defined correlation between genotype and phenotype in CBS deficiency.

The cDNA sequence of the CBS gene was published in 1993 [3]. To date, more than 80 mutations have been detected in the CBS gene in CBS-deficient patients (Kraus et al., unpublished), the majority being missense mutations. Using different *in vitro* expression systems, the pathogenic property has been determined for some of the mutations [5,6]. So far, analyses of these defects have mainly been performed using fibroblast-derived cDNA. No results of a complete screening of the CBS gene on genomic DNA have as yet been published, although such an analysis would facilitate detection of genetic variations such as splice mutations and mutations leading to a null allele.

We therefore established a screening method based on genomic DNA for detection of mutations in the CBS gene. This was made possible by the elucidation of the intron-exon boundaries of the 15 coding exons. Knowledge of those boundaries enabled the design of specific intronic primers for amplification of all coding exons. These PCR products were subsequently sequenced directly. We here report the detection of a novel deletion and a previously identified missense mutation in two Danish siblings with partly pyridoxine-responsive homocystinuria due to CBS deficiency. Using the conventional method based on sequencing of cDNA only the missense mutation would have been identified.

2. Materials and methods

2.1. Patients

Patients 1 and 2 were two Danish siblings with severe hyperhomocysteinemia (Fig. 1). No diagnosis of CBS deficiency had been made prior to referral. Patient 1 is a 34-year-old male who suffered a spontaneous deep venous thrombosis (DVT) at the age of 19, and recurrence at the age of 28 years. Since the first thrombotic episode he has been treated for symptoms of psychosis. At present, treatment consists of clozapine. Furthermore, specialist eye investigation revealed strabismus, myopia and astigmatism, but no signs of dislocated optic lenses. There is no mental retardation or radiographic signs of osteoporosis. Patient 2 is a 22-year-old female who developed a spontane-

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Abbreviations: CBS, cystathionine β -synthase; DVT, deep venous thrombosis; tHcy, total homocysteine in plasma; PLP, pyridoxal 5'-phosphate; AdoMet, S-adenosylmethionine

Nucleotide sequence data reported in this paper together with the additional intron sequences obtained have been submitted to the EMBL, GeneBank and DDBJ data bases, and have been assigned the accession numbers AJ005133–AJ005156.

ous incident of DVT, complicated by pulmonary embolism, at the age of 21 years, using no hormonal contraceptives. She has never presented with mental retardation or other sequelae of CBS deficiency, and her bone structure as assessed by X-ray is normal.

2.2. Determination of total homocysteine in plasma

Total homocysteine in plasma was measured by gas chromatography-mass spectrometry, using stable isotope dilution. Samples were collected in tubes containing heparin as an anticoagulant and with sodium fluoride added to a final concentration of 4 g/l [7]. Plasma was separated by centrifugation within 2 h.

2.3. Isolation of RNA, cDNA synthesis and DNA isolation

RNA was isolated from phytohaemagglutinin-stimulated (72 h) lymphocytes using a modified phenol-chloroform-based method, the RNeasy B method (WAK-Chemie Medical GmbH, Bad Homburg v.d.H., Germany). cDNA synthesis was performed using an oligodT₍₁₈₎ primer and the First Strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). For analysis of genomic DNA, we isolated DNA from 300 µl EDTA-stabilised blood using the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA).

2.4. PCR amplification of CBS genomic DNA

For elucidation of the intron-exon boundaries, PCR was carried out in a volume of 50 µl containing ~200 ng of isolated DNA, 7.5 pmol of each primer, 120 µM of each dNTP, 6% DMSO, 2.5 units *Taq* and Pwo polymerase (Expand long template PCR system, Boehringer Mannheim) in the buffer supplied with the enzyme. Amplifications were performed in an OmniGene automatic temperature cycler (Hybaid, Middlesex, UK) using the following conditions: 2 min at 94°C, 1 min at 65°C and 2 min 30 s at 68°C for one cycle followed by 35 cycles of 94°C for 20 s, 65°C for 30 s and 68°C for 2 min 30 s, using the tube control mode of the thermal cycler.

The exon-specific PCR analyses were carried out in a volume of 50 µl containing ~200 ng of isolated DNA, 7.5 pmol of each primer (Table 3), 120 µM of each dNTP, 6% DMSO, 1 unit *Taq* polymerase (Amplitaq Gold, Perkin Elmer) in the buffer supplied with the enzyme. Amplifications were performed in an OmniGene automatic temperature cycler using the following conditions: 10 min at 94°C, 1 min at the appropriate annealing temperature (Table 3) and 45 s at 72°C for one cycle followed by 35 cycles of 94°C for 30 s, annealing for 45 s and 72°C for 45 s, using the tube control mode of the thermal cycler.

2.5. Sequencing of PCR products

We used the prism AmpliTaq FS DNA polymerase dye primer sequencing kit (–21M13 and M13 reverse) (Perkin Elmer Cetus, Norwalk, CA) for sequencing of the PCR products. PCR products were subjected to automated cycle sequencing using a Catalyst 800 Molecular Biology LabStation (Perkin Elmer Cetus). The following cycle conditions were used: 15 cycles of 95°C for 20 s, 55°C for 50 s and 68°C for 60 s followed by 15 cycles of 95°C for 25 s and 68°C for 60 s.

Sequencing products were run in a 4.75% polyacrylamide gel using an ABI Prism 377 DNA Sequencer (Applied Biosystems).

2.6. CBS enzyme assay

Skin biopsy fibroblast cultures were established from both patients for enzyme activity measurements according to [8]. CBS activity was measured in patient fibroblasts as described elsewhere [9]. Assays were performed with and without addition of 1 mM PLP to the incubation mixture, and after stimulation with 100 µM *S*-adenosylmethionine (AdoMet).

3. Results

3.1. Elucidation of the intron-exon boundaries of the CBS gene

Using primers specific to the human CBS cDNA sequence, we amplified several PCR products containing intronic sequences between contiguous exons, using M13 tailed primers. These fragments were sequenced directly in both directions using M13 sense or antisense sequencing primers, and the intron-exon boundaries of the 15 coding exons were determined (Table 1). At least 30 bp of each, or the entire intron sequence is listed in Table 2.

3.2. Amplification and sequencing of genomic DNA

Exon-specific primers were designed from the intron sequences for amplification of the 15 CBS exons (Table 3). By designing intron-specific primers, amplification of the intron-exon boundaries was made possible. This enables the detection of splice mutations in the subsequent sequencing analysis. The addition of an M13 tail, sense and antisense, respectively (Table 3) to the 5' end of each primer, allows sequencing of all 15 exons using the same sequencing primers. Following PCR amplification of exons 1–15 from genomic DNA, the PCR products were sequenced directly using a semi-automated sequencing procedure and M13 dye primers.

3.3. Detection of CBS mutations from patients

The established method was used for detection of mutations in the CBS gene in patients 1 and 2 that were referred for thrombophilia investigation at our department. Both had severe hyperhomocysteinemia (> 100 µmol total homocysteine/l plasma) [11]. Initially, the search for CBS mutations was performed by the conventional method involving sequencing of

Table 1
Intron-exon boundaries of the human cystathionine β-synthase gene

Exon/Intron No.	5' Exon sequence	Exon size (bp)	3' Exon sequence ^a	5' Intron splice site (donor)	Intron size ^b	3' Intron splice site (acceptor)
1	ATGCCTTCTG	209 ^c	ACACTGCCCC (209)	gtaagt	3367	tttcag
2	GGCAAAATCT	107	TGTGAGCTCT (316)	gtgagt	2132	tcccag
3	TGGCCAAGTG	135	GGGAACACCG (451)	gtgggt	548	cctcag
4	GGATCGGGCT	80	CTCCGAGAAG (531)	gtgggt	94	ctgcag
5	GTGGACGTGC	135	CCTAGACCAG (666)	gtgagg	114	atgcag
6	TACCGCAACG	70	CAGTGTGATG (736)	gtgcgt	1212	caccag
7	GGAAGCTGGA	92	TGGATGCAGG (828)	gtgagt	821	ctgcag
8	ATCATTGGGG	126	GGACAGGACG (954)	gtagg	556	gtcag
9	GTGGTGGACA	85	CTGCTGTGCG (1039)	gtgagt	1761	ctgcag
10	GTGGCAGTGC	106	GGAACACAT (1145)	gtaaga	1132	ccgcag
11	GACCAAGTTC	78	AGAAGCCCTG (1223)	gtaaga	257	cctcag
12	GTGGTGGCAC	135	ATGAGGCGGG (1358)	gtcagt	580	ccacag
13	GGTAATCCTG	109	GTTCAAACAG (1467)	gtaccc	1258	ttgcag
14	ATCCGCCTCA	85	CAGATCCAGT (1552)	gtgagt	2076	cctcag
15	ACCACAGCAC	101 ^d	(GGACCAGAAG) (1653)			

^aNumbers in parentheses indicate cDNA positions [3].

^bAccording to [10].

^cThe coding part of exon 1 contains 209 bp.

^dThe coding part of exon 15 contains 101 bp.

Table 2
CBS intron sequences

Intron	5' Intron sequence (50 bp listed) 5' to 3'	3' Intron sequence (50 bp listed) 5' to 3'
1	gtaagtcctgcttttgcacacagagggtgatccataacctg-gagggca	tttctgaataattgtggactcctctgtttcag
2	gtgagtgccctggctttggagaggggttccctccatcta- cactgggctcc	tgggggggtgggtcaggggtccctcctgtgattcatctctgcctccag
3	gtgggtgccaggccagaggggtggccggggtggtgtcccc- taccat	gcagggttgggggtcactggccctctcaccctctgtgtgccctcag
4	gtgggtggcggtggccagggggtgcatagggcaggggatga- gggtgc ^a	ctgggcccaggcgaaggtgcagggccacctttccctcctgcag ^a
5	gtgaggcacctggggcctggagacagggcatccctaggaggg- ctcggtcagg ^a	gatgtgggggtgactgaggtgtcaggggtcagctcagggctctgtctc- tttctatgcag ^a
6	gtgctgcccctgttccatcttgattgcatt	cagggaccaagaagttatccgtccaccag
7	gtgagtggtgtggccgggcccggagaggggc	ctggccttgagccctgaagccgcgcctctgcag
8	gtaggtcagtgccagagcccgccacagtgccggtgcagcctg- taactacccc	cgtgggagcggctcccacgctgacgggtgtggtggggctcctgctcag
9	gtgagtggtggcgggcacgggggtatggg	ggcggtctgactcgtggccctctctgcag
10	gtaagacacgggttccctcccaggtcctctctc- catcctgccacc	cgggggcccgggtggggtcgcgggactcgggtgactcccccatcccgag
11	gtaagaccgctcgccggagacccgctgctctgtgctggccctg- cccagtg	cgagagcgtttgtccttactgacccccgcacctgcctcttccctcag
12	gtcagttcagccccctgctcagagct- cagtcctgggttctgtctgccagctcc	tgggaggggtgaggtatgagcgtgacctctgctgcccccgctccacag
13	gtaccagtcacctacaggcagctcaaacagatgctgcagtcacc- tacagg	aaactcgtggggccatgttccccctgccactgaccacgcttcccttgcag
14	gtgagtggggcccctgctctgtgctggtgggttctcactgggt- caggccac	accagcctccacaggcactgcaaaccactgcctcgttctccctcag
3'UTR	agtccggagcgtggggcgtgtggagcgggcccgcacccttg- cccactt	

^aThe entire intron sequence is listed.

cDNA. This analysis revealed homozygosity for the 833T→C mutation (I278T) [12]. However, amplification of exon 8 and mutation-specific restriction enzyme analysis of genomic DNA [13] showed that both patients were heterozygous for the mutation (data not shown), indicating that the sequencing analysis using the conventional method comprised only one allele. cDNA being synthesised from only one allele can be due to lacking transcription or an unstable transcript from the second allele, or preferential PCR amplification of one allele, most likely due to low levels of mRNA transcribed from the second allele and subsequent competition between the alleles during PCR. To determine a presumed second disease-causing mutation on the allele not seen in the obtained cDNA sequence, we analysed genomic DNA comprising the entire coding region of the CBS gene as described above. The sequenc-

ing analysis revealed a novel 22 bp deletion of exon 4 (493–514del), resulting in the introduction of a stop codon immediately after the deletion. Gel electrophoretic analysis of the PCR products prior to sequencing also showed that PCR products of exon 4 from both patients consisted of two bands of different mobility (Fig. 1). Furthermore, genomic DNA sequencing analysis showed that the patients were heterozygous for the 833T→C mutation in exon 8 in accordance with the restriction enzyme analysis, and both were heterozygous for the 699C→T polymorphism (Y233Y) and heterozygous for the 1080T→C polymorphism (A360A) [3]. The cDNA sequence, however, showed that both patients were homozygous for the 699C→T polymorphism and the 1080T→C polymorphism which further confirms the absence of one allele in the cDNA pool.

Table 3
Primers used for amplification of exon 1–15 of the CBS gene^a

Exon	Sense primer (5'→3')	Antisense primer (5'→3')	Annealing temperature (°C)
1	GGAACCCACAGCATCCGAG	TCAGCCCTCTTGATCAAAAGC	62
2	TTCTGAATAATGTGGACTCC	GAAGCCCTCTCCAAAGCC	58
3	GGGTGAGCAGGAATCAATGGG	CTGGCCACCCCTCTGGGC	62
4	GGGCCCCCTCACCCCTCTG	TGACCCCGCCCTTGGCCAC	64
5	GGTGCAGGCCACCGCTTTCCC	GGAATGCCTGTCTCCAGGCCCC	64
6	GCTCACGGGCTCTGCTCTC	ACGCAATCAAGATGGACAGAGG	64
7	CATTTACCCAAGAAGTTATCCGTCC	CCTCTCCGGCCCGGCCACAC	58
8	CTTGAGCCCTGAAGCCGCGCC	GTGGCCGGGCTCTGGACTCG	64
9	CTGTGGTGGGGTCTCTGCT	CCGTGCCCCGCCACCCAC	64
10	GCGCGTCTGACTCGTGGCCC	GCAGGATGGAGAGGACAGGG	64
11	CGGGGACTCGGTGACTCCC	CAGCGGTCTCCGGCCGAG	62
12	CCCCGACCTGCCCTCTTCC	CTGAGCTCTGAGCAGGGGCTG	64
13	GTGCTGACCCCTGCCTGCCC	CTGCCTGTAGGTGACTGGGTAC ^b	68
14	CATGTTCCCCCTGCCACTGAC	CGCAGGGGATGGGGGGCC	64
15	CCCATGTTGCACAGGTGCC	CACACGCCCGAGCGCTCCG	64

^aTo the listed primers, an M13 sequence was added 5' to the gene-specific sequence. These are: M13-21: TGTAACACGACGGCCAGT (sense primer) and M13 rev: CAGGAAACAGCTATGACC (antisense primer).^bThis primer was designed to span the intron boundary because a large number of repetitive sequences is present in intron 13.

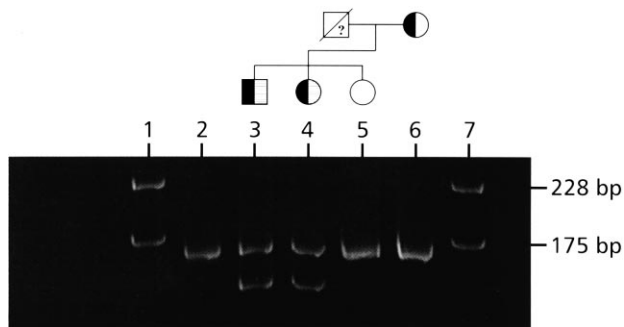


Fig. 1. Genotypes of investigated family members. Upper panel shows the pedigree of the family. 3: patient 1; 4: patient 2; 5: sister; 6: mother; filled figure: 833T→C mutation; hatched figure: 22 bp deletion. Lower panel shows the confirmation of the presence of the 22 bp deletion by gel electrophoretic analysis of PCR products of exon 4. Lane 1: DNA size marker; lane 2: control; lane 3: patient 1; lane 4: patient 2; lane 5: sister; lane 6: mother; lane 7: DNA size marker.

The asymptomatic sister and the mother of the patients were subsequently investigated. Both displayed normal total homocysteine in plasma (tHcy), 9.1 and 8.0 μM respectively. Sequence analysis of genomic DNA showed a normal genotype in the sister (Fig. 1). The polymorphic 699C→T substitution was not present, whereas she was homozygous for the polymorphic 1080T→C substitution. The mother was heterozygous for the 833T→C mutation, heterozygous for the 699C→T polymorphism and homozygous for the 1080T→C polymorphism. No samples were available for analysis of the genotype of the father who died several years ago from myocardial infarction at the age of 58 years. The results of the genetic analysis indicate that the 22 bp deletion of exon 4 present in the patients was the paternal CBS allele. By combining the sequences of cDNA and genomic DNA, we could determine the haplotype of the investigated alleles. The haplotype of the maternal disease-causing allele was determined to be: 699T, 833C and 1080C, and the haplotype of the paternal allele, presumably carrying the 22 bp deletion, was: 699C, 833T and 1080T. The two normal alleles of the sister both had the haplotype: 699C, 833T and 1080C.

3.4. Measurement of CBS activity in fibroblasts

CBS enzyme activity was measured in extracts of cultured fibroblasts. Without addition of PLP, activities were 0.46 and 0.66 nmol cystathionine/mg protein/h, respectively (normal activity range: 2.3–18.2). In the presence of 1 mM PLP, the activity was 0.92 and 1.04, respectively (normal activity range: 3.5–22.3). Addition of AdoMet even lowered the activity to 0.16 and 0.55, whereas in controls the activity is increased three-fold. On admission, tHcy was 295 μM and 257 μM , respectively. Folic acid therapy using 5 mg daily for 2 weeks did not change the level of tHcy, whereas pyridoxine therapy using 250 mg/day in combination with 5 mg folic acid for 6 additional weeks resulted in lowering of tHcy to 80 μM and 37 μM , respectively. Although reduced, tHcy levels after pyridoxine treatment were still within the range of intermediate hyperhomocysteinemia (30 μM < tHcy < 100 μM) [11], and the patients were considered partly pyridoxine-responsive. Treatment with betaine is planned but results are not available at the present time.

4. Discussion

Determinations of the genetic defects that cause homocystinuria due to CBS deficiency have revealed a wide heterogeneity of disease-causing mutations. Because of the heterogeneity of mutations in CBS deficiency, detection of the molecular defects that cause CBS deficiency requires appropriate screening methods including the entire coding region of the gene. This analysis is essential for improved understanding of the molecular mechanisms involved in CBS deficiency, and for further studies of genotype-phenotype relationships. In addition, detection of disease-causing, in several cases private, mutations can provide useful information for the treatment of CBS-deficient patients, which includes treatment with one or several of the homocysteine-lowering agents pyridoxine, betaine and folic acid [14].

To obtain an improved and facilitated method for mutation analysis, we elucidated the intron-exon boundaries of the CBS gene. This has enabled the design of specific primers that are each located in an intron, immediately 5' or 3' to the exon in question. This enabled the detection of mutations within the entire coding region of the CBS gene, including the intron-exon boundaries. We compared the established method with the conventional method to detect the disease-causing mutations in two Danish siblings with severe hyperhomocysteinemia. Genetic analysis using direct sequencing of cDNA indicated homozygosity for the 833T→C mutation. Subsequent analysis of genomic DNA by PCR amplification of exon 8 and mutation-specific restriction enzyme analysis revealed heterozygosity for the mutation, indicating that only one allele had been expressed and sequenced. Using the method described in this study, heterozygosity for the 833T→C mutation was confirmed. Furthermore, on the second allele we detected a novel 22 bp deletion in exon 4 (493–514del), followed immediately by an introduced nonsense codon. The mother of the patients was heterozygous for the 833T→C mutation, whereas the sister had none of the mutations. Presumably, the deleted allele was inherited from the father. The deletion, resulting in a stop codon, in exon 4 obviously makes the allele undetectable by the conventional method. Generally, the closer a nonsense codon resides relative to the initiation codon, the more likely the nonsense codon will reduce mRNA abundance [15]. Likely, the mRNA from the deleted allele is unstable and degraded rapidly.

Our patients were considered partly pyridoxine-responsive according to Wilcken et al. [16]. In addition, the fibroblast enzyme activity of the patients was low, but not zero, and no stimulation with AdoMet was obtained. This is in accordance with results of Kluijtmans et al. [17] who also detected CBS activity in the heterozygous range and no stimulation with AdoMet in a CBS-deficient patient. Often, no fibroblast residual activity is present in pyridoxine non-responsive patients, whereas pyridoxine-responsive patients often have some residual activity [18]. Conflicting data on the pyridoxine responsiveness of the 833T→C mutation have been presented. Hu et al. [19] reported that the 833T→C mutation usually confers pyridoxine responsiveness, whereas results from a yeast functional assay indicated pyridoxine non-responsiveness [20]. This may be due to different folding and processing mechanisms in yeast. It has been observed that different combinations of allelic variants can result in a modification of the

phenotype conferred by one variant when combined with a second allelic variant [5].

We determined the haplotypes of the normal and the pathogenic alleles to deduce whether the 699C→T or the 1080T→C polymorphism was linked to the 833T→C mutation. In this family the 833T allele was always associated with the 699C variant, whereas the 833C allele co-segregated with the 699T variant. The 1080T variant was only present on the allele containing the 22 bp deletion. Furthermore, the haplotype analysis confirmed the lack of cDNA sequence from the allele with the deletion.

Taken together, our established method has several advantages compared to the existing methods based on analysis of cDNA from fibroblasts or lymphocytes. The analysis of genomic DNA can be performed using a small amount of peripheral blood (300 µl) as sample material. Furthermore, the use of intronic primers permits the detection of splice mutations, of which only one has previously been reported among CBS-deficient patients [5]. It is well known that many genetic diseases are attributable to mutations that result in splicing defects [21]. Consequently, further analysis of genomic DNA from CBS-deficient patients in whom only one allele was seen in the cDNA sequencing, as reported [22], may reveal the presence of a splicing defect on the other allele. Alternatively, mutations resulting in a null allele or an unstable and rapidly degraded mRNA could be present. Relatively few splice mutations and deletions have been detected among CBS-deficient patients. This may be due to the lack of appropriate screening methods until now. Automated sequencing is made possible by the use of the Catalyst Molecular Biology LabStation and by using only two different sequencing primers for all exons. This is time-saving and facilitates the sequencing procedure. In addition, the presence of frequently occurring CBS mutations can now be investigated in a large number of patients in routine analysis for detection of heterozygotes or homozygotes for CBS deficiency. The designed intronic primers reported here can be applied to other screening methods as well, such as single-strand conformation polymorphism analysis. The amplified PCR products are small (160–300 bp) and thus well suited for this kind of mutation analysis.

The present method permits a facilitated and improved detection of heterozygotes for CBS deficiency. To date, determination of carrier state has been performed by measurement of fibroblast CBS activity or methionine loading tests [23]. Now, we can perform the analysis using a peripheral blood sample. The facilitated detection of heterozygotes for CBS deficiency may be valuable in the further elucidation of a possible implication of heterozygosity for CBS deficiency in occlusive peripheral and cerebral arterial disease [24]. Especially, the characterisation of obligate heterozygotes is facilitated: when the pathogenic mutations have been detected in a patient with CBS deficiency, it is sufficient to analyse for the presence of these mutations in the parents to determine whether they are carriers of one of those mutations, or whether the detected mutations are de novo mutations.

In conclusion, by elucidating the intron-exon boundaries of the 15 coding CBS exons we have established a novel screening method based on genomic DNA for mutation analysis in the CBS gene. Using this method, we have identified a novel 22 bp deletion of exon 4, which was undetectable by sequencing of cDNA. Thus, our method is preferable to the existing screening methods because a minute amount of sample is

used, and the sensitivity is higher than that of the conventional method because the detection of splice mutations and mutations resulting in no or a low amount of transcript is enabled.

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