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High Prevalence of a Mutation in the Cystathionine β-Synthase Gene

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Summary

We found that a mutation previously described by Sebastio et al., involving a 68-bp insertion in the coding region of exon 8 of the cystathionine-β-synthase (CBS) gene in a single patient with homocystinuria, is highly prevalent. In our control population, 11.7% (9/77) of the individuals were heterozygous carriers of this mutation. In contrast to the previous report, which assumed that the 68-bp insertion introduced a premature-termination codon and resulted in a nonfunctional CBS enzyme, we found that the presence of this mutation is not associated with hyperhomocysteinemia. Assay of CBS activity in transformed lymphocytes from individuals who were heterozygous or homozygous for this mutation showed normal activity. Furthermore, reverse-transcripion–PCR showed that individuals carrying this mutation have normal size mRNA. Our results suggest that the insertion creates an alternate splicing site, which eliminates not only the inserted intronic sequences but also the $T_{833}C$ mutation associated with this insertion. The net result is the generation of both quantitatively and qualitatively normal mRNA and CBS enzyme. Although the mutation does not seem to affect the activity of the CBS enzyme, the prevalence is somewhat increased in patients with premature coronary-artery disease, although the difference is not statistically significant.

Introduction

Many case-control (Boers et al. 1985; Clarke et al. 1991; Engbersen et al. 1995) and several large epidemiological studies (Stampfer et al. 1992; Selhub et al. 1995) have demonstrated that mild hyperhomocysteinemia is an important risk factor for cerebral artery disease, peripheral artery disease, and coronary-artery disease (CAD). In the absence of overt deficiencies of vitamin B12, folate, or pyridoxal phosphate, the most important causes of mild hyperhomocysteinemia are genetic defects affecting either the enzyme cystathionine β-synthase (CBS; E.C.4.2.1.22) in the transsulfuration pathway or 5,10-methylene tetrahydrofolate reductase (E.C.1.5.1.20), a critical enzyme in the remethylation of homocysteine to methionine.

The screening for mutations in homocystinuric patients with deficiency of CBS enzyme activity has led to the identification of ≥17 mutations in the CBS gene (Kraus 1994; Kluijtmans et al. 1995; Sebastio et al. 1995; Sperandeo et al. 1995). Two mutations, a $T_{833}C$ transition that substitutes threonine for isoleucine at codon 278 and a $G_{919}A$ transition that substitutes serine for glycine at codon 307, are found to be most prevalent (Hu et al. 1993; Shih et al. 1995; Tsai et al. 1996*a*). The majority of these mutations were found by use of reverse-transcription–PCR (RT-PCR) of mRNA coding for the CBS enzyme in cultured fibroblasts. The need for cell culture makes this method laborious and not suitable for screening a large number of individuals.

In the current study, we used genomic DNA isolated from peripheral blood and primers complementary to intronic sequences, to screen for mutations in the CBS gene by simple PCR-based methods. In studying a group of patients with premature CAD, we found an unexpectedly high prevalence of the mutation involving a 68-bp insertion in the coding region of exon 8 of the CBS gene. The mutation does not seem to affect the activity of the CBS enzyme. Screening of controls showed that the mutation also was prevalent in the control population. The prevalence of this mutation was somewhat greater in patients with premature CAD than in the control population, although the difference did not reach statistical significance.

Subjects and Methods

Study Population

We screened 182 patients (146 males [age range 34–55 years; mean age 47.6 years] and 36 females [age range 36–55 years; mean age 48.6 years) with premature CAD as documented by angiographically proved atherosclerosis and/or one or more episodes of myocardial infarction or coronary-artery bypass graft before the age of 55 years. For the control group, we recruited 77 apparently healthy individuals (39 males [age range

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34–60 years; mean age 45.4 years] and 38 females (age range 39–57 years; mean age 46.6 years). There was no statistical difference in the mean ages of the patients and controls. Patients and controls were of mixed ancestry common to the upper midwestern region of the United States and belonged to no particular ethnic group. This study was approved by the Human Studies Committee of the University of Minnesota Institutional Review Board.

Methionine Loading and Assay of Plasma Total Homocysteine (tHcy)

A fasting heparinized blood sample was drawn, and plasma was separated in $< \frac{1}{2}$ h for the measurement of baseline tHcy level. Methionine (100 mg/kg body wt) was administered orally, and a second blood sample was collected 4 h after loading. Both samples were assayed for tHcy concentrations, as described elsewhere (Tsai et al. 1996*a*): protein-bound homocysteine and homocysteine in the form of homocystine and mixed homocysteine/cysteine disulfide were reduced by 3% DTT, deproteinized by sulfosalicylic acid, and quantitated on a Beckman 6300 Amino Acid Analyzer.

CBS Assay

Since we found no CBS activity in normal lymphocytes, we performed the assay on transformed lymphocytes obtained from fresh acid-citrate-dextrose–anticoagulated blood. We transformed the lymphocytes into lymphoblastic cell lines by infecting them with Epstein-Barr virus in the presence of 0.5 μg cyclosporine A/ml, as described elsewhere (Anderson and Gusella 1984). After transformation of cell lines was complete, an aliquot containing $12–15 \times 10^6$ cells/ml was suspended in 100 mM Tris-HCl buffer (pH 8.3) and was sonicated, and protein was determined. CBS enzyme activity of the lymphocytes was assayed in a reaction mixture containing ^{14}[C]-serine, S-adenosyl-methionine, pyridoxal phosphate, serine, DL-homocysteine, cystathionine, and propylglycine, as described elsewhere (Tsai et al. 1996*a*). CBS activity was measured as the rate of condensation of serine and homocysteine to cystathionine; the radioactive cystathionine formed was quantitated by liquid scintillation after separation by thin-layer chromatography.

Mutation Analysis

Genomic DNA was extracted from peripheral leukocytes isolated from acid-citrate-dextrose–anticoagulated blood by use of a commercially available DNA isolation kit (Puregene; Gentra Systems). A 184-bp DNA fragment containing exon 8 of the CBS gene was selectively amplified by PCR as previously described by Tsai et al. (1996*b*). PCR reactions were performed with ~200 ng of genomic DNA, 2.5 units of AmpliTaq® DNA polymerase (Perkin Elmer Cetus), 10 mmol Tris (pH 8.3)/liter, 1.5 mmol $MgCl_2$/liter, 50 mmol KCl/liter, 0.2 mmol of all four deoxynucleotide triphosphates/liter, and 0.2 μmol of each primer/liter (sense, 5′-CTGGCCTTGAGCCCTGAA-3′, derived from intron 7; and antisense, 5′-GGCCGGGCTCTGGACTC-3′, derived from intron 8), in a volume of 100 μl. After denaturation at 95°C for 3 min, temperature was cycled 30 times (at 95°C for 1 min, at 60°C for 1 min, and at 72°C for 2 min), followed by extension at 72°C for 3 min to amplify the target DNA. The PCR products were electrophoresed on a 2% low-melting-point agarose gel (NuSieve GTC; FMC Bioproducts) containing ethidium bromide and were visualized on a UV transilluminator (Fotodyne). To determine the presence or absence of the $T_{833}C$ mutation, the PCR product of exon 8 was digested with *Bsr*I restriction enzyme according to the manufacturer's (New England BioLabs) instructions: 4 μl of PCR product was digested with 2.5 U of *Bsr*I enzyme and 1 μl of buffer, at 65°C for 4.5 h, and was visualized after electrophoresis on a 12% polyacrylamide gel (2%C) stained by a silver-staining method.

RT-PCR

Total RNA was extracted from immortalized lymphocytes by use of a commercially available RNA isolation kit (PUREscript; Gentra Systems). Synthesis of cDNA was performed with 5 mg of total RNA by reverse transcription according to the manufacturer's (Perkin Elmer) protocol.

To obtain adequate amplification of the RNA, we performed two PCR reactions with two sets of nested primers and amplification conditions as described above for exon 8. For the first PCR, 3 ml of total cDNA product was amplified in a 50-ml volume, by use of 2.5 units of AmpliTaq DNA polymerase, 1 mmol $MgCl_2$/liter, and 0.1 μmol of each primer/liter (sense, 5′-CTGAAGAACGAAATCCCCAA-3′, derived from exon 5; and antisense, 5′-GCCTCCTCATCGTTGCTCTT-3′, derived from exon 9). A 364-bp product was obtained. For the second PCR, 3 ml of the amplified product from the first PCR was amplified in a 50-ml volume, by use of 0.05 ml of all four deoxynucleotide triphosphates, 2.5 units of AmpliTaq DNA polymerase, 1 mmol $MgCl_2$/liter, and 0.05 μmol of each primer/liter (sense, 5′-CACCATCACGGGCATTGCCA-3′, derived from exon 7; and antisense, 5′-TCTGCTCCGTCTGGTTCAGCT-3′, derived from exon 8). The final PCR product was 118 bp.

Direct Nucleotide Sequencing

PCR products obtained by RT-PCR were gel purified and were sequenced in both the forward and reverse direction, by use of the Applied Biosystems (ABI) Prism™ Dye Terminator Cycle Sequencing Ready Reac-

Table 1

Prevalence of 68-bp Insertion in Controls and in Patients with Premature CAD

	No. (%) with 68-bp Insertion		
Group (*n*)	NN	NM[a]	MM
Controls (77)	68 (88.3)	9 (11.7)	0 (0)
Patients (182)	152 (83.5)	29 (15.9)	1 (.5)

[a] $P = .16$ for comparison of the two proportions in the two groups.

tion Kit with AmpliTaq DNA Polymerase FS (Perkin Elmer) on an ABI 373 DNA Sequencer.

Results

Identification of Mutation

By means of primers chosen from sequences in introns 7 and 8, as described in Subjects and Methods, a 184-bp DNA fragment containing exon 8 was amplified selectively by PCR and was visualized on agarose gel containing ethidium bromide. In 29 of the 182 patients and in 9 of the 77 controls (table 1), amplification resulted in a larger-molecular-weight, more slowly moving band in addition to the expected 184-bp product (fig. 1*A*). DNA from one patient showed only the more slowly moving band.

We isolated this more slowly moving band in six of the individuals, and, on DNA sequencing, found that in all six cases this product represents exon 8 plus a 68-bp insertion between nucleotides 843 and 844. As indicated in figure 2*A*, this insertion consists of 53 bp of intron 7 and 15 bp of exon 8. Figure 2*B-1* and figure 2*B-2* compare the sequence of exon 8 from a normal allele (fig. 2*B-1*) with that of an individual with the 68-bp insertion (fig. 2*B-2*). Within the insertion sequence (fig. 2*B-2, shaded box*), the 15 bp at the 3′ end are identical to the first 15 bp of exon 8 of a normal allele (nucleotides 829–843). The 53 bp of the inserted sequence at the 5′ end show a large degree of sequence homology with the 55 nucleotides of the 3′ end of intron 7 of a normal allele. Compared with the sequence of intron 7 of a normal allele, the insertion has two missing nucleotides—thymine and guanine, nucleotides 51 and 52, respectively, as counted from the 3′ end of intron 7 (see fig. 2*B-1*, where missing nucleotides are indicated by a dot [·] above the nucleotides)—and two nucleotides that are substituted—thymine for cytosine at nucleotide 45 and guanine for cytosine at nucleotide 30, from the 3′ end of intron 7 (both indicated by a dot [·] above the nucleotides in fig. 2*B-1*). Sequencing showed that the T→C transition at nucleotide 833 (indicated by the asterisk [*] in fig. 2*B-2*) is present in individuals with the insertion. However, within the insertion sequence, there is no substitution of nucleotide 833.

To determine whether all individuals with the 68-bp insertion carried the $T_{833}C$ transition in the same allele that included the 68-bp insertion, the PCR product of exon 8 was digested with *Bsr*I restriction enzyme. The T→C transition at nucleotide 833 creates a new *Bsr*I digestion site (fig. 2*B-2, underlined*). Before digestion, DNA from an individual without the insertion shows a 184-bp product, whereas that from an individual heterozygous for the insertion shows a 252-bp band in addition to the expected 184-bp product. On digestion, the 252-bp product is cleaved to form 209- and 43-bp fragments; however, the 184-bp band is not cleaved. All individuals carrying the insertion had this characteristic pattern; thus the T→C transition at nucleotide 833 of the CBS gene is a consistent feature present in the same allele that includes the 68-bp insertion.

RT-PCR

Figure 1*B* shows RT-PCR results obtained across the insertion region, by amplification of RNA from a control, a patient heterozygous, and a patient homozygous for the insertion. A single band of 118 bp was obtained in all cases. The band was gel purified and sequenced. Sequencing showed that mRNA did not contain the 68-bp insertion or the $T_{833}C$ transition.

CBS Enzyme Assay

We performed the CBS enzyme assay on five controls not carrying the insertion, on two CAD patients heterozygous for the insertion, on one CAD patient who was

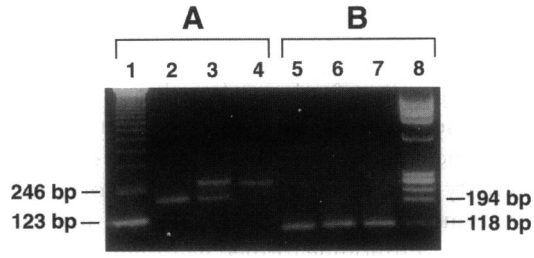

Figure 1 Ethidium bromide–stained agarose gel containing PCR-reaction products from three individuals. *A*, Electrophoretic pattern of exon 8 of the CBS gene from genomic DNA from a control without the insertion and having only the expected 184-bp DNA fragment of exon 8 (lane 2), from a CAD patient heterozygous for the insertion and having a 252-bp band in addition to the 184-bp fragment (lane 3), and from a CAD patient homozygous for the insertion and having only the 252-bp fragment (lane 4). *B*, Electrophoretic pattern after RT-PCR using RNA from the control not carrying the insertion (lane 5), from the CAD patient heterozygous for the insertion (lane 6), and from the CAD patient homozygous for the insertion (lane 7). A 123-bp DNA ladder (Gibco BRL) and ØX174 RF, DNA *Hae*III fragment (Gibco BRL), were used as size markers (lanes 1 and 8, respectively).

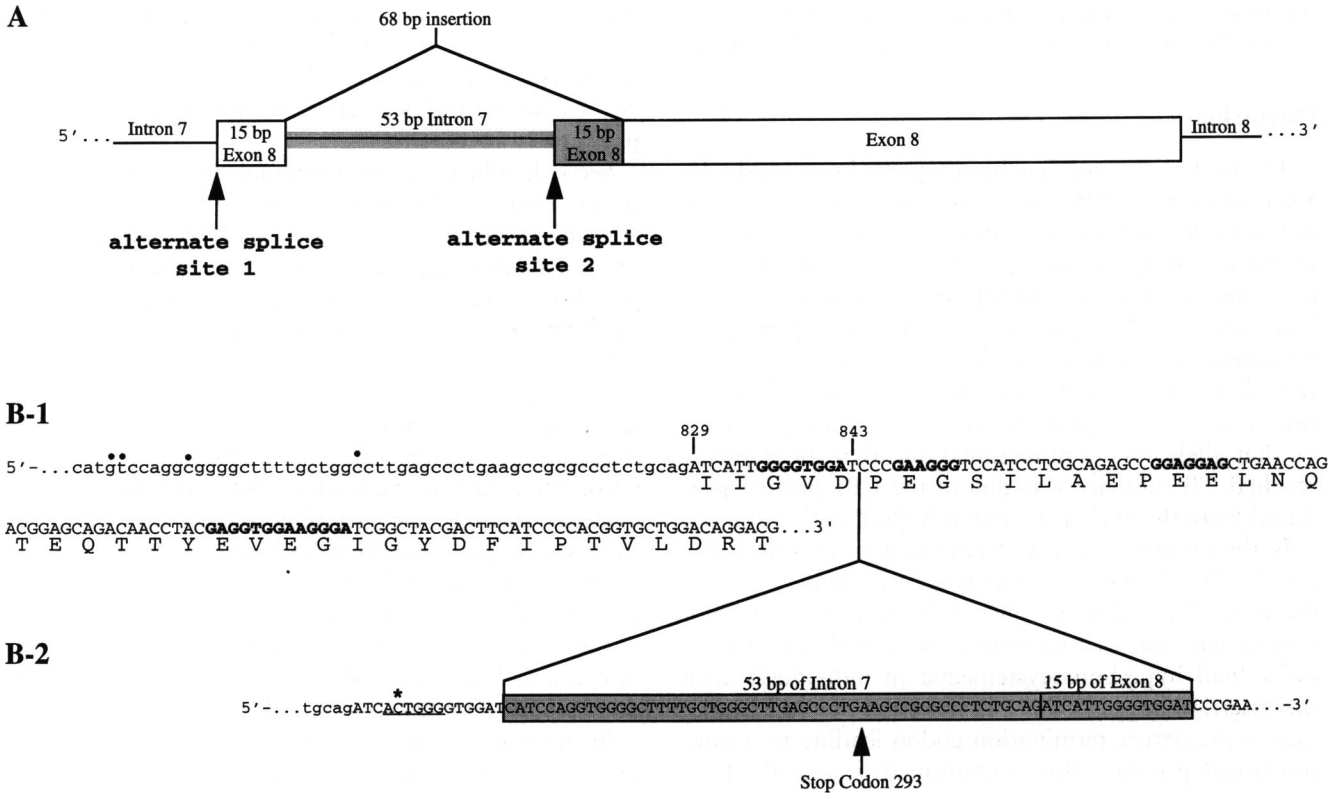

Figure 2 Characterization of mutation. *A*, Location of 68-bp insertion and alternate splice sites within exon 8 of the CBS gene. *B*, Comparison of the sequences of exon 8 from a normal allele (*B-1*) and an individual with the 68-bp insertion (*B-2*). The insertion is represented as a shaded box. The sequence of the first 53 nucleotides of the insertion is identical to that of the 55 nucleotides of the 3′ end of intron 7 in a normal allele, with three exceptions (indicated in the intronic sequence of the normal allele by a dot [·] above the nucleotides). The sequence of the remaining 15 nucleotides is identical to that of the wild-type sequence of the first 15 nucleotides (829–843) from the 5′ end of exon 8 in the normal allele. The $T_{833}C$ transition (indicated by an asterisk [*]) precedes the inserted sequence and is always present in the same allele that carries the 68-bp insertion. The *Bsr*I digestion site (ACTGGN) created by the T→C transition is underlined. The four purine-rich sequences in exon 8 of the normal allele are in boldface.

homozygous for the insertion, and, as CBS-deficient controls, on two known homocystinuric patients without the insertion. The CBS enzyme activities of the patients heterozygous for the insertion (6.2 and 3.8 nmol/h/mg protein) and homozygous for the insertion (4.7 nmol/h/mg protein) were comparable to the CBS activities of the five controls without the insertion (mean 5.6 nmol/h/mg protein; range 4.0–8.7 nmol/h/mg protein), whereas there was no activity in patients with known homocystinuria.

Mutation Frequency: Association with Elevated tHcy

Table 1 shows that, although the heterozygous state of the 68-bp insertion is slightly more prevalent in patients with premature CAD than in controls (15.9% vs. 11.7%, respectively), the difference between the two groups is not statistically significant ($P = .16$, when the two proportions are compared). Only one patient was homozygous for the insertion.

We found no significant difference in the mean ± SD fasting tHcy concentration of the CAD patients carrying the insertion (10.9 ± 9.4 μmol/liter), compared with CAD patients without the insertion (11.0 ± 6.4 μmol/liter). The mean ± SD rise (calculated as the difference between post–methionine load and fasting tHcy concentrations) in post–methionine-load tHcy concentration of individuals with the insertion (males, 16.9 ± 4.9 μmol/liter; and females, 21.0 ± 5.8 μmol/liter) is slightly lower compared with that in patients without the insertion (males, 20.8 ± 8.3 μmol/liter; and females, 27.8 ± 1.3 μmol/liter), although the differences did not reach statistical significance ($P = .93$).

Of the 29 patients heterozygous for the insertion, 4 had elevated (>12 μmol/liter, upper 95th percentile; mean ± SD = 8.1 ± 1.8 μmol/liter) fasting plasma tHcy (13, 13, 14, and 58 μmol/liter), and none had an elevated (>28 μmol/liter increase over fasting level) post–methionine-loading plasma tHcy. Both fasting and post–me-

thionine-load tHcy concentrations in the patient homozygous for the insertion were within the normal range.

Discussion

The 68-bp insertion has been reported previously, by Sebastio et al. (1995), in a single patient with classic homocystinuria due to CBS deficiency. That patient was reported to be homozygous for the $T_{833}C$ transition and to be heterozygous for the 68-bp insertion. The patient had dislocation of optic lenses and was responsive to treatment with pyridoxine. In that report, it was assumed that, because the insertion introduced a premature-termination codon, the truncated protein produced by that allele was nonfunctional and that the patient's residual CBS activity was due to the CBS protein produced from the allele carrying only the $T_{833}C$ mutation.

In the present study, we found that this 68-bp insertion is a highly prevalent mutation present in ~12% of the control population. The 68-bp insertion does not lead to hyperhomocysteinemia. The lack of post–methionine-load hyperhomocysteinemia in individuals with the insertion suggests that the insertion does not introduce a premature-termination codon leading to a nonfunctional protein. This is confirmed by our RT-PCR results in the transformed lymphocytes, which demonstrate the existence of only normal size mRNA.

Although the inserted 53 bp of intron 7 differ from the normal intron 7 by both a deletion of two bases and two single-base substitutions, the exonic sequence is identical to the first 15 bp of exon 8 of a normal allele. The net result of the insertion is to create two intron 7–exon 8 borders and, thus, two competing splice sites. The finding of only normal size mRNA implies either that splicing is taking place exclusively at the distal 3′ splice site or that the message resulting from splicing at the proximal 3′ splice site (which would introduce a premature-termination codon) is undetectable or unstable (Cheng and Maquat 1993). We believe that splicing takes place exclusively at the distal 3′ splice site, on the basis of the following reasons. First, an examination of the two splice sites showed that the proximal site may be lacking exonic sequences critical for optimal splicing. Splice-site selection is determined by factors such as proximity and strength of splicing signals, formation of RNA secondary structures, intronic sequences other than those specifying splicing signals, and exonic sequences (Lavigueur et al. 1993). Also, purine-rich sequences located in the exon may enhance splicing (Xu et al. 1993) by binding serine/arginine-rich proteins, which promotes interaction of U2 snRNP with the branch-site region, and may involve U1 snRNP (Lavigueur et al. 1993). As figure 2*B-1* (*boldface*) illustrates, exon 8 of the CBS gene contains four purine-rich sequences. We postulate that these sequences may be splicing enhancers and are required for the effective splicing of exon 8. Three of four of these sequences are absent in the proximal site, which contains only the first 15 bp of exon 8, and thus effective splicing at that site is precluded.

Second, splicing at the proximal site or at both sites should result in quantitatively diminished expression of the CBS protein. Our results in the CBS assay in transformed lymphocytes demonstrate that the presence of the 68-bp insertion, either in heterozygous carriers or in the homozygous form, does not lower the CBS enzyme activity. In vitro assays of enzyme activities are subject to the conditions of cell culture. However, the lack of deficiency of CBS activity is confirmed by the lack of hyperhomocysteinemia in individuals carrying this mutation after methionine loading. Although the available evidence strongly suggests that splicing takes place at the distal 3′ splice site, this conclusion must be tempered by the limitation of the accuracy of the enzyme assay in cultured lymphocytes. Currently, our laboratory is developing methods for quantitating CBS mRNA levels to confirm that individuals with the insertion mutation do not have diminished CBS mRNA levels.

In summary, we have found that the 68-bp insertion in the CBS gene, elsewhere described in a single homocystinuric patient, is highly prevalent. We demonstrate that the insertion is likely to be a benign mutation, since normal mRNA is restored through preferential splicing at one of the two intron 7–exon 8 splice sites. This mutation thus provides a natural example of the importance, for effective splicing, of critical sequences in an exon. The biological significance, if any, of the presence of the insertion is unknown. In the current study, the insertion is slightly more prevalent, although not of statistical significance, in patients with premature CAD than in controls. However, this finding needs to be confirmed in large epidemiological studies.

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