Hyperhomocysteinemia in premature arterial disease: examination of cystathionine β-synthase alleles at the molecular level

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Hyperhomocysteinemia occurs in approximately 30% of the patients with premature occlusive arterial disease (POAD). Some of these exhibit significantly reduced fibroblast cystathionine β-synthase (CBS) activities, suggesting that they may be heterozygous for CBS deficiency. To test this possibility, we studied cDNA derived from four well characterized patients with POAD, exhibiting hyperhomocysteinemia and reduced CBS activities, from four normal controls, and from four obligatory heterozygotes for CBS deficiency. Lysates of individual colonies of E.coli, containing full-length PCR-amplification products in the expression vector, pKK388.1, were tested for CBS activity. cDNA from at least seven of the eight possible independent POAD alleles encoded catalytically active, stable CBS which exhibited normal response to both PLP and AdoMet. The sequences of all 3'-untranslated regions of all seven isolated POAD alleles were identical to the normal, 'wild-type' CBS sequences. The results of the expression studies were confirmed for one POAD patient by determining the full-length cDNA sequences for both alleles; these were entirely normal over the complete length of the cDNA. In contrast, the screening method correctly distinguished mutant from normal alleles in all four obligatory heterozygotes studied. We conclude that CBS mRNAs from POAD individuals are free from inactivating mutations, including all 33 previously identified in heterozygous carriers and homocystinuric patients.

INTRODUCTION

Mild hyperhomocysteinemia (HHC) is found in approximately one-third of cases of premature occlusive arterial disease (POAD) (1) whereas vascular complications such as stroke and thromboembolus are common among homocystinuric individuals (2). As a consequence, elevated plasma levels of homocysteine are widely recognized as an independent risk factor for such vascular disorders (1). The biochemical and cellular participation of homocysteine in this condition is unclear, but may involve stimulation of clotting factors V (3) and XII (4) together with transcriptional induction of factor III (5) and inhibition of protein C conversion (6) as well as endothelial cell damage by peroxides (7).

Homocysteine is formed subsequently to transmethylation from AdoMet. Normally about half of this amino acid is resynthesized into methionine while the remainder is trans-sulfurated to serine by cystathionine β-synthase (CBS), forming cysteine. While blocks in either pathway can lead to elevated plasma concentrations of homocysteine, CBS deficiency is acknowledged to be the most frequent cause of homocystinuria in humans (2). This enzyme is a homotetramer of 63 kDa, encoded by a gene located at human 21q22.3 (8). Heme and pyridoxal 5'-phosphate (9) are required for activity and the enzyme is regulated allosterically by AdoMet (10). Homocystinuria which results from CBS deficiency is inherited as an homozygous autosomal recessive trait, in which the enzyme activity is virtually extinguished (2). We have examined cDNAs from a number of homocystinuric patients and identified 33 distinct pathogenic mutations to date, using a bacterial CBS expression system which localizes the position of the mutation (11; unpublished).
Owing to the wide range of normal fibroblast CBS controls, it is difficult to differentiate between low normal and heterozygous-deficient enzyme activities; nonetheless, we were interested in the potential link between heterozygous CBS deficiency and HHC/POAD. We therefore decided to employ molecular methods to differentiate between normal and mutant CBS alleles. These methods circumvent ambiguities originating from the normal/heterozygous activity overlap when assayed in fibroblast lysates.

To ascertain whether such a link existed, we examined cDNA preparations representing seven alleles which could be positively identified from four separate patients exhibiting HHC/POAD. These patients were selected from 30 candidates exhibiting POAD with accompanying HHC, exacerbated by methionine loading (12,13). Some of these also possessed persistent low levels of CBS in fibroblast cultures. A new screening method was developed in which allelic products were tested by expression of full-length cDNAs in bacteria rather than the segmental replacement method described earlier (14). Identification of synonymous mutations facilitated identification of the separate alleles. Lastly, we sequenced the 3' untranslated regions. We now report that all of the allelic products were free from pathogenic mutations and that the cDNA preparations expressed normal amounts of CBS in the bacterial expression system. It appears highly unlikely that pathogenic mutations in the coding region or the 3'-untranslated region are responsible for the HHC observed in these patients.

RESULTS

Subjects studied

Relevant biochemical and clinical findings for the patients studied in this report are listed in Table 1. Arteriosclerosis was found in all four of the HHc/POAD patients. Peak plasma homocysteine levels following a methionine load (12,13) were well above normal for each of the individuals, approximating the values observed for obligate heterozygotes. Fibroblast cultures from three of the four exhibited CBS activities below the normal range.

Screening strategy

We previously developed a bacterial expression method to localize pathogenic mutations in cDNA segments from homocystinuric patients (14). The aim of this study was to test for the presence of any inactivating mutations rather than to localize them. Therefore, we expressed the entire coding region of each allele from each patient. The screening strategy is summarized in Figure 1.

To date, 33 pathogenic mutations have been identified and expressed in E. coli. Only one of these produces appreciable CBS activity after expression, approaching 50% of the control values. The overwhelming majority, however, completely inactivate the enzyme. Figure 2 illustrates the wide separation in CBS activity produced by normal and mutant alleles. Coupled with Western analysis of the bacterial proteins, the method is highly sensitive; it has never failed to localize mutations in CBS from known homozygotes.

Screening for inactive CBS alleles among patients with POAD/HHC

The infidelity of Taq-catalyzed polymerase chain reaction arises principally from the absence of proofreading in Taq DNA polymerase. Pyrococcus DNA polymerase proofreads its product; accordingly, its substitution for Taq DNA polymerase significantly improved PCR fidelity. Whereas PCR of control cDNAs with Psp polymerase produced only seven inactive clones out of 78 (9%, Fig. 3), similar experiments with Taq polymerase yielded five inactive clones out of 14. This 36% error rate was too high to allow us to use the Taq enzyme for full-length cDNA amplification, accordingly, Psp DNA polymerase was used for all experiments.

To ascertain the suitability of this method as a screen for heterozygosity, we first tested cDNA derived from known heterozygotes (Fig. 3). The frequency of inactive clones from four obligate heterozygotes (30/70; 43%) was significantly greater than for the controls (7/78; 9%), thus the method sensitively distinguishes between these groups. In contrast, the frequency of inactive CBS colonies (7/63; 11%) from the four HHc/POAD patients was not significantly different from the controls. These functional data suggest that it is unlikely that the low CBS activities in this group of POAD/HHC patients result from mutations in the coding region of the CBS gene, assuming that both parental alleles were equally represented in the clones which had been analyzed.

Segregation of CBS activity with allelic polymorphisms

To eliminate the possibility that only one allelic product had been tested in the expression studies, we examined the cloned cDNAs for synonymous mutations in the coding regions (15). These markers permitted us to identify both parental alleles in

Table 1. Subjects reported in this study

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Fasting HCY (µM)</th>
<th>Peak HCY (µM)</th>
<th>Site of arteriosclerosis</th>
<th>Severity of disease</th>
<th>CBS U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>3014</td>
<td>F/P</td>
<td>8.9</td>
<td>54.3</td>
<td>Internal carotid and anterior cerebral arteries</td>
<td>Transient ischaemic attacks</td>
<td>3.5</td>
</tr>
<tr>
<td>3022</td>
<td>F/P</td>
<td>5.7</td>
<td>66.8</td>
<td>Renal, mesenteric and iliac arteries</td>
<td>Severe reno-vascular hypertension</td>
<td>17</td>
</tr>
<tr>
<td>3024</td>
<td>F/P</td>
<td>5.3</td>
<td>37.8</td>
<td>Abdominal aorta and femoral arteries</td>
<td>Severe dysbasia</td>
<td>3.2</td>
</tr>
<tr>
<td>3030</td>
<td>F</td>
<td>9.1</td>
<td>51.0</td>
<td>Cerebral vessels</td>
<td>Unknown</td>
<td>5.9</td>
</tr>
<tr>
<td>Obligate heterozygotes (n = 10)</td>
<td></td>
<td>4.2±2.8</td>
<td>41.0±24.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 30)</td>
<td></td>
<td>0.0±0.9</td>
<td>6.8±1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Homocysteine (HCY) levels before (fasting) and after (peak) methionine challenge (12) are listed for each of the experimental subjects as well as normal controls and obligate heterozygotes for CBS deficiency (mean ± 1 standard deviation). F/P: Premenopausal female patients. CBS activities for controls and obligate heterozygotes represent the ranges found in cultured fibroblasts by this laboratory. The majority of controls range from 20–25 U/mg protein.
individual clones exhibiting normal CBS activity. To carry out this test, we analyzed clones derived from obligate heterozygotes as well as from the POAD/HHC patients who were informative for these polymorphisms. The results of these experiments are summarized in Table 2.

We first tested clones from two obligate heterozygotes for silent T/C substitutions at position 699. The triplets encoded at that position (TAC/TAT) both encode tyrosine, therefore the protein sequence is unaffected. Of the 12 allelic clones tested for one patient (3056) all eight active ones exhibited ‘T’ alleles (i.e. T699), whereas all four inactive clones contained ‘C’ alleles (C699). These results demonstrate that these polymorphisms segregated with active and inactive CBS alleles for this individual. Similarly, all five active clones from 3065 exhibited ‘C’ alleles (C699) while all five inactive clones were ‘T’ alleles. These results demonstrate that the PCR-based RFLP analytical method used (15) clearly identifies the segregated parental alleles.

Both parental alleles were identified among active CBS clones derived from three patients with POAD/HHC (Table 2). The T/C (699) polymorphism was found in allelic products from patients 3022 and 3030 whereas another synonymous mutation, C1080T (GCC/GCT; Ala 360), was employed to distinguish between the allelic products from patient 3014.

The fourth patient, 3024, did not exhibit these polymorphisms, thus it is not certain that both parental alleles were represented in the tested clones. The presence of both these alleles in active clones unequivocally demonstrates that both parental alleles encode enzyme which is catalytically functional, therefore neither parental CBS allele encodes protein with pathogenic mutations in these patients. These results clearly differentiate between the obligate heterozygotes whose active clones are all of the same haplotype and the POAD patients whose active clones were not.

In addition, less than 15% difference was observed in CBS activity in C and T alleles from each informative patient. The mildest pathogenic mutation studied to date, A114V (unpublished), decreases the activity of CBS by at least half when the allele is expressed in E.coli. The statistically insignificant differences between parental alleles (C and T) of each vascular patient suggest that it is quite unlikely that these individuals are carriers for any known mutation, even one so mild as A114V.

**DNA sequencing**

*Primer portion of the cloned cDNAs*

The method described in this paper effectively tests the majority of the CBS coding region, however, the sequences of the primer portions of the cDNA escape definitive scrutiny. Accordingly, we sequenced these regions to ascertain whether the abnormally low fibroblast levels of enzyme activity stemmed from cryptic mutations in the 5’- or 3’-ends of the coding sequence. These regions were first amplified by PCR.
In the 5'-portion, between six and eight clones derived from each patient were chosen for sequencing to limit the probability that only one parental allele was tested. The 5'-ends were sequenced from nts -1 to 40, covering the entire region occupied by the sense primer. All of the clones in all four patients possessed normal cDNA sequence in this region.

Sequencing of the 3' untranslated region
We next searched for mutations in the 3'-sequence of the cDNA using a similar strategy. To ensure that both parental alleles were represented in the clones, we tested the individual colonies for a frequent Msp I polymorphism (T2199C) in the 3'-untranslated region. Three of the patients were polymorphic, thus only two clones (each representing one allele) were chosen for DNA sequencing. The sequence of the plasmids harboring these cDNAs was indistinguishable from normal in the region homologous to the antisense primer as well as in the 3'-untranslated region. Four plasmids originating in patient 3024, who did not exhibit the MspI polymorphism, were also sequenced through the antisense primer region further into the 3'-untranslated region and were also fully congruent with the normal allele. These results demonstrate the absence of cryptic mutations in both the 5'- and 3'-coding regions and 3' untranslated region of CBS cDNA.

DNA sequencing of full length cDNA in patient 3014
To search for other DNA mutations which might be undetectable in our screening system, we sequenced the entire coding region of CBS cDNA from both alleles of one patient (3014). The Ala360 synonymous mutation in the coding region was used to identify cDNA clones originating from separate alleles. The sequence of both alleles were completely normal over their entire coding regions.

Activation of patient CBS by S-adenosylmethionine
Incubation of ‘wild-type’ CBS with AdoMet stimulates the enzyme’s activity by increasing its affinity for homocysteine (10,14). Subtle mutations in the AdoMet binding region, displacing the effector from its binding site, might reduce the enzyme activity to the levels observed in the fibroblasts from the four patients studied. These mutations would be accompanied by a lack of response of the enzyme to AdoMet. We measured CBS activity in fibroblast lysates, and in bacterial extracts, incubated with added AdoMet varying from 0–500 μM and calculated the activation constant, Kₐₙ₉, for AdoMet to determine whether such mutations might exist. Figure 4 depicts the results of the fibroblast study. Although the basal activities differed considerably, CBS in each patient cell extract responded to AdoMet similarly. Both the 15 μM AdoMet activation constant and the 2.5-fold increase in enzyme activity were identical for the patients and controls. These results indicate that the AdoMet binding domains for CBS in all patient lines are intact and that the endogenous AdoMet concentration in the cell cultures is similar in each cell line.

CBS, expressed in E.coli, behaved similarly when incubated with varying concentrations of AdoMet (data not shown). These results demonstrate unequivocally that both alleles of each patient encode CBS with identical behavior towards AdoMet; accordingly, the wide variation in the CBS activities of these patients cannot be ascribed to defective AdoMet binding.

Analysis of cloned CBS by Western blotting
Since all of the CBS clones were free from pathogenic mutations, we suspected that the low intracellular CBS activities might be attributed to decreased amounts of normal enzyme subunits. The volumes of fibroblast extracts were adjusted so that each sample contained equal amounts of enzyme activity.
Immunoprecipitates of these samples were analyzed by Western blotting to estimate the actual amount of enzyme in each. Comparable band intensities resulted from identical amounts of enzyme activity even though 20-fold more total cellular protein was required for the defective cell lines (Fig. 5, upper panel). We conclude that the specific activity of the pure enzyme molecules is constant in each fibroblast line and that the low CBS activity found in these lines results from decreased amounts of enzyme molecules rather than impaired catalytic activity. These conclusions were further supported by the following bacterial studies.

All seven separated alleles were examined by Western blotting of the 
coli lysates. Whereas the quantity of crude fibroblast protein required to produce a constant (2 U) amount of CBS was quite variable, bacterial lysates were far more consistent, even when the colonies expressed separate CBS alleles (Fig. 5, lower panel). These results demonstrate that the specific activity of the cloned CBS subunits does not vary in clones derived from separated parental alleles. Additionally, they reveal that the enzyme proteins encoded by each allele are equally stable in 
coli, as would be expected if each allele encoded an identical product.

DISCUSSION

The purpose of this research was to ascertain whether the hyperhomocysteinemia observed in the four patients studied originated from mutations in the CBS coding region. At least seven of the eight possible alleles were examined and did not contain mutations. We therefore conclude that the HHC observed in these four individuals does not stem from defective CBS protein. CBS binds three cofactors in addition to its two substrates: PLP (16), AdoMet (10,14), and heme (9). All of these affect CBS activity. PLP is an active participant in the reaction (16), AdoMet stimulates the enzyme allosterically (10,14), while heme is required for both PLP binding and enzyme activity (9). Fibroblast cultures from three of the four patients exhibited low enzyme activities upon repeated assay. It is unlikely that these resulted from reduced PLP affinity since the enzyme responded normally when assayed in both the presence and absence of this cofactor (data not shown); additionally, altered PLP affinity is associated with mutations in the coding region (17) and none were found. AdoMet sensitivity, likewise, was unaffected for all of the preparations (Fig. 4). CBS specific activity depends upon its saturation with heme (9). This porphyrin binds tightly to CBS and is evidently inserted either concurrently with or shortly after synthesis. Addition of heme to enzyme incubations is without effect on either crude or pure preparations of the enzyme (9) so it is not possible to test directly the affinity of CBS for heme. Western analyses of the enzymes from fibroblasts, however, demonstrated that the amount of CBS protein closely correlated to the levels of CBS activity (Fig. 5) so it is likely that the heme saturation is equal for the normal and HHC/POAD samples. These results imply that the reduced amounts of CBS in the fibroblast samples resulted from altered enzyme expression rather than a mutation in its structure.

The clear separation of activity between normal and mutant alleles in this screening method (Fig. 2) greatly simplifies identification of bacterial colonies harboring plasmids containing mutant CBS cDNAs. The substitution of Psp DNA polymerase, which proofreads its products, for Taq, which does not, significantly improved the sensitivity of the method. Fidelity of the screening method depends directly upon fidelity
of the polymerase. This was tested by PCR of normal CBS cDNA sequences. Using Taq polymerase, less than 2/3 of the PCR products encoded active CBS when a normal plasmid template was amplified. More than 90% of the PsP polymerase control products were active.

All of the known mutations associated with homocystinuria impact considerably on CBS activity expressed in transformed bacteria (11; unpublished). There was no significant difference in the frequency of inactive clones between the HHC/POAD patient and the normal control groups; although considerable variation was observed between individuals in the obligate heterozygote group. In addition, screening for synonymous mutations allowed us to show that individual parental CBS alleles were tested for pathogenic mutations in three of the patients. All of the coding regions of these sequences were free from pathogenic mutations. This was not the case for the obligate heterozygotes which were unequivocally differentiated from the controls. We subsequently separated the allelic products from the obligate heterozygotes and identified the mutations in the abnormal strand (14,21; unpublished). This method permits unequivocal identification of heterozygous carriers for homocystinuria for the first time.

Since at least three of these patients are homozygous for normal CBS, their hyperhomocysteinemias evidently stem from other factors. Homocysteine, once formed, can either serve as a substrate for transsulfuration or be resynthesized into methionine (2). Although the most frequent cause of homocystinuria is CBS deficiency (2), the condition may arise from inadequate methionine resynthesis as well (18,19). Defects in cobalamin metabolism (19) or methylenetetrahydrofolate reductase insufficiency (18) could precipitate hyperhomocysteinemia in these subjects. Other defects could include vitamin malnutrition with or without attenuated heme biosynthesis, or concurrent low expression of key enzymes such as methylenetetrahydrofolate reductase and CBS. The rate of methionine resynthesis is currently under investigation to evaluate its contribution to these patients’ condition.

Lastly, it is important to note that there is a strong overlap of the normal and heterozygote CBS activity ranges in cultured fibroblasts (20). Three of the patients studied were below the normal range while the fourth patient was well within it. Our initial intent was to evaluate whether they were heterozygous for CBS deficiency. With this publication, we demonstrate that they were all homozygous for normal CBS. In many cases, fibroblast cultures have been in passage for some time. In others, the phase of the culture may not be reliably determined. Each of these conditions can affect the level of enzyme assays, including those for CBS. Considerable variability in CBS activity may occur within cultures of apparently healthy fibroblasts as well. These considerations together with our results indicate that fibroblast CBS activities alone cannot be used to assess the CBS zyosity of a patient, even when accompanied by hyperhomocysteinemia; accordingly, the diagnosis of individuals suspected of being carriers of homocystinuria must be based on analysis of their nucleic acid sequences.

MATERIALS AND METHODS

Patients and cell lines

Cell lines 3014, 3022, 3024, and 3030 from POAD/HHC patients used in these studies originated from two clinical and biochemical studies described elsewhere (12,13). Cell lines 2047, 2103 and 3720 were from controls and 367, 3009, 3056 and 3065 were from mothers of homocystinuric patients. The mutations in these obligate heterozygotes were 1278T, E239K, G307S, and R336C, respectively (14,21; unpublished). Fibroblasts were cultured in MEM with 10% fetal calf serum, nonessential amino acids, glutamine, 100 U/ml of kanamycin and 100 μg/ml of streptomycin.

PCR amplification of cDNA

DNA was amplified using Pneumococcus sp. GB-D DNA polymerase (Psp, Deep Vent Polymerase, New England Biolabs, Beverly, MA) or with Taq polymerase as noted in the results section. Complementary DNA was synthesized from mRNA as described previously (14). Dilute target strands were subsequently denatured prior to PCR by heating to 95°C for 3 min and rapidly chilling in an ice/water bath.

Each 100 μl reaction mixture for PCR with the Deep Vent enzyme contained 1 μl of cDNA, 1×Deep Vent buffer (NEB), 300 μM of each dNTPs, 80 pmol of both sense and antisense primers (described above), 4% dimethylsulfoxide and 8 mM MgSO₄. Conditions for Taq-mediated PCR were: 2.5 μl cDNA, 10 mM TRIS.HCl (pH 8.4), 50 mM KCl, 0.1% gelatin, 200 μM of each dNTPs, 1.5 mM MgCl₂ and 80 pmol of both sense and antisense primers contained in a total of 100 μl. Primer sequences for both sets of PCRs were: sense: 5'-GGGATCCATCGGATTAGGAGGATTTAAC-3'; antisense: 5'-AGACCCCCCAG-3'. The bold portion of the sense primer hybridized to the 5' coding region of CBS cDNA. The underlined codon following the initiator ATG was changed from proline to glycine to complete a Nco I site; this change coincidentally increased the activity of CBS. The nonhybridizing portion of the primer contained a prokaryotic ribosome binding site and a Cla I site; Antisense primer 5'-TACGGGTACCCCGCTCGGAGAAGGCTCCAG-3'. The bold portion was complementary to the 3' region of the cDNA whereas the remainder contained an artificial Kpn I site.

PCR was conducted in a Techne FHC-1 thermocycler with block temperature control when the Deep Vent enzyme was used. The reaction mixture was preheated to 80°C/5 min and 1 U of Psp polymerase was added to each tube. The temperature was cycled 30 times (96°C/1 min; 61°C/1 min; 72°C/2.3 min) to amplify target DNA. The Thermocycler produced uniform results with this enzyme whereas the reactions did not proceed well with the Hybrid TR1, used for Taq-amplified PCR.

The Hybrid TR1 thermal reactor with tube temperature monitoring was employed for Taq DNA polymerase PCR. Enzyme was added after preheating to 80°C/5 min, then the mixture was cycled as follows: 1 cycle (94°C/3 min, 50°C/1 min, 72°C/3 min), 4 cycles (94°C/1.5 min, 50°C/1 min, 72°C/3 min), 24 cycles (94°C/1.5 min, 50°C/1 min, 72°C/2 min) and final cycle (94°C/1.5 min, 55°C/1 min, 72°C/4 min).

PCR products were purified, restricted with Cla I and Kpn I, gel purified, and ligated in gel into similarly prepared pKK388.1 (14). Individual, ampicillin-resistant colonies of transformed DH5α/FβI/FQ E.coli were subsequently used to express CBS or prepare plasmid DNA.

CBS expression in E.coli

Enzyme activity was measured in 20 μl lysates of transformed cells grown from individual colonies after incubation with IPTG (isopropylthio-β-D-galactoside, GIBCO BRL, Gaithersburg, MD) as fully described elsewhere (14). One unit of CBS activity was defined as 1 nmol of cystathionine produced in 1 h at 37°C. This radiometric assay can reliably detect enzyme activity as low as 0.03 U (100 CPM above background).

Analysis of synonymous mutations

Three synonymous mutations in the coding region of CBS cDNA and one polymorphism in the 3' untranslated region (G573A; C699T; T1080C; T2199C) were analyzed by a PCR-based RFLP method described elsewhere (15).

cDNA sequencing

A 5’-portion of CBS cDNA in all four PAD/HHC patients was amplified by PCR between nucleotides –2 and 272 using the sense primer 5’-CGTGAATTCACGCACCATCTCTGTCGGTCCCA-3’, together with the antisense primer 5’-TACGACATGCTACGG-CAGGGACTCTTCTCCCAAT-3’. Reaction conditions were identical to those described for Psp PCR except that 4 mM MgSO₄ was used and 30 cycles of amplification (96°C, 1 min; 55°C, 1 min; 75°C, 30 s) were carried out. PCR products were restricted with Cla I/Eco RI, subcloned into a pBluescript KS(+/-) cartridge and the resulting plasmid preparations used for sequencing. A 3’-portion of CBS cDNA of all four patients between nucleotides 1462 and 2316 was similarly amplified with...
Gibco BRL.

ACKNOWLEDGMENTS

CircumVent kit (NEB). Competent cells (DH5αFTQ) were obtained from Magic MiniPrep kits (Promega, Madison, WI) were used to prepare plasmid DNA. Restriction endonucleases and T4 ligase were purchased from NEB (Beverly, MA). DNA was sequenced by the dideoxy method (23) using the DNA polymerase using the sense primer P5, 5'-CGTAGAATTCAAGTCATCCTCAAGCGATTTC-3', and the antisense primer P6, 5'-TACGATCG-ATGTTGACATTTATGGAAATGTCGTC-3'. Thermocycling conditions were: 9°C, 1 min; 64°C, 1 min; and 72°C, 54 s. The entire CBS cDNA coding regions between nucleotides 22 and 1646 from both alleles of patient 2014 were cloned and sequenced.

Other methods

Magic MiniPrep kits (Promega, Madison, WI) were used to prepare plasmid DNA. Restriction endonucleases and T4 ligase were purchased from NEB (Beverly, MA). DNA was sequenced by the dideoxy method (23) using the DNA polymerase using the sense primer P5, 5'-CGTAGAATTCAAGTCATCCTCAAGCGATTTC-3', and the antisense primer P6, 5'-TACGATCG-ATGTTGACATTTATGGAAATGTCGTC-3'. Thermocycling conditions were: 30 cycles of 96°C, 1 min; 64°C, 1 min and 72°C, 54 s. The entire CBS cDNA coding regions between nucleotides 22 and 1646 from both alleles of patient 2014 were cloned and sequenced.

REFERENCES