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The Molecular Basis of Homocystinuria Due to Cystathionine β-Synthase Deficiency in Italian Families, and Report of Four Novel Mutations

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Summary

Four new mutations in the cystathionine β-synthase (CBS) gene have been identified in Italian patients with homocystinuria. The first mutation is a G-to-A transition at base 374 in exon 3, causing an arginine-to-glutamic acid substitution at position 125 of the protein (R125Q). This mutation has been found in homozygosity in a patient partially responsive to pyridoxine treatment. The second mutation is a C-to-T transition at base 770 in exon 7, causing a threonine-to-methionine substitution at amino acid 257 of the protein (T257M). This mutation has been observed in homozygosity in a patient nonresponsive to the cofactor treatment. The third mutation, found in heterozygosity in a patient responsive to pyridoxine treatment, is an insertion of 68 bp in exon 8 at base 844, which introduces a premature termination codon. The fourth mutation is C-to-T transition in exon 2 at base 262, causing a proline-to-serine substitution at position 88 of the protein (P88S). This mutation is carried on a single allele in three affected sisters responsive to the cofactor treatment. In addition, six previously reported mutations (A114V, E131D, P145L, I278T, G307S, and $A_{1224\text{-}2}C$) have been tested in 14 independent Italian families. Mutations A114V and I278T are carried by three and by seven independent alleles, respectively. The other four mutations—including G307S and $A_{1224\text{-}2}C$, common among northern European patients—have not been detected.

Introduction

Homocystinuria due to cystathionine β-synthase deficiency, CBS (L-serine hydro-lyase [adding homocysteine] EC4.2.1.22), is the most common inborn error of sulfur amino acid metabolism. CBS deficiency is inherited as an autosomal recessive disorder (MIM 236200) and is characterized by dislocated optic lenses, central nervous system involvement, skeletal abnormalities, and vascular disease with severe thromboembolic complications. Two clinical forms can be distinguished on the basis of patient's responsiveness to the treatment with the coenzyme precursor pyridoxine: a more severe clinical picture is observed in the nonresponsive patients than in the responsive ones (Mudd et al. 1989).

Heterozygosity for CBS deficiency is considered an important cause of hyperhomocysteinemia that strongly relates to premature vascular disease (Clarke et al. 1991). In fact, obligate heterozygotes for CBS deficiency show early vascular lesions at a significant higher rate than in controls (Rubba et al. 1990).

Human CBS cDNA has been isolated and characterized; the gene maps to chromosome 21q22.3 and encodes for a 63-kDa subunit of 551 amino acid residues (Kraus et al. 1993). CBS, a tetramer of this subunit, binds two substrates, homocysteine and serine, and three additional ligands: the coenzyme pyridoxal 5′-phosphate, an allosteric activator S-adenosylmethionine, and heme, whose function is not clear yet (Kery et al. 1994). The human CBS amino acid sequence displays a close homology, >90%, with the rat enzyme, and 52% when compared with bacterial O-acetylserine (thiol)-lyase (CYSK) and with spinach cysteine synthase (CSYN) (Swaroop et al. 1992). The human CBS gene has yet not been fully characterized, but preliminary data indicate a close homology with the rat CBS gene structure.

The screening for mutations in homocystinuric patients has led to the identification of two relatively common mutations, I278T (Kozich and Kraus 1992) and G307S (Hu et al. 1993). The former (I278T) accounts for 15% of affected alleles from patients of different ethnic origin. The latter (G307S) accounts for 58% of affected alleles screened so far (Hu et al. 1993) and 71% of affected alleles in Irish nonresponsive patients. Other mutations have been detected in single or very few pa-

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Table 1

CBS Mutations

Mutation	Exon	References
$C_{233}G$ (P78R) (linked) G_{306} (K102N)	2	de Franchis et al. 1994
$C_{262}T$ (P88S)	2	Present study
$C_{341}T$ (A114V)	3	Kozich et al. 1993
$G_{374}A$ (R125Q)	3	Present study
$G_{374}A$ (R125Q) (linked) $G_{393}C$ (E131D)	3	Marble et al. 1994
$C_{434}T$ (P145L)	3	Kozich et al. 1993
$G_{715}A$ (E239K)	6	de Franchis et al. 1994
$C_{770}T$ (T257M)	7	Present study
$T_{833}C$ (I278T)	8	Kozich and Kraus 1992
844ins68	8	Present study
$G_{919}A$ (G307S)	8	Hu et al. 1993
$C_{1006}T$ (R336C)	9	Kraus 1994
$G_{1106}A$ (R369H)	10	Kraus 1994
ag→cg (intron 11) Δ exon 12	12	Kozich and Kraus 1992
Δ 29 bp	7	Kraus 1994

tients (Ward et al. 1994). All mutations found so far are located in exons 2–12, most of them clustered in exon 3 and 8 (table 1) (Kraus 1994). In the present survey of 14 Italian independent families, we have tested the occurrence of six known mutations and have found four novel mutations. Finally, our data contribute to the genotype/phenotype correlation and to the distribution of CBS mutations in Europe.

Patients, Material, and Methods

Patients

Clinical findings of 18 patients belonging to 14 unrelated families, all originating from southern Italy, are listed in table 2. Minimal criteria for the diagnosis of homocystinuria due to CBS deficiency were: homocystinuria and hypermethioninemia, associated with dislocation of optic lenses (Mudd et al. 1985). All patients met these criteria except 3b and 3c, who have not developed the ocular finding due to early pyridoxine treatment. CBS activity in skin fibroblasts was tested (Andria et al. 1989) and was found to be <1% of normal values in patients 1, 2, 4a, 4b, 7, and 8. All patients were responsive to pyridoxine treatment except patients 2, 9, and 11, who were nonresponsive, and patient 1, who has shown a partial response.

Reverse Transcriptase (RT)–PCR, Subcloning, and Sequencing

Total RNA was extracted from cultured skin fibroblasts by using the guanidinium thiocyanate method (Chirgwin et al. 1979). Synthesis of cDNA was carried out using 5 μg of denatured total RNA by reverse-transcriptase (Gibco BRL) at 37°C for 60 min according to the manufacturer's protocol. One-tenth of total cDNA product was amplified in a 50-μl final volume with 250 μM of each dNTP, 2.5 U of *Taq* DNA polymerase (Boehringer-Mannheim), and 100 pmol of each primer. PCR conditions were as follows: denaturation for 1 min at 94°C, annealing for 1 min at 57°C–62°C, and elongation for 1–2 min at 72°C. Primers used for amplification are listed in table 3. PCR products were subcloned and sequenced in both directions by the dideoxy chain–termination method (Sanger et al. 1977) using the Sequenase kit (USB) and [α-^{32}P] dCTP as the labeled deoxyribonucleotide.

Genomic DNA Preparation, PCR Amplification, Restriction Analysis, and Direct Sequencing

Genomic DNA was isolated from peripheral blood samples and from cultured skin fibroblasts by standard methods (Maniatis et al. 1989). DNAs were amplified using the same conditions reported for the RT-PCR, and primers are described in table 3. PCR products were digested with specific enzymes to test the novel and the previously reported mutations: R125Q, T257M, P88S, A114V, E131D, P145L, I278T, G307S, and $A_{1224\text{-}2}C$ (Kraus, 1994). Restriction analysis conditions are reported in table 4. Restriction digests were analyzed by 10%–12% PAGE. All mutations were confirmed by di-

Table 2

Clinical Findings

	Patients																	
	1	2	3a	3b	3c	4a	4b	5	6a	6b	7	8	9	10	11	12	13	14
Initials	LG	ON	SS	SR	SG	ML	MA	PV	DM	DA	PA	QE	TG	SA	TL	CG	MN	TR
Age at diagnosis (years)	4	1	6	2	0, 5	9	3	29	12	10	12	25	4	7	6	33	15	35
Ectopia lentis	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Osteoporosis	+	+	+	−	−	+	+	+	+	+	+	−	+	−	+	−	+	+
Vascular disease	+	−	−	+[a]	−	+[a]	−	−	−	+[a]	+[a]	−	+[a]	−	−	−	−	−
Mental retardation	+	+[b]	−	−	−	+	−	−	−	+	+	+	+	+[b]	+	−	−	−
Pyridoxine responsiveness	PR[c]	−	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+

[a] ECHO-Doppler evidence only.
[b] Mental retardation and epilepsy.
[c] PR = partially responsive.

rect sequencing using single-stranded DNA obtained by asymmetrical PCR.

PCR-SSCP Analysis

For SSCP analysis, 0.4 μl of [α-^{32}P] dCTP (3,000 Ci/mmol) were added to the PCR reaction. Primers used for PCR-SSCP are listed in table 4, and the conditions are the same as those described for RT-PCR. SSCP was carried out on 6% polyacrylamide nondenaturing gels containing 10% glycerol (Orita et al. 1989).

Expression of Mutations and Western Analysis

The two regions containing the mutations R125Q and T257M (nt −32–595 and 260–977, respectively) were amplified by PCR using the CBS cDNA from fibroblasts of patients 1 and 2. Primers used were: 5′TGAACTGTCAGCACCATCTGTCCGG3′ and 5′AGTCGAACCTGGCATTGGT3′ for the R125Q mutation; and 5′CCCCTATGGTCAGAATCAACAA3′ and 5′CTCTTGAACCACTTGTCCACC3′ for the T257M mutation.

Each amplified segment was subcloned into the expression vector $pHCS_3$ to construct a hybrid CBS cDNA containing the segments of patient cDNA in the context of otherwise normal CBS cDNA sequence, as reported elsewhere (Kozich and Kraus 1992; de Franchis et al. 1994). Five clones and three clones were expressed from patients 1 and 2, respectively. To confirm the presence of the mutation and to exclude the possibility of PCR artefacts, two inactive clones from each patient were sequenced. The *Escherichia coli* lysate from one inactive clone of patient 2 was also used for western analysis, as reported elsewhere (Kozich and Kraus 1992).

Results

A Missense Mutation at Codon 125 (R125Q)

In patient 1, we have identified a G-to-A transition ($G_{374}A$) changing an encoded arginine (CGG) to glutamic acid (CAG) at codon 125 in exon 3 (R125Q). The remainder of the cDNA in the coding and 3′-UTRs is identical to the wild-type sequence. This mutation has been confirmed by direct sequencing of both alleles of patients' genomic DNA (fig. 1, *left*). As R125Q creates a new *Pst*I site, the PCR fragment containing the mutation (141 bp) is cleaved into two segments (83 and 58 bp), while the normal fragment is not cleaved (fig. 1, *right*). This approach confirms that patient 1, born to first-cousin parents, is homozygous for R125Q and that his mother is heterozygous (the father is not available for testing) (fig. 1, *right*). No other patient of the present survey carries this mutation (data not shown). The region containing the mutation R125Q (nt −32–595 of the cDNA) was used to replace the wild-type sequence in the bacterial expression vector $pHCS_3$. Five independent clones did not show any measurable CBS enzymatic activity. In addition, very little immunoprecipitable CBS product was observed by western analysis in another patient carrying the same mutation (Marble et al. 1994).

A Missense Mutation at Codon 257 (T257M)

In patient 2 we have identified a C-to-T transition ($C_{770}T$) changing an encoded threonine (ACG) to methionine (ATG) at codon 257 in exon 7 (T257M). The remainder of the cDNA in the coding and 3′-UTR is identical to the wild-type sequence, except for a synonymous change T1080C (data not shown). The T257M mutation has been confirmed by direct sequencing of both alleles of patient's genomic DNA (fig. 2, *upper left*). Because the T257M creates a new *Nla*III site, the PCR fragment (101 bp) containing the mutation is cleaved in three segments (68, 21, and 12 bp), while the normal fragment is cleaved into two segments (89 and 12 bp) (fig. 2, *upper right*). This approach confirms that patient 2, born to first-cousin parents, is homozygous for T257M; his brothers and parents are heterozygous

Table 3

Primers Used in the Present Study

No.	Primers[a]	Sequence[b]	Position
Primers pair for RT-PCR amplification and direct sequencing:			
1	S	5′-TGAACTGTCAGCACCATCTGTCCGG-3′	Exon 1
2	A	5′-AAAGGTGAACGCCTCCTCATCGTT-3′	Exon 9
3	S	5′-AAGCTGGACATGCTGGTGGCTTCA-3′	Exon 7
4	A	5′-AATCCACTCTGCGCAGTCATTGCC-3′	3′-UTR
5	A	5′-TCTTCAGAATATCTGGCAAG-3′	Exon 2
6	S	5′-TCATCGTGATGCCAGAGAAGATG-3′	Exon 4
7	A	5′-CATCCAGGACACTTCTCCTTC-3′	Exon 7
8	A	5′-CTCAGGAACTTGGTCATGTAG-3′	Exon 11
9	S	5′-ATTCTGCCCGACTCAGTGCGGAA-3′	Exon 10
10	A	5′-ATCTCCAGGATGTGCGAGACG-3′	Exon 15
11	S	5′-AGTCATCTACAAGCAGTTCAAACAG-3′	Exon 14
Primers pair for PCR amplification of genomic DNA and direct sequencing:			
1	S	5′-GGCAAAATCTCCAAAATCTTGCCA-3′	Exon 2
2	A	5′-tccaaagccagggcactcacAGA-3′	Exon 2-intron 2
3	S	5′-TGGCCAAGTGTGAGTTCTTCAA-3′	Exon 3
4	A	5′-CGATCCCGGTGTTCCCGGATGT-3′	Exon 3
5	S	5′-AAGCTGGACATGCTGGTGGCTTCA-3′	Exon 7
6	A	5′-acaccactcacCCTGCATCCA-3′	Exon 7-intron 7
7	S	5′-ctgccttgagccctgaagcc-3′	Intron 7
8	A	5′-ctggactcgacctacCGTCCT-3′	Exon 8-intron 8
9	S	5′-CGTAGAATTCCTGAGCGACAGGTGGTGC-3′	Exon 11
10	A	5′-ACGATCGATCGATGGTGTGCCCACAGGTGA-3′	Exon 12
Primers pair for PCR-SSCP amplification of genomic DNA:			
Exon 2:			
1	S	5′-GGCAAAATCTCCAAAATCTTGCCA-3′	Exon 2
2	A	5′-tccaaagccagggcactcacAGA-3′	Exon 2-intron 2
Exon 3:			
3	S	5′-ctgggatcagaaaattcgctgg-3′	Intron 2
4	A	5′-caccctctgggcctggcacc-3′	Intron 3
Exon 4:			
5	S	5′-ctctcaccctctgtgtgccctca-3′	Intron 3
6	A	5′-gccccggccacgcccacccac-3′	Intron 4
Exon 5:			
7	S	5′-caggccaccgctttccctcctg-3′	Intron 4
8	A	5′-CTGGTCTAGGATGTGAGAATT-3′	Exon 5
Exon 6:			
9	S	5′-TACCGCAACGCCAGCAACCCC-3′	Exon 6
10	A	5′-aacgcaatcaagatggacagag-3′	Intron 6
Exon 7:			
11	S	5′-AAGCTGGACATGCTGGTGGCTTCA-3′	Exon 7
12	A	5′-acaccactcacCCTGCATCCA-3′	Exon 7-intron 7
Exon 9:			
13	S	5′-ctgacgggctgtggtggggtcc-3′	Intron 8
14	A	5′-CGCACAGCAGCCCCTCTTGCGC-3′	Exon 9

[a] S = sense primer; A = antisense primer.
[b] Sequence corresponding to introns are indicated in lowercase letters.

Table 4

Experimental Conditions Used for Restriction Analysis

Mutation	Exon	Primers[a]	Annealing Temperature (°C)	Size[b] (bp)	Enzyme[c]	Fragment Size[b] (bp)
P88S	2	1, 2	57	127	*Mnl*I	66 and 61
A114V	3	3, 4	60	141	*Mae*II	141 and 119
R125Q	3	3, 4	60	141	*Pst*I	83 and 58
E131D	3	3, 4	60	141	*Dde*I	141
P145L	3	3, 4	60	141	*Alu*I	117 and 24
T257M	7	5, 6	61	101	*Nla*III	68, 21, and 12
I278T	8	7, 8	62	174	*Bsr*I	132 and 42
G307S	8	7, 8	62	174	*Alu*I	81, 49, and 44
$A_{1224\text{-}2}C$	Skipping 12	9, 10	60	428	*Msp*I	94, 93, 79, and 62

[a] Sequences of primers are listed in table 2 (PCR amplification of genomic DNA).
[b] Expected size of amplified fragment.
[c] Restriction enzyme that can be used to confirm the mutation.
[d] Sizes of fragments resulting from restriction-enzyme digestion of amplified products.

for this change (fig. 2, *upper right*). T257M has not been found in any other patient in the present survey (data not shown). When inserted into the bacterial expression vector $pHCS_3$, containing the wild human CBS sequence, the T257M mutation led to a sharp decrease of both the CBS enzymatic activity and the amount of the immunoprecipitable specific product, as seen by western analysis (fig. 2, *lower left*).

A 68-bp Insertion in Exon 8 (844ins68)

In patient 12, a 68-bp insertion has been found in exon 8. PCR amplification of exon 8 from genomic DNA yielded two different products (174 and 242 bp). The insertion interrupts the normal sequence of the codon 282 (proline, at nt 844 in the cDNA). The 68-bp fragment encodes for 11 amino acids in frame, followed by a premature termination codon (TGA). The sequence of the insertion perfectly matches the intron-exon junction at the 5′ of exon 8 (53 and 15 nt upstream and downstream of the splicing site, respectively) (fig. 3, *top*). On the same allele before the insertion, a T-to-C transition ($T_{833}C$) has been found at codon 278, which causes an isoleucine-to-threonine (ATT-to-ACT) change (I278T) (fig. 3, *top*). This mutation has been reported

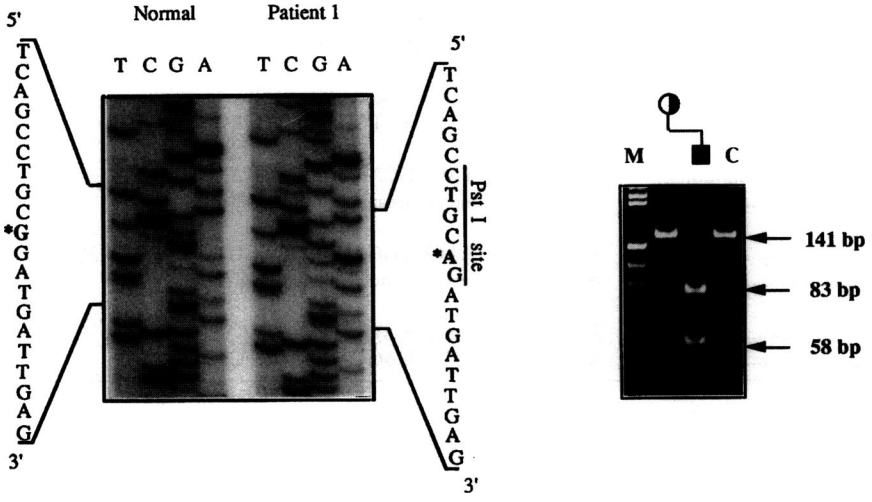
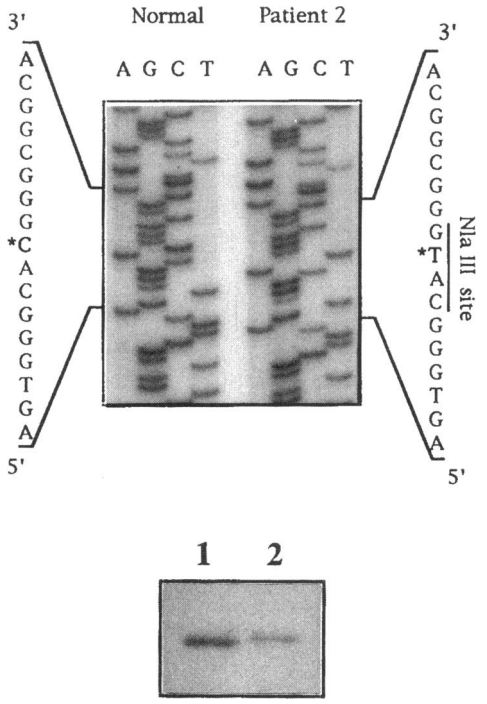


Figure 1 *Left,* Sequences of genomic DNA of patient 1 and a normal control, 3′ to 5′ from the bottom, demonstrating the substitution of a G (marked with an asterisk [*]) found in the normal sequence by an A (*) in patient 1. This mutation introduces a new *Pst*I site and changes the arginine codon CGG to glutamate codon CAG, at position 125. *Right,* PCR fragments analyzed by 12% PAGE after digestion with *Pst*I. *Lane M,* DNA MW (Marker V; Boehringer-Mannheim). *Lane C,* Normal control.



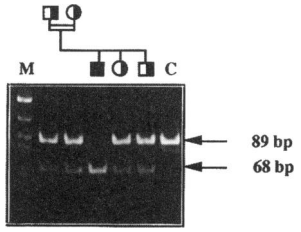


Figure 2 *Upper left,* Genomic DNA sequences of patient 2 and a normal control, 5′ to 3′ from the bottom, demonstrating the substitution of a C (*) found in the normal sequence by a T (*) in patient 2. This mutation introduces a new *Nla*III site and changes the threonine codon ACG to methionine codon ATG, at position 257. *Upper right,* Pedigree of patient 2 and PCR fragments analyzed by 12% PAGE after digestion with *Nla*III. *Lane M,* DNA MW (Marker V; Boehringer-Mannheim). *Lane C,* Normal control. *Lower left,* Western analysis of *Escherichia coli* lysate. Lysate of *Escherichia coli,* transformed with human CBS expression construct, were immunoprecipitated with polyclonal anti-CBS antibodies and submitted to western analysis. *Lane 1,* Normal human CBS. *Lane 2,* CBS from patient 2 with the T257M mutation.

elsewhere (Kozich and Kraus 1992). DNA sequencing of cloned PCR products of exon 8 also reveals that the other allele carries the I278T mutation (fig. 3, *bottom left*). Therefore, patient 12 is homozygous for the I278T mutation and heterozygous for a 68-bp insertion. It is interesting that the sequence of the insertion contains the normal isoleucine 278 codon (ATT). Parents were not investigated, as they had died.

A Missense Mutation at Codon 88 (P88S)

In family 3 (three affected sisters 3a, 3b, and 3c), we have identified a C-to-T transition ($C_{262}T$) changing a proline (CCT) to serine (TCT) at codon 88 in exon 2 (P88S). This mutation has been found by SSCP of exon 2 on genomic DNA (fig. 4, *upper left*). Direct sequencing of exon 2, amplified from genomic DNA, has revealed that the P88S mutation is present on one allele of the three affected sisters (fig. 4, *upper right*). As this mutation introduces a *Mnl*I site, the PCR product of exon 2 (127 bp) is cleaved into two fragments (66 and 61 bp). This approach confirms that patients 3a, 3b, and 3c are heterozygous for P88S, inherited from the father, while the healthy sister does not carry it (fig. 4, *lower left*). The P88S mutation is not present in any other patient of the present survey or in 100 unrelated normal alleles (data not shown).

Screening for Known Mutations

Six mutations, reported elsewhere (Kozich and Kraus 1992; Hu et al. 1993; Kozich et al. 1993; Marble et al. 1994) have been tested to screen the patients of the present survey (table 4). These mutations introduce or destroy a restriction site in the CBS coding region. By using *Mae*II restriction analysis of exon 3, the A114V mutation has been detected in 3/26 independent alleles (patients 6a, 6b, 8, and 9) (fig. 5). Expression of clones carrying the A114V mutation from patient 6a yelded CBS activity of ~50% of the control (data not shown). Using *Bsr*I restriction analysis of exon 8, the I278T mutation has been detected in 7/26 independent alleles (2 homozygous and 3 heterozygous patients) (fig. 6). The three affected sisters, 3a, 3b, and 3c, carrying the P88S mutation on the paternal allele, also show I278T, inherited from the mother (data not shown). All the other mutations (E131D, P145L, G307S, and $A_{1224\text{-}2}C$) have not been found in the present survey (data not shown). Molecular data found in the present study are summarized in table 5.

Discussion

We have investigated 18 patients (14 unrelated Italian families) affected by homocystinuria due to CBS deficiency. This study has led to the identification of four novel mutations. The missense mutation at codon 125 (R125Q) has been found in patient 1, in homozygosity. His clinical features are severe and resemble those of a nonresponsive patient, though a partial response to pyridoxine treatment is present. R125Q has been independently reported by us in patient 1 (Sebastio et al.

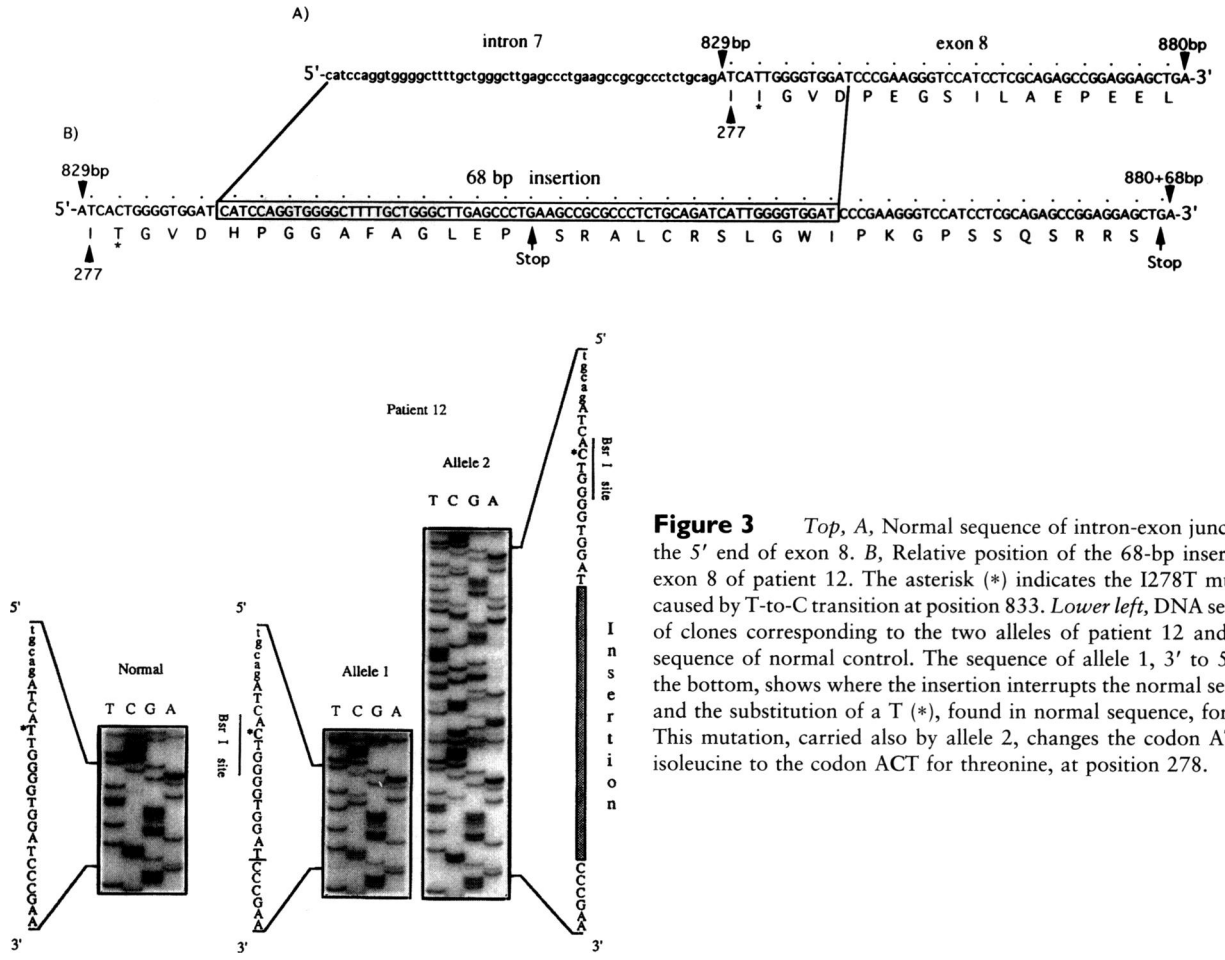


Figure 3 *Top, A,* Normal sequence of intron-exon junction at the 5′ end of exon 8. *B,* Relative position of the 68-bp insertion in exon 8 of patient 12. The asterisk (*) indicates the I278T mutation caused by T-to-C transition at position 833. *Lower left,* DNA sequence of clones corresponding to the two alleles of patient 12 and direct sequence of normal control. The sequence of allele 1, 3′ to 5′ from the bottom, shows where the insertion interrupts the normal sequence and the substitution of a T (*), found in normal sequence, for C (*). This mutation, carried also by allele 2, changes the codon ATT for isoleucine to the codon ACT for threonine, at position 278.

1993) and by Marble et al. (1994) in a nonresponsive patient of Irish origin who carries on both alleles an additional mutation, E131D, not found in patient 1. When tested in vitro, R125Q abolishes the CBS enzymatic activity. As to the genotype-versus-phenotype relationship, when present in homozygosity, R125Q is responsible for a severe clinical picture and reduced responsiveness to pyridoxine treatment.

The missense mutation at codon 257 (T257M) has been found in patient 2 in homozygosity. When tested in vitro, T257M completely inactivates CBS enzymatic activity and drastically reduces the immunoprecipitable specific product. In addition, the threonine may be a critical amino acid residue at position 257, as it is conserved from human and rat enzymes to homologous sequences of bacterial CYSK and spinach CSYN. Patient 2 presents clinical and biochemical findings of the nonresponsive form of the disease, including a severe CNS involvement.

The 68-bp insertion in exon 8 (844ins68) is a genomic rearrangement found in a patient homozygous for the I278T mutation. This insertion introduces premature termination codons. The origin of the insertion is likely to be the result of an unequal crossing-over in either of the parents. I278T is considered a mutation not affecting the responsiveness to coenzyme treatment and associated with a milder phenotype (Kozich and Kraus 1992). As a matter of fact, patient 12 displays a mild clinical picture (dislocation of optic lenses only). This suggests that the amount of CBS subunit, produced by the allele carrying the I278T mutation only, is consistent with a mild phenotype.

The missense mutation at codon 88 (P88S) has been found in three affected sisters (patients 3a, 3b, and 3c). The segregation analysis in the family shows that P88S is inherited from the father and I278T from the mother. Neither of the two mutations is present in the unaffected sister, as also shown by the intragenic *MspI* RFLP analysis elsewhere reported (see fig. 5 in Kraus et al. 1993). The proline at position 88 is conserved in homologous proteins from human and rat to bacteria and plants, thus suggesting a critical role of this amino acid in the

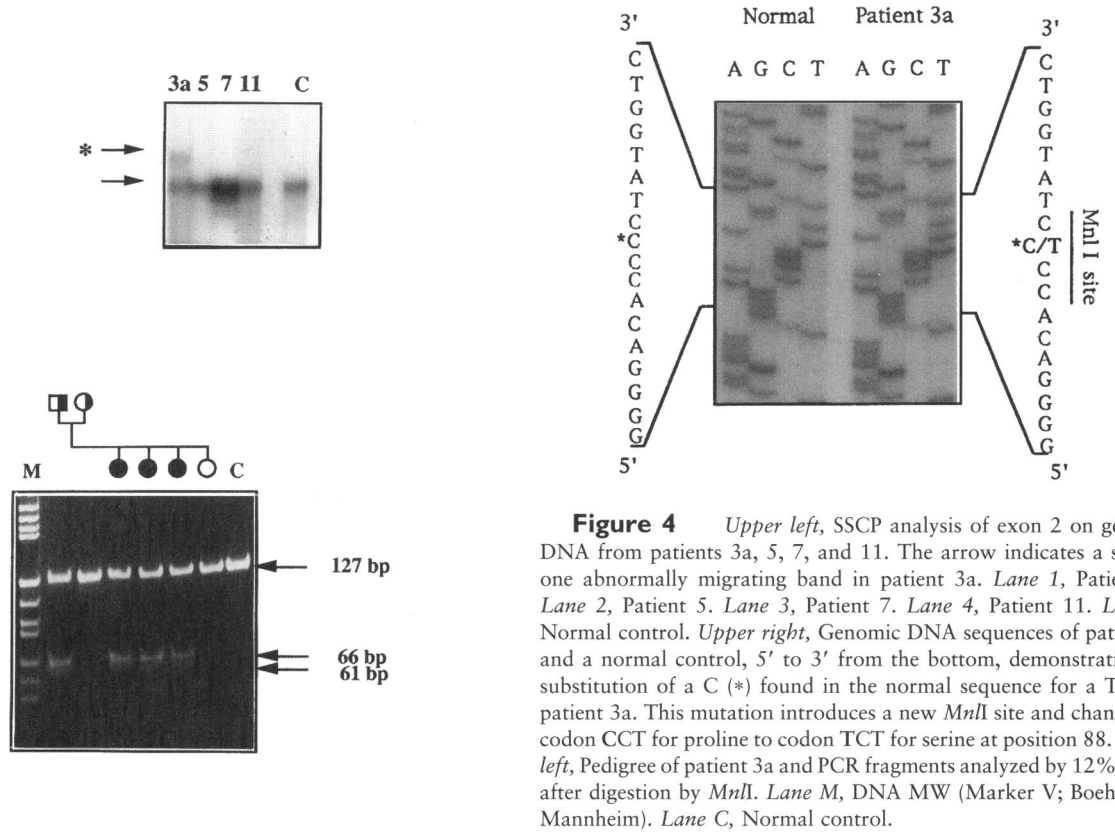


Figure 4 *Upper left,* SSCP analysis of exon 2 on genomic DNA from patients 3a, 5, 7, and 11. The arrow indicates a shift of one abnormally migrating band in patient 3a. *Lane 1,* Patient 3a. *Lane 2,* Patient 5. *Lane 3,* Patient 7. *Lane 4,* Patient 11. *Lane C,* Normal control. *Upper right,* Genomic DNA sequences of patient 3a and a normal control, 5′ to 3′ from the bottom, demonstrating the substitution of a C (*) found in the normal sequence for a T (*) in patient 3a. This mutation introduces a new *MnlI* site and changes the codon CCT for proline to codon TCT for serine at position 88. *Lower left,* Pedigree of patient 3a and PCR fragments analyzed by 12% PAGE after digestion by *MnlI*. *Lane M,* DNA MW (Marker V; Boehringer-Mannheim). *Lane C,* Normal control.

structure of the protein. We do not have a formal proof that P88S is a disease-causing mutation, but genetic and evolutionary considerations strongly support it.

As to the screening for known mutations, I278T has been found in 7 (27%) of 26 independent mutant alleles. I278T has been previously detected in 9 (12.5%) of 62 mutant alleles of different origin (Polish, French, German, Norwegian, English, and Jewish), thus indicating that it is a widespread mutation, quite common in Italian patients, too. The I278T mutation, when found in homozygosity, is associated with the pyridoxine-responsive form of the disease.

The A114V mutation, previously found in a single allele of a responsive patient of Irish-German origin, may be considered as a relatively frequent mutation in Italian patients (4 patients and 3 independent alleles). Three of them, carrying this mutation in heterozygosity (6a, 6b, and 8), have a pyridoxine-responsive form of the disease. This indicates that A114V, although very

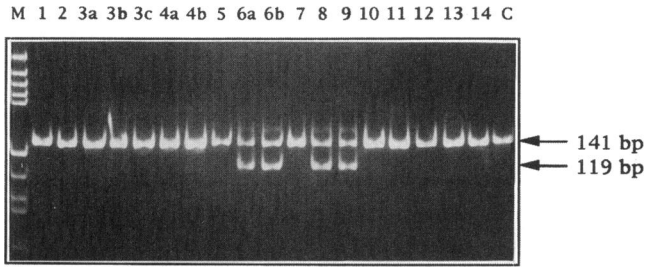


Figure 5 PCR fragment, amplified from genomic DNA of patients 1–14 and analyzed by a 12% PAGE after digestion with *MaeII*, showing the presence of the A114V mutation in patients 6a, 6b, 8, and 9. Lane M, DNA MW (Marker V; Boehringer-Mannheim). *Lane C,* Normal control.

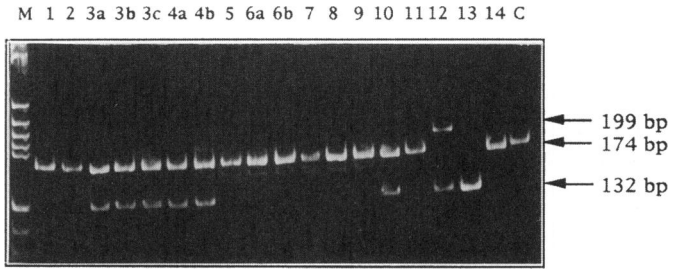


Figure 6 PCR fragment, amplified from genomic DNA of patients 1-14 and analyzed by a 10% PAGE after digestion by *BsrI*, showing the presence of the I278T mutation in patients 3a, 3b, 3c, 4a, 4b, 10, 12, and 13. Patients 12 and 13 are homozygous, while 3a, 3b, 3c, 4a, 4b, and 10 are heterozygous. *Lane M,* DNA MW (Marker V; Boehringer-Mannheim). *Lane C,* Normal control.

Table 5

Genotypes of Patients with Homocystinuria Due to CBS Deficiency

Patient No.	Allele 1	Allele 2	Codon	Consequence	Exon
1	$G_{374}A$	$G_{374}A$	125	R125Q	3
2	$C_{770}T$	$C_{770}T$	257	T257M	7
3a	$C_{262}T$	$T_{833}C$	88/278	P88S/I278T	2/8
3b	$C_{262}T$	$T_{833}C$	88/278	P88S/I278T	2/8
3c	$C_{262}T$	$T_{833}C$	88/278	P88S/I278T	2/8
4a	$T_{833}C$	...[a]	278/?	I278T/?	8/?
4b	$T_{833}C$	...[a]	278/?	I278T/?	8/?
6a	$C_{341}T$	...[a]	114/?	A114V/?	3/?
6b	$C_{341}T$	...[a]	114/?	A114V/?	3/?
8	$C_{341}T$	...[a]	114/?	A114V/?	3/?
9	$C_{341}T$	...[a]	114/?	A114V/?	3/?
10	$T_{833}C$	...[a]	278/?	I278T/?	8/?
12	$T_{833}C$+ins68bp	$T_{833}C$	278+ins/278	I278T+Stop/I278T	8/8
13	$T_{833}C$	$T_{833}C$	278/278	I278T/I278T	8/8
5-7-11-14	...[a]	...[a]			

[a] The allele was negative for four frequent mutations $C_{341}T$ (A114V), $T_{833}C$ (I278T), $G_{919}A$ (G307S), and $A_{1224\text{-}2}C$ (Δ exon 12). It was also negative for two private mutations $G_{393}C$ (E131D) and $C_{434}T$ (P145L).

close to the putative binding site lysine at position 119, probably does not abolish the coenzyme binding. As a matter of fact, when expressed in $pHCS_3$ the allele carrying the A114V mutation (patient 6a) yields ~50% of the normal CBS activity. On the contrary, the fourth patient (9 in table 2) has a nonresponsive form of the disease, suggesting that the second mutation, not found yet, should cause a severe reduction of CBS activity.

In the present survey, none of the alleles carries the G307S mutation, which is very frequent in nonresponsive patients of "Celtic" origin and occurs in 71% of Irish alleles, specifically (Hu et al. 1993; Ward et al. 1994). A likely explanation is that G307S has a more recent origin and has not yet spread. A similar consideration can be made for the mutation $A_{1224\text{-}2}C$ causing the exon 12 skipping. This mutation has been detected in nonresponsive mutant alleles of German and Turkish origin (Koch et al. 1994) and in a responsive patient of Jewish origin (Kozich and Kraus 1992), but it was not found in the present survey.

Our molecular data nicely correlate with clinical and biochemical evidence of a lower occurrence of the nonresponsive form of CBS deficiency in Italy (Andria et al. 1989). Mutations E131D and P145L, previously found in single alleles, have not been detected in Italian patients, thus probably representing very rare or *private* mutations.

In conclusion, 50% of the affected alleles of Italian patients (from southern Italy) have been characterized. Mutant alleles have been identified in five independent patients (four homozygotes and one compound heterozygote) and in one allele in five patients. In four patients, neither of the mutant alleles has been characterized. Data of the present study confirm that CBS mutations cluster in exon 3 or 8 (11 of 26 independent alleles).

Our results indicate that I278T and A114V are frequent and widespread mutations in Europe and should be always considered for the molecular diagnosis of the responsive form of CBS deficiency. On the other hand, G307S and $A_{1224\text{-}2}C$ are frequent among nonresponsive patients and are probably restricted to those of northern European origin. Finally, a better knowledge of the molecular basis of CBS deficiency may improve the screening of CBS deficient heterozygotes who are at higher risk for premature thromboembolic disease.

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