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Identification and Functional Analyses of *CBS* Alleles in Spanish and Argentinian Homocystinuric Patients

Mónica Cozar,[1†] Roser Urreizti,[1†] Laura Vilarinho,[2] Carola Grosso,[3] Raquel Dodelson de Kremer,[3] Carla G. Asteggiano,[3] Jaime Dalmau,[4] Ana María García,[4] María Antonia Vilaseca,[5] Daniel Grinberg,[1†] and Susana Balcells[1*†]

[1]Departament de Genètica, Universitat de Barcelona, IBUB, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Barcelona, Spain; [2]Instituto de Genética Médica Jacinto Magalhaes, Porto, Portugal; [3]Centro de Estudio de las Metabolopatías Congénitas (CEMECO), Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Hospital de Niños de la Santísima Trinidad, Córdoba, Argentina; [4]Unidad de Nutrición y Metabolopatías, Hospital Infantil La Fe, Valencia, Spain; [5]Servei de Bioquímica. Hospital Sant Joan de Déu, Barcelona, Spain

ABSTRACT: Homocystinuria due to CBS deficiency is a rare autosomal recessive disorder characterized by elevated plasma levels of homocysteine (Hcy) and methionine (Met). Here we present the analysis of 22 unrelated patients of different geographical origins, mainly Spanish and Argentinian. Twenty-two different mutations were found, 10 of which were novel. Five new mutations were missense and five were deletions of different sizes, including a 794-bp deletion (c.532−37_736+438del794) detected by Southern blot analysis. To assess the pathogenicity of these mutations, seven were expressed heterologously in *Escherichia coli* and their enzyme activities were assayed in vitro, in the absence and presence of the CBS activators PLP and SAM. The presence of the mutant proteins was confirmed by Western blotting. Mutations p.M173del, p.I278S, p.D281N, and p.D321V showed null activity in all conditions tested, whereas mutations p.49L, p.P200L and p.A446S retained different degrees of activity and response to stimulation. Finally, a minigene strategy allowed us to demonstrate the pathogenicity of an 8-bp intronic deletion, which led to the skipping of exon 6. In general, frameshifting deletions correlated with a more severe phenotype, consistent with the concept that missense mutations may recover enzymatic activity under certain conditions.
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KEY WORDS: CBS; homocystinuria; heterologous expression

Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that occupies a major regulatory branch point in methionine metabolism. Homocysteine may be either remethylated to methionine or catabolized to form cysteine through the transsulfuration pathway. The first step of transsulfuration involves the condensation of homocysteine with serine to form cystathionine, a reaction catalyzed by the enzyme cystathionine β-synthase (CBS; EC 4.2.1.22).

Mutations in the *CBS* gene (MIM♯ 613381) cause classical homocystinuria (Hcu; MIM♯ 236200), an autosomal recessive disease characterized by severe hyperhomocysteinemia and homocystinuria, decreased plasma levels of cysteine and, often, hypermethioninemia. At the clinical level, classical homocystinuria mainly affects the eye, the skeleton, the vascular system, and the central nervous system (CNS). Symptoms usually include ectopia lentis, osteoporosis, scoliosis, Marfanoid features, premature arteriosclerosis, thromboembolism, and mental retardation [Mudd et al., 1985]. Age of onset and disease severity are highly variable, ranging from dramatically affected children to asymptomatic adults [Magner et al., 2011; Skovby et al., 2010; Walter et al., 1998; Yap and Naughten, 1998]. Treatments that lower tHcy, such as B vitamins, dietary methionine restriction, and betaine supplementation, can significantly reduce the incidence of vascular events (the main cause of death in these patients) and improve the neurological problems [Wilcken and Wilcken, 1997; Yap and Naughten, 1998; Yap et al., 2001].

Homocystinuria is a rare disease with variable incidence. Although the worldwide incidence is estimated as 1/344,000 born alive [Mudd et al., 1995], in Qatar it is 1/3,124 [El-Said et al., 2006] and in Japan 1/800,000 [Mudd et al., 1995]. In Northern Europe the incidence may be of 1/20,500 to 1/6,400, as estimated from the high number of p.I278T mutation carriers found in some populations [Gaustadnes et al., 1999; Refsum et al., 2004].

To date, more than 150 different *CBS* mutations have been found worldwide, some of them common. Mutation p.I278T is considered panethnic and is particularly frequent in Northern Europe [Janosik et al., 2001b; Kraus et al., 1999; Moat et al., 2004]. In Ireland, the p.G307S mutation accounts for 71% of the mutant alleles [Gallagher et al., 1995], and in the Iberian Peninsula and Colombia, mutation p.T191M represents between 40 and 75% [Urreizti et al., 2006a] of the mutant alleles.

A truncated form of the human CBS enzyme lacking the C-terminal regulatory domain has been crystallized [Janosik et al.,

2001a; Meier et al., 2001] and structurally characterized [Banerjee and Zou, 2005; Kabil et al., 2001; Kery et al., 1999; Meier et al., 2001; Taoka et al., 2002]. The active form of this cytoplasmic enzyme is a homotetramer of four 63-kDa subunits. Each subunit combines one heme group and one pyridoxal phosphate (PLP), the latter acting as a cofactor in the reaction. In addition, each subunit binds the allosteric activator S-Adenosil-Methionine (S-AdoMet or SAM), an intermediate in the methionine cycle. Heterologous expression in *Escherichia coli* has been widely used to test the functionality of the *CBS* mutant alleles independently from the patient's genetic background [de Franchis et al., 1994; Katsushima et al., 2006; Maclean et al., 2002]. It has been proven to be a useful tool since the *E. coli*-expressed human CBS is indistinguishable from that obtained from cultured fibroblasts [Bukovska et al., 1994; Kozich and Kraus, 1992]. In addition, in vitro, its relative activity in response to PLP and SAM is comparable to that of the enzyme obtained from human tissues [Bukovska et al., 1994; Kozich and Kraus, 1992].

This study updates the *CBS* mutation spectrum of the homocystinuric patients from the Iberian Peninsula by presenting the analysis of 16 new cases. It also includes one Norwegian, one Indian, and four Argentinian patients. In this cohort of 22 cases, 22 mutations were found, 10 of which were novel. The new mutations include a deletion of 794 bp (c.532-37_736+438del794) detected by Southern blot analysis and an intronic deletion that leads to the skipping of exon 6, which was characterized using a minigene strategy. The pathogenic role of seven of the changes was assessed by heterologous expression of these mutant proteins, and their stability and activity were analyzed.

Materials and Methods

Patients

Twenty-six patients with classical homocystinuria from 22 unrelated pedigrees were involved in this study. Patients were initially diagnosed by their physicians on the basis of clinical manifestations suggestive of homozygous CBS deficiency. Biochemically, these patients presented with a combination of severe hyperhomocysteinemia (typically above 150 μmol/l), and hypermethioninemia (typically above 40 μmol/l). Thirteen Spanish, three Portuguese, one Norwegian, one Indian, and four Argentinian patients were included in the study. Our research was conducted in accordance with the tenets of the Declaration of Helsinki. The nature and possible consequences of the study were first explained to all patients and/or their parents, before their informed consent for inclusion in the research project was obtained.

PCR Amplification and DNA Sequencing

Genomic DNA was prepared from peripheral blood leukocytes, using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

All 16 *CBS* coding exons (including exon 15) and their intronic flanking regions, were amplified by PCR and sequenced as described previously [Urreizti et al., 2006a] with some modifications. Briefly, PCR reactions were performed on a final volume of 50 μl with 50 ng gDNA, 0.2 mM of each dNTP, 0.4 mM of each primer, 1.5–2.5 mM MgCl$_2$, and 1.25 U of GoTaq$^®$ Flexi DNA Polymerase (Promega). All mutations detected were confirmed by restriction analysis of the PCR products with the appropriate restriction enzyme, and the presence of all new mutations was assessed in 100 control chromosomes from Spanish anonymous donors. Primer sequences and PCR conditions have been described previously [Urreizti et al., 2006a]. *MTHFR* c.677C>T (rs1801133) was analyzed in all patients as described in Frosst et al. [1995].

To characterize the deletion found in patient 87, genomic DNAs of the patient and her parents were PCR-amplified using primers 4F and 7R as described above with the addition of a final concentration of 5% DMSO. The PCR fragments were purified and sequenced.

For naming the mutations the following reference sequences were used: Genomic, GenBank NG_008938.1; cDNA, ENST-00000352178. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Southern Blot

A Southern blot to analyze a 36-kb fragment of the *CBS* genomic region (Fig. 1), between two *Drd*I sites, encompassing all 23 exons, plus 6 kb of the 5′ flanking region and 11.5 kb of the 3′ region, was performed as follows: 10 μg of patient and control gDNA were double digested by *Drd*I and *Afl*II (NEBiolabs, Ipswich, MA), electrophoresed on a 0.9% agarose gel, blotted onto Amersham Hybond^{TM}-N+ (GE Healthcare, Waukesha, WI) membrane using standard protocols, and fixed by UV crosslinking. The *Drd*I/*Afl*II double digestion of the 36 kb yields, in a wild-type setting, four fragments of around 15, 10, 8, and 3 kb, respectively (Fig. 1A).

Probe Design, Preparation, and Hybridization

Four probes were designed by selecting one unique sequence of approximately 600 bp within each restriction fragment. The sequences were aligned to the whole genome by BLAST, to confirm their specificity. These four sequences were PCR-amplified from total genomic DNA (as described above), cloned into pUC19 vector (Fermentas, Burlington, ON, Canada) and sequenced. Probes were obtained from the clones by using digoxigenin-dUTP and the "PCR DIG Probe Synthesis Kit" (Roche, Mannheim, Germany), according to the manufacturer's instructions. Primer sequences for the probes are available on request. Labeled probes were subsequently purified using Illustra^{TM} GFX^{TM} PCR DNA, and a Gel Band Purification Kit (GE Healthcare).

All subsequent steps of the Southern protocol (prehybridization, hybridization, and developing of the filters) were performed using reagents (Dig Easy Hyb) and protocols (Dig Application Manual) from Roche (Mannheim, Germany).

Site-Directed Mutagenesis, Heterologous Expression, and In Vitro Enzyme Activity Assays of the *CBS* Mutations

All *CBS* mutant constructs were derived from the wild-type *CBS* expression plasmid pHCS3 [Kozich and Kraus, 1992], a gift from the authors of that study. Each mutation was introduced into the wild-type expression plasmid using a Quik Change II XL^{TM} Site-Directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA) and expressed in XL-Gold *E. coli* cells as described in Urreizti et al. [2006b]. Expression of pHCS3 was used as a positive control, and that of the empty vector pKK388.1 was included as a negative control. Protein extracts were obtained and CBS activity was measured as described in Kraus [1987] with some modifications [Urreizti et al., 2006b].
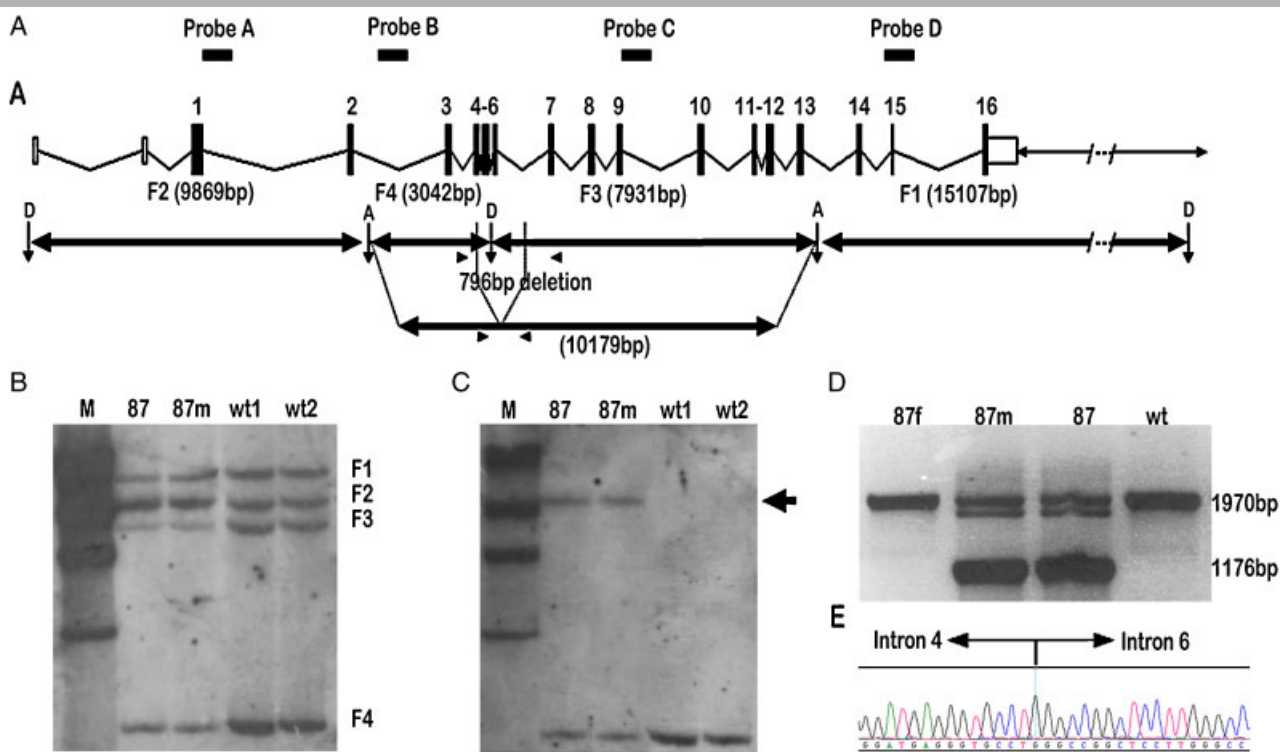
Figure 1. Southern Blot analysis of the *CBS* gene. **A:** Scheme of the human *CBS* gene. The *Drd*I (**D**) and *Afl*II (**A**) restriction sites and the corresponding restriction fragments (F1–F4) are indicated below the scheme. The positions of the probes (**A–D**) are indicated above. **B:** Southern Blot analysis of patient ♯87 and her mother (♯87m) hybridized with the whole set of probes. Two wild-type DNAs were included in the analysis. M, molecular weight marker. **C:** Southern blot analysis hybridized with probe B. The arrow indicates the new band found in the patient and her mother. **D:** PCR amplification of the fragment from intron 3 to intron 7. An extra band of reduced size was found in patient 87 and her mother, corresponding to a 794-bp deletion. The extra band in 87m and 87 corresponds to the heteroduplex. **E:** Chromatogram of patient 87 showing the deletion boundaries. [Color figures can be viewed in the online issue, which is available at www.wiley.com/humanmutation.]

CBS Protein Analysis

To assess the presence and relative amount of wild-type and mutant CBS proteins, Western blot analysis of the soluble fraction of the crude cell lysates was performed under denaturing conditions as described in Janosik et al. [2001b], with some modifications [Urreizti et al., 2006b].

Minigene Construction and Splicing Assay

Genomic DNA from patient 68a (heterozygote for mutation c.667−14_−7del8) was amplified using *CBS* primers 4F and 6R to obtain a fragment of 626 or 618 bp (from the last 29 bp of intron 3 to the first 103 bp of intron 6). This PCR product, containing both wild-type and mutant alleles, was purified using Illustra™ GFX™ PCR DNA and a Gel Band Purification Kit (GE Healthcare) and cloned into the pGLB1 vector [Diaz-Font et al., 2005]. Plasmid pGLB1 is based on a pcDNA3.1 vector and contains exons 7, 8, and 9 and introns 7 and 8 of the *GLB1* gene, where intron 7 contains a *Pme*I restriction site, which was used to clone the *CBS* fragment. The resulting plasmids, named pGLB1-CBS_wt and pGLB1-CBS_mut, were confirmed by sequencing.

The splicing assay was performed by transfecting 1 μg of each minigene plasmid with 5 μl of Lipofectamine 2000 Reagent (Life Technologies, Basel, Switzerland) into 90% confluent HeLa cells. Total RNAs were isolated 24 hr after transfection using the QIAshredder and the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was determined spectrophotometrically, integrity

was verified by gel electrophoresis and RNA quality was assessed by OD 260/230 and 260/280 ratios.

Two micrograms of each RNA was reverse-transcribed in a final volume of 20 μl using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

PCR amplification of each cDNA product was performed using primers T7 and SP6 as previously described [Diaz-Font et al., 2005; Santamaria et al., 2008]. The PCR products were electrophoresed and each band of interest was purified and analyzed by sequencing.

Results

Screening for *CBS* Mutations

Twenty-two different mutations (Table 1), 10 of them novel, were found in 22 unrelated patients of different geographical origin (Table 2). Forty-two mutant alleles were identified through sequencing of coding exons and flanking intronic regions, while an additional mutant allele was discovered by Southern blot analysis (see below). Only one allele remained unidentified. Five of the new mutations were missense while five were deletions.

After sequencing the complete *CBS* coding sequence, only mutation c.1566delG was found in heterozygosis in patient 87, which she inherited from her father. A Southern blot analysis of this patient and her mother, hybridized with a set of four probes covering the entire *CBS* genomic region (Fig. 1A), revealed the

Table 1. List of the 22 Different Mutations Found in This Cohort

DNA	Deduced protein change	Exon–intron	Alleles	Restr. enz.[a]	References
c.146C > T	p.P49L	Ex 1	1		de Franchis et al., 1998
c.253G > A	p.G85R	Ex 2	1		Maclean et al., 2002
c.361C > T	p.R121C	Ex 3	1		Katsushima et al., 2006
c.374G > A	p.R125Q	Ex 3	1		Marble et al., 1994
c.518delTGA	**p.M173del**	**Ex 4**	**2**		**This study**
c.532−37_736+438del794	**p.V178GfsX23**	**In 4–In 6**	**1**		**This study**
c.572C > T	p.T191M	Ex 5	7		Urreizti et al., 2003
c.599C > T	**p.P200L**	**Ex 5**	**1**	*Msp*I−	**This study**
c.667−14_667−7del8	**p.Y223GfsX23**	**In 5**	**1**		**This study**
c.676G > A	p.A226T	Ex 6	2		Shan and Kruger, 1998
c.689delT	**p.L230RfsX39**	**Ex 6**	**1**	*Msp*I+	**This study**
c.824G > A	p.C275Y	Ex 7	1		Urreizti et al., 2003
c.833T > G	**p.I278S**	**Ex 8**	**1**	*Tsp*Ri+	**This study**
c.833T > C	p.I278T	Ex 8	4		Kozich and Kraus, 1992
c.841C > T	**p.D281N**	**Ex 8**	**1**	*Bam*HI−	**This study**
c.862_866del5	**p.E289GfsX39**	**Ex 8**	**2**		**This study**
c.962A > T	**p.D321V**	**Ex 9**	**1**	*Hpy*8I−	**This study**
c.1085C > T	p.T353M	Ex 10	2		Dawson et al., 1997
c.1136G > A	p.R379Q	Ex 10	1		Urreizti et al., 2003
c.1330G > A	p.D444N	Ex 12	4		Kluijtmans et al., 1996
c.1336G > T	**p.A446S**	**Ex 12**	**1**	*Bst*NI+	**This study**
c.1566delG	p.K523SfsX18	Ex 16	5		Castro et al., 1999

Mutations not previously described are indicated in bold.

[a]Novel missense mutations were checked in control chromosomes using the indicated enzyme. +: the mutation creates a new site; −: the mutation destroys a site. Reference sequences: Genomic, GenBank NG_008938.1; cDNA, ENST00000352178. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Table 2. Patients, CBS, and MTHFR C.667C > T Genotypes, Disease Severity, and Geographical Origin

Patient	Genotype	MTHFR (c.677C > T)	Severity	Country
74	p.[T353M]+[D444N]	TT	Severe	Spain
79	p.[**P200L**]+[**?**]	CT	Asymptomatic	Spain
80a	p.[R125Q]+[P49L]	TT	Mild	Spain
80b	p.[R125Q]+[P49L]	CT	Asymptomatic	
82a	p.[T191M]+[**D281N**]	CC	Mild	Spain
82b	p.[T191M]+[**D281N**]	CC	Mild	
82c	p.[T191M]+[**D281N**]	CC	Mild	
83	p.[T353M]+[D444N]	TT	Mild	Spain
84	p.[I278T]+[R121C]	CT	Mild	Spain
88	p.[D444N]+[D444N]	CT	Mild	Spain
92	p.[T191M]+[T191M]	CT	Mild	Spain
93	p.[T191M]+[T191M]	CT	Severe	Spain
94	p.[T191M]+[**I278S**]	CT	Severe	Spain
100	p.[T191M]+[R379Q]	CT	Mild	Spain
107	c.[1566delG]+[1566delG]	CT	NA	Spain
108	p.[I278T]+[C275Y]	TT	Mild	Spain
81	c.[1566delG]+[1566delG]	TT	Severe	Portugal
87	c.[1566delG]+[**532−37_736+438del794**]	CC	Neonatal screening (asymptomatic)	Portugal
109	p.[**E289GfsX39**]+[**E289GfsX39**]	CT	Severe	Portugal
68a	p.[G85R]+**c.[667−14_667−7del8]**	CT	Severe	Argentina
68b	p.[G85R]+c.[667−14_667−7del8]	CT	Severe	
89	**c.[689delT]**+[**689delT**]	CT	Severe	Argentina
90	p.[**D321V**]+[**A446S**]	CT	Mild	Argentina
91	p.[A226T]+[A226T]	CT	Severe	Argentina
105	p.[I278T]+[I278T]	ND	NA	Norway
55	p.[**M173del**]+[**M173del**]	ND	Mild	Indian

ND, not determined; NA, not available.

same pattern of bands observed in control samples but the relative intensities of fragments 2, 3, and 4 were altered (Fig. 1B). Hybridization of the same membrane separately with each individual probe revealed an additional band of nearly 10 kb detected by probes B and C (Fig. 1C). This result was consistent with a deletion removing the *Drd*I site present in intron 5. To test this hypothesis, a PCR amplification from intron 3 to intron 7 was performed. As shown in Figure 1D, a new band of 1,176 bp was observed in the samples of the patient and her mother. Sequencing revealed a 796-bp deletion (c.532−37_736+438del794), which spans from the last 37 bp of intron 4 to the first 438 bp of intron 6 (Fig. 1E). This deletion causes a frameshift starting from a valine to glycine substitution at position 178 and leading to a stop codon at residue 201 (p.V178GfsX23).

Analyses of Pathogenicity of Mutant *CBS* Enzymes

None of the new mutations found was present in 100 control chromosomes, ruling out the possibility of common polymorphisms.

We assessed the pathogenicity of all five new missense mutations and of the novel in-frame deletion p.M173del using an *E. coli* heterologous expression system followed by Western blot and in vitro enzyme activity analysis (Fig. 2). Mutation p.P49L, previously described by de Franchis et al. [1998] was also analyzed.

All the proteins from the mutant alleles were found in amounts similar to those of the wild type (Fig. 2, upper panel). The activity of these mutant enzymes was assayed in vitro, either in the presence or absence of the cofactor PLP, or in the joint presence of PLP and SAM (Fig. 2, lower panel). As previously described [Kluijtmans et al., 1996; Maclean et al., 2002], we found that the wild-type CBS activity was strongly stimulated by both PLP and the combination of PLP and SAM. Mutations p.M173del, p.I278S, p.D281N, and p.D321V showed null activity, either in the absence or presence of the activators. In contrast, mutations p.49L, p.P200L, and p.A446S retained different amounts of activity and also the ability to be stimulated.

In particular, mutation p.P49L displayed null activity in the absence of any of the activators, retained 71% of the wild-type activity in the presence of PLP and showed reduced activation by SAM. In contrast, in the absence of any of the activators, mutation p.P200L displayed around a third of the activity of the wild type (15 vs. 43%) and retained the ability to be induced by PLP and SAM. SAM stimulation was twofold, comparable to that of the wild type. Finally, p.A446S displayed highly variable levels of activity in different tests, in the absence or presence of activators, but in general the values where in the range of the wild type or higher.

Effect of a Small Intronic Deletion on RNA Splicing

The small deletion c.667-14_667-7del8 involved intron 5 sequences within the acceptor site, without affecting the conserved AG dinucleotide. Because no RNA was available from patients with this mutation (68a and 68b), a minigene assay was performed to test the effect of this mutation on RNA splicing (Fig. 3A). The genomic region spanning from the 3′ part of intron 3 to the 5′ part of intron 6 of the *CBS* gene of the mutant and wild-type alleles was PCR-amplified and cloned within intron 7 of a construct containing several *GLB1* exons (see Materials and Methods for details). Wild-type and mutant constructs were transfected into HeLa cells and splicing was assayed by RT-PCR using the T7 and SP6 primers (Fig. 3B). The major band observed in the wild-type sample corresponds to the inclusion of all three *CBS* exons, whereas in the mutant, exon 6 was skipped, as assessed by sequencing of all the bands.
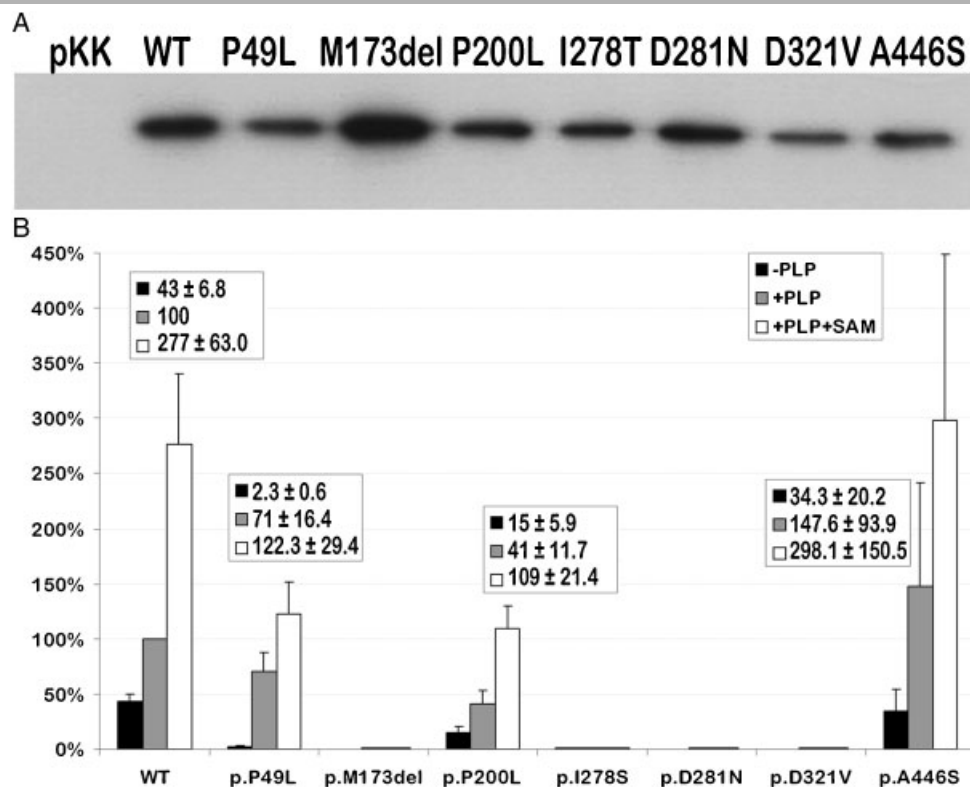

Figure 2.   Heterologous expression and activity of seven *CBS* mutations. **A:** SDS-PAGE followed by Western blot immunodetection of wild-type and mutant CBS proteins expressed in *E. coli* XL Gold. Ten micrograms of total protein extract was loaded in each lane. Six replicates were performed for each mutation. pKK, empty vector used as negative control. **B:** In vitro enzyme activity of wild-type and mutant *CBS* alleles from *E. coli* XL Gold extracts in the absence of both PLP and SAM (black boxes), in the presence of PLP (gray boxes), and in the presence of both PLP and SAM (white boxes). Wild-type CBS activity in the presence of PLP was taken as the reference value (100%). Activity values greater than 4% are indicated in the figure. Values are the means of six replicates. Error bars represent SD.
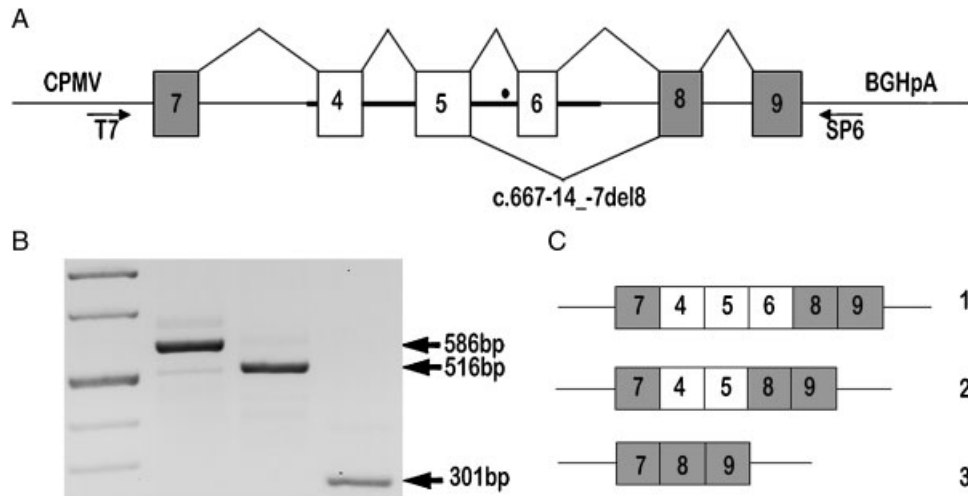
Figure 3. Splicing analysis of mutation c.667–14_-7del8 included in a minigene construction. **A:** Scheme of the minigene: gray boxes represent *GLB1* exons and white boxes are *CBS* exons. The thick horizontal line represents the *CBS* introns and the thin line the *GLB1* introns. PCMV, CMV promoter; BGHpA, BGH polyadenylation site. Wild-type and mutant splicing patterns are indicated above and below the gene, respectively. The dot indicates the position of the mutation. The wild-type and mutant constructs were transiently transfected into HeLa cells. Total RNA was extracted 24 hr after transfection, reverse-transcribed, and PCR-amplified with primers T7 and SP6 (indicated by arrows). **B:** PCR amplification of the wild-type (1) mutant (2) and the minigene construct without the *CBS* exons (3). The asterisk points to a minor band lacking exon 4 observed in the wild-type construct. **C:** Diagrams showing the three main PCR products.

Discussion

Mutation p.T191M was identified in 21.8% of the alleles of the Spanish and Portuguese patients included in this study (Table 2). Taking into consideration all 50 homocystinuric patients from the Iberian Peninsula included in this and in our previous studies [Urreizti et al., 2003, 2006a], mutation p.T191M accounts for 44% of the mutant chromosomes. Mutation c.1566delG is the second most prevalent change (7% overall), and is particularly common in Portugal (21%). In the present study, we found the panethnic mutation p.I278T for the first time in two unrelated Spanish patients, both in the heterozygote state, and in a Norwegian patient, in homozygosis (Table 2). Missense mutations are the most common alterations in the *CBS* gene in the patients of the present study (73%). We also found six deletions of different sizes (1–794 bp), five of them novel.

Southern blot analysis allowed us to identify a large deletion of nearly 800 bp in the heterozygous state (c.532−37_736+ 438del794, p.V178GfsX23) in patient 87 and in her mother. Additionally, a qmfPCR assay was performed on the patient's sample yielding a consistent result [C. Ged, personal communication]. This patient, diagnosed through neonatal screening, presented with dramatically increased plasma methionine (700 μM) and total homocysteine (145 μM). She has been on a normal diet and treated with pyridoxal, folate, and betaine since her birth. Currently aged 4 years, her plasma homocysteine and methionine levels are 76 and 302 μM, respectively, and she is clinically asymptomatic, highlighting the importance of an early diagnosis.

An intronic 8-bp deletion (c.667–14_-7del8) was found in two siblings (♯68a–♯68b, Table 2). Prediction algorithms indicated a subtle reduction in the score of the splicing acceptor site of intron 5. However, a minigene analysis clearly showed that it led to the skipping of exon 6, and produced a frameshift. This is consistent with the severity of the patients' phenotypes. We also found a 5-bp deletion in exon 8 in patient ♯109, and a 1-bp deletion in exon 6 in patient ♯89, both in homozygosis (Table 2). The severity of

these genotypes is in accordance with the severe clinical presentation of both patients. In general, frameshift-generating deletions correlate with a more consistently severe phenotype, as expected.

Seven mutations (six missense and a 3-bp deletion) were expressed in *E. coli* and analyzed by Western blot under denaturing conditions (Fig. 2, upper panel). Their presence in amounts similar to those of the wild-type CBS suggests that the mutations do not affect protein integrity. Alternatively, they may affect the catalytic core, proper protein folding, or the ability to form tetramers. In this regard, several authors have studied the involvement of certain chaperones in the rescue of missense CBS mutants, and demonstrated that the regulation of these chaperones could lead to almost complete recovery of the mutant protein activity [Kopecka et al., 2011; Majtan et al., 2010; Singh et al., 2007, 2010].

Four mutations showed null activity, which supports their pathogenic character. These mutations, p.D281N, p.I278S, p.D321V, and p.M173del, were found in four unrelated patients, ♯82a (and his siblings), ♯94, ♯90, and ♯55, respectively. No correlation between their null activity and the phenotypes of the patients could be established because all but patient ♯94 presented with mild disease.

The other three mutations, displaying different degrees of enzyme activity, were p.P49L, p.P200L, and p.A446S, present in patients ♯80, ♯79, and ♯90, respectively, all in the heterozygous state. In these cases, a correlation between their residual activity and the mild phenotype of the patients was observed.

We found the activity of p.P49L to be 75% of that in the wild type. Since our work was ongoing, Kozich et al. [2010] published an analysis of mutation p.P49L expressed in *E. coli*. They found it to be an active enzyme, indistinguishable from the wild type. Mutation p.P49L was found in two siblings: one with adult onset, and the other asymptomatic. Genetic analysis of the proband (♯80a in Table 2 and subject II.1 in Fig. 4), showed compound heterozygosity for p.P49L and p.R125Q (Table 2). Genotyping of
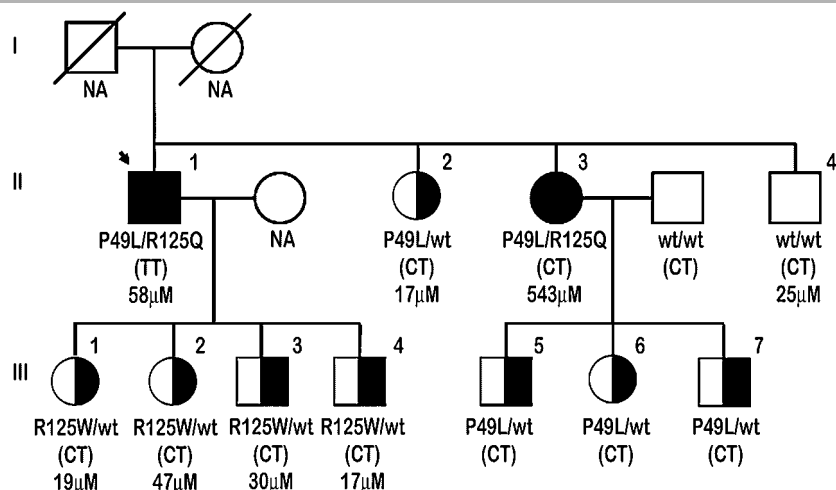
Figure 4. Pedigree of family 80. Patient 80a (II.1), the proband, is marked with an arrow. Patient 80b is member II.3. *CBS* genotypes are indicated below the pedigree symbols; the *MTHFR* c.677C > T genotypes are also included in brackets. Total plasma homocysteine levels (μmol/l) are indicated when available. NA, not available.

his siblings revealed an asymptomatic sister also carrying both mutations (♯80b; II.3 in Fig. 4). The proband had suffered a myocardial infarction at age 53. Blood testing revealed moderate hyperhomocysteinemia and hypermethioninemia (58 and 655 μM, respectively) and he responded well to PLP (tHcy: 28 μM after treatment). On the other hand, his asymptomatic sister, aged 54, presented with severe hyperhomocysteinemia (543 μM) and hypermethioninemia (1,723 μM) but had no clinical sign of classical homocystinuria and had given birth to three healthy offspring without complications. As the two *CBS* mutations were inherited separately by the offspring, we know that they were not in *cis* in the mother or in his affected brother. Mutation p.R125Q, the other mutation in family ♯80, was originally described as a null activity mutation [Sebastio et al., 1995]. In their recent publications, Kozich et al. [2010] and Majtan et al. [2010] expressed it in *E. coli*. In both studies the reduced activity was improved under permissive conditions or in the presence of chaperones. Taken together, these new results on p.P49L and p.R125Q help explain the mild or null affectation of patients ♯80a and ♯80b. In addition, the *MTHFR* 677C > T polymorphism may act as a phenotype modifier in this family. However, the discrepancy between the blood markers and the phenotype of patient ♯80b remains unexplained.

Mutation p.P200L was identified as the sole mutation in patient ♯79. The patient's symptoms are limited to hypertension and severe hyperhomocysteinemia (183 μM), which does not respond to vitamin B6 and folate treatment. A second *CBS* mutation may be deep intronic, located in a regulatory region far from the gene, or involved in a complex rearrangement not detected by our screening methods. Mutation p.A446S was found in a mildly affected patient (♯90, Table 2), in compound heterozygosis with the null mutation p.D321V (Fig. 2). The activity of p.A446S was very similar to that of the wild type, and this correlates with the mild phenotype of the patient, who only presented with lens dislocation at 45 years, despite a high tHcy level (105 μM, which was reduced to 40 μM after 2 months of treatment with folic acid and vitamins B6 and B12).

The poor genotype–phenotype correlation widely observed by us and others among the homocystinuric patients and the demonstration that some patients carrying two *CBS* mutant alleles are asymptomatic, call for environmental and genetic modifiers

involved in the pathophysiology of the disease. Some of these genetic factors may be involved in the folding and degradation of the mutant proteins.

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