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Molecular Basis of Phenotype Expression in Homocystinuria

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Summary: Cystathionine β-synthase (CBS) deficiency is the most common cause of homocystinuria in humans. The human gene maps to chromosome 21q22.3 and encodes the CBS subunit of 551 amino acid residues (63 kDa). CBS, a tetramer of these subunits, binds its two substrates, homocysteine and serine, and three additional ligands: pyridoxal 5'-phosphate, *S*-adenosylmethionine, and haem. Screening for mutations by expressing patient cDNA segments in *E. coli* permitted us to separate the parental CBS alleles, localize each mutation within one third of the cDNA, and functionally analyse the mutant protein. Using this method we identified the first 14 mutations in homocystinuria. The most common mutation in patients of predominantly 'Celtic' origin is the $G_{919}A$ transition which substitutes serine for glycine 307.

The metabolic fates of homocysteine, arising from *S*-adenosylmethionine, are either resynthesis of methionine or transsulphuration to cysteine. Defects in either of these pathways may precipitate homocystinuria. However, the chief cause for this inherited disorder is the deficiency of cystathionine β-synthase (CBS) (L-serine hydrolyase (adding homocysteine) EC 4.2.1.22). This condition is characterized by clinical symptoms affecting the central nervous system, the skeleton, the eye and the vascular system. Mental retardation, psychotic behaviour and seizures are often seen as manifestations of changes in the CNS; scoliosis, arachnodactyly, elongation of the long bones, and osteoporosis are examples of the skeletal malformations. Dislocated optic lenses are very typical of homocystinuria and the problems affecting the vascular system include vascular occlusions, malar flush and livedo reticularis. Biochemical findings include elevated methioine and homocysteine and low cysteine in body fluids (Mudd et al 1989).

GENE ORGANIZATION

The human CBS gene maps to chromosome 21 (Skovby et al 1984a), specifically to 21q.22.3 (Munke et al 1988). The rat gene has been extensively characterized. It is ~25 kbp long and its transcribed sequence is broken up into 18 exons and 17 introns (Swaroop et al 1992). Exon 13 and 16 are alternatively spliced, and consequently there are two major active enzyme isoforms in rat tissues (Roper and Kraus 1992).

HUMAN CBS cDNA

Human CBS cDNA has been cloned and sequenced (Kraus et al 1993). Two populations of cDNAs have been recognized, differing in their 3′-untranslated regions. Most CBS mRNAs retain a 214 bp insert in the 3′-untranslated region, bounded by the consensus intron dinucleotides GT/AG, while some transcripts lack this sequence. The open reading frame predicts a CBS polypeptide consisting of 551 amino acid residues. The similarity of its amino acid sequence exceeds 90% when compared to rat CBS and 52% when compared to the bacterial *cysK* gene product, *O*-acetylserine (thiol)-lyase (Swaroop et al 1992).

ENZYME CHARACTERISTICS

CBS is a tetramer of identical 63 kDa subunits (Skovby et al 1984b). The enzyme was initially purified from human liver in its activated, proteolysed form as a dimer of 48 kDa subunits (Kraus and Rosenberg 1983). CBS binds its two substrates, homocysteine and serine, as well as three additional ligands: the coenzyme pyridoxal 5′-phosphate (Kraus et al 1978), an allosteric activator *S*-adenosylmethionine (Roper and Kraus 1992) and a haem moiety whose function is not yet clear (Bukovska et al 1994). Recently, the coding region of human cDNA was expressed in *E. coli*. This allowed for the first time purification of the enzyme to homogeneity in the form in which it exists in human tissues, a tetramer of 63 kDa subunits. The cloned enzyme is indistinguishable from its hepatic counterpart in its kinetic and immunological properties and in its affinity for pyridoxal 5′-phosphate and *S*-adenosylmethionine and haem (Bukovska et al 1994).

SCREENING FOR MUTATIONS

Identification of disease-causing mutations usually involves screening single- or double-stranded fragments of genomic DNA or cDNA for migration differences in native or denaturing gels. These methods detect alterations in the nucleotide sequence but they do not differentiate pathogenic mutations from silent ones. Our approach to screening for mutations in homocystinuric patients starts with the expression of hybrid CBS cDNAs in *E. coli*. These hybrid cDNAs consist of different segments of patient cDNA in the context of otherwise normal CBS cDNA sequence. Pathogenic mutation(s) in the test segment extinguish CBS activity in the transformed *E. coli* host. The strategy used for the functional screen for CBS mutations is depicted in Figure 1. As a starting material we constructed plasmid pHCS3, which produces normal human CBS in *E. coli*, by inserting the coding region of human CBS cDNA into the bacterial expression vector pKK 388.1. Next, three separate cartridges were prepared by removing the 5′-portion, the middle segment, or the 3′-portion of the CBS coding region. The corresponding sections generated by PCR amplification of patient cDNA were then substituted for these regions. Prior to preparing the cartridges, each was inactivated by deleting a portion of the respective test segment. These deletions ensured that CBS activity could only arise from cDNA segments without a mutation rather than from incomplete digestion of the original plasmid.

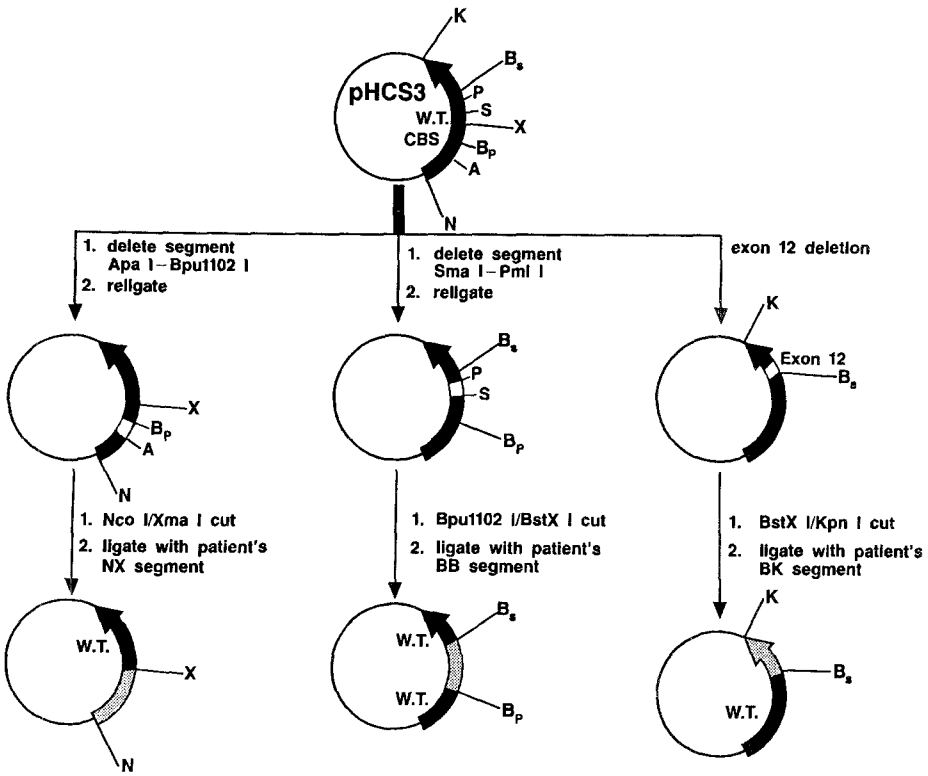

Figure 1 Mutation screening method. The coding region of CBS in pHCS3 is indicated by the wide, solid arrow. Inactivating deletions were created in the 'wild-type' (WT) human CBS expression vector pHCS3 (top) by the manipulations indicated. The exon 12 deletion indicated was derived from a naturally occurring mutation (Kožich and Kraus 1992). These deletions are indicated by breaks in the solid arrow in the plasmids depicted in the middle row. Separate expression cartridges were subsequently prepared by digesting these vectors as indicated: Nco I(N)/Xma I(X) for the vector NX; Bpu1102 I(Bp)/BstX I(Bs) for BB; and BstX I(Bs)/Kpn I(K) for BK. Hybrid constructs (bottom) were prepared by substituting segments from patient cDNA (stippled arcs) for the corresponding inactivated region of the plasmid. After transforming *E. coli* with these constructs, individual clones were tested for CBS activity

This screening method permits separation of individual alleles and approximate localization of the mutation(s). Additionally, it affords functional analysis of the mutant protein (Kozich and Kraus 1992; de Franchis et al 1994).

CBS MUTATIONS

Nature of the mutations: We have identified 14 mutations in homocystinuric patients to date (Kožich and Kraus 1992; de Franchis et al 1993; Hu et al 1993; Koch et al 1993; Kožich et al 1993; Marble et al 1993; Sebastio et al 1993; Gallagher et al 1994). Table 1 lists these mutations by type, by number of independent alleles discovered, by exon, and by ethnic origin of the patients. Some of the mutations occur more

frequently than others. Thus the $G_{919}A$ transition, leading to a substitution of ser for gly at position 307 (G307S), is undoubtedly the leading cause of homocystinuria in Ireland (71% of affected alleles) (Gallagher et al 1994). It has also been detected frequently in US and Australian patients of 'Celtic' origin including families with Irish, Scottish, English and French roots (Hu et al 1993). Of the 62 independent alleles characterized to date, 36 (58%) carried this mutation (Table 1). However, it should be emphasized that the patient population was biased towards patients of Irish origin. A second frequent mutation is the $T_{833}C$ transition (I278T), found in patients of very diverse ethnic and racial backgrounds. The $G_{374}A$ (R125Q) mutation was found in three alleles in patients of Romanic origin, while in an Irish patient it was found together with another mutation on the same allele, namely $G_{393}C$ (E131D). Another linked pair of mutations found in an Irish patient was the $C_{233}G$ (P78R) and $G_{307}C$ (K102N) pair. When reproduced separately by *in vitro* mutagenesis of expression plasmids, each was found to be deleterious to CBS activity (Marble et al 1993; de Franchis et al 1994).

Most mutations are missense and are localized to the amino terminal half of the polypeptide in exons 2 through 10, clustered mainly in exons 3 and 8. One splicing error was confirmed in four mutant alleles; the AG acceptor site of intron 11 was changed to CG, leading to 'in-frame' deletion of exon 12.

Genotype and pyridoxine reponsiveness: The majority of homocystinuric patients are compound heterozygotes. Table 2 depicts the combinations of different allelic genotypes identified so far and how these affect patient response to B_6 therapy. The 'Celtic' mutation G307S appears to be incompatible with B_6 responsiveness whether

Table 1 Incidence, location and origin of CBS mutations

Mutation	*Alleles*	*Exon*	*Origin*
$C_{233}G$ (P78R) (linked) $G_{306}C$ (K102N)	1	2	Irish
$C_{341}T$ (A114V)	1	3	Irish/German
$G_{374}A$ (R125Q)	3		Italian, Portuguese
$G_{374}A$ (R125Q) (linked) $G_{393}C$ (E131D)	1	3	Irish
$C_{434}T$ (P145L)	1	3	Irish/German
$G_{715}A$ (E239K)	1	6	Irish
$C_{770}T$ (T257M)	1	7	Italian
$T_{833}C$ (I278T)	9	8	Jewish, Polish, French, German, Norwegian, English, Italian
$G_{919}A$ (G307S)	36	8	Irish/German/Dutch/ French/Scottish/English
$C_{1006}T$ (R336C)	1	9	English
$G_{1106}A$ (R369H)	2	10	Irish
ag → cg(intron 11) Δ exon 12	4	12	Jewish, German
Δ 29bp	1	7	German

Table 2 Genotypes in CBS deficiency

Patient	*Allele 1*	*Allele 2*	B_6
676	$C_{341}T$ (A114v)	$C_{434}T$ (P145L)	+
366	$T_{833}C$ (I278T)	intron 11, ag → cg Δ exon 12	+
CM	exon 7, Δ29bp	intron 11, ag → cg Δ exon 12	−
MK	intron 11, ag → cg Δ exon 12	intron 11, ag → cg Δ exon 12	−
L209	$T_{833}C$ (I278T)	$T_{833}C$ (I278T)	+
L227	$T_{833}C$ (I278T)	?	−
L238, L264, L265	$T_{833}C$ (I278T)	?	+
3055	$G_{919}A$ (G307S)	$G_{919}A$ (G307S)	−
3038	$G_{919}A$ (G307S)	?	−
1873	$G_{715}A$ (E239K)	$C_{233}G$ (P78R) $G_{306}C$ (K102N)	+
578	$G_{374}A$ (R125Q) $G_{393}C$ (E131D)	$G_{374}A$ (R125Q) $G_{393}C$ (E131D)	−
GL, SG	$G_{374}A$ (R125Q)	$G_{374}A$ (R125Q)	+/−
NO	$C_{770}T$ (T257M)	$C_{770}T$ (T257M)	−
3065	$C_{1006}T$ (R336C)	?	−
?	$G_{1106}A$ (R369H)	$G_{1106}A$ (R369H)	

it is present in one or two copies in the patient. The nature of the other mutation in patients who are compound heterozygous for the G307S is not known, however. On the other hand, I278T usually confers pyridoxine responsiveness, whether in homozygotes or compound heterozygotes (Hu et al 1993).

Lack of correlation between genotype and phenotype: A number of patients have no measurable CBS activity in their fibroblasts, and CBS subunits themselves are undetectable in fibroblast extracts of some of these individuals. Many of these patients, however, are pyridoxine responsive. The clinical phenotypes range from normal to severe. These include three siblings (brother and two sisters) with an identical genotype, $G_{715}A$, on the maternal CBS allele and two mutations, $C_{233}G$ and $G_{306}C$, on the paternal CBS allele (Table 2, patient 1873). The brother has normal intelligence and has had a single episode of calf thrombosis at the age of 34 years. In contrast, his sisters are mentally retarded and have suffered from skeletal abnormalities and other clinical complications since an early age. It should be noted that their asymptomatic mother also lacks CBS subunits in her fibroblasts. She is, however, a heterozygote and has one normal CBS allele and one carrying the $G_{715}A$ mutation (de Franchis et al 1994). Similarly, patient 366 (Table 2) has no detectable fibroblast CBS protein but is affected with a very mild pyridoxine-responsive homocystinuria (Kožich and Kraus 1992). Taken together these observations indicate that the absence of detectable CBS enzyme protein in fibroblasts does not preclude an *in vivo* response to pyridoxine, and that an identical genotype does not always result in the same phenotype even within a family.

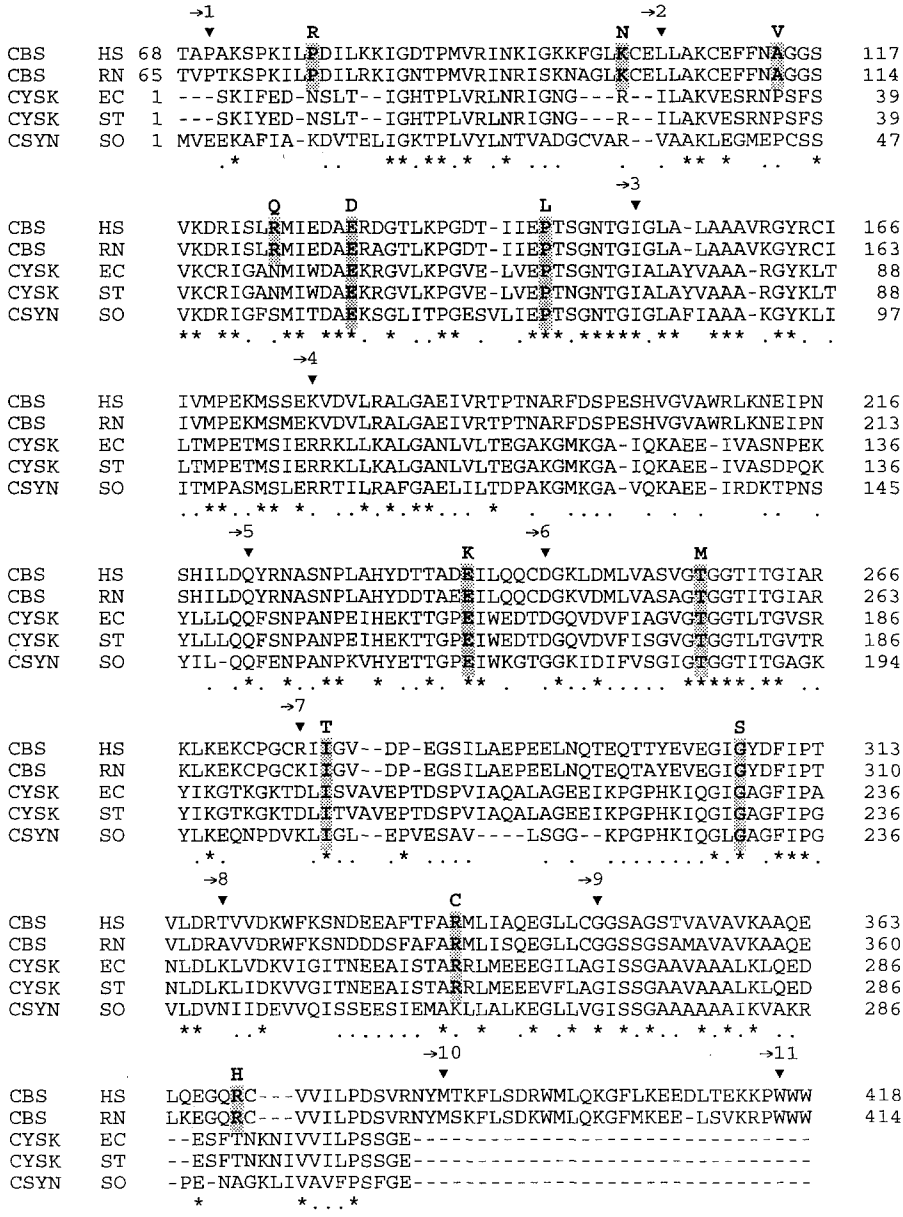

Figure 2 Evolutionary conservation of CBS sequence and human mutations. CBS, HS, human CBS; CBS RN, rat CBS; CYSK EC, *E. coli O*-acetylserine lyase; CYSK ST, *Salmonella typhimurium O*-acetylserine lyase; CSYN SO, spinach cysteine synthase. Amino acids in each sequence are numbered on both sides. Asterisks (*) signify absolute conservation of amino acid residues; dots (·) show conservative replacements. Approximate positions of introns are indicated by solid, numbered triangles (▼). The human exonic CBS mutations are noted above the sequences by bold letters

Apparently, the absence of CBS in fibroblast extracts does not imply complete enzyme deficiency in other body tissues. The variation in phenotypes between patients with the same CBS genotypes may be due to the dependence of CBS activity on other pathways such as those involved in the synthesis of its cofactors *S*-adenosylmethionine, pyridoxal 5′-phosphate and haem.

Mutations are present in the most conserved region of CBS: Figure 2 illustrates comparison of the human CBS amino acid sequence to the rat enzyme and to the sequences of *O*-acetylserine lyase (cysteine synthase) from bacteria and plants. It is obvious from the figure that exons 2–6 are the most highly conserved, with less conservation in exons 7–9. All mutations identified to date, with the exception of the intron 11 mutation, are located in exons 2–10, the majority of them are found in exons 3 and 8. Exon 3 contains lysine 119, which is thought to be the PLP binding residue. In all cases the human mutation changes an amino acid conserved between the human and rat CBS, and in many cases, a residue shared between all the enzymes has been altered. For example, the frequently seen G307S and I278T mutations change absolutely conserved glycine and isoleucine residues.

In conclusion, rapid progress has been made in identifying the first 14 mutations in the CBS gene from patients with homocystinuria. The mutations are somewhat heterogeneous and most patients are compound heterozygotes. The G307S mutation found in 50–70% of alleles in patients of Irish descent appears to be incompatible with pyridoxine responsiveness *in vivo*.

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