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ARTICLE

Functional modeling of vitamin responsiveness in yeast: a common pyridoxine-responsive cystathionine β-synthase mutation in homocystinuria

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Cystathionine β-synthase (CBS) deficiency is an autosomal recessive disorder which results in extremely elevated levels of total plasma homocysteine (tHcy) and high risk of thromboembolic events. About half of all patients diagnosed with CBS deficiency respond to pyridoxine treatment with a significant lowering of tHcy levels. We examined 12 CBS-deficient patients from 10 Norwegian families for mutations in the *CBS* gene and identified mutations in 18 of the 20 *CBS* alleles. Five of the seven patients classified as pyridoxine-responsive contain the newly identified point mutation, $G_{797}A$ (R266K). This point mutation is tightly linked with a previously identified 'benign' 68 bp duplication of the intron 7–exon 8 boundary within the *CBS* gene. We tested the effect of all of the mutations identified on human CBS function utilizing a yeast system. Five of the six mutations had a distinguishable phenotype in yeast, indicating that they were in fact pathogenic. Interestingly, the $G_{797}A$ allele had no phenotype when the yeast were grown in high concentrations of pyridoxine, but a severe phenotype when grown in low concentrations, thus mirroring the behavior in humans. These studies show that the $G_{797}A$ mutation is an important cause of pyridoxine-responsive CBS deficiency and demonstrate the utility of yeast functional assays in the analysis of human mutations.

INTRODUCTION

A goal of modern human genetics is to use our knowledge of specific mutations to help understand and treat genetic disease. In most cases after a disease gene is isolated, investigators identify DNA alterations in this gene and attempt to correlate these alterations with phenotypic variation in the disease. However, the results of such studies can be confusing. It is difficult to prove that a DNA alteration actually causes disease and is not a rare polymorphism in the patient. What is needed is a combination of traditional mutation detection methods with systems that allow examination of the effect of a DNA alteration on the function of the coded protein. Such functional systems are also needed for getting at the mechanistic questions of how mutations actually disturb protein function. The ability to answer these questions could potentially open new avenues of therapy.

The genetic disorder we have chosen to study is cystathionine β-synthase (CBS) deficiency. CBS deficiency is an autosomal recessive disorder which results in extremely elevated levels of total plasma homocysteine (tHcy) and methionine (1). In individuals with this disease, the major clinical complication is an increased incidence of thromboembolic events, although other problems such as lens dislocation, osteoporosis and neurological defects are frequently observed. Several studies suggest high plasma homocysteine levels may be directly responsible for the vascular lesions observed in patients (1,2).

There is a wide spectrum of phenotypic variation in individuals with CBS deficiency. About 50% of patients respond to high

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doses of pyridoxine with a marked reduction in their homocysteine level, and have a better clinical prognosis. Studies on CBS activity in cell lines from these patients demonstrate some residual enzyme activity, whereas cells from pyridoxine non-responders have no detectable activity (1).

The cause of CBS deficiency is mutations in the human *CBS* gene. Over 20 different point mutations have been identified in patients with CBS deficiency (3–9). However, studies on the *CBS* gene also provide a good example of the problems inherent in distinguishing disease-causing mutations from polymorphisms. A 68 bp duplication of the region containing the intron 7–exon 8 junction was initially reported as a mutation in an Italian CBS deficient patient (8). However, more recent studies indicate that this duplication is in fact a benign polymorphism found on 3–6% of all *CBS* containing chromosomes (10,11).

In this study we have combined conventional mutation screening with our previously developed yeast functional assay for human CBS to analyze 12 CBS deficient patients from 10 unrelated Norwegian families. The major finding of this study is the identification of a novel point mutation, $G_{797}A$, found in the majority of pyridoxine-responsive patients. When expressed in yeast, this mutation shows a pyridoxine-responsive phenotype. These results demonstrate the usefulness of yeast functional assays in human genetic analysis.

RESULTS

Patient characteristics

Our patient population consisted of 12 Norwegian individuals from 10 unrelated families. The patients all had homocystinuria due to suspected CBS deficiency. Details of each of the patients are shown in Table 1. Given that there were 10 families, there were at most 20 different *CBS* alleles present in this population.

Mutation screening

Initially, we screened for two point mutations, $G_{919}A$ (G307S) and $T_{833}C$ (I278T), which have been observed in multiple patients in other studies (9,12). For the screening we used PCR amplification of DNA isolated from frozen whole blood followed by restriction digest with specific enzymes to detect the mutations (PCR/RFLP, see Materials and Methods and Table 4). We found that four of the 20 alleles in the patients contained the $G_{919}A$ mutation and two of the alleles contained the $T_{833}C$ mutation.

The assay used to detect $T_{833}C$ also allowed us to assay for the presence or absence of the 68 bp duplication of the intron 7–exon 8 boundary which was previously described as a benign polymorphism (Fig. 1). We discovered that nine of the 20 *CBS* alleles present (45%) contained the 68 bp duplication allele. This frequency was much higher than the 6% allele frequency reported by Tsai *et al*. (10) in their study population from the upper Midwest. Because the allele frequency of the 68 bp duplication in Norwegians has not been previously determined, we examined the allele frequency in unaffected Norwegian chromosomes and found it to be ~5.5% (2/36).

Table 1. Patient characteristics

Patient	Sex	Age at diagnosis (y)	Treatment daily dose	tHcy[a] relative to treatment before (μmol/l)	after (μmol/)l	B6 response[d]	Complications/symptoms
N1	Male	17	15 mg FA, 450 mg B6	151.0	7.6	R	Lens luxation
N2	Female	12	15 mg FA, 900 mg B6 and 6–12 g betaine	152.0	104.1[b]	N	Lens luxation, marfanoid features, developmental delay
N3	Male	7	15 mg FA, 900 mg B6 10 g betaine	pos	32.7[c]	N	Lens luxation, convulsions, developmental delay
N4a	Male	25	40 mg B6	pos	11.6	R	Lens luxation
N4b	Male	20	40 mg B6	pos	11.6	R	Lens luxation, marfanoid features, thromboembolic events, severe psychiatric illnesss responding to B6
N5	Female	20	10 mg FA, 80 mg B6 and 12 g betaine	273.2	111.4	N	Small cerebrovascular event
N6a	Female	33	0.4 mg FA, 40 mg B6	245.0	8.0	R	None
N6b	Male	27	0.4 mg FA, 40 mg B6	130.0	18.0	R	Severe psychiatric illness not responding to treatment
N7	Male	19	5 mg FA, 600 mg B6	315.0	230.0	N[c]	Lens luxation, marfanoid features
N8	Female	9	60 mg FA, 600 mg B6	pos	6.2	R	Lens luxation, thromboembolic event, cerebral oedema, fully recovered after therapy
N9	Male	23	40 mg B6	221.0	15.8	R	Lens luxation
N10	Male	42	15 mg FA, 450 mg B6 12 g betaine	336.0	50.9	N	Lens luxation, small cerebrovascular event

[a]tHcy, total plasma homocysteine; pos indicates positive cyanide-nitroprusside test.
[b]tHcy was measured on several occasions, initially it decreased to 41.0 μmol/l, then gradually increased to ~100 μmol/l.
[c]tHcy was measured on several occasions, and during a period of 4 years the level increased to 81.6 μmol/l.
[d]R, B6 responsive; N, B6 non-responsive.
[e]Non-compliant, needs betaine.

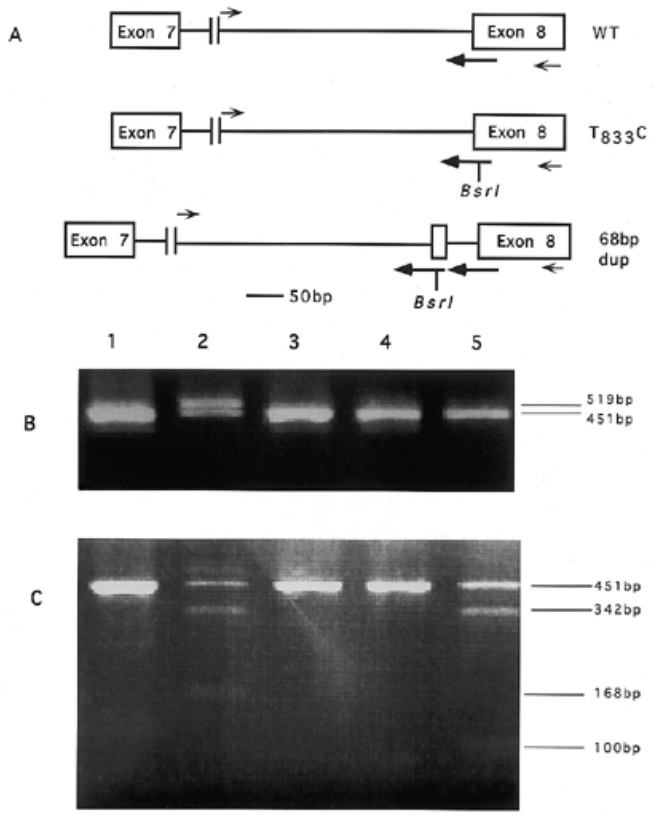

Figure 1. Detection of wt, $T_{833}C$ and 68 bp duplication alleles. (**A**) Schematic of the structure of wild-type, $T_{833}C$ and 68 bp duplication alleles. Small arrows indicate primers used for PCR assay and the large arrow indicates the 68 bp region that is duplicated in the 68 bp duplication. The $T_{833}C$ mutation creates a new *Bsr*I restriction site within this same region. The 5′ repeat in the 68 bp duplication contains the same single basepair change as observed in $T_{833}C$. (**B**) and (**C**) PCR detection of $T_{833}C$ and 68 bp duplication for five patients. (B) The amplified fragments before digestion; (C) the same samples after digestion with *Bsr*I. Individuals containing the wild-type or $T_{833}C$ allele contain a 451 bp fragment before digestion, while individuals containing the 68 bp duplication have a 519 bp fragment. Lane 2 shows a patient heterozygous for the 68 bp insertion, while lane 5 shows a patient heterozygous for the $T_{833}C$ mutation. Lanes 1, 3 and 4 are wild-type for both the insertion and the $T_{833}C$ mutation. After digestion, individuals containing the $T_{833}C$ mutation have 342 and 100 bp fragments present, while individuals with the 68 bp duplication have 342 and 168 bp fragments present.

Using PCR/RFLP methodology, we screened for two other mutations originally identified in non-Norwegian CBS deficient patients (WDK, unpublished). These mutations included $C_{1105}T$ (R369C) and $T_{959}C$ (V320A). We found one $C_{1105}T$ allele and two $T_{959}C$ alleles in this CBS-deficient population (Fig. 2). No $C_{1105}T$ or $T_{959}C$ alleles were identified in 36 control chromosomes screened.

In one of the probands (patient N2) DNA of exon 7 was sequenced. We found that this patient was homozygous for a novel mutation $C_{785}T$ (T262M) (Fig. 3). With a PCR/RFLP method, the rest of the patients as well as 100 control chromosomes were screened for the $C_{785}T$ mutation. No mutations were observed in either group.

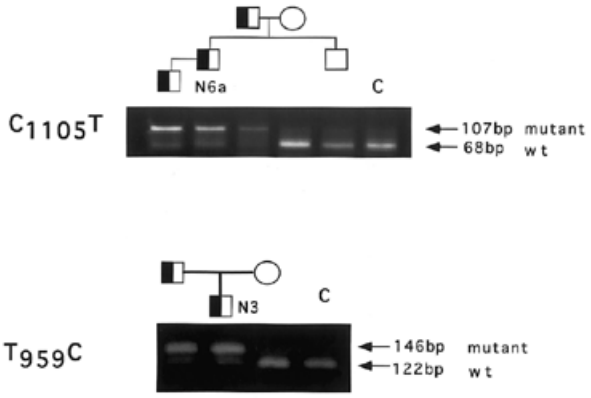

Figure 2. PCR/RFLP assays used to detect $C_{1105}T$ and $T_{959}C$ mutations. The last lane in both panels marked 'C' is a wild-type control. Upper panel shows PCR/RFLP assay of patient N6a and some family members. Half-filled in squares or circles show carrier status for each individual. The presence of the mutation makes the DNA resistant to digestion with *Hae*II. It should be noted that the $T_{833}C$ allele is also segregating in this family (data not shown). Bottom panel shows $T_{959}C$ assay for patient N3 and parents. The presence of the mutation makes the DNA resistant to digestion with *Msc*I. The $G_{919}A$ mutation is also segregating in this family (data not shown).

A novel $G_{797}A$ mutation in association with the 68 bp duplication

The high frequency of the 68 bp duplication on the diseased chromosomes suggested that there might be linkage disequilibrium between a disease causing mutation and this polymorphism. Therefore, we sequenced the DNA of exon 7 in one of the probands homozygous for the 68 bp duplication (patient N1) (Fig. 3), and found that he was homozygous for a novel mutation, $G_{797}A$, which results in an arginine to lysine change at position 266 of the CBS protein.

We developed a PCR/RFLP method for detecting the $G_{797}A$ mutation and screened the rest of our probands (Materials and Methods). Notably, seven of the 20 affected chromosomes in our population contained the $G_{797}A$ allele, and all of them were found in association with the 68 bp duplication. Patients N1, N4ab and N9 were homozygous for both $G_{797}A$ and the 68 bp duplication, indicating physical linkage between the two alleles. Patient N8 was heterozygous for both alleles, but we lacked family material to establish linkage. We did not observe any $G_{797}A$ mutations in 100 control chromosomes.

We also tested to see if a significant fraction of 68 bp duplication containing chromosomes also contained $G_{797}A$. We screened DNA obtained from 242 non-homocystinuric individuals for the 68 bp duplication and found 15 individuals who contained at least one 68 bp duplication allele. None of these individuals contained the $G_{797}A$ mutation. These findings indicate that the $G_{797}A$ mutation is rare in both the general population and in individuals containing the 68 bp duplication.

Single strand conformation polymorphism (SSCP)

In parallel with this work we also screened all of the probands by SSCP analysis. Exons 1–10 and 12 were analyzed (exon 11 could not be amplified.) These studies confirmed the presence of the

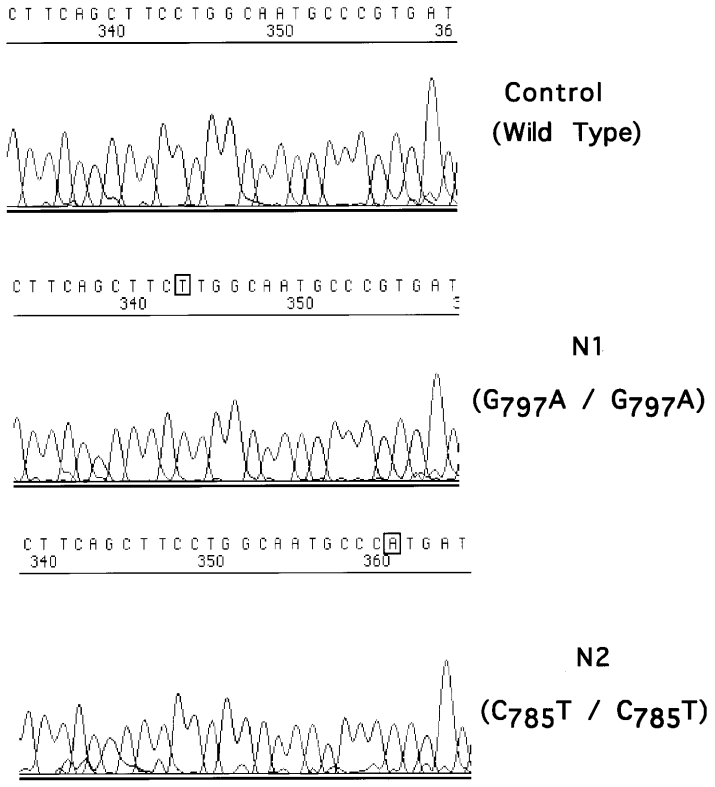

Figure 3. Identification of the $G_{797}A$ and $C_{785}T$ alterations. Sequence trace file from ABI377 automated sequencer showing homozygosity of the $G_{797}A$ mutation in patient N1 and homozygosity of the $C_{785}T$ mutation in patient N2. The same region from a control individual is shown at the top.

$G_{797}A$, $C_{785}T$, $G_{919}A$ and $T_{833}C$ alleles (data not shown). The $C_{1105}T$ and $T_{959}C$ alleles were not identified by SSCP.

By using all the methods described, we identified mutations which alter the predicted CBS amino acid sequence in 18 of the 20 alleles present in our CBS deficient population (Table 2).

Table 2. Patient genotype and pyridoxine-responsiveness

Patient	68 bp duplication	Mutations	Clinical pyridoxine responsiveness
N1	68/68	$G_{797}A/G_{797}A$	Yes
N2	wt/wt	$C_{785}T/C_{785}T$	No
N3	wt/68	$G_{919}A/T_{959}C$	No
N4a/N4b	68/68	$G_{797}A/G_{797}A$	Yes
N5	wt/68	$G_{919}A/T_{959}C$	No
N6a/N6b	wt/wt	$T_{833}C/C_{1105}T$	Yes
N7	wt/wt	$T_{833}C/?$	No
N8	68/wt	$G_{797}A/?$	Yes
N9	68/68	$G_{797}A/G_{797}A$	Yes
N10	wt/wt	$G_{919}A/G_{919}A$	No

Functional analysis of CBS mutations in yeast

To determine the functional consequences of these *CBS* missense mutations we expressed them in a strain of *Saccharomyces cerevisiae* in which the endogenous *CBS* gene is deleted (WK63ΔyCBS) (5). This strain cannot synthesize cysteine and thus requires media with cysteine for growth (Cys^+ media). Expression of a functional human CBS protein allows cysteine production and thus allows growth on Cys^- media. All of the identified mutations, $G_{797}A$, $C_{785}T$, $C_{1105}T$, $T_{833}C$, $G_{919}A$ and $T_{959}C$, were engineered by site-directed mutagenesis into the human CBS encoding cDNA. Each mutant cDNA was subsequently cloned into an expression vector and transformed into WK63ΔyCBS (see Materials and Methods).

Strains containing the $T_{833}C$, $G_{919}A$ and $C_{785}T$ mutations were unable to form colonies on standard Cys^- media (Fig. 4). This result indicates that these mutations severely affect CBS enzyme function. Cells containing the $T_{959}C$ mutation were able to form single colonies on standard Cys^- media, but these colonies were significantly smaller than those formed by yeast cells expressing wild-type human CBS. Yeast containing the $G_{797}A$ and the $C_{1105}T$ mutation grew just as well as wild-type. These results indicate that the R369C and R266K alterations in the CBS protein have less severe effects on enzyme function than the G307S, I278T, V320A and T262M alterations.

All five of the patients with the $G_{797}A$ allele and the one patient with the $C_{1105}T$ allele were pyridoxine-responsive. We reasoned that our failure to observe a phenotype in yeast carrying these alleles might be due to the abundance of pyridoxine present in standard yeast media. Therefore, we examined the growth behavior of yeast on media with reduced amounts of pyridoxine. Cells expressing all three alleles formed colonies of approximately equal size on Cys^- media containing 1.9 μM pyridoxine (Fig. 5A). When the pyridoxine level was reduced to 2.6 nM, cells containing the $G_{797}A$ allele formed significantly smaller colonies compared with wild-type (Fig. 5B). On plates containing 0.86 nM pyridoxine,

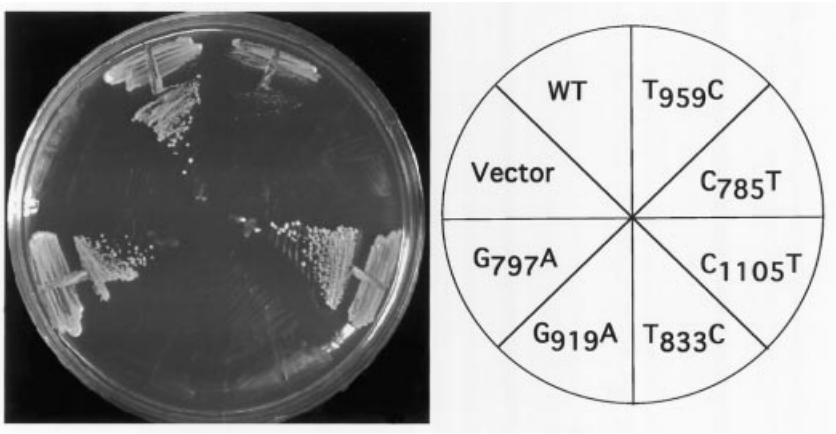

Figure 4. Expression of mutant CBS proteins in yeast. Yeast strain WK63ΔyCBS containing pHCBS with the indicated mutations were plated out on synthetic complete media lacking tryptophan (to select for vector) and cysteine.

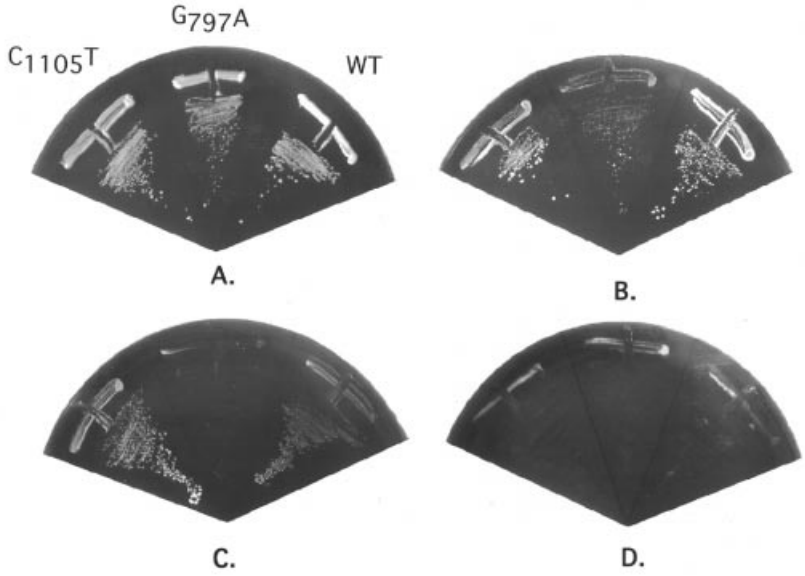

Figure 5. Pyridoxine responsiveness of $G_{797}A$ alteration in yeast. Each sector shows (from left to right) $C_{1105}T$, $G_{797}A$ and wild-type expressing yeast on synthetic complete plates lacking cysteine with varying levels of pyridoxine. All images are after 72 h. (**A**) 1.9 μM pyridoxine; (**B**) 2.6 nM pyridoxine; (**C**) 0.86 nM pyridoxine; (**D**) no pyridoxine.

$G_{797}A$ containing cells could not form single colonies (Fig. 5C). Unexpectedly, at low pyridoxine concentrations, yeast containing the $C_{1105}T$ allele formed larger colonies than wild-type. On plates with no pyridoxine, no growth was observed for any allele (Fig. 5D).

These observations were confirmed by growth rate experiments in liquid media. Doubling times were determined for each strain in Cys^- media containing defined amounts of pyridoxine (Table 3). Under high pyridoxine conditions (1.9 μM) all three strains had similar doubling times. As the pyridoxine concentrations were reduced all three strains showed an increase in doubling times, but the $G_{797}A$ had significantly longer doubling times than the other two strains (Table 3, bottom row). As observed in the colony forming assay, yeast containing the $C_{1105}T$ allele had faster doubling times than the wild-type under low pyridoxine conditions.

Table 3. Liquid growth rates

Pyridoxine conc.	Allele		
(nM)	Wild-type	$G_{797}A$	$C_{1105}T$
1900	4.6 h	4.5 h	4.6 h
2.60	6.3 h	6.3 h	5.1 h
0.86	8.7 h	11.9 h	6.9 h
0.29	9.5 h	17.3 h	8.3 h

DISCUSSION

In this study we analyzed 12 CBS-deficient probands from 10 unrelated Norwegian families using a combination of conventional mutation detection methods and a yeast functional assay for the

human *CBS* gene. We were able to identify six different missense mutations present in 18 of the 20 alleles in the patient group: $G_{919}A$, $C_{785}T$, $T_{959}C$, $T_{833}C$, $G_{797}A$ and $C_{1105}T$. Two of the mutations, $G_{919}A$ and $T_{833}C$, have been previously reported, while the other four are novel.

We expressed all of the mutant human CBS proteins above in our previously described yeast system to examine their function (5). In this system the mutant CBS proteins are expressed in a yeast strain deleted for the endogenous CBS gene, and growth behavior of the yeast is observed on standard yeast media lacking cysteine. The two known mutations, $G_{919}A$ and $T_{833}C$, along with one new mutation, $C_{785}T$, exhibited a very strong no growth phenotype. The $T_{959}C$ allele gave a distinct slow growth phenotype. Our results from the yeast assay strongly suggest that these four DNA alterations are disease causing mutations and not benign polymorphisms. Interestingly, almost all of these mutant alleles (with the exception of one $T_{833}C$ mutation) are found in the pyridoxine non-responsive patients.

Two other mutations detected, $G_{797}A$ and $C_{1105}T$, did not give distinguishable phenotypes in yeast under the standard assay conditions. These two mutations were found exclusively in pyridoxine-responsive patients. Since standard yeast media is rich in pyridoxine (1.9 μM in yeast media compared with ~0.3 nM in human serum), we examined the phenotype of these two mutations at low pyridoxine levels. When grown under these conditions yeast expressing the wild-type allele are able to form colonies, while strains carrying the $G_{797}A$ allele are not. In liquid cultures with high pyridoxine the doubling time of yeast carrying the $G_{797}A$ allele was similar to wild-type, but growth became noticeably slower with decreasing pyridoxine. Thus, $G_{797}A$ allele expressed in yeast is pyridoxine responsive, similar to what is observed for this allele in humans.

The $C_{1105}T$ mutation did not exhibit a no growth phenotype in yeast, even when grown at low pyridoxine concentrations. In fact, yeast carrying this allele actually seem to grow slightly better than yeast carrying the wild-type allele under these conditions. Thus our functional data would suggest that the $C_{1105}T$ alteration is not pathogenic, but is simply a polymorphic variant of the protein. In support of this, we have found two additional alleles of $C_{1105}T$ in screening of 200 non-homocystinuric individuals (WK, unpublished). Thus, it may in fact be a rare polymorphism. If this were the case, we would assume that there must be another mutation in *cis* with this alteration that we failed to discover during mutation screening. Certainly our mutation screening was not exhaustive as we failed to discover at least two other mutant alleles in this group of patients. Alternatively, the allele may be pathogenic, but is not properly modeled in yeast. It is possible that some mutations which affect protein stability or folding may not be properly modeled in the yeast system. In addition, mutations which affect the interaction of CBS with co-factors other than pyridoxine (e.g., heme or S-adenosylmethionine) may not be modeled well because these factors are present in different concentrations in yeast versus mammalian cells.

Our functional data in yeast suggest that the bonefida $T_{833}C$ mutation is not pyridoxine-responsive. However, several reports have found an association between the presence of the $T_{833}C$ mutation and pyridoxine responsiveness in patients (9,13,14). Our findings concerning linkage disequilibrium between the 68 bp duplication and the $G_{797}A$ allele could explain this apparent contradiction. The 68 bp duplication of the intron 7–exon 8 border is not a perfect duplication. There is a single nucleotide change in the 5′ repeat which is identical to the mutation found in patients with the $T_{833}C$ alteration. This nucleotide change creates a novel *Bsr*I restriction site (8). However, individuals with this duplication produce fully functional CBS mRNA because only the second splice acceptor site is utilized and thus the mutation is not introduced into the mRNA (10,11). We presume that the bonefida $T_{833}C$ mutation arose due to intra-chromosomal recombination between the two repeats. All of the reports showing association of pyridoxine-responsiveness with $T_{833}C$ were done before the existence of the 68 bp duplication was known. Thus, it is possible that some of the $T_{833}C$ alleles identified in these studies were actually 68 bp duplication alleles. We suggest that these patients should be reexamined in light of this information.

The spectrum of mutations discovered in Norwegian homocystinurics is different than that described in other populations. In Ireland, which has a relatively high rate of homocystinuria, the $G_{919}A$ allele accounts for 71% of the mutant alleles (12). In our Norwegian group this allele accounts for only 20%. In Italian patients the $G_{919}A$ allele was not observed at all (15). In Norwegians the $G_{797}A$ allele appears to be quite frequent (35%), but this allele has not been reported elsewhere. The differential distribution of disease-causing mutations in different populations implies that most of the mutations causing CBS deficiency are of relatively recent origin. Consistent with this hypothesis is the linkage disequilibrium observed between the $G_{797}A$ mutation and the 68 bp duplication. Presumably, the original $G_{797}A$ mutation formed on a chromosome containing the 68 bp duplication, and then expanded in the population due to genetic drift.

The work described in this paper has potential implications in regard to two facets of CBS deficiency. The ethnic differences in the distribution of different mutations should have important implications for genetic testing. As testing for *CBS* mutations becomes more widespread, knowledge of an individual's ethnic background will allow genetic testing to be done more efficiently and cost-effectively. More important are the implications for the functional modeling of mutant CBS proteins in yeast. We have shown here that it is possible to identify a substance, i.e., pyridoxine, that can rescue a specific allele in yeast, and this substance can also have beneficial effects in humans. By having model systems available for other mutant alleles it may be possible to identify other substances which can rescue mutant alleles and potentially be beneficial to patients suffering from CBS deficiency.

More generally, this study demonstrates the utility of combining a yeast functional assay with conventional mutation detection in the analysis of human genetic disease. As more human genes are functionally modeled in yeast, this type of analysis should be applicable to a wide range of human diseases.

MATERIALS AND METHODS

Patients and controls

Eleven patients were recruited from different medical practices in the south, west and middle part of Norway. The one remaining patient was diagnosed in the Hordaland Homocysteine Study (16). The present study was approved by the Regional Ethics Committee and all of the patients signed informed consent agreements. Details about the patients are given in Table 1.

Table 4. PCR-RFLP assay conditions

Mutation	Primers	Buffer[a]	Annealing temp. (°C)	Enzyme	Sizes (bp)
$G_{919}A$	5′-ATCATTGGGGTGGATCCCGA-3′	J	55	PvuII	112-wt
	5′-ACCGTGGGGATGAAGTCGCAG-3′				81, 32-mut
68bp dup	5′-GTCCCCAAAGGCTCTGCTGC-3′	B	62	None	519-dup
	5′-GTGGGGATGAAGTCGTAGCC-3′				451-wt
				*Bsr*I	451-wt
					342, 168-dup
$T_{833}C$	Same as above	B	62	*Bsr*I	451-wt
					342, 100-mut
$C_{785}T$	5′-GGGCACGGGCGGCACCACCA-3′	E	68	*Nco*I	193-wt
	5′-AACACCTCCCAGGCAGCGCA-3′				172,21-mut
$G_{797}A$	5′-AAGCTGGACATGCTGGTGGC-3′	C	65	*Bst*NI	98-mut
	5′-CCACTCACCCTGCATCGAGG-3′				57, 41-wt
$T_{959}C$	1st PCR	D	65		
	5′-CCAGGTGGCACAGGCAGGGA-3′				
	5′-CATCGTTGCTCTTGAACCACTTGGCC-3′				146-mut
	2nd PCR	C	65	*Msc*I	122-wt
	5′-GAGACCTCTGGGGTCCTACC-3′				
	5′-CATCGTTGCTCTTGAACCACTTGGCC-3′				
$C_{1105}T$	5′-GTGGCAGTGCTGGCAGCACG-3′	A	60	*Hae*II	107-mut
	5′-ATGTAGTTCCGCACTGAGTC-3′				68, 38-wt

[a]Buffers were obtained using PCR Optimizer™ kit from Invitrogen.

Table 5. Primers for SSCP analysis

Exon	Oligo	Sequence	Size (bp)	Annealing temp. (°C)	$[MgCl_2]$ (mM)
1 (a)	S: I21	5′-tgaaccgacgcctctctcctt-3′	98	60	1.0
	A: 36	5′-atgctccgagcaggtgcacct-3′			
1 (b)	S: 35	5′-agaggataaggaagccaagga-3′	132	60	1.5
	A: I22	5′-tgtgatcaaaagcaggactta-3′			
2 (a)	S: 3	5′-ggcaaaatctccaaaaatct-3′	127	55	2.5
	A: I9	5′-ctccaaagccagggcact-3′			
2 (b)	S: I8	5′-ataattgtggactcctct-3′	75	55	1.5
	A: 4	5′-gtgtccccgattttcttcag-3′			
3	S: 3F	5′-ggtcccctctgtgattcatactct-3′	222	55	1.5
	A: 3R	5′-cggcatgggtaggggacaac-3′			
4	S: I11	5′-ctctcaccctctgtgtgccctca-3′	82	60	2.0
	A: 6	5′-tggcatcacgatgatgcagc-3′			
5	S: I24	5′-aaggtgcaggccaccgcttt-3′	166	60	1.5
	A: 37	5′-ctggtctaggatgtgagaatt-3′			
6	S: 32	5′-taccgcaacgccagcaacccc-3′	100	55	1.0
	A: I13	5′-aacgcaatcaagatggacagag-3′			
7	S: 7F	5′-ccaggcagggacccaagaat-3′	170	60	1.5
	A: 7R	5′-ccactccgcactgtccctct-3′			
8	S: I3	5′-gcagttgttaacggcggtat-3′	248	55	2.5
	A: I7	5′-ggctctggactcgaccta-3′			
9	S: I17	5′-ctgacgggctgtggtggggtcc-3′	115	55	1.0
	A: 33	5′-cgcacagcagcccctcttgcgc-3′			
10	S: 10F	5′-gcacgtgcacaattcatgcata-3′	278	60	1.5
	A: 10R	5′-ggctgccggttctcaggtga-3′			
12	S: 12F	5′-gcgagagcgtttgtccttat-3′	~230	60	1.5
	A: 12R	5′-ggcagacagaacccaggact-3′			

In eight patients, pretreatment tHcy levels were available. These were categorized as pyridoxine responsive if the tHcy level showed a 50% or greater reduction after initiation of pyridoxine (250–500 mg/day) combined with low dose folic acid (0.4 mg/day) and intramuscular B_{12} injections. In four patients (diagnosed >10 years ago) the pre-treatment levels of plasma tHcy are not known. In these patients a positive cyanide-nitropusside test in the urine combined with clinical features were considered sufficient for the diagnoses. Two of these patients (N4a and b) have a normal tHcy level with only pyridoxine substitution and are considered pyridoxine responsive. One patient (N8) uses high doses of both pyridoxine and folic acid, but her particularly low tHcy level indicates pyridoxine responsiveness. The fourth patient has had varying tHcy concentrations (30–80 μmol/l) and uses high doses of pyridoxine, folic acid and betaine and is therefore considered non-responsive.

We had two control groups. A Norwegian control group consisting of 50 controls, 25 males and 25 females, was randomly picked from a selection of 329 anonymous subjects participating in the Hordaland Homocysteine Study. A second control group consisting of 242 subjects from the the Portland Oregon area is described in ref. 17.

Mutation identification

DNA was isolated from frozen whole blood using Instagene (Biorad). For PCR-RFLP assays, 10 μl of DNA was used in 50 μl PCR reactions. Table 4 shows the conditions and enzymes used for each individual assay. After PCR, products were precipitated with ethanol, resuspended in 20 μl TE with restriction buffer, and then digested for 3 h. Products were then analyzed on 3% agarose gels.

For the direct sequencing of mutations, the PCR products were purified after gel electrophoresis using QIAquick Gel Extraction Kit™ (Qiagen), and sequenced at the Automated Sequencing Facility at the Fox Chase Cancer Center. The primers for PCR amplification of the direct sequencing products are as follows. For exon 7 ($G_{797}A$ and $C_{785}T$ mutations) we amplified with 5′-AAGCTGGACATGCTGGTGGC-3′ and 5′-CCACTCAC-CCTGCATCGAGG-3′. For the $T_{959}C$ mutation (exon 9) the primers used were 5′-ATCATTGGGGTGGATCCCGA-3′ and 5′-CGTTGCTCTTGAACCACTTG-3′. For the $C_{1105}T$ mutation, the primers used were the same as in Table 4.

SSCP was adapted from ref. 18. Initial PCR reactions were prepared using the primer sequences given in Table 5 and a second PCR was then performed using the same primer pairs and 2 μl of the initial PCR product as template. Amplification was performed in a 20 μl volume containing 20 pM of each primer, 200 mM each of dATP, dGTP, dTTP, 0.2 mM of dCTP and 0.1 μl [α-^{32}P]dCTP (6000 Ci/mmol), 100 mM Tris–HCl (pH 8.3), 500 mM KCl, 1–5 mM $MgCl_2$ (depending on optimum conditions for each primer pair) and 1 U *Taq* polymerase. Cycle conditions were the same as for the primary PCR. The resulting radioactive PCR products were then diluted in formamide loading buffer (1 ml in 10 ml buffer). Samples were denatured for 2 min at 95°C, immediately put on ice for 2 min, and 2.5 ml were loaded onto the SSCP gel. A commercial acrylamide, MDE Gel Solution (Flowgen Instruments Ltd.), specifically for SSCP analysis, was used in the preparation of the SSCP gels. SSCP gels were run overnight (14 h) in 6× TBE buffer at 4°C. Voltage was maintained at a constant 200 V. Gels were transferred to 3 MM Whatman filter paper (Alchem), dried at 80°C under vacuum, and exposed to autoradiograph film for 4 h or overnight at –70°C.

Testing mutations in yeast

All of the point mutations described were engineered in pUC:HCBS (5). Mutations were confirmed by sequencing. Mutant CBS cDNAs were cloned into pHCBSΔ and introduced into yeast strain WK63yCBSΔ by gap repair as described (5). Glutathione (30 μg/ml) was used to supply cysteine to the cells.

Yeast nitrogen base with varying amounts of pyridoxine was made using the recipe in ref. 19. Doubling times were determined as follows. Cells (100 ml) were grown to an OD_{600} of ~1 in SC-Trp media supplemented with glutathione. The cells were washed three times with dH_2O and diluted in 5 ml of SC-Trp media with defined pyridoxine levels to a calculated OD_{600} of 0.25. Cells were incubated at 30°C for 8 h to deplete existing cellular pyridoxine pools, and then OD followed for an additional 12 h and doubling times were calculated.

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