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# Identification and Functional Analysis of Three Distinct Mutations in the Human Galactose-1-Phosphate Uridyltransferase Gene Associated with Galactosemia in a Single Family

J. L. Fridovich-Keil,[1] S. D. Langley,[2] L. A. Mazur,[1] J. C. Lennon,[1] P. P. Dembure,[2] and L. J. Elsas II[2]

[1]Department of Genetics and Molecular Medicine and [2]Division of Medical Genetics, Department of Pediatrics, Emory University School of Medicine, Atlanta

## Summary

We have identified three mutations associated with transferase-deficiency galactosemia in a three-generation family including affected members in two generations and have modeled all three mutations in a yeast-expression system. A sequence of pedigree, biochemical, and molecular analyses of the galactose-1-phosphate uridyltransferase (GALT) enzyme and genetic locus in both affected and carrier individuals revealed three distinct base substitutions in this family, two (Q188R and S135L) that had been reported previously and one (V151A) that was novel. Biochemical analyses of red-blood-cell lysates from the relevant family members suggested that each of these mutations was associated with dramatic impairment of GALT activity in these cells. While this observation was consistent with our previous findings concerning the Q188R mutation expressed both in humans and in a yeast-model system, it was at odds with a report by Reichardt and colleagues, indicating that in their COS cell–expression system the S135L substitution behaved as a neutral polymorphism. To address this apparent paradox, as well as to investigate the functional significance of the newly identified V151A substitution, all three mutations were recreated by site-directed mutagenesis of the otherwise wild-type human GALT sequence and were expressed both individually and in the appropriate allelic combinations in a GALT-deficient strain of the yeast *Saccharomyces cerevisiae*. The results of these yeast-modeling studies were fully consistent with the patient data, leading us to conclude that, at least within the context of the cell types studied, in the homozygous state Q188R is a mutation that eliminates GALT activity, and S135L and V151A are both mutations that impair GALT activity to <6% of wild-type values.

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Address for correspondence and reprints: Dr. Judith L. Fridovich-Keil, Department of Genetics and Molecular Medicine, Room 429B, Emory University School of Medicine, 1462 Clifton Road, NE, Atlanta, GA 30322.



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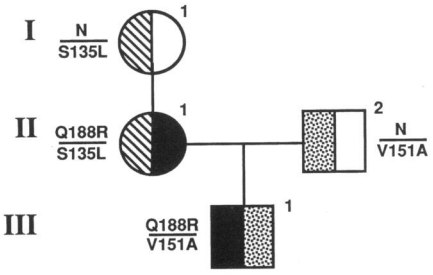
## Introduction

Classic galactosemia is a potentially lethal inborn error of metabolism, which results from impairment of the enzyme galactose-1-phosphate uridyltransferase (GALT), which normally is responsible for catalyzing the second step of the Leloir pathway of galactose metabolism (Segal 1989). Recently, the cDNA and gene encoding human GALT have both been cloned and sequenced (Reichardt and Berg 1988; Flach et al. 1990; Leslie et al. 1992), paving the way for identification of candidate mutations ostensibly associated with the disorder in patients and families. Indeed, more than two dozen candidate mutations have now been reported, as summarized elsewhere (Elsas et al. 1995 [in this issue]); some affect highly conserved sites, whereas others affect divergent sites. While all of these candidate mutations have been identified in the GALT sequences of galactosemic patients, the question of whether the identified base changes represent the (only) causal agents of GALT impairment in these individuals remains unanswered. Indeed, some candidate mutations have been identified in the GALT sequences of both patients and ostensibly normal controls (Leslie et al. 1992), lending evidence to the concern that some of these candidate mutations may be neutral polymorphisms rather than functionally significant mutations.

Reichardt and colleagues were the first to address this concern experimentally, recreating candidate mutations by site-directed mutagenesis and expressing the encoded proteins transiently in a COS cell system (Reichardt and Woo 1991). By these studies, several candidate mutations were reported to be functionally significant mutations, while others were declared to be neutral polymorphisms (Reichardt et al. 1991, 1992; Reichardt and Woo 1991). Unfortunately, at times the quantitative and, sometimes, even the qualitative results of these studies were at odds with the corresponding patient data (Leslie et al. 1992; Fridovich-Keil and Jinks-Robertson 1993; present report), suggesting that the COS cell system might not be fully reliable for use in assessing the functional capacity of defined GALT alleles. While the cause(s) of the apparent disparities remains uncertain, one reasonable possibility is that COS cells, like most mammalian cells studied to date, express endogenous GALT. Considering that GALT functions as a dimer, the presence of endogenous subunits could potentially lead to




| individual | RBC GALT (umol/gm Hb/ h) |
|---|---|
| I 1 | (7.5), (6.0) |
| II 1 | (2.4), (1.7) |
| II 2 | (13.5) |
| III 1 | (0.0), (0.5) |
| normal range | 17.0 - 43.5 (n=12) |

**Figure 1** Family pedigree. Deduced GALT genotypes are indicated to the side of each symbol. RBC GALT values also are indicated.

the formation of mixed heterodimers, thereby confounding the interpretation of any data obtained. In response to this concern, we developed a yeast-expression system for human GALT, which makes use of a *gal7*-deficient strain of the simple eukaryote *Saccharomyces cerevisiae* (Fridovich-Keil and Jinks-Robertson 1993). We have used this system elsewhere to investigate the relative activities associated with both the wild-type and Q188R (glutamine→arginine at codon 188) alleles of GALT and have found that the results obtained bear striking similarity to those observed for the corresponding homozygous patient samples (Fridovich-Keil and Jinks-Robertson 1993).

Here we have applied a modified version of our original yeast system to model each of three candidate mutations identified in a single family with galactosemia. Through the use of single-transformation versus cotransformation experiments, we have modeled each mutation in the homozygous, heterozygous, and compound-heterozygous states and have compared the results obtained with those observed for the corresponding patient samples. The results of both the yeast and patient cell assays were fully consistent and demonstrated that, at least within the context of the cell types involved, the Q188R mutation was associated with null activity and that both the S135L (serine→leucine at codon 135) and V151A (valine→alanine at codon 151) mutations were associated with severely diminished, albeit detectable, activity.

## Patients and Methods

### *Patients*

The proband (III-1; fig. 1) was detected by Georgia's newborn screening program using the Beutler GALT method (Beutler et al. 1965; Beutler and Baluda 1966). He was the firstborn child of an 18-year-old mother (II-1) and exhibited prolonged neonatal jaundice, vomiting, and poor feeding during his 1st wk of life. His erythrocyte galactose-1-phosphate level was 25.8 mg/dl (normal ≤1) at age 7 d. After 1 wk on a lactose-free diet, his erythrocyte galactose-1-phosphate level had fallen to 8.3 mg/dl, and it has ranged between 1.3 and 5.1 mg/dl over the past 7 years. At age 7.5 years he is now normal with respect to growth, development, speech, and neurological status. By contrast, his mother (II-1) is mentally retarded, with a clinical history of seizures poorly controlled by anticonvulsants. She was not known to have galactosemia prior to the diagnosis of her son and evaluation of her family (fig. 1). This study was performed with the approval of the Emory University Human Investigations Committee.

### *Biochemical Assays of Patient Samples*

*Red-blood-cell (RBC) GALT assays.*—RBCs pelleted from fresh heparinized blood were washed twice with two volumes of 0.9% saline and then lysed with two volumes of 13.3 mM sodium phosphate pH 7.0 and 3.75 mM DTT. Hemoglobin concentration was determined by the cyanmethemoglobin method, as described elsewwhere (Van Kampen and Zijlstra 1974). GALT activity was determined by a modification of methods described elsewhere (Mellman and Tedesco 1965; Ng et al. 1967; Lee and Ng 1982). In particular, 100 µl of RBC lysate, 50 µl of 0.5 M glycylglycine pH 8.7, 50 µl of 4 mM UDPG, and 50 µl of 8 mM $^{14}$C-gal-1-p (0.075 µCi) were mixed and incubated at 37°C for 10 min. Reactions were then stopped by boiling (5 min) and were clarified by centrifugation in a Beckman microfuge, and 50 µl of the supernatant was subjected to descending diethylaminoethyl (DEAE) paper chromatography. The radioactivity corresponding to uridine-5′-diphosphate (UDP)–galactose was expressed as a fraction of total radioactivity in each sample and was used to determine the micromoles of UDP-galactose produced in the assay. Enzyme activity was then converted into µmol of UDP-galactose produced/g hemoglobin/h.

*Lymphoblast GALT assays.*—All lymphoblast lines were obtained by Epstein-Barr virus (EBV) immortalization of patient lymphocytes at the Clinical Research Center, Emory University. Twenty microliters of cells in suspension culture were collected by centrifugation, were washed twice with 5 ml of 0.9% saline, and then were lysed by sonication in 400 µl of 13.3 mM sodium phosphate pH 7.0 and 12.5 mM DTT. Protein concentration was determined using the Bio-Rad protein reagent. GALT activity was determined as follows: 50 µl cell lysate, 25 µl of 0.5 M glycylglycine, 25 µl of 4 mM uridine 5′-diphosphoglucose (UDPG), and 25 µl of 8 mM $^{14}$C-gal-1-p (0.0375 µCi) were mixed together and incubated at 37°C for 1 h. Reactions were stopped by boiling and were analyzed as described above. Enzyme activity was expressed as pmol of UDP-galactose produced/µg total cell protein/min.

### *PCR, SSCP, and Direct Sequencing of Patient GALT Sequences*

Samples of DNA extracted either from fresh, heparinized blood or from cultured lymphoblasts representing each of the individuals indicated in figure 1 were screened first for the presence or absence of the common Q188R and N314D mutations, by using a combination of PCR and restriction-endonuclease digestions to be described elsewhere (Elsas et al. 1995). Since a comparison of these PCR results with the outcome of the GALT enzyme assays led us to suspect that additional unidentified GALT mutations were likely to be present in the family, all DNA samples were subjected to full SSCP analysis of all exons, as described elsewhere (Elsas et al. 1995). Finally, all GALT-coding regions from both the mother and child (pedigree designations II-1 and III-1, respectively) were analyzed by direct sequencing (Elsas et al. 1995), while only exons 5 and 6 were sequenced from the father and grandmother (pedigree designations II-2 and I-1, respectively) to confirm the familial origin of the mutations identified in the child and mother.

### *Yeast Strains, Plasmids, and Expression Studies*

All yeast-expression studies, including culture manipulations, extract preparations, and GALT enzymatic assays, were performed using the GALT-deficient strain of *Saccharomyces cerevisiae,* yJFK1, essentially as described elsewhere (Fridovich-Keil and Jinks-Robertson 1993). The plasmids employed in these studies—pLMy2, pPEy1, and their respective GALT-encoding derivatives—will be described in greater detail elsewhere (J. P. Elsevier and J. L. Fridovich-Keil, unpublished data). In brief, both are low-copy-number *CEN* plasmids that are identical except that they carry different nutritional markers (*TRP1* and *LEU2,* respectively) to complement auxotrophies in the host strain, facilitating both the single-transformation and cotransformation studies needed to model the expression of defined GALT alleles in both the homozygous and heterozygous (or compound-heterozygous) states. Because both plasmids carry centromeric (CEN) sequences, each should be maintained at 1–2 copies/cell (Guthrie and Fink 1991), thereby avoiding complications due to significant fluctuations in plasmid copy number. Furthermore, as described elsewhere (Fridovich-Keil and Jinks-Robertson 1993), prior to harvest all yeast cultures were grown in synthetic medium containing glycerol/ ethanol rather than galactose as the carbon source, so that no selective pressure associated with GALT activity was exerted on the cells. Finally, control experiments have demonstrated that parallel derivatives of both plasmids (pLMy2 and pPEy1) facilitate the expression of indistinguishable amounts of human GALT, as determined both by activity assays and by western blot analyses (J. P. Elsevier and J. L. Fridovich-Keil, unpublished data).

Quantitative GALT assays were performed in triplicate or quadruplicate, involving samples prepared from at least two independent colonies, as described elsewhere (Fridovich-Keil and Jinks-Robertson 1993). In all cases assays were performed within the linear range as defined for that sample.

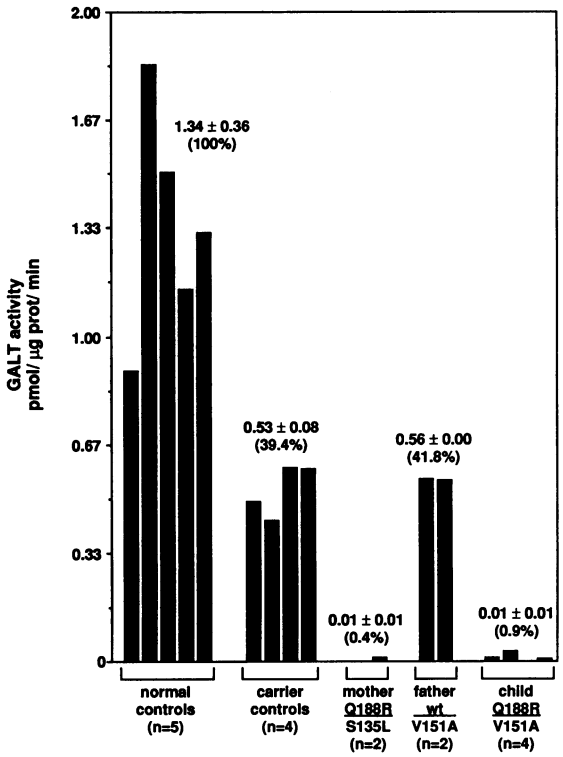



**Figure 2** GALT levels in patient lymphoblasts. GALT enzymatic assays (see Patients and Methods) were performed on extracts of lymphoblastoid cells corresponding to each of the family members indicated, as well as to normal and carrier controls. Bar heights represent actual sample values. Averages and SDs for each set are indicated above the appropriate bars, and averages normalized to the respective wild-type value are indicated in parentheses. Molecular genotypes (where known) and numbers of observations for each sample are listed below each set.

All plasmid manipulations were performed using standard techniques (Sambrook et al. 1989) and bacterial strains (XL1-Blue, from Stratagene; and RY2504 and 71.18, gifts from Ms. Kathy Tatti and Dr. Charles Moran, Emory University). All site-directed mutagenesis procedures were performed as described elsewhere (McClary et al. 1989).

## Results

### *Pedigree Analysis and Biochemical Evaluation of Patient Samples*

Fresh peripheral blood was collected from each of the family members illustrated in figure 1 and was used both for analysis of RBC GALT activity levels and for the preparation of EBV-immortalized lymphoblast cell lines, as described in Patients and Methods. Isoform patterns in the patient (III-1) and his mother (II-1) were consistent with homozygosity for classical G alleles (no staining was observed), while those from the father (II-2) were consistent with his being a heterozygote for the G allele (Elsas et al. 1994). Figure 1 presents the results of single or duplicate

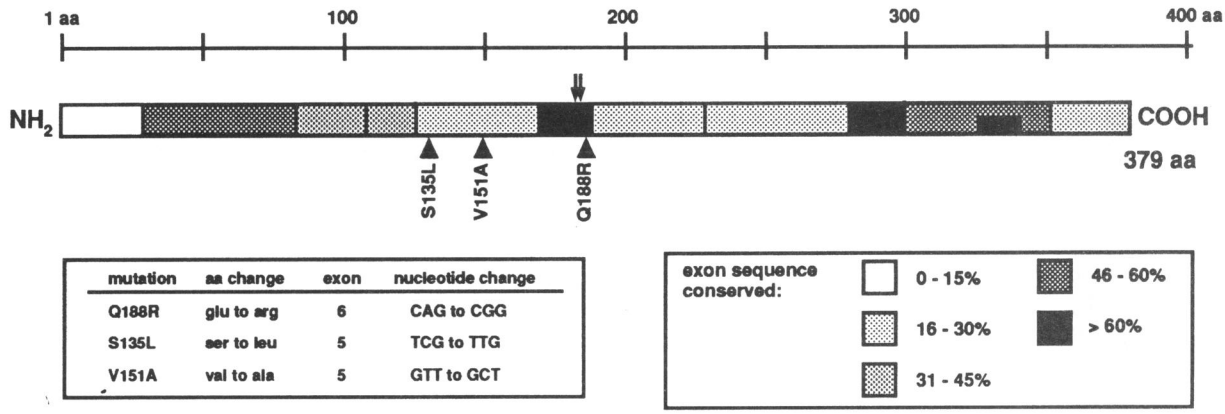



**Figure 3** Diagram of the human GALT protein, with relevant substitutions indicated. The human GALT protein is illustrated with shading to represent the relative degree of evolutionary conservation (between human, yeast, and bacterial GALT) associated with the sequence encoded by each human exon. The double arrows represent the putative active site of the enzyme (his-pro-his). The three substitution mutations identified in the family reported here are indicated.

RBC GALT analyses of these samples; figure 2 presents the results of repeated analyses of the GALT activity levels in cultures of immortalized lymphoblasts representing the proband and both parents, as well as five normal controls and two carrier controls (each analyzed twice). Combined, these data demonstrate that in both cell types the GALT activities found in both the proband and his mother were very low ($\leq$1% of normal for the lymphoblast data), while the activity levels found in the father and maternal grandmother were within the expected carrier range.

### *Identification of Candidate Mutations: Q188R, S135L, and V151A*

Intrigued by the observation that both the mother and child in this family demonstrated significant, albeit less than absolute, GALT impairment, we proceeded to isolate genomic DNA from the available samples (individuals I-1, II-1, II-2, and III-1), for molecular analysis. Applying a rapid PCR-based screen for the most common mutations associated with galactosemia, Q188R and N314D (asparagine→aspartate at codon 314) (see Patients and Methods), we determined that none of the four individuals tested carried the N314D substitution but that both the mother and child (II-1 and III-1) carried Q188R in the heterozygous state.

Next, DNA samples from each of the four individuals were investigated by SSCP analysis (see Patients and Methods), in an effort to identify other regions of the gene that might carry candidate mutations to account for the observed degree(s) of GALT impairment. The striking result was that exon 5 demonstrated two distinct and altered patterns of migration, one seen in DNAs from both the child and father and a second seen in DNAs from both the mother and maternal grandmother (data not shown). Exon 6 showed the expected heterozygous Q188R-associated pattern in DNAs from both the mother and child. All other exons from all four samples were unremarkable. Direct sequencing (see Patients and Methods) of exon 5 from each sample subsequently confirmed that both the mother and maternal grandmother carried single copies of the S135L substitution, which had been reported elsewhere (Reichardt et al. 1992), while both the child and father carried single copies of a novel V151A substitution (fig. 3). As a precaution, all remaining exons were sequenced from both the mother and child, confirming the presence of the Q188R mutation in each and revealing no other apparent variations. Considering that the mother (and child) carried the Q188R mutation while the maternal grandmother did not, we presume that this allele was inherited from the maternal grandfather, who was unavailable for study.

### *Modeling Individual Candidate Mutations in Yeast*

To assess the functional capacity of each of the three identified candidate mutations in a defined and homozygous context, each was recreated by site-directed mutagenesis in the otherwise wild-type human GALT sequence and was introduced into a GALT-deficient strain of yeast, yJFK1, essentially as described elsewhere (Fridovich-Keil and Jinks-Robertson 1993). Biochemical analyses of extracts from the resultant homozygous transformants (fig. 4) clearly demonstrated that Q188R-GALT had no detectable activity, as reported elsewhere (Fridovich-Keil and Jinks-Robertson 1993); that S135L-GALT had ~5% of normal activity; and that V151A-GALT had ~3% of normal activity.

### *Modeling Mutations in Yeast in the Heterozygous and Compound-Heterozygous States*

Considering that many galactosemic patients are not true molecular homozygotes, as was the case for the individuals of interest in this family, we next modeled each of the three defined candidate mutations in both the heterozygous and compound-heterozygous states. To accomplish this goal, the appropriate GALT sequences, both wild-type and mu-

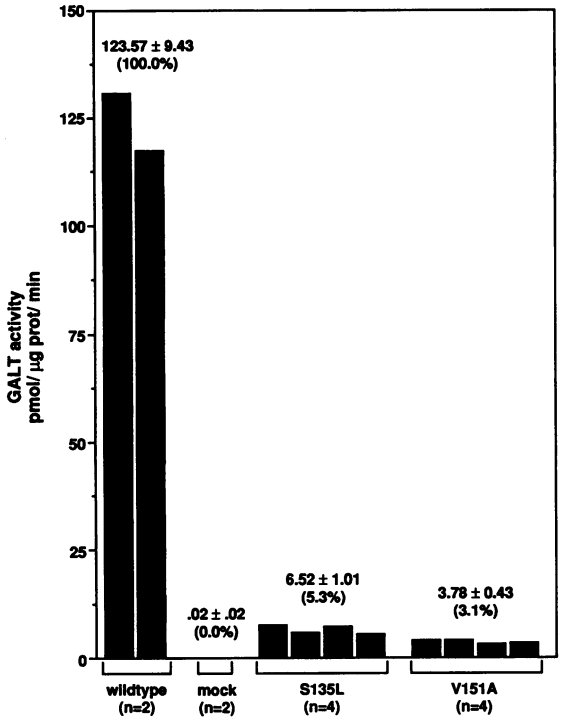



**Figure 4** Activities associated with the wt, S135L, and V151A human GALT alleles expressed in the homozygous state in yeast. Activity assays were performed as described in Patients and Methods, on extracts of mock-transformed cells as well as yeast expressing each of the human GALT alleles indicated. Bar heights represent actual sample values. Averages and SDs for each set are indicated above the appropriate bars, and averages normalized to the respective wild-type value are indicated in parentheses. Molecular genotypes and numbers of observations for each sample are listed below each set.

tant, were introduced into a second yeast-expression plasmid, pPEy1, identical to pLMy2 except that it carries a marker conferring host leucine prototrophy rather than tryptophan prototrophy (J. P. Elsevier and J. L. Fridovich-Keil, unpublished data). Yeast was then cotransformed with the appropriate combinations of plasmids, was maintained under selection for both tryptophan and leucine, and was analyzed as described above.

The results of these experiments (fig. 5) clearly demonstrated the anticipated outcome—namely, that all three mutations were associated, in the heterozygous (carrier) state, with between 20% and 50% of wild-type levels of activity, while both compound-heterozygous samples were associated with <1% of wild-type activity. As a negative control, yeast homozygous for the Q188R null-activity allele of GALT were analyzed in parallel. Like mock-transformed cells (fig. 4), these cells demonstrated *no* detectable GALT activity, thereby validating the low levels of activity observed in the compound heterozygotes. All of these data were remarkably consistent with the results of the patient lymphoblast studies (fig. 2) and, together with the results from the single-transformation studies, strongly indicated that, at least within the context of the cells investigated, all three identified candidate mutations are indeed functionally significant mutations.

## Discussion

We report here the identification and modeling in yeast of three separate substitution mutations in the human GALT gene that are associated with galactosemia in a single family. Two of these mutations, Q188R and S135L, have been reported elsewhere (Reichardt et al. 1991, 1992); Q188R represents the most common mutation identified in Caucasian Americans with classic galactosemia (Elsas et al. 1993, 1994), while S135L has been reported in a much smaller number of samples, also derived from patients with galactosemia (Reichardt et al. 1992). Previous studies of lymphoblasts derived from patients homozygous for the Q188R mutation have demonstrated essentially no GALT activity in these cells (Fridovich-Keil and Jinks-Robertson 1993). Similarly, yeast-modeling studies involving the Q188R mutation recreated within the context of either the human GALT sequence or the yeast GALT sequence have demonstrated complete impairment of GALT activity (Fridovich-Keil and Jinks-Robertson 1993). In contrast, Rei-

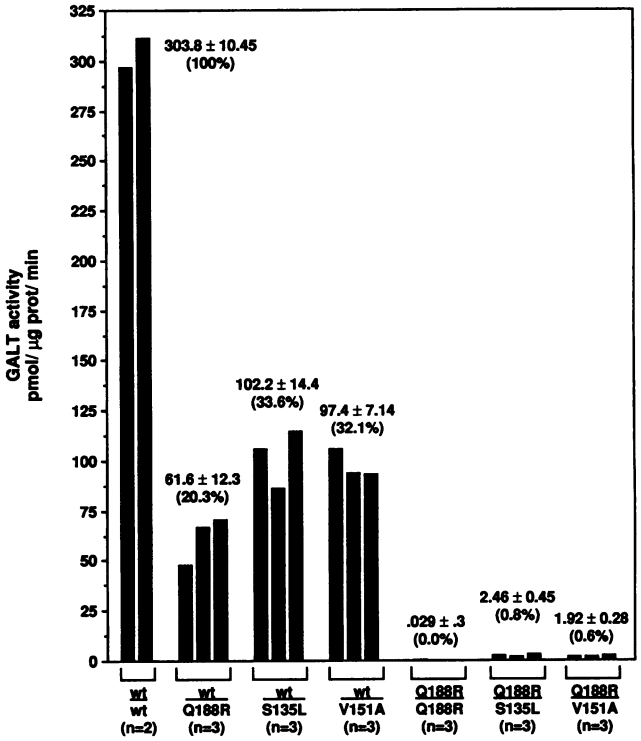



**Figure 5** Activities associated with the Q188R, S135L, and V151A human GALT alleles expressed in the heterozygous or compound-heterozygous states in yeast. Activity assays were performed as described in Patients and Methods, on extracts of yeast expressing each of the combinations of human GALT alleles indicated. Bar heights represent actual sample values. Averages and SDs for each set are indicated above the appropriate bars, and averages normalized to the respective wild-type value are indicated in parentheses. Molecular genotypes and numbers of observations for each sample are listed below each set.

chardt et al. (1991) reported from their COS cell–expression studies that the Q188R mutation is associated with 10% residual activity. The reason for this disparity remains unknown, but it may reflect either the influence of wild-type endogenous GALT present in the host COS cells, some other properties of these cells, or complications in quantitation that result from the transient-expression assay. The results reported here both confirm and extend our earlier report concerning GALT impairment by the Q188R mutation. Again we have found that, in the homozygous state in yeast, Q188R-GALT has no detectable activity, and, furthermore, we have observed that in both the heterozygous state with wild-type GALT and the compound-heterozygous state with either S135L-GALT or V151A-GALT, Q188R-GALT contributes no detectable activity.

In contrast to Q188R, the S135L substitution was reported originally, by Reichardt et al. (1992), to be a neutral polymorphism, on the basis of the results of their COS cell–expression studies together with the observation that the amino acid encoded at position 135 is not fully conserved across species. The results reported here stand in sharp contrast to those findings. Our analyses both of patient cells and of homozygous and heterozygous (or compound-heterozygous) yeast all demonstrate significant impairment of activity associated with the S135L mutation. As with Q188R, the explanation for this apparent disparity remains unknown, but it may reflect either differences in the properties of the host cells or differences between transient- and stable-expression assays. It seems unlikely in this instance that endogenous GALT present in the COS cells could fully account for the disparity, especially considering the results of our heterozygous modeling studies, in which impairment of activity was observed despite the fact that the S135L-GALT was expressed in cells coexpressing large levels of wild-type GALT. Nonetheless, some other properties of the host cells may certainly account for the observed differences; for example, COS cells are derived originally from African green monkey kidney cells (Gluzman 1981), and our studies have involved either human RBCs, human lymphoblasts, or yeast. Considering the role that tissue-specific expression or modifications may play in modulating GALT expression and activity, it seems reasonable to assume that some combination of tissue-specific or species-specific factors may be involved here.

Another interesting point is that Reichardt et al. (1992) first identified the S135L substitution in each of two different cell lines in conjunction with a second substitution, F171S (phenylalanine→serine at codon 171) and therefore hypothesized that these two mutations might be linked. In the family reported here, the substitution F171S was not observed, suggesting, at least in this one example, that these two substitutions need not always be linked.

In considering the outcome of the heterozygous modeling studies, it is interesting to note that in the heterozygous state with wild-type GALT, Q188R-GALT consistently demonstrated markedly less activity than did either S135L-GALT or V151A-GALT. While these alleles demonstrated slightly different activities when modeled in the homozygous states, the explanation for this "heterozygous observation" may be more complex and, at least in theory, may be influenced both by whether each mutant allele remains competent to combine in the homo- or heterodimer state and by whether any heterodimer formed is active. Experiments are now underway to address these issues.

Finally, the results reported here concerning the S135L substitution suggest that the commonly invoked assumption (e.g., see Reichardt et al. 1992)—i.e., that, because an amino acid position is not highly conserved through evolution, it may be "unimportant" for function—may require reconsideration. Indeed, through higher-resolution studies of the residual activity observed in this and other partial impairment alleles, we hope to learn more about the nature of the enzyme and its structure-function relationships. As demonstrated here, the yeast-expression system should prove very useful for this work.

## Acknowledgments

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