Relationship between genotype, activity, and galactose sensitivity in yeast expressing patient alleles of human galactose-1-phosphate uridylyltransferase

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SUMMARY
Impairment of the human enzyme galactose-1-phosphate uridylyltransferase (GALT) results in the potentially lethal disorder galactosemia; the biochemical basis of pathophysiology in galactosemia remains unknown. We have applied a yeast expression system for human GALT to test the hypothesis that genotype will correlate with GALT activity measured in vitro, and with metabolite levels and galactose sensitivity measured in vivo. In particular, we have determined the relative degree of functional impairment associated with each of sixteen patient-derived hGALT alleles; activities ranged from null to essentially normal. Next, we utilized strains expressing these alleles to demonstrate a clear inverse relationship between GALT activity and galactose sensitivity. Finally, we monitored accumulation of galactose-1-P, UDP-gal, and UDP-glc in yeast expressing a subset of these alleles. As reported for humans, yeast deficient in GALT, but not their wild-type counterparts, demonstrated elevated levels of gal-1-P and diminished UDP-gal upon exposure to galactose. These results present the first clear evidence in a genetically and biochemically amenable model system of a relationship between GALT genotype, enzyme activity, sensitivity to galactose, and aberrant metabolite accumulation. As such, these data lay a foundation for future studies into the underlying mechanism(s) of galactose sensitivity in yeast, and perhaps other eukaryotes, including humans.
INTRODUCTION

The enzyme galactose-1-phosphate uridylyltransferase (GALT) catalyzes the second step of the Leloir pathway of galactose metabolism, converting UDP-glucose (UDPG) and galactose-1-phosphate (gal-1-P) to glucose-1-phosphate (glu-1-P) and UDP-galactose (UDP-gal) (1, 2). Impairment of human GALT (hGALT) results in the potentially lethal disorder classic galactosemia (2, 3).

Currently, most infants with classic galactosemia born in industrialized nations are detected in the neonatal period by mandated newborn screening procedures. Dietary restriction of galactose initiated early and maintained throughout life for these patients prevents the potentially lethal sequelae of the disorder. Unfortunately, despite treatment, the long-term outcome for these patients is mixed: 85% of girls with galactosemia experience primary ovarian failure, and 30-50% of patients of both genders demonstrate learning disabilities and speech and/or motor dysfunction, among other complications (4). Although aberrant accumulation or depletion of key galactose metabolites, including gal-1-P, UDP-gal, galactitol, and others, are hypothesized to underlie the observed complications (reviewed in 2, 3), the biochemical mechanism of pathophysiology in galactosemia remains unknown.

One of the fundamental questions with regard to classic galactosemia concerns the identification of predictive factors that might be used to distinguish those patients who will thrive long-term, from those who will experience complications. Waggoner and colleagues addressed this issue a decade ago with an international retrospective questionnaire study, and found no clear correlation between long-term outcome and three of the most obvious candidate extrinsic factors: age at diagnosis, presence of neonatal complications prior to treatment, or strict dietary compliance (4). These data suggested that some intrinsic factor(s) might serve a predominant role in defining outcome.

With regard to intrinsic factors, perhaps the most obvious is GALT genotype. The number of candidate mutations identified in patient alleles now exceeds 150, the majority of which are missense point mutations (5). Indeed many, if not most, galactosemia patients studied are
compound heterozygotes, further complicating the picture. Although most naturally-occurring
mutant alleles of hGALT have not been well characterized with regard to function, high-
sensitivity biochemical studies of large groups of ungenotyped patients have demonstrated clear
biochemical heterogeneity in the patient population (6, 7). Furthermore, differences in the
prevalence of specific hGALT genotypes also have been observed in populations of patients with
vs. without detectable GALT activity (8). These data support the hypothesis that genotype may
correlate with activity, which in turn may influence metabolite levels and phenotypic outcome.
Indeed, ungenotyped patients with detectable GALT activity have been reported to accumulate
lower levels of gal-1-P, and to experience a milder clinical course, and than do their counterparts
without detectable GALT activity (6, 7). Similarly, a number of studies have reported decreased
levels of UDP-gal, and altered ratios of UDP-glc/UDP-gal in samples from galactosemic
patients compared to controls (2). Nonetheless, there has been no direct test of the relationship
of these parameters. Furthermore, while some retrospective outcome studies of patients with
galactosemia have reported a statistically significant relationship between genotype and outcome,
others have not (9-12), perhaps reflecting the complications of confounding variables and limited
sample sizes.

We report here the first quantitative and allele-specific test of the hypothesis that there is a
relationship between hGALT genotype, activity, metabolite levels, and sensitivity to galactose in
a eukaryotic system. In particular, we have used yeast, extending from initial observations by
Douglas and Hawthorne (13), who reported that GALT-deficient yeast, but not their wild-type
counterparts, were growth-arrested by the addition of small amounts of galactose to the media,
despite the presence of other metabolizable carbon sources. We have applied a previously-
described null-background yeast expression system (14) to study sixteen naturally-occurring
patient alleles of human GALT (R67C, S135L, L139P, V151A, F171S, P183T, Q188R, R201H,
R231H, R259W, K285N, E291K, N314D, R333W, Y323D, and T350A). In particular, we have
characterized each allele in terms of both abundance and activity of the encoded human GALT
protein. Three main groups of alleles were identified: those with <1% wild-type activity, those
with 1-5% wild-type activity, and those with ≥~10% wild-type activity. Monitoring the impact of galactose-exposure on strains of yeast expressing each mutant allele, we observed that, with few exceptions, yeast expressing the lowest activity hGALT proteins demonstrated the most significant sensitivity to galactose, and the most prolonged accumulation of gal-1-P, an indicator of galactose metabolic imbalance. Strains expressing the intermediate activity alleles of hGALT demonstrated intermediate galactose sensitivity and transient gal-1-P accumulation. Finally, those strains expressing the highest activity alleles of hGALT demonstrated no sensitivity to galactose, and no detectable accumulation of gal-1-P. Furthermore, studies of UDP-gal and UDP-glc accumulation in samples prepared from yeast expressing wild-type human GALT vs. no GALT showed a specific and significant loss of UDP-gal, but not UDP-glc, only in the GALT-deficient cells in response to the addition of galactose. These results present the first clear evidence in a biochemically and genetically amenable model system of a relationship between hGALT genotype, encoded enzyme activity measured in vitro, aberrant metabolite accumulation, and sensitivity to galactose measured in vivo. As such, these data lay a foundation for future studies into the underlying mechanism(s) of galactose toxicity in yeast, and perhaps other eukaryotes, including humans.

EXPERIMENTAL PROCEDURES

Plasmids
All hGALT mutations were recreated by site-directed mutagenesis of the otherwise wild-type sequence, as described previously (15). The primers used to generate alleles R67C, L139P, P183T, R201H, R231H, R259W, K285N, E291K, Y323D, and T350A were hGR67CF (5’-GAAGACAGTGCCCTGCCATGACCCTCTC-3’), hGL139PF (5’-GGATGTAACGCCGCCACTCATGACCCCTCTC-3’), hGP183TF (5’-GGATGAAACGCCGCCACTCATGTC-3’), hGR201HF (5’-GCTGTTCTAAACACCCACCCCCACT-3’), hGR201HF (5’-
GATATTGCCCAGCATGAGGAGCGA-3'), hGR231H (5’-TCAGGAAGGAAACATCTGGTCTAACC-3’), hGR259WF (5’-GCTGCCCGTGTGACATGTGCAGCGG-3’), hGK285NF (5’-GCTTGGACCAATTATGACAACCTC-3’), hGE291KF (5’-GACAACTCTTTAAGACGTCCTTTCCC-3’), hGY323DF (5’-CACGCTCATTACGACCCTCCGCTC-3’), hGT350AF (5’-GAGGGACCTCGCCCTGAGCAGGCT-3’), respectively. All resultant mutant alleles were confirmed by dideoxy sequencing. Recreations of the mutations S135L, V151A, F171S, Q188R, N314D, and R333W have been described previously (14, 16-19).

For expression at low copy number, each allele was subcloned using the enzymes Eco R1 and Sal 1 into the centromeric yeast vector pMM22 (20). For expression at high copy number, alleles were subcloned into the 2micron yeast vector pYEP-GAP (generous gift of Dr. Warren Kruger, Fox Chase Cancer Ctr.). Both plasmids facilitate expression of the introduced open reading frame from the constitutive yeast GAP promoter.

**Yeast strains and Manipulations:** All yeast manipulations were carried out according to standard techniques as described previously (21). All YEP-GAP and MM22 plasmids were transformed into yJFK1, a previously described haploid strain of *Saccharomyces cerevisiae* deficient in GAL7, the endogenous yeast GALT (14). Transformants were selected and maintained on the basis of tryptophan prototrophy, conferred by the plasmid. Except where otherwise noted, cells were cultured in media containing dextrose as the sole carbon source to prevent any selective pressure for GALT activity.

Soluble cell lysates were prepared from 30mL cultures grown at 30°C to OD600=1.5, essentially as described previously (14, 22). Briefly, cell pellets were resuspended in 500µl lysis buffer (20mM HEPES, 1mM dithiothreitol, 0.3mg/ml bovine serum albumin, 0.3mM aprotinin, 1mM pepstatin A, 2mM antipain, 1mM phosphoramidon, 0.2µg/ml chymostatin, 8mM E64, and 1mM PMSF) and transferred to 2 mL tubes. 100µl acid washed glass beads (0.5mm diameter)
were added to each tube and the cells were disrupted with 6 cycles of agitation (45 s on high followed by 45 s on ice) using a multihead Vortex at 4°C. Each disrupted cell suspension was then transferred to a 1.5mL tube and centrifuged in a microfuge on high speed for 10 minutes at 4°C to pellet insolubles. Finally, each clarified supernatant was transferred to a fresh tube, and assessed for protein concentration using the Biorad protein assay reagent as recommended by manufacturer, with bovine serum albumin as a standard.

GALT activity assays: GALT activity was determined in soluble yeast lysates as described previously (14, 22). For lysates prepared from yeast expressing low copy number plasmids (MM22 derivatives), between 1-20ug total protein was included in each reaction, adjusted to maintain linearity of the assay. 1ug protein was used for assays of wild-type hGALT under these conditions. For lysates prepared from yeast expressing high copy number plasmids (YEP-GAP derivatives), between 0.3 to 180ug total protein was included in each reaction, adjusted to maintain linearity of the assay. 0.3 to 0.5ug protein was used for assays of wild-type hGALT under these conditions. All assays were performed in triplicate (or greater), as indicated (Table 1), representing extracts prepared from independent colonies, and adjusted according to total protein before being normalized to the appropriate wild-type values.

Western Blot Analyses: Western blot analyses were performed as described previously (18). SDS-PAGE gels to be blotted were loaded with 5ug/lane protein representing yeast expressing low copy number plasmids (MM22 derivatives), and either 1ug (wild-type) or 5ug (mutants) protein per lane representing yeast expressing high copy number plasmids (YEP-GAP derivatives). Both wild type and mutant forms of human GALT were detected using a rabbit polyclonal antiserum raised against hexahistidine tagged hGALT at a dilution of 1:100,000. As a control for loading, blots also were probed with an antiserum against the endogenous yeast protein cyclophilin (at a dilution of 1:30,000) (23). Signals were visualized using an HRP-conjugated antiserum against rabbit Ig (Amersham Pharmacia Biotech, 1:5000 dilution),
followed by reaction with the enhanced chemiluminescence (ECL) system from Amersham Pharmacia Biotech, as recommended by the manufacturer.

**Sensitivity of yeast to galactose:** Cultures inoculated from colonies were grown initially in synthetic medium containing 2% dextrose overnight to an OD$_{600}$ between 1 and 2. Cells were then diluted into 6mls fresh medium containing 2% glycerol/2% ethanol in place of dextrose at an OD$_{600}$ = 0.1. These cultures were allowed to grow to an OD$_{600}$ of about 1, at which point they were again diluted in duplicate into 6ml of fresh synthetic medium containing 2% glycerol/2% ethanol at an OD$_{600}$ = 0.1 to begin growth curves. Time points were taken at 0, 7, 22, and 31 hours, at which point the OD$_{600}$ of each culture was close to 0.4. At that point, galactose was added to a final concentration of 0.05% to one culture from each pair, and all tubes were returned to the rotator at 30°C. Finally, measurements of OD$_{600}$ were followed for each culture periodically over the next 2-4 days, as indicated (Figures 2-4).

**Gal-1-P measurements:** Samples were prepared from duplicate 30 ml cultures of cells grown as described above (prior to the addition of galactose). At 31 hours after inoculation, a 6ml sample was removed from each culture (zero time point), and galactose was added to the remaining volume of one culture from each pair to a final concentration of 0.05%. At 7, 15, and 63 hours following the addition of galactose, 6ml samples from each culture were harvested, pelleted, and frozen. Finally, cell pellets were lysed as described above, except that protease inhibitors were not included in the lysis buffer. Protein concentrations were measured, as described above, using the Biorad protein assay reagent as recommended by manufacturer, with bovine serum albumin as a standard.

Next, each lysate was cleared of proteins by vigorous extraction with methanol (500µl methanol/ 200µl aqueous sample), followed by centrifugation to pellet the protein precipitate. Finally, each clarified supernatant was transferred to a fresh 1.5mL tube and dried under vacuum. Pellets were resuspended in sterile, deionized water for further analysis.
Gal-1-P levels were quantitated using a coupled spectrophotometric assay described previously (17, 22) with slight modifications. In particular, the assay buffer contained 100mM glycyl-glycine, pH 8.7, 6mM dithiothreitol, 5μM glucose-1,6-diphosphate, 5mM MgCl₂, 0.8mM NADP, 0.1μg of phosphoglucomutase and 0.06μg of glucose-6-phosphate dehydrogenase in a total reaction volume of 400μl. All assays were carried out using 300ng of purified HIS6-tagged hGALT isolated from a yeast expression system. A standard curve was generated using UDP-glucose at a concentration of 0.6mM with varying concentrations of galactose-1-phosphate (0.075mM to 1.2mM). To determine the levels of galactose-1-phosphate present in each test sample, 50μl of deproteinated extract (representing 20μg- 416μg of original lysate protein) were used in place of a known quantity of galactose-1-phosphate, so that the amount of gal-1-P present in that sample could be determined by interpolation from the standard curve. Final values presented (Figures 3 and 4) were normalized according to total protein in each original extract.

To ensure that our measurements of gal-1-P were comparable to those of other groups using the method of Bergmeyer (24), that utilizes alkaline phosphatase and galactose dehydrogenase; we assayed a set of test samples, some with high gal-1-P, and others with low gal-1-P, by both methods. In all cases comparable values were obtained from both assays (data not shown).

**UDP-gal and UDP-glc measurements:** Samples were prepared from cultures of yeast expressing either no GALT, or wild-type human GALT, grown in the presence vs. absence of galactose, harvested, lysed, and cleared of proteins, as described above. UDP-gal was measured against a standard curve established using a coupled reaction with purified UDP-gal-4 epimerase (kind gift of Drs. Jim Thoden and Hazel Holden, Wisconsin) and UDP-glc dehydrogenase (Sigma), as described previously (25). UDP-glc was measured directly in each sample in the absence of epimerase using UDP-glc dehydrogenase (Sigma), and also quantitated by comparison with a standard curve. Final values presented (Figure 4) were normalized according to total protein in each original extract.
RESULTS

Expression and analysis of patient-derived alleles of human GALT in yeast: We have used site-directed mutagenesis of the wild-type human GALT sequence to recreate each of sixteen naturally occurring mutations: R67C, S135L, L139P, V151A, F171S, P183T, Q188R, R201H, R231H, R259W, K285N, E291K, N314D, Y323D, R333W, T350A. Following confirmation, each allele was introduced into both low copy number (CEN, MM22) and high copy number (2µ, YEP-GAP) yeast expression plasmids containing the constitutive GAP promoter, and transformed into the previously described null background strain of *Saccharomyces cerevisiae*, yJFK1 (14). Plasmids encoding the wild-type hGALT sequence, and empty plasmids alone, were also included in all experiments as positive and negative controls, respectively. Unless otherwise noted, all cultures were maintained in dextrose-containing medium to prevent selective pressure based on encoded hGALT activity.

To confirm expression, and to determine the relative abundance of each substituted hGALT protein in yeast, soluble lysates prepared from cells expressing each hGALT allele from a centromeric plasmid were subjected to western blot analysis with the rabbit polyclonal anti-hGALT antiserum, EU70. As a control for loading of lanes, each filter was also probed with a polyclonal antiserum that recognizes yeast cyclophilin (23), an abundant endogenous yeast protein. As illustrated in Figure 1, wild-type hGALT was readily detectable in this system, and the negative control was clean. A faint cross-reacting band that runs just above the position of hGALT was also visible in all lanes, providing an additional internal control for loading. Twelve of the sixteen substituted hGALT proteins demonstrated abundance comparable to that of the wild-type protein, while four (V151A, R231H, R259W, K285N) were below the threshold of detection. To increase sensitivity of the assay, lysates were prepared from yeast expressing each of these four alleles from high copy number plasmids. Western blot analyses of these samples (Figure 1, right panel), again in parallel with wild-type hGALT also expressed from a high copy
number plasmid, confirmed that each of the four mutant proteins was expressed, albeit at markedly decreased levels (>20-fold) relative to the wild-type protein.

Lysates representing each hGALT allele expressed from both low copy and high copy number plasmids also were analyzed for GALT activity using a standard in vitro assay, as described in Experimental Procedures. As reported previously, lysates from yJFK1 expressing no hGALT demonstrated no detectable GALT activity in these assays (14, data not shown). As illustrated in Table 1, the sixteen mutant alleles tested displayed a spectrum of activity levels, ranging from below the threshold of detection, to nearly wild-type. Grouped roughly according to in vitro activity, six (F171S, Q188R, R231H, R259W, K285N, R333W) were below the threshold of detection, four (R67C, S135L, L139P, V151A) displayed low but detectable activities (between 1-5% wild-type levels), and six (P183T, R201H, E291K, N314D, Y323D, T350A) displayed close to 10% or greater wild-type levels of activity.

Sensitivity of yeast expressing mutant alleles of human GALT to galactose: More than 30 years ago, Douglas and Hawthorne (13) demonstrated that yeast deficient in gal7, the endogenous yeast GALT, were sensitive to the presence of low levels of galactose added to their culture medium, despite the presence of other metabolizable carbon sources such as glycerol and ethanol. We have repeated these results with our strains and applied this system to probe the relationship between hGALT activity and galactose toxicity in yeast.

In brief, yJFK1 expressing wild-type hGALT, no GALT, or each of the sixteen substituted hGALT alleles described above were grown in duplicate cultures of synthetic medium containing glycerol/ethanol as the carbon source. At approximately 31 hours following inoculation, when all cultures were growing well (OD$_{600} = \sim 0.4$), galactose was added to 0.05% final concentration to one of each of the pairs of cultures. All cultures were then returned to incubation at 30°C with periodic monitoring of OD$_{600}$ over the course of approximately four days. As expected, all cultures grew indistinguishably in the absence of galactose (data not shown). In the presence of galactose, however, marked differences were apparent in the growth profiles of the different
strains (Figure 2), and these correlated well with the levels of \textit{in vitro} GALT activity associated with each strain.

In particular, those strains expressing $\geq 10\%$ GALT activity (left panel) grew well despite the addition of galactose to their medium, although the two lowest GALT activity strains in this set, Y323D and T350A, did show a transient slow-down in growth. In contrast, cells expressing mutant hGALT alleles associated with between 1-5\% wild-type activity demonstrated a marked slow-down in growth rate following the addition of galactose that lasted for several days (Figure 2, center panel). All but one of these cultures (R67C) eventually resumed essentially wild-type growth rates. Finally, cells expressing either no GALT, or inactive alleles of hGALT, demonstrated a profound and prolonged cessation of growth following the addition of galactose. These cultures did not recover to normal growth rates within the time frame of the experiment (Figure 2, right panel).

**Relationship between galactose sensitivity and metabolite accumulation in yeast:** As an initial step toward probing the biochemical basis of galactose-sensitivity in yeast, we repeated growth studies of two cultures representing each of the three hGALT activity groups described above (high, intermediate, and very low), and monitored intracellular gal-1-P levels at four time points following the addition of galactose to each culture. The times plotted (Figure 3) correspond to 0, 7, 15, and 63 hours after the addition of galactose. As illustrated in Figures 3A and D, cells expressing high levels of GALT activity (wild-type or T350A) demonstrated no detectable accumulation of gal-1-P at any time. Cells expressing intermediate (1-5\% wild-type) levels of GALT activity (S135L and L139P) demonstrated no detectable gal-1-P at 0 hours but did show accumulation of gal-1-P at the 7 and 15 hour time points. By 63 hours after the addition of galactose, again no gal-1-P was detected in these cells. Finally, cultures expressing no detectable GALT activity (null, or Q188R) demonstrated no detectable gal-1-P at 0 hours, but marked accumulation of the metabolite at both the 7 and 15 hour time points. By 63 hours after the addition of galactose, the gal-1-P levels in both cultures had dropped precipitously, although both
remained within the detectable range. As expected, in duplicate samples of all cultures that received no galactose, no gal-1-P was detected at any time point tested (data not shown).

As an additional test of potential metabolite imbalance, we grew cultures of yeast expressing either no GALT or wild-type human GALT, in medium containing 2% glycerol/2% ethanol to early log phase, and then added galactose to 0.05% to half of the cultures and harvested time points, as described above. Measurements of gal-1-P, UDP-gal, and UDP-glc were performed as described in Experimental Procedures. Consistent with clinical reports (2), we observed a clear decrease in UDP-gal levels in samples prepared from GALT-deficient yeast exposed to galactose, but not in corresponding samples prepared from yeast expressing wild-type hGALT (Figure 4, panels E, F). In contrast, only minor changes in the levels of UDP-glc were observed in these same samples (Figure 4, panels G, H). As expected, gal-1-P accumulated to high levels only in the GALT-deficient cells exposed to galactose (Figure 4, panels C, D).

DISCUSSION

The results reported here are significant for two reasons. First, although many patient-derived alleles of human GALT have been identified (5), few have been well characterized in terms of degree or nature of resulting enzyme impairment. Of the sixteen mutant alleles studied here, although seven have been analyzed previously (S135L (19, 26-28), V151A (19), F171S (16, 26), Q188R (14,17,29-32), R231H (33), N314D (18, 34), and R333W (17, 29, 30)), nine have not (R67C, L139P, P183T, R201H, R259W, K285N, E291K, Y323D, T350A). These results therefore represent the first clear demonstration that these nine mutations are functionally significant, and not simply polymorphisms that occur in linkage with some distinct but unknown causal mutation.

Second, the data reported here present the first clear evidence in a genetically and biochemically amenable model system of a relationship between hGALT genotype, encoded enzyme activity, \textit{in vivo} galactose toxicity, and aberrant metabolite accumulation. These data lay
a foundation for future studies into the biochemical basis of galactose toxicity in yeast, and perhaps other eukaryotes, including humans.

**hGALT expression, abundance, and activity:** Our data presented here concerning the expression, abundance, and activity of patient-derived alleles of hGALT demonstrate that, as recognized previously in patients (6, 7), not all hGALT alleles derived from patients with galactosemia are completely null. They represent a spectrum, albeit generally at the low end of activity.

Three-dimensional homology modeling of the human GALT sequence onto the *E. coli* GALT crystal structure (35) predicts that all four mutations associated with marked reduction in protein abundance (V151A, R231H, R259W, and K285N) impact residues near the surface of the protein. None of these residues is within 13 angstroms of either the active site, the metals, or the dimer interface. Although compromised stability seems likely, the actual cause of the low abundance for each of these substituted proteins remains unknown.

Similarly, in terms of activity, with few exceptions, all of the mutations associated with very low or no detectable GALT activity impact either residues near the active site (S135L, L139P, F171S, Q188R), near the dimer interface (R333W), or represent low abundance proteins (V151A, R231H, R259W, and K285N). One notable exception is R67C. Homology modeling predicts that the R67 residue lies in a loop that protrudes from the surface of the GALT protein. The underlying explanation for why this substitution causes such marked catalytic impairment, but no apparent loss in abundance, remains unclear. Yeast expressing this same mutation also exhibit anomalous galactose sensitivity relative to the degree of catalytic impairment observed *in vitro*, as will be discussed below.

**Comparison with prior studies:** Comparisons between data presented here and those reported elsewhere reveal that, to the resolution of these studies, the yeast system recapitulates accurately what has been seen in humans carrying the corresponding hGALT mutations. For example, F171S (8), Q188R (8, 14, 36), R231H (33), R259W (37), K285N (38), and R333W (8) have all
been associated with null or close to null GALT activity in hemolysates and/or lymphoblasts of patients homozygous or compound heterozygous for these mutations. In contrast, the mutations R67C (39, 40), S135L (8,27), and V151A (19) have all been associated with low but non-zero activity in patient samples. The substitutions P183T (41) and T350A (37) have both been associated with milder GALT impairment in patient hemolysates and/or lymphoblasts. In addition, with regard to N314D, recent reports have described a collection of both coding and non-coding sequence variations that are found in cis with this substitution and that distinguish the mildly under-expressed Duarte (D2), from the mildly over-expressed Los Angeles (LA, D1) GALT alleles (42), both of which carry N314D (41, reviewed in 5). These data reinforce the hypothesis that N314D may be a polymorphism (34) that causes the electrophoretic mobility shift associated with both the Duarte and LA alleles (18), but not the abundance/activity differences observed between them (reviewed in 5). Finally, although R201H, L139P, E291K, and Y323D, have all been identified in samples derived from patients with galactosemia, no data have been reported assigning specific GALT activity levels to these substitutions (9, 44).

Comparisons between model system-derived data and relevant patient data are clearly important. Unfortunately, the fact that many galactosemia mutations have been identified in patients only in the compound heterozygous state confounds accurate assignment of catalytic impairment to each allele. Modeling studies in COS cells and in yeast have previously attempted to address this caveat. For several of the substitutions modeled in both systems, comparable results were obtained (e.g. F171S (16, 26), R333W(17, 29, 30), and N314D (18, 34)). For other substitutions, however, disparate results were observed (S135L (19, 26, 28), Q188R (14, 29), and R231H (33)). In each of these cases, the data obtained in yeast more closely corresponded to those derived from patient samples. The basis for the disparity between the COS and yeast-derived results remains unclear; however, considering that GALT functions as a dimer, one possibility may be interaction between the exogenous human protein and endogenous wild-type GALT subunits in the COS cells. The yeast system has been genetically modified to eliminate endogenous GALT protein (see Experimental Procedures).
Galactose-sensitivity in yeast:

Our results concerning an inverse relationship between GALT activity \textit{in vitro} and galactose-sensitivity \textit{in vivo} are fully consistent with prior studies of galactosemia patients (6), and demonstrate several points. First, although we have presented the data (Figure 2) in terms of low, intermediate, and higher activity alleles, these are groupings of convenience. The alleles themselves represent a spectrum, as does the degree of galactose-sensitivity observed, with an inverse correlation between the two. Additional studies will be required to define more accurately the exact nature of this relationship.

Second, with regard to the “intermediate” alleles demonstrating between 1-5% wild-type activity (Figure 2, middle panel), the galactose sensitivity observed is transient. By about the 96 hour time point, the cultures begin to recover, resuming growth rates reminiscent of the “higher activity” cultures. Two possible scenarios may explain this apparent recovery, either (a) the environment has changed, presumably galactose in the cultures has been consumed over time and fallen below some threshold of toxicity, or (b) the cells have changed -- they have adapted. Studies are currently underway to distinguish between these possibilities.

The third point demonstrated by the data in Figure 2 is that there are exceptions to the relationship between GALT activity measured \textit{in vitro} and sensitivity to galactose measured \textit{in vivo}: R67C is a case in point. The explanation for why this allele exhibits intermediate levels of activity \textit{in vitro}, but behaves more like a null allele \textit{in vivo}, will be a focus of future study. The implication, that GALT activity measured \textit{in vitro} under defined buffer conditions with excess substrates may not accurately reflect function \textit{in vivo}, is not only possible but likely. Other options, such as disrupted enzyme sequestration (45) or macromolecular interactions normally required for function \textit{in vivo}, may also contribute to the discrepancy, and will be explored.

Metabolite accumulation and galactose-sensitivity: The results presented in Figures 3 and 4 illustrating a relationship between galactose-sensitivity and aberrant accumulation of gal-1-P and
UDP-gal in yeast are consistent with earlier patient studies (2, 6) and also raise several important questions. First, are elevated levels of gal-1-P or depleted levels of UDP-gal actually causal in mediating galactose-toxicity in either yeast or humans, or are these changes merely correlated? Future studies will be required to distinguish between these possibilities. It is important to note that studies of a mouse GALT knock-out model have demonstrated, at least in that system, that biochemical absence of GALT does not always result in clinical pathology, despite accumulation of gal-1-P (46, 47). It is particularly interesting to note, however, that GALT-deficient mice maintained on a diet including galactose did not demonstrate any detectable depletion of UDP-gal (47) relative to their wild-type counterparts, while both humans (2) and yeast (Figure 4) do. Another important consideration involves aldose reductase, an enzyme that converts galactose to galactitol, and that is normally expressed in humans at significantly higher levels than in mice. Ai and colleagues have recently reported studies of a mouse knock-out model for galactokinase deficiency galactosemia (48), demonstrating that despite absence of galactokinase and abnormal accumulation of representative metabolites, these mice failed to exhibit cataracts (the predominant feature of the human disorder) until a human aldose reductase transgene was introduced into the mouse genetic background. Clearly, similar modification of the GALT-deficient mouse may also impact outcome. It should be noted that yeast do express aldose reductase (GRE3). Further studies will be required to explain the underlying biochemical basis of the apparent outcome differences observed between the mouse, human, and yeast GALT-deficient systems.

A second question concerns the quantitative relationship between gal-1-P levels and culture growth rates in yeast. It is important to recognize that although the gal-1-P levels detected in yeast null for GALT at the latest time point measured (Figure 3, panel F) are markedly lower than those detected earlier in the experiment, they are non-zero. In contrast, yeast expressing “intermediate activity” alleles of hGALT (panel E) had no detectable gal-1-P at the corresponding time point. This small but significant difference may account for the absence of clear growth in the GALT-null cultures within the time frame of the experiment. It is also
important to note, however, that as illustrated in panels B and E, there was a lag of close to 48 hours between loss of detectable gal-1-P and recovery of growth in the cultures. Further studies will be required to explore more fully the relationship between gal-1-P, or other metabolites, and galactose-toxicity in yeast.

A final question raised by the data presented here (Figure 3) concerns the observation that even in yeast expressing no detectable GALT activity, although gal-1-P levels rise precipitously upon exposure to galactose, they do come down again. Prior studies (31,32) have reported minute, but non-zero, levels of activity associated with Q188R-hGALT, and with its Q168R E. coli GALT counterpart. While this residual activity may be invoked to explain the decline of accumulated gal-1-P over time in yeast expressing Q188R-hGALT, the same decline also was observed in yeast completely devoid of GALT (panel F). These data suggest that some GALT-independent pathway for the metabolism of gal-1-P must exist in yeast. This observation is consistent with the recent results of others (49, 50, 51), although the biological significance of this alternative pathway under normal (GALT-proficient) conditions remains to be clarified.

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FIGURE LEGENDS

Figure 1: Western blot analyses of soluble whole cell lysates from yeast expressing human GALT. 5ug of total protein derived from cells expressing each of the indicated hGALT alleles from low copy number plasmids were loaded in each lane of the gels (left, middle panels). 1ug of total protein (WT) or 5ug (all others) derived from cells expressing each of the indicated hGALT alleles from high copy number plasmids were loaded in each lane of the gel depicted in the panel on the right. The lower sections (cyc) of both panels are presented as loading controls, as described in Experimental Procedures.

Figure 2: Galactose sensitivity of gal7- yeast expressing patient alleles of human GALT. Yeast expressing each of the indicated alleles of hGALT were cultured in synthetic medium containing glycerol/ethanol, with galactose added to 0.05% final concentration at 31 hours (arrows). Growth was monitored for each culture by OD600. Yeast expressing higher activity alleles of hGALT are presented in the left-most panel, yeast expressing intermediate activity alleles (+ WT as a control) as presented in the middle panel, and yeast expressing the lowest activity alleles (+ WT as a control) are presented in the right-most panel. All values plotted represent averages ± SD (n=3). Parallel samples of all cultures maintained in medium containing dextrose grew well (data not shown).

Figure 3: Transient accumulation of gal-1-P in yeast experiencing galactose-toxicity. As in Figure 2, yeast devoid of endogenous gal7 but expressing the indicated patient alleles of hGALT were cultured in synthetic medium containing glycerol/ethanol, with galactose added to 0.05% final concentration at 31 hrs (arrows). In addition to monitoring OD600s at the indicated times, samples of each culture were harvested and analyzed for intracellular gal-1-P accumulation. As illustrated, cells expressing the higher activity alleles of hGALT demonstrated no measurable
accumulation of gal-1-P, while those cells expressing either intermediate and lower activity alleles exhibited notable accumulations of gal-1-P.

**Figure 4:** Impact of galactose exposure on accumulation of UDP-gal and UDP-glc in yeast. As in Figure 3, yeast expressing either no GALT or wild-type hGALT were cultured in synthetic medium containing glycerol/ethanol, with galactose added to half of the cultures at 0.05% final concentration at 40 hrs (arrows). In addition to monitoring OD\textsubscript{600}, at the indicated times (panels A, B), samples of each culture also were harvested and analyzed for intracellular levels of gal-1-P (panels C, D), UDP-gal (panels E, F), and UDP-glc (panels G, H). In all panels, samples representing cultures with galactose are represented by open circles, and samples representing cultures without galactose are represented by filled circles. All values plotted represent average +/- SD (n=3). In panel C, samples representing yeast cultured in both the presence and absence of galactose are plotted, although only one set is visible because the values at each time point were coincident (all 0). Similarly, gal-1-P measurements at the 40 hr time point (panel D) representing yeast cultured in both the presence and absence of galactose were both 0, so only one is visible in the figure.
| allele   | relative GALT activity (n) |
|----------|-----------------------------|
| wild-type | 100.0 +/- 5.8 (9)            |
| N314D    | 102.5 +/- 33.0 (3)          |
| E291K    | 62.8 +/- 9.8 (3)            |
| R201H    | 62.8 +/- 9.8 (3)            |
| P183T    | 45.2 +/- 6.8 (4)            |
| T350A    | 9.9 +/- 1.3 (3)             |
| Y323D    | 9.6 +/- 0.9 (3)             |
| V151A    | 4.6 +/- 1.1 (5)             |
| S135L    | 2.7 +/- 0.4 (6)             |
| R67C     | 2.3 +/- 0.4 (3)             |
| L139P    | 1.9 +/- 0.6 (3)             |
| F171S    | < 0.2 +/- 0.0 (3)           |
| Q188R    | < 0.2 +/- 0.0 (3)           |
| R231H    | < 0.2 +/- 0.0 (6)           |
| R259W    | < 0.2 +/- 0.0 (12)          |
| K285N    | < 0.2 +/- 0.0 (5)           |
| R333W    | < 0.2 +/- 0.0 (9)           |

**TABLE 1:** Activity assays of soluble lysates from yeast expressing the indicated alleles of human GALT. All values were normalized to the corresponding wild-type GALT activity level, and are presented as average +/- SD (n). Activity levels in extracts demonstrating <5% wild-type activity were determined using cells expressing the indicated hGALT alleles from a high copy number plasmid, while those demonstrating ≥10% activity were determined using cells expressing the indicated hGALT alleles from centromeric plasmids. In each case activity values associated with the mutant hGALT proteins were normalized against wild-type hGALT expressed from the same vector backbone.
FIGURES:

Figure 1

[Image of a gel electrophoresis with gels labeled hGALT and cyc, showing bands for different genotypes under conditions of low and high copy number plasmids CEN and 2μ.]

Figure 2

[Image of a graph showing OD₆₆₀ over time for different genotypes and phenotypes, with error bars indicating variability.]
Figure 3
Figure 4