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Characterization of Two Mutations Associated with Epimerase-Deficiency Galactosemia, by Use of a Yeast Expression System for Human UDP-Galactose-4-Epimerase

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Summary

UDP-galactose-4-epimerase (GALE) is a highly conserved enzyme that catalyzes the interconversion of UDP-galactose and UDP-glucose. Impairment of this enzyme in humans results in one of two clinically distinct forms of epimerase-deficiency galactosemia—one benign, the other severe. The molecular and biochemical distinction between these disorders remains unknown. To enable structural and functional studies of both wild-type and patient-derived alleles of human GALE (hGALE), we have developed and applied a null-background yeast expression system for the human enzyme. We have demonstrated that wild-type hGALE sequences phenotypically complement a yeast *gal10* deletion, and we have biochemically characterized the wild-type human enzyme isolated from these cells. Furthermore, we have expressed and characterized two mutant alleles, L183P-hGALE and N34S-hGALE, both derived from a patient with no detectable GALE activity in red blood cells but with ~14% activity in cultured lymphoblasts. Analyses of crude extracts of yeast expressing L183P-hGALE demonstrated 4% wild-type activity and 6% wild-type abundance. Extracts of yeast expressing N34S-hGALE demonstrated ~70% wild-type activity and normal abundance. However, yeast coexpressing both L183P-hGALE and N34S-hGALE exhibited only ~7% wild-type levels of activity, thereby confirming the functional impact of both substitutions and raising the intriguing possibility that some form of dominant-negative interaction may exist between the mutant alleles found in this patient. The results reported here establish the utility of the yeast-based hGALE-expression system and set the stage for more-detailed studies of this important enzyme and its role in epimerase-deficiency galactosemia.

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Introduction

UDP-galactose-4-epimerase (GALE; E.C.5.1.3.2) catalyzes the third step of the Leloir pathway of galactose metabolism (Frey 1996) (fig. 1). Impairment of this enzyme in humans results in one of two clinically distinct forms of autosomal recessive epimerase-deficiency galactosemia (MIM 230350). A clinically benign, or "peripheral," form was first reported by Gitzelmann (Gitzelmann 1972; Gitzelmann and Steimann 1973), on the basis of studies of asymptomatic patients who exhibited no detectable GALE activity in red blood cells (RBC) but who exhibited normal activity in other tissues. A second, much rarer and "generalized" form of the disorder was first reported by Holton et al. (1981), on the basis of studies of a severely affected patient demonstrating undetectable levels of GALE activity in all tissues examined. Limited population studies suggest that the peripheral form may be quite common, at least in some ethnic groups (Alano et al., in press). In contrast, only two cases of generalized epimerase-deficiency have been reported (Holton et al. 1981; Sardharwalla et al. 1988). The molecular and biochemical distinction between the peripheral and generalized forms of epimerase deficiency remains unknown.

Wild-type GALE enzymes have been isolated and/or characterized in a variety of species and tissues (e.g., see Maley and Maley 1959; Wilson and Hogness 1964, 1969; Darrow and Rodstrom 1968; Salo et al. 1968; Tsai et al. 1970; Langer and Glaser 1974; Piller et al. 1983 Frey 1996; Thoden et al. 1996), including humans (Bergren et al. 1973; Mitchell et al. 1975). All of these GALE enzymes are believed to function as homodimers (e.g., see Frey 1996; Langer and Glaser 1974; Thoden et al. 1996), and both biochemical and structural evidence from studies of the *Escherichia coli* enzyme now indicate that one NAD^+ is bound per subunit (Frey 1996; Thoden et al. 1996). It is interesting to note that, although the enzymes isolated from *E. coli* and yeast do not require exogenously added NAD^+ for function, all mammalian GALE enzymes do require exogenous NAD^+ (Langer and Glaser 1974). In addition, although the GALE enzymes isolated from bacteria and yeast are able

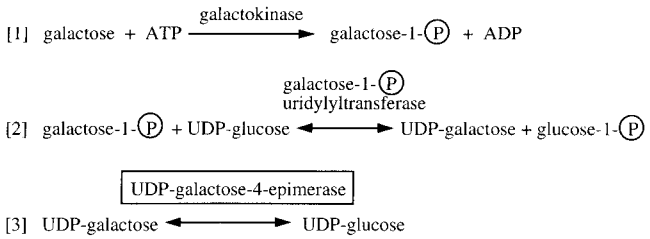


Figure 1 Leloir pathway of galactose metabolism

to interconvert only UDP-glucose and UDP-galactose (Darrow and Rodstrom 1968; Salo et al. 1968; Piller et al. 1983), purified mammalian GALE enzymes appear to be bifunctional, demonstrating the ability to interconvert both UDP-glucose/UDP-galactose and UDP-N acetylglucosamine/UDP-N acetylgalactosamine (Maley and Maley 1959; Piller et al. 1983; Kingsley et al. 1986). Considering the pivotal role of these latter substrates in cellular assembly of complex polysaccharides and other glycosylated macromolecules, the biological significance of the mammalian GALE enzyme clearly extends beyond the catabolism of dietary galactose.

A human cDNA encoding wild-type human GALE (hGALE) was recently cloned and characterized by Daude et al. (1995), thereby enabling molecular studies of mutations associated with epimerase-deficiency galactosemia. To facilitate studies of both wild-type and patient-derived mutant forms of hGALE, we have developed a null-background yeast expression system for the human enzyme. We have utilized this system to express, isolate, and characterize wild-type hGALE, as well as two patient-derived mutant alleles each modeled in the homozygous, heterozygous, and compound-heterozygous states, comparing the results with those observed in corresponding family members. The results reported here represent the first biochemical characterization of mutant forms of hGALE associated with epimerase-deficiency galactosemia.

Subjects and Methods

Patient

The proband is a 5-year-old male born to a Caucasian mother and a Pakistani father. His neonatal course was unremarkable, and he remained on a lactose-containing diet. Results of newborn screening at 6 and 9 d of age revealed abnormally elevated galactose sugars but normal UDP-galactose-1-phosphate uridylyltransferase (GALT) activity. Follow-up studies confirmed normal erythrocyte GALT activity, elevated levels of galactose-1-phosphate, negative urinary reducing sugars, and no detectable erythrocyte GALE activity (A. Alano, S. Almashanu, J. M. Chinsky, P. Costeas, M. G. Blitzer, E. A. Wulfsberg, T. M. Cowan, unpublished data). The child remained clinically well on a lactose-containing diet, with no hepatomegally, liver disease, vomiting, or acidosis, and although RBC galactose-1-phosphate was elevated in infancy, with time these values decreased to the normal range. Urinary galactitol, measured at age 26 mo, was within normal limits, consistent with a diagnosis of peripheral epimerase deficiency. At ~1 year of age the proband began experiencing delays in gross motor development, and at age 5 years he exhibits mild to moderate mental retardation with global delays in language and cognitive abilities, but otherwise he is healthy and well. Blood samples from the proband and both parents were collected as described elsewhere (A. Alano, S. Almashanu, J. M. Chinsky, P. Costeas, M. G. Blitzer, E. A. Wulfsberg, T. M. Cowan, unpublished data), with appropriate informed consent. This research project was approved by the institutional review board at Emory University.

Yeast Strains, Plasmids, and Expression Studies

All recombinant DNA manipulations were performed according to standard techniques (Sambrook et al. 1989) and utilized *E. coli* strain XL1-Blue (Stratagene). The yeast strain yBBQ1, which carries a disruption of the genomic *gal80* locus, was derived from strain YM366 (*MATa gal10-120 ura3-52 his3-200 ade2-101 lys2-801 tyr1-501;* generously donated by Dr. Mark Johnston, Washington University School of Medicine, St. Louis) by one-step gene replacement via homologous recombination (Rothstein 1991). The *gal80* disruption cassette used in this procedure carried the Alani-Kleckner *URA3* cassette (Alani et al. 1987) subcloned in place of a 600-bp *Bgl*II fragment, which had been removed. The wild-type hGALE-coding sequence was obtained by standard PCR amplification of cDNA isolated from control lymphoblasts by use of a 16:1 mixture of *Taq* and *Pfu* DNA polymerases and the primers 5′-GCCGGAATTCATAAAAATGGCAGAGAAGGTGCTGG-3′ (forward) and 5′-GCGGTCGACGCTGCTTTTCCTGGTCCTTGG-3′ (reverse). The wild-type rat GALE-coding sequence was obtained similarly by PCR amplification using as template the rat cDNA clone (Zeschnigk et al. 1990) generously donated by Drs. Zeschnigk and Starzinski-Powitz, with primers 5′-GCCGGAATTCATAAAAATGGAGGAGAAGGTGCTCGTC-3′ (forward) and 5′-GCGGTCGACTGTAGGGTCCTGTGGCGGCTGC-3′ (reverse). These two sets of primers introduced unique 5′ *Eco*RI and 3′ *Sal*I sites onto the ends of both amplified fragments to facilitate subcloning. Sequences encoding the HIS6 and 12CA5 epitope tags also were introduced onto the 3′ end of the hGALE-coding sequence by PCR (reverse primers 5′-GCCGTCGACTCAATGGTGATGGTGATGGTGGGCTTGCGT GCCAAAGCCTG-3′ and 5′-GCCGTCGACTCAAGCGTAGTCTGGGACGTCG TATGGGTAGGCTTGCGTGCCAAAGCCTG-3′, respectively); all resultant subclones were confirmed by dideoxy sequencing.

All GALE alleles to be transformed singly into yeast were subcloned into the low-copy-number expression plasmid pBQy1, which was derived from pPEy1 (Fridovich-Keil et al. 1995*b*) by replacement of the *LEU2* marker by *HIS3*. To enable cotransformation and therefore coexpression of pairs of hGALE alleles in yeast, the appropriate hGALE sequences were introduced into both pBQy1 and pBQy4, which are identical except for the markers that they carry (*HIS3* and *URA3,* respectively). All yeast transformations, culture manipulations, and extract preparations were performed according to standard protocols as described elsewhere (Fridovich-Keil and Jinks-Robertson 1993; Fridovich-Keil et al. 1995*a*; Fridovich-Keil et al. 1995*b;* Elsevier et al. 1996; Quimby et al. 1996).

Lymphoblast Transformation, Culture, and Analysis

Transformed lymphoblasts were established by use of a modification of the procedure of Anderson and Gusella (1984). White blood cells were separated in a Ficoll gradient, and the buffy coat containing mononuclear leukocytes was resuspended in RPMI 1640 (containing FBS, L-glutamine, antibiotic-antimycotic, phytohemagglutinin-M, and interleukin-2) and was transformed with Epstein-Barr virus (EBV). For biochemical analysis, expanded cultures of cells were pelleted by centrifugation, were washed once in 0.85% NaCl, and were stored at −80°C until assay. For enzyme assays, thawed cell pellets were disrupted by sonication in deionized water.

GALE Activity Assays

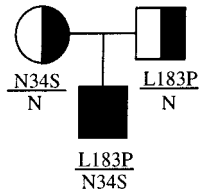
From RBC hemolysates.—GALE was assayed in RBC by use of a two-step fluorometric method (modified and adapted from Beutler [1975] and Gitzelmann and Steimann [1973]) that involved (1) epimerization of UDP-galactose to UDP-glucose, followed by (2) conversion of the UDP-glucose and exogenous NAD^+ to UDP-glucuronate and NADH, which was monitored fluorometrically. The first reaction (0.1 ml patient hemolysate in 300 mM glycine and 1.67 mM NAD^+, with 0.8 mM UDP-galactose in a final volume of 0.3 ml) was incubated at 37°C for 20 min and was stopped by boiling. After centrifugation to pellet insolubles, 0.03 ml supernatant was added to the second reaction, which also contained 0.74 mM NAD^+, 74 mM glycine, and 0.00825 U UDP-glucose dehydrogenase (Sigma U5500), in a total volume of 1.335 ml. This second reaction was incubated at room temperature for 1 h, and fluorescence was read at excitation-wavelength 360 nm and emission-wavelength 460 nm. Data obtained were then converted into units of μmol UDP-glu/g hemoglobin/h, as described elsewhere (A. Alano, S. Almashanu, J. M. Chinsky, P. Costeas, M. G. Blitzer, E. A. Wulfsberg, T. M. Cowan, unpublished data). Hemoglobin concentration was determined by use of Drabkins reagent (Sigma 525-2) as described by the manufacturer.

From lymphoblasts.—GALE activity levels in extracts of cultured lymphoblasts were evaluated essentially as described above, except that the first reaction contained 0.22 mM UDP-gal, 1 mM NAD^+, 10 mM sodium pyruvate, 100 mM glycyl glycine pH 8.7, and 0-0.08 ml cell lysate (at 1.5–2.0 mg total protein/ml). To stop each reaction, samples were boiled for 2 min and were centrifuged, and 0.15 ml supernatant was then used for the second reaction. Total protein concentration in each extract was determined by the method of Lowry et al. (1951).

From yeast extracts.—GALE activity was evaluated in yeast extracts by determination of the conversion of substrate to product, as modified from the method of Merrill et al. (1976). Except where otherwise noted, each 25-μl reaction mixture contained 5 μl premix (3.3 μM [0.1 μCi] (^{14}C)-UDP-galactose; Amersham CFB129), 2 mM UDP-galactose (cold), 2 M glycine buffer pH 8.7), 5 μl 20 mM NAD^+, and 15 μl yeast extract diluted in a buffer of 20 mM Hepes/KOH, pH 7.5, 1 mM DTT, and 0.3 mg BSA/ml. All reactions were incubated at 37°C for 30 min, were stopped by boiling for 5 min, and then the product was centrifuged for 5 min at room temperature to pellet insolubles. Finally, 10 μl each reaction mixture was spotted onto a PEI-cellulose thin-layer chromatography (TLC) plate (Baker) and dried in a 37°C incubator for 30–60 min. The chromatogram was run in a single dimension for 7-20 h, with a solvent of 1.5 mM $Na_2B_4O_7$, 5 mM H_3BO_4, and 25% ethylene glycol. Paper toweling was clipped at the top of each TLC plate to extend the effective "run time" of each assay. After chromatography, each plate was removed from the solvent and was air-dried, and areas containing radioactive material were visualized and quantitated by use of a Molecular Dynamics Phosphorimager.

Enzyme Purification and Kinetics

Yeast extracts were prepared from 1-liter cultures grown at 30°C to midlogarithmic phase in YPGal, were harvested by centrifugation at 4°C, and were lysed as described elsewhere (Fridovich-Keil and Jinks-Robertson 1993; Quimby et al. 1996). Nickel-affinity purification of HIS6-tagged proteins was performed essentially as described elsewhere (Quimby et al. 1996), except that the binding buffer contained 50 mM imidizole, the wash buffer contained 60 mM imidizole, and the protein-elution buffer contained 200 mM imidizole. Near homogeneity of each sample was demonstrated by one dimensional SDS-PAGE followed by staining with Coomassie blue (data not shown). Concentrations of both crude yeast lysates and purified proteins were determined by use of the Bio-Rad protein-assay reagent, with BSA as the standard. Kinetic analyses of each protein were performed in duplicate over a range of 10 different concentrations of substrate (UDP-galactose) (with NAD^+ held



	RBC GALE (μmol UDP-glu/g Hb/h)	lymphoblast GALE (μmol UDP-glu/mg prot/min)
mother	6.3	not done
father	7.9	not done
proband	0.0, 0.0	0.0002, 0.0002
controls	13.9-20.8	0.0014, 0.0015

Figure 2 Family pedigree. Deduced GALE genotypes are indicated below each symbol. RBC and lymphoblast GALE activity levels also are indicated, where available.

constant at 4 mM) and eight different concentrations of NAD^+ (with UDP-galactose held constant at 0.4 mM). To control for potential differences between individual purifications, analyses were performed on samples derived from each of three separate purifications for each protein.

Western Blot Analysis

Western blot analyses were performed as described elsewhere (Fridovich-Keil et al. 1995*b;* Elsevier et al. 1996; Quimby et al. 1996). Proteins were detected by use of the 12CA5 monoclonal antibody (Boehringer Mannheim) at 1 mg/ml. To control for loading of lanes, a rabbit polyclonal antiserum generated against yeast cyclophilin A (Zydowsky et al. 1992) also was included, at a dilution of 1:10,000. The secondary antibodies used were sheep anti-mouse Ig (for 12CA5) and goat anti-rabbit Ig (for cyclophilin), both coupled to horseradish peroxidase (Amersham). Signals were visualized by use of the ECL kit from Amersham and were quantitated by use of a Molecular Dynamics Personal Densitometer SI scanning laser densitometer.

Results

Pedigree Analysis and Biochemical Evaluation of Patient Samples

Fresh peripheral blood was collected from each of the individuals illustrated in figure 2; RBC GALE activity for the proband was undetectable; and the corresponding levels in both parents were significantly reduced, consistent with their status as obligate carriers. Preliminary GALE activity assays also were performed on extracts of EBV-immortalized lymphoblasts from the proband (fig. 2).

Molecular analyses of the GALE-coding sequences in the patient and both parents revealed three base substitutions relative to the published sequence (Daude et al. 1995): an A→G transition at 194, predicting the substitution N34S; a T→C transition at 632, predicting the substitution V180A; and a T→C transition at 641, predicting the substitution L183P (A. Alano, S. Almashanu, J. M. Chinsky, P. Costeas, M. G. Blitzer, E. A. Wulfsberg, T. M. Cowan, unpublished data). Direct sequencing of cDNA samples, followed by mismatch PCR analyses of genomic DNA, identified single copies of the N34S substitution in both the proband and his mother and identified single copies of the L183P substitution in both the proband and his father (fig. 2). DNA mutations corresponding to both the N34S and L183P predicted substitutions were not seen in any of eight control alleles. In contrast, the T→C transition at 632 was found in all alleles tested, representing both control and affected individuals (A. Alano, S. Almashanu, J. M. Chinsky, P. Costeas, M. G. Blitzer, E. A. Wulfsberg, T. M. Cowan, unpublished data). This base change also was found in the wild-type hGALE allele used in the yeast expression system. We therefore conclude that the transition at position 632 is likely to be a polymorphism and, indeed, that the sequence reported here may actually reflect the predominant allele.

A Yeast Expression System for hGALE

We have exploited the technical facility of *Saccharomyces cerevisiae* to create a null-background haploid strain of yeast, yBBQ1, that carries a genomic deletion in the endogenous *gal10* locus and that therefore expresses no endogenous UDP-galactose-4-epimerase. yBBQ1 also carries genomic disruptions of its *his3* and *ura3* loci, to enable selection of transformants, and of the *gal80* repressor locus, to enable high-level expression from the plasmid-borne *GAL10* promoter even in the absence of galactose (Johnston 1987).

As a first test of the ability of plasmid-encoded mammalian GALE enzymes to function in yeast, yBBQ1 cells were transformed with yeast low-copy number plasmids encoding either wild-type rat (Zeschnigk et al. 1990) or hGALE. Both wild-type yeast *GAL10* and plasmid backbone alone also were introduced as controls. All transformants were selected initially on the basis of plasmid-encoded histidine prototrophy, and GAL^+ phenotype was scored by growth in liquid medium containing galactose as the sole carbon source (Sgal-his). All GALE-expressing transformants grew well; the negative control did not (fig. 3). These data confirm that both the rat and wild-type hGALE sequences produce functional products in yeast.

As a more direct test of the functional capacity of hGALE expressed in yeast, whole-cell lysates were prepared from the appropriate transformants and were assayed for enzymatic activity in the presence or absence of exogenous NAD^+ (fig. 4). As expected (Darrow and Rodstrom 1968), yeast GALE demonstrated full activity even in the absence of exogenous NAD^+, whereas hGALE activity demonstrated a strong dependence on exogenously added NAD^+ (fig. 4).

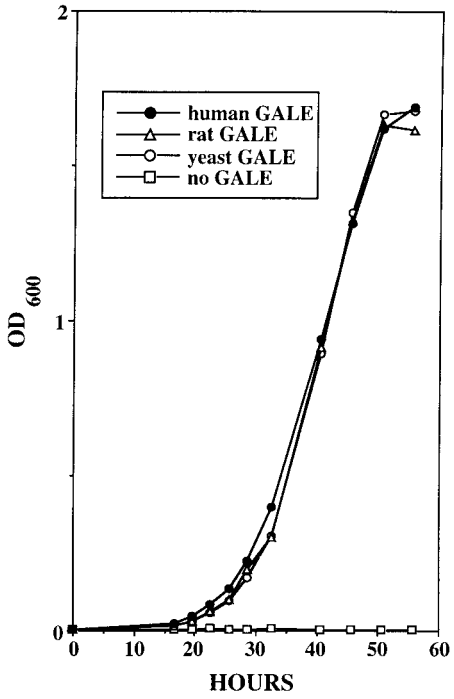


Figure 3 Yeast growth in liquid medium containing galactose as the sole carbon source. yBBQ1 cells transformed with pBQy1-derivative plasmids encoding each of the GALE alleles indicated were inoculated into selective medium at time 0, incubated at 30°C with shaking, and monitored over time by optical density at 600 nm. All four strains grew well in medium containing glucose (data not shown).

Isolation and Characterization of Wild-Type hGALE Expressed in Yeast

To facilitate recognition and isolation of hGALE expressed in yeast, we engineered two small tags, (HA)12CA5 (Wilson et al. 1984) and HIS6 (e.g., see Gentz et al. 1989), onto its C-terminus. To ensure that neither tag impaired activity of the corresponding fusion protein, activity assays were performed on extracts of yeast expressing either untagged, 12CA5-tagged, or HIS6-tagged hGALE. As illustrated, all three forms of hGALE demonstrated indistinguishable levels of activity (fig. 5). Specificity of the 12CA5 antibody was confirmed by SDS-PAGE western blot analysis of crude yeast lysates (fig. 6).

Kinetic studies were performed by use of the wild-type HIS6-tagged hGALE protein purified to near homogeneity. Kinetic constants were determined by fitting the data to a Lineweaver-Burke plot (Robyt and White 1990) (table 1).

The effect of exogenous NAD^+ on activity of the purified hGALE protein was evaluated over an NAD^+ concentration range of 0–40 μM, with UDP-galactose held constant at 0.4 mM. In the absence of exogenous NAD^+, no GALE activity was detected. As increasing amounts of NAD^+ were added, the apparent GALE activity rose dramatically (fig. 7).

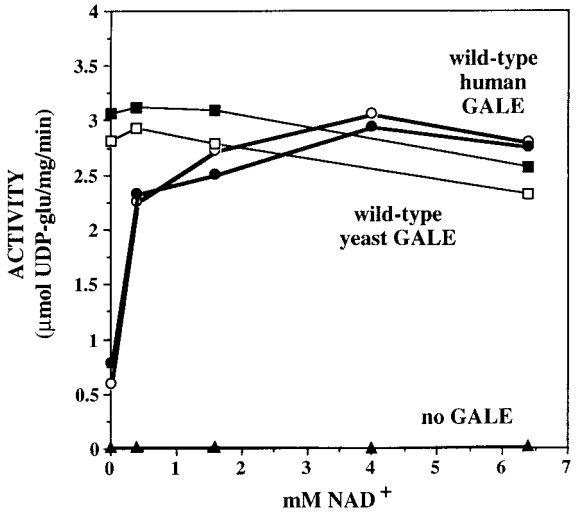


Figure 4 Effect of NAD^+ on GALE activity in extracts of yeast expressing either wild-type hGALE (*circles*), wild-type yeast GALE (*squares*), or no GALE (*triangles*). Duplicate extracts (*unblackened and blackened symbols*) were assayed for GALE activity at each of the (exogenous) NAD^+ concentrations indicated, as described in Subjects and Methods. The concentration of exogenous UDP-galactose was held constant at 0.4 mM.

Characterization of N34S-hGALE and L183P-hGALE Expressed in Yeast

To assess the functional significance of both the L183P and N34S substitutions, each corresponding mu-

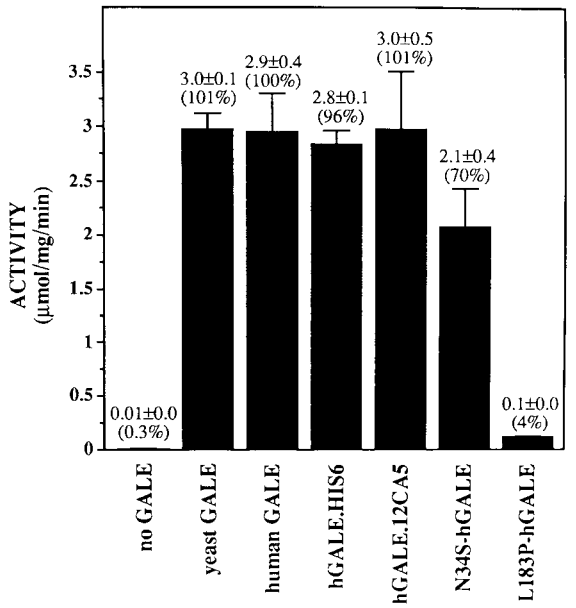


Figure 5 Activities of wild-type and mutant hGALE proteins expressed in yeast. Crude lysates prepared from cultures expressing each of the GALE proteins indicated were analyzed for activity as described in Subjects and Methods. Values plotted represent averages ± SD (n = 8). Average activity levels normalized to the human wild-type (untagged) are given in parentheses.

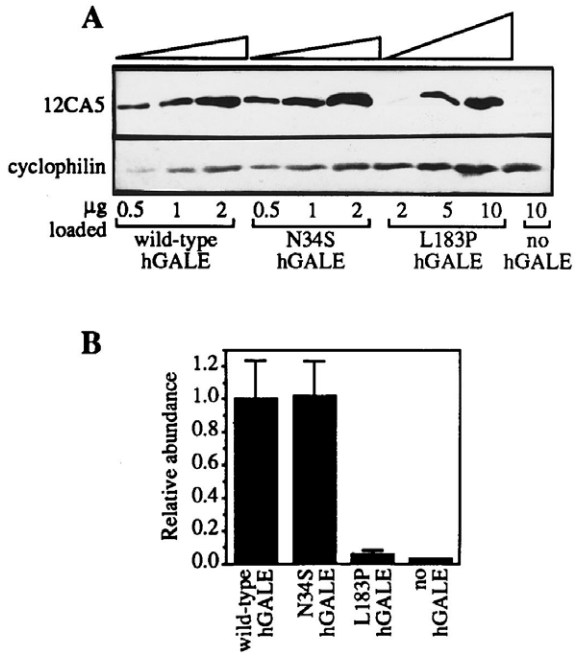


Figure 6 *A,* Western blot analysis of wild-type, N34S-hGALE, and L183P-hGALE proteins expressed in yeast. As indicated, increasing levels of the appropriate yeast extracts were loaded onto adjacent lanes of an SDS-polyacrylamide gel, were electrophoresed, and then were analyzed with respect to GALE abundance by western blot using a mouse monoclonal antibody against 12CA5, as described in Subjects and Methods. As a control for loading, a rabbit polyclonal antiserum directed against the endogenous yeast protein cyclophilin (Zydowsky et al. 1992) also was included in this experiment. *B,* Quantitative analysis of the results presented in *A*. Values plotted represent normalized averages ± SD of the 12CA5:cyclophilin signal ratio for each extract.

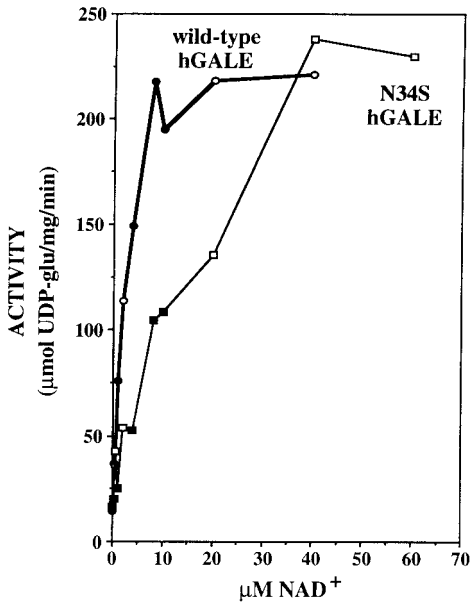


Figure 7 Effects of NAD^+ on purified preparations of wild-type and N34S-hGALE. Assays were performed as described in Subjects and Methods, with exogenous UDP-galactose held constant at 0.4 mM and with exogenous NAD^+ concentration varied as indicated. For each protein, unblackened and blackened symbols represent data derived from independent experiments.

tation was introduced into the otherwise wild-type hGALE sequence, and the resultant alleles were expressed in yeast. Biochemical analyses of crude cell extracts demonstrated that, under normal assay conditions, N34S-hGALE retained ~70% wild-type activity and L183P-hGALE retained ~4% wild-type activity (fig. 5). Both mutant proteins also were both 12CA5 tagged and HIS6 tagged, and, as has been described for the wild-type enzyme, no detectable perturbations of activity were observed (data not shown).

Western blot analyses of whole-cell lysates representing 12CA5-tagged mutant and wild-type hGALE proteins were performed to evaluate the relative impact of each substitution on hGALE steady-state abundance (fig. 6). As a control for loading of lanes, each blot also was probed with a rabbit polyclonal antiserum directed against endogenous yeast cyclophilin (Zydowsky et al. 1992). As illustrated in figure 6, steady-state N34S-hGALE levels were indistinguishable from those of the wild-type protein, suggesting that the mild activity impairment associated with this substitution in crude cell lysates (fig. 5) reflected a difference in function of the enzyme, rather than abundance of the protein. In contrast, L183P-hGALE abundance was reduced to only ~6% of wild-type levels, thereby accounting for most if not all of the activity loss (fig. 5).

To characterize more precisely any kinetic effects of the N34S substitution on hGALE function, HIS6-N34S-hGALE/N34S-hGALE was purified from yeast lysates to near homogeneity. Kinetic analyses of the purified enzyme under conditions of fixed exogenous NAD^+ (4 mM) demonstrated both Michaelis constant (K_m) and specific-activity values indistinguishable from those of the wild type (table 1). Because the *E. coli* GALE residue N32, corresponding to N34 in hGALE, is predicted to form a hydrogen bond with the adenine ring of bound NAD^+ (Thoden et al. 1996), potential effects of the

Table 1

Specific Activity and Apparent K_m Values for Purified Wild-Type and N34S-hGALE Proteins Isolated from Yeast

hGALE Derivative	Apparent UDP-gal K_m (mM)	Specific Activity (μmol UDP-glu/mg protein/min)
Wild type	0.23 ± 0.06	265 ± 81
N34S	0.33 ± 0.08	275 ± 100

NOTE.—Assays to determine the UDP-galactose K_m were performed at a fixed concentration of 4 mM NAD^+. All values are averages ±SD of three or more independent analyses.

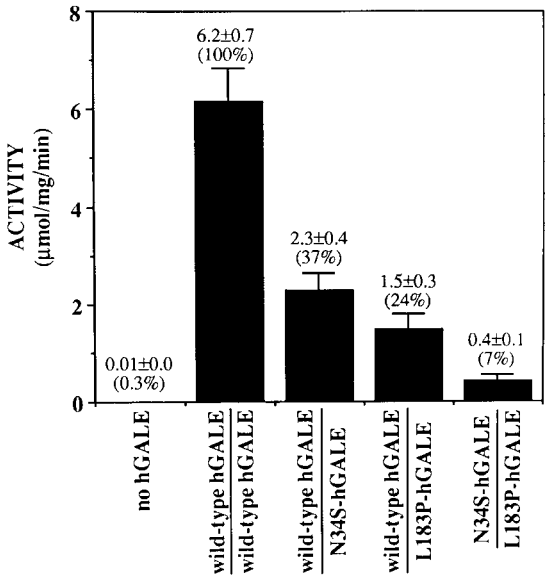


Figure 8 Activities of coexpressed wild-type and mutant hGALE proteins expressed in yeast. Crude lysates prepared from cultures expressing each of the pairs of GALE alleles indicated were analyzed for activity as described in Subjects and Methods. Values plotted represent averages ± SD ($n \geq 6$). Average activity levels normalized to the human wild type are given in parentheses.

N34S substitution on NAD^+ binding also were investigated. Kinetic analyses were performed under conditions of fixed substrate concentration and varying amounts of exogenous NAD^+. The results of these assays revealed a more-than-fourfold increase over wild type in the amount of exogenous NAD^+ required to achieve half-maximal activity (fig. 7).

Coexpression of Mutant and Wild-Type Alleles

To evaluate potential interactions between GALE alleles, we have coexpressed both untagged N34S-hGALE and untagged L183P-hGALE in yeast, modeling both the heterozygous (parents) and compound-heterozygous (proband) states. Control experiments demonstrated that both low-copy number plasmids used in these studies expressed indistinguishable levels of GALE protein (data not shown).

Activity assays on crude lysates demonstrated that, as expected, cells coexpressing two copies of wild-type hGALE expressed twice the level of activity seen in cells expressing hGALE from a single copy (figs. 5 and 8). In contrast, cells coexpressing either each mutant allele together with the wild type or the two mutant alleles in combination demonstrated activities that were significantly reduced (fig. 8). These data raise the intriguing possibility that some form of dominant-negative interaction may exist between the mutant alleles identified in this family. To test the possibility that diminished activity was an artifactual consequence of double rather than single transformation, we cotransformed yeast with two different plasmids each encoding N34S-hGALE; GALE activity detected in these cells was 61% (±0.01; $n = 4$) of that in cells coexpressing two wild-type alleles, thereby eliminating this possibility.

Discussion

That GALE activity was detectable in immortalized lymphoblasts prepared from the proband reported here is not surprising; Mitchell et al. (1975) observed essentially normal GALE activity in lymphoblasts derived from a number of different patients with peripheral epimerase-deficiency galactosemia. Contrary to that report, however, we detected reduced rather than normal levels of activity, thereby raising the possibility that the epimerase deficiency in this patient may not be entirely peripheral. Clearly, studies of the GALE activities in additional tissues will be required to address this issue.

Published values for the specific activities of mammalian GALE enzymes range from 0.65 μmol UDP-glu/mg protein/min (Maxwell 1957) to 315 ± 25.2 μmol UDP-glu/mg protein/min (Piller et al. 1983). Presumably this range reflects differences in either purity or partial inactivation of the enzymes during isolation. We report here a specific activity of 265 μmol UDP-glu/mg protein/min (table 1), demonstrating that expression of the human enzyme in yeast did not compromise the catalytic capacity of the enzyme.

Differences are apparent in the substrate K_m values reported here and elsewhere. The UDP-galactose K_m values reported for GALE enzymes from calf liver (Maxwell 1957), bovine mammary gland (Tsai et al. 1970), porcine submaxillary gland (Piller et al. 1983), and human fibroblasts (Chacko et al. 1972) are 0.02–0.05 mM; the apparent UDP-galactose K_m reported here is 0.23 ± 0.06 mM (table 1). Variations in either sample purity or assay method may account for much of this difference. For example, in some cases crude fibroblast lysates rather than purified enzymes were analyzed, so that contributions from endogenous pools of substrate or other factors could not be discounted (Chacko et al. 1972). It is interesting to note that the UDP-galactose K_m reported for purified *E. coli* GALE is 0.16 mM (Wilson and Hogness 1964), a value similar to the one reported here.

The results reported here (figs. 4 and 7) clearly demonstrate that the previously observed differential dependencies of yeast and mammalian GALE enzymes on exogenous NAD^+ (Maxwell 1957; Darrow and Rodstrom 1968; Salo et al. 1968; Tsai et al. 1970; Langer and Glaser 1974) reflect properties intrinsic to these proteins, rather than differences in the cellular environments in which these enzymes normally are synthesized. As described above for specific activity and K_m, differences in sample preparation, purity, and/or methods of analysis also may account for apparent differences between previous reports and our observations concerning the

level of exogenous NAD^+ required for half-maximal activity of hGALE (fig. 7).

Our observation that purified N34S-hGALE required more than four times as much NAD^+ to achieve half-maximal activity as did the wild-type enzyme is particularly intriguing, especially considering that Mitchell et al. (1975) reported >25 years ago, on the basis of their studies of hGALE partially purified from control and patient lymphoblasts, that, to achieve comparable levels of heat stability, the patient-derived enzyme required ~10 times as much NAD^+ as did the control. We presume that the apparent discrepancy between levels of exogenous NAD^+ required by crude yeast lysates and purified hGALE enzymes to achieve half-maximal activity (fig. 4 vs. fig. 7) reflects largely the combined impacts of two factors: (1) the presence of many NAD^+-binding proteins in crude yeast lysates and (2) the likely presence of at least trace quantities of NADH, a potent inhibitor of GALE (Maxwell 1957; Langer and Glaser 1974), in crude lysates.

Differential dependence of the wild-type and N34S-hGALE enzymes on exogenous NAD^+ also likely explains the observation that crude cell lysates expressing N34S-hGALE demonstrated only 70% wild-type activity when assayed at 4 mM NAD^+ (fig. 5). Similar assays performed at higher NAD^+ concentrations (data not shown) demonstrated less-pronounced differences between samples.

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References

Alani E, Cao L, Kleckner N (1987) A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. Genetics 116: 541–545

Alano A, Almashanu S, Maceratesi P, Reichardt J, Panny S, Cowan TM (1997) UDP-galactose-4-epimerase deficiency among African-Americans: evidence for multiple alleles. J Invest Med 45:191

Anderson MA, Gusella JF (1984) Use of cyclosporin A in establishing Epstein-Barr virus transformed human lymphoblastoid cell lines. In Vitro 20:856–858

Bergren WR, Ng WG, Donnell GN (1973) Uridine diphosphate galastose 4-epimerase in human and other mammalian hemolysates. Biochim Biophys Acta 315:464–472

Beutler E (1975) Red cell metabolism: a manual of biochemical methods, 3d ed. Grume & Stratton, Orlando

Chacko CM, McCrone L, Nadler HL (1972) A study of galactokinase and glucose 4-epimerase from normal and galactosemic skin fibroblasts. Biochim Biophys Acta 284:552–555

Darrow RA, Rodstrom R (1968) Purification and properties of uridine diphosphate galactose 4-epimerase from yeast. Biochemistry 7:1645–1654

Daude N, Gallaher TK, Zeschinigk M, Starzinski-Powitz A, Petry KG, Haworth IS, Reichardt JKV (1995) Molecular cloning, chracterization, and mapping of a full-length cDNA encoding human UDP-galactose 4′-epimerase. Biochem Mol Med 56:1–7

Elsevier JP, Wells L, Quimby BB, Fridovich-Keil JL (1996) Heterodimer formation and activity in the human enzyme galactose-1-phosphate uridylyltransferase. Proc Natl Acad Sci USA 93:7166–7171

Frey PA (1996) The Leloir pathway: a mechanistic imperative for three enzymes to change the sterochemical configuration of a single carbon in galactose. FASEB 10:461–470

Fridovich-Keil JL, Jinks-Robertson S (1993) A yeast expression system for human galactose-1-phosphate uridylyltransferase. Proc Natl Acad Sci USA 90:398–402

Fridovich-Keil JL, Langley SD, Mazur LA, Lennon JC, Dembure PP, Elsas LJ II (1995*a*) Identification and functional analysis of three distinct mutations in the human galactose-1-phosphate uridyltransferase gene associated with galactosemia in a single family. Am J Hum Genet 56:640–646

Fridovich-Keil JL, Quimby, BB, Wells L, Mazur LA, Elsevier JP (1995*b*) Characterization of the N314D allele of human galactose-1-phosphate uridylyltransferase using a yeast expression system. Biochem Mol Med 56:121–130

Gentz R, Chen CH, Rosen CA (1989) Bioassay for transactivation using purified human immunodeficiency virus tat-encoded protein: trans-activation requires mRNA synthesis. Proc Natl Acad Sci USA 86:821–824

Gitzelmann R (1972) Deficiency of uridine diphosphate galactose 4-epimerase in blood cells of an apparently healthy infant. Helv Paediatr Acta 27:125–130

Gitzelmann R, Steinmann B (1973) Uridine diphosphate galactose 4-epimerase deficiency. II. Clinical follow-up, biochemical studies and family investigation. Helv Paediatr Acta 28: 497–510

Holton JB, Gillett MG, MacFaul R, Young R (1981) Galactosemia: a new severe variant due to uridine diphosphate galactose-4-epimerase deficiency. Arch Dis Child 56:885–887

Johnston M (1987) A model fungal gene regulatory mechanism: the GAL genes of *Saccharomyces cerevisiae*. Microbiol Rev 51:458–476

Kingsley D, Kozarsky KF, Hobbie L, Krieger M (1986) Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. Cell 44:749–759

Langer R, Glaser L (1974) Iteraction of nucleotides with liver uridine diphosphate-glucose-4′-epimerase. J Biol Chem 249: 1126–1132

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin reagent. J Biol Chem 193: 265–274

Maley F, Maley GF (1959) The enzymatic conversion of glu-

cosamine to galactosamine. Biochim Biophys Acta 31:577–578

Maxwell ES (1957) The enzymic interconversion of uridine diphosphogalactose and uridine diphosphoglucose. J Biol Chem 229:139–151

Merrill CR, Das AK, Lapolla RJ, Prissovsky I (1976) Microassay for UDP-galactose 4-epimerase activity. Anal Biochem 72:606–613

Mitchell B, Haigis E, Steinmann B, Gitzelmann R (1975) Reversal of UDP-galactose 4-epimerase deficiency of human leukocytes in culture. Proc Natl Acad Sci USA 72:5026–5030

Piller F, Hanlon MH, Hill RL (1983) Co-purification and characterization of UDP-glucose 4-epimerase and UDP-N-acetylglucosamine 4-epimerase from porcine submaxillary glands. J Biol Chem 258:10774–10778

Quimby BB, Wells L, Wilkinson KD, Fridovich-Keil JL (1996) Functional requirements of the active site position 185 in the human enzyme galactose-1-phosphate uridylyltransferase. J Biol Chem 271:26835–26842

Robyt JF, White BJ (1990) Biochemical techniques: theory and practice. Waveland Press, Prospect Heights, IL

Rothstein R (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol 194:281–301

Salo WL, Nordin JH, Peterson DR, Bevill RD, Kirkwood S (1968) The specificity of UDP-glucose 4-epimerase from yeast *Saccharomyces fragilis*. Biochimm Biophys Acta 151:484–492

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2d ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sardharwalla IB, Wraith JE, Bridge C, Fowler B, Roberts SA (1988) A patient with severe type of epimerase deficiency galactosemia. J Inherit Metab Dis 11:249–251

Thoden JB, Frey PA, Holden HM (1996) Crystal structures of the oxidized and reduced forms of UDP-galactose 4-epimerase isolated from *Escherichia coli*. Biochemistry 35:2557–2566

Tsai CM, Holmberg N, Ebner KE (1970) Purification, stabilization, and properties of bovine mammary UDP-galactose 4-epimerase. Arch Biochem Biophys 136:233–244

Wilson DB, Hogness DS (1964) The enzymes of the galactose operon in *Escherichia coli*. J Biol Chem 239:2469–2481

——— (1969) The enzymes of the galactose operon in *E. coli*: the subunits of uridine diphosphogalactose-4-epimerase. J Biol Chem 244:2132–2136

Wilson I, Niman H, Houghten R, Cherenson A, Connolly M, Lerner R (1984) The structure of an antigenic determinant in a protein. Cell 37:767–778

Zeschnigk M, von Wilcken-Bergmann B, Starzinski-Powitz A (1990) cDNA from rat cells with reconstitutive galactose-epimerase activity in *E. coli*. Nucleic Acids Res 18:5289

Zydowsky LD, Ho SI, Baker CH, McIntyre K, Walsh CT (1992) Overexpression, purification, and characterization of yeast cyclophilins A and B. Protein Sci 1:961–969