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Genetics

Molecular basis of galactosemia: Mutations and polymorphisms in the gene encoding human galactose-1-phosphate uridylyltransferase

(missense mutation/inborn error of metabolism/evolution)

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ABSTRACT **We describe the molecular characterization of two mutations responsible for galactosemia, an inherited disorder of galatose metabolism that causes jaundice, cataracts, and mental retardation in humans. The coding region of galactose-1-phosphate uridylyltransferase (GALT; UDPglucose:α-D-galactose-1-phosphate uridylyltransferase, EC 2.7.7.12) was amplified by the polymerase chain reaction from total cDNA of a classic galactosemic individual and was characterized by direct sequencing of the products. Two missense mutations were identified: (*i*) replacement of valine-44 by methionine and (*ii*) replacement of methionine-142 by lysine. These mutations led to a drastic reduction in GALT activity when individual mutant cDNAs were overexpressed in a mammalian cell system, although full-length protein is synthesized in this assay. The two galactosemia mutations account for 3 of the 15 galactosemia alleles analyzed. These results suggest that galactosemia is caused by a variety of mutations, which might be responsible for the observed clinical heterogeneity of this disorder. We also present the molecular characterization of two GALT polymorphisms: (*i*) replacement of leucine-62 by methionine and (*ii*) replacement of asparagine-314 by aspartate. It appears that galactosemia mutations tend to occur in regions that are highly conserved throughout evolution while the polymorphisms change variable residues.**

Classic galactosemia (McKusick 23040), an inborn error of human galactose metabolism, is caused by deficiency of galactose-1-phosphate uridylyltransferase (GALT; UDPglucose:α-D-galactose-1-phosphate uridylyltransferase, EC 2.7.7.12), the second enzyme of the Leloir pathway. It is inherited as an autosomal recessive disorder (1). The disease, which occurs with a frequency of ≈1:50,000 liveborn infants (2), is characterized in its early stages by vomiting, diarrhea, jaundice, and failure to thrive. Later on, most children develop cataracts and mental retardation. In the past, death due to *Escherichia coli* sepsis was not an uncommon outcome (1). Most states and many foreign countries have instituted newborn screening programs, since many of the more severe symptoms can be avoided by placing afflicted individuals on a galactose-restricted diet. However, some symptoms such as chronic neurologic complications and ovarian failure persist despite dietary therapy (3).

By using molecular techniques, a number of human genetic diseases have been analyzed and many mutations have been characterized. This type of work is clinically useful in the diagnosis of the disease in question and may also answer questions about its pathophysiology. Finally, mutations can pinpoint functionally important regions in the affected protein. In genetic diseases, all conceivable mutations have in fact been detected. For example, in the hemoglobinopathies deletions, splicing mutations, premature translation stops, and missense mutations have been identified (5).

By using the cloned GALT cDNA and antibodies to the protein as probes, all 12 galactosemic patients previously analyzed have been GALT mRNA$^+$ and GALT antigenically cross-reactive, suggesting that this disease is caused by a preponderance of missense mutations (6). In order to determine whether there are multiple mutant GALT alleles, we analyzed a classic galactosemic individual in detail at the molecular level.

MATERIALS AND METHODS

Tissue Culture. GM148, -2795, and -2796 cells were obtained from the National Institute of General Medical Science Human Mutant Cell Repository (Camden, NJ) and cultured in RPMI 1640 medium (Hazleton, Lenexa, KS) supplemented with 10% bovine calf serum (HyClone). Skin biopsies were kindly provided by Harvey Levy (Harvard) and grown as primary fibroblasts in high-glucose Dulbecco's modified Eagle's medium (GIBCO) with 20% serum. Transformed adherent cells (COS, GM637, and -639) were grown in the same medium supplemented with 10% serum. Lymphoblastoid cells were transformed with Epstein–Barr virus (8) from blood samples obtained from John McReynolds (Orlando, FL) and Seymour Packman (University of California San Francisco). Lymphoblastoid line TB (Duarte allele) was the kind gift of Louis Elsas (Emory University, Atlanta). The uneven number, 15, of galactosemia alleles shown in Table 2 comes from this last compound heterozygote. The lymphoblastoid cells represent both Caucasian and African-American patients.

PCR Amplification of GALT cDNA. Total cDNA was synthesized from RNA prepared as described (9) with a Boehringer Mannheim kit using the oligo(dT) primer as directed by the manufacturer, except that prior to reverse transcription the poly(A)$^+$ RNA was denatured for 5 min at 65°C in 10 mM Tris·HCl/1 mM EDTA, pH 7.5. The GALT coding region was amplified with primers hG5′-19 (TTTTTCCAGCGGATCCCCC) and hG3′-23 (CTTAATTCAGCAAGACTGTTGAA) (dissociation temperature, 62°C; ref. 10) by repeating the following cycle 30 times: (*i*) denaturation of 92°C for 30 s, (*ii*) annealing for 2.5 min at 50°C, and (*iii*) polymerization for 4 min at 72°C in an Ericomp TwinBlock machine (San Diego). The reaction mixture contained 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/250 μM dNTP/5 mM 2-mercaptoethanol/7 μM EDTA/0.3 μg of each primer, ≈0.3 μg (total) of cDNA (1/4th of the cDNA synthesis reaction) and 2.5 units of Ampli-Taq DNA polymerase (Perkin–Elmer/Cetus). Finally, the termini of the PCR products were made flush for 4 min at 72°C by DNA polymerase. The amplified DNA was gel purified with Geneclean from Bio 101 (La Jolla, CA) to remove the PCR primers.

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Abbreviation: GALT, galactose-1-phosphate uridylyltransferase.

Direct Double-Stranded Sequencing. Purified double-stranded PCR products (≈100 ng in 7 μl) were sequenced with the Sequenase 2.0 kit (United States Biochemical) as recommended by the manufacturer with the following modifications: (*i*) the PCR products were denatured in 10 mM Tris·HCl/1 mM EDTA, pH 7.5, by boiling for 5 min; (*ii*) 0.5 μg of 17-mer internal sequencing primer or PCR primer was annealed to the denatured cDNA at 37°C for 15 min; (*iii*) the dideoxynucleotide stop reaction was carried out at 45°C for 5 min; and (*iv*) denaturation prior to loading on a sequencing gel was for 5 min at 70°C.

All oligonucleotides described in this study were purchased from Genosys (Houston) and were used without further purification.

Site-Directed Mutagenesis. pcDGALT (11) was modified by inserting as a 500-base-pair *Cla* I piece the phage f1 origin of replication [excised from pEN-oriCla (12), obtained from Marjorie Russel, Rockefeller University, New York] to yield pJR16. The putative GALT mutation was reconstructed in this phagemid with the MutaGene kit from Bio-Rad as directed by the manufacturer except that R408 (Stratagene) was used as helper phage. *In vitro* DNA synthesis was primed with the mutagenic 19-mer primer on uracil-containing single-stranded pJR16 DNA and the desired mutation was confirmed by sequencing single-stranded DNA rescued from colonies.

GALT Expression and Assay. COS cells were electroporated as described (11) with a Promega X-Cell 450 instrument at 600 V with 1000-μF capacitance and a time constant of 10 ms. Cell extracts were prepared as described (11) except that the sonication step was omitted in the extract preparation. GALT enzyme activity was measured as described and is expressed as micromoles of uridine diphosphoglucose consumed per hour (11). GALT immunoreactive protein was quantitated with anti-GALT serum and ^{125}I-labeled protein A (6). Mutant enzyme activities are corrected for the endogenous (mock) background.

Allele-Specific Oligonucleotide Hybridization of Normal and Mutant GALT cDNAs. PCR-amplified cDNA was transferred to a Nytran membrane (Schleicher & Schuell) as recommended by the manufacturer and prehybridized in 0.84 M NaCl/60 mM sodium phosphate/6 mM EDTA, pH 7.5/0.5% SDS, 1× Denhardt's solution containing denatured salmon sperm DNA at 100 μg/ml at 50°C for 30 min. ^{32}P-end-labeled oligonucleotides (19-mers spanning each mutation) were added in fresh hybridization mix at 10 ng/ml and annealed for 15 hr to the blotted cDNAs. Blots were washed with 3 M tetramethylammonium chloride (Aldrich) (13)/0.5% SDS/0.1 mM Tris/0.1 mM EDTA twice at room temperature for 10 min, once at 64°C for 10 min, and finally once more at room temperature.

RESULTS

Amplification and Sequencing of the GALT Coding Region. The strategy for identification of the GALT mutations involved six steps: (*i*) mRNA was purified from cells of interest (IM9 and UC, both normal lines, and GM2795, a lymphoblastoid line from a Caucasian galactosemic patient). (*ii*) The mRNA preparations were reverse transcribed into total cDNA. (*iii*) The entire GALT coding region was amplified by PCR from total cDNA with specific primers resulting in a single band. (*iv*) The PCR products were purified on preparative agarose gels to remove the amplification primers and then directly sequenced with nested internal primers. (*v*) Putative mutations were reconstructed in normal GALT cDNA by *in vitro* mutagenesis, and their properties were assayed by transient expression in COS cells. Finally, the frequencies of each mutation were determined by allele-specific oligonucleotide hybridization to both normal and galactosemia alleles.

The entire GALT coding region was amplified as a single 1.2-kilobase (kb) band from total cDNA by using PCR technology (Fig. 1). The amplified band was shown to be GALT by virtue of its hybridization to the cloned probe (Fig. 1*B*). On both the ethidium bromide-stained gel (Fig. 1*A*) and the Southern blot of the same gel (Fig. 1*B*) only one band of 1.2 kb was detected, suggesting that the amplification product was mainly GALT.

We sequenced the entire population of PCR products directly. This procedure eliminates artificial *Taq* DNA polymerase errors because the entire population of amplified DNA products is analyzed and also allows for the simultaneous sequencing of both mutant alleles in the patient. The sequences obtained from purified PCR product with internal primers are shown in Fig. 2. We identified three nucleotide substitutions in the GALT coding region of galactosemic patient GM2795 and one in the normal control line UC. (*i*) In the patient (GM2795) we identified a G → A transition at base pair 158, replacing valine-44 by methionine (Fig. 2*A*). (*ii*) Also in the galactosemia cell line we observed a C → A transversion at position 212, which replaced leucine-62 by methionine (Fig. 2*B*). (*iii*) In the same patient we identified at nucleotide 453 a T → A transversion leading to the replacement of methionine-142 by lysine (Fig. 2*C*). (*iv*) In the normal cell line (UC) we identified a fourth GALT mutation: an A → G transition at nucleotide 968, which replaced asparagine-314 by aspartate (Fig. 2*D*).

Reconstruction and Expression of the GALT Mutations. To establish which GALT mutations in the patient are causal to galactosemia, we reconstructed each by *in vitro* mutagenesis in pJR16, a derivative of pcDGALT that overexpresses normal GALT in mammalian cells. Electroporation of COS cells with either pcDGALT or pJR16 in a transient expression assay led to a 25- to 31-fold stimulation of GALT activity over the endogenous background (Table 1). However, when the methionine-44 and lysine-142 mutant constructs (pJR23 or pJR17, respectively) were introduced into these cells, little or no increase at all in GALT activity was observed (Table 1). The activity for the mutant enzymes was so close to the background activity from the endogenous monkey enzyme that an accurate quantitation of the residual activity of the mutants was difficult. Therefore, the residual activity reported for the mutants is only an approximation. The mutant cDNAs expressed an equivalent amount of immunoreactive protein that was also of normal size (Table 1). Thus, the reduction in enzymatic activity seen with the methionine-44

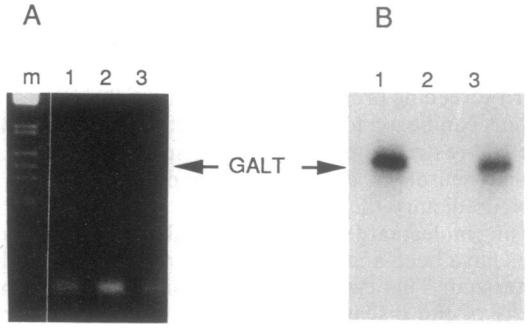

FIG. 1. PCR amplification leads to a single GALT-encoding band. (*A*) Ethidium bromide-stained 1.1% agarose gel loaded with PCR products from cDNAs synthesized from IM9 (normal, lane 1) and from GM2795 (G/G, lane 3) cells. Lane 2, no cDNA was added; lane m, molecular weight markers (*Hin*dIII-cleaved λ and *Hae* III-cleaved φX174 DNAs). (*B*) The gel was blotted and hybridized to the cloned GALT probe. Both cases showed only a single 1.2-kb band, suggesting that the procedure is highly specific.

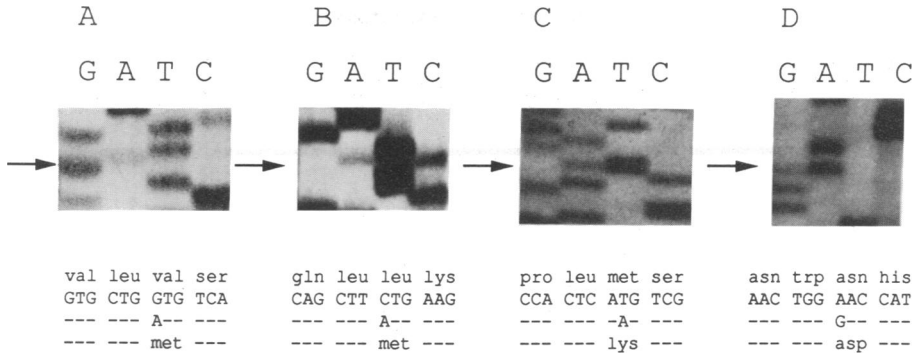

FIG. 2. Characterization of four GALT mutations by direct sequencing of PCR products. (*A–C*) DNAs from GM2795, a classic galactosemic patient. (*D*) DNA from UC, a normal cell line. The sequence in *C* was obtained on the antisense strand. The normal sequences are shown in the upper two lines below each sequencing gel and the lower two lines are the GALT mutations.

and lysine-142 mutations is not the result of unstable proteins. In contrast, when either the methionine-62 or aspartate-314 GALT mutations were electroporated into COS cells, a dramatic increase was observed in GALT activity (Table 1). Essentially normal amounts of immunoreactive material were synthesized as well. Thus, the specific activity of these two GALT mutants is either normal (in the case of the methionine-62 mutation) or slightly higher (for the aspartate-314 mutation).

Allele-Specific Oligonucleotide Studies on the Frequency of the GALT Mutations. To examine the frequency of the GALT mutations, we hybridized both normal and mutant allele-specific oligonucleotides to a panel of normal and galactosemia cDNA populations. Our sample contained four normal cell lines (i.e., eight normal alleles), seven galactosemia lines (14 galactosemia alleles), and one Duarte/galactosemia compound heterozygote (one Duarte and one galactosemia allele, giving us a total of 15 galactosemia alleles). The galactosemia cell lines were obtained from unrelated patients and represent both Caucasian and African-American patients. Although the normal sequence was seen in all cells analyzed, mutations were observed only in lymphocytes from the galactosemic patient (GM2795) for the methionine-44 and the methionine-62 mutations (Table 2). The aspartate-314 replacement was observed only in the normal (UC) cell line and the lysine-142 mutation was identified in two galactosemia cell lines, GM2795 and GM148, a lymphoblastoid line from another Caucasian patient. Thus, the two galactosemia mutations account for 3 of the 15 galactosemia alleles analyzed by allele-specific oligonucleotide hybridization (Table 2). The two polymorphisms are also very rare, accounting for either 1 of 15 or one of eight alleles. Furthermore, the Duarte polymorphism, a common GALT electrophoretic variant with reduced enzymatic activity (1), does not appear to be caused by any one of the mutations we studied (Table 2).

Table 1. Biochemical analysis of four GALT mutations

Plasmid	Enzyme activity, μmol/hr	Protein, cpm	Specific activity, $\mu mol \cdot hr^{-1} \cdot cpm^{-1}$	% normal
	Experiment 1			
None (mock)	19	39	0.49	0
pcDGALT (normal)	480	899	0.53	100
pJR16 (normal)	538	996	0.54	100
pJR17 (Lys-142)	38	848	0.02	4
	Experiment 2			
None (mock)	14	29	0.48	0
pJR16 (normal)	492	912	0.54	100
pJR18 (Asp-314)	675	963	0.70	129
pJR23 (Met-44)	13	779	0	0
pJR24 (Met-62)	512	873	0.59	108

DISCUSSION

A number of human genetic diseases such as cystic fibrosis (7), Lesch–Nyhan syndrome (14), muscular dystrophy (15), phenylketonuria (4), the thalassemias (5), hemophilia (16), color blindness (17), and α1-antitrypsin deficiency (18) have been studied at the molecular level. The picture that has emerged from these and other studies is that all imaginable mutations, including deletions, insertions, and point mutations, are observed. It has been proposed, on the basis of appropriate Southern, Northern, and Western blotting experiments, that galactosemia is caused by a preponderance of missense mutations because all 12 patients analyzed previously are GALT mRNA$^+$ and GALT antigenically cross-reactive (6). We report in this paper the molecular analysis of a classic galactosemic patient that has led to the biochemical and genetic characterization of two galactosemia missense mutations and two GALT polymorphisms.

Reconstruction of two of the four GALT mutations, methionine-44 in pJR23 and lysine-142 in pJR17, results in low or undetectable enzymatic activity although normal levels of protein are made (Table 1). These two GALT mutations are also found only on galactosemia alleles (Table 2). We surmise that these two mutations are present on different chromosomes. These two sets of experiments are biochemical and genetic proof that these two mutations are causative of galactosemia. We also identified a third substitution in patient GM2795, the methionine-62 mutation (Fig. 2*B*). We found the

Table 2. Genetic analysis of four GALT mutations

Probe	Number of alleles, mutant/total		
	Normal	Galactosemia	Duarte
Val-44 → Met			
Normal (Val-44)	8/8	14/15	1/1
Galactosemia (Met-44)	0/8	1/15	0/1
Leu-62 → Met			
Normal (Leu-62)	8/8	14/15	1/1
Polymorphic (Met-62)	0/8	1/15	0/1
Met-142 → Lys			
Normal (Met-142)	8/8	13/15	1/1
Galactosemia (Lys-142)	0/8	2/15	0/1
Asn-314 → Asp			
Normal (Asn-314)	7/8	15/15	1/1
Polymorphic (Asp-314)	1/8	0/15	0/1

The allele-specific oligonucleotides used were 19-mers spanning the GALT mutation (cf. Fig. 2). The uneven number of galactosemia alleles, 15, result from a Duarte/galactosemia compound heterozygote.

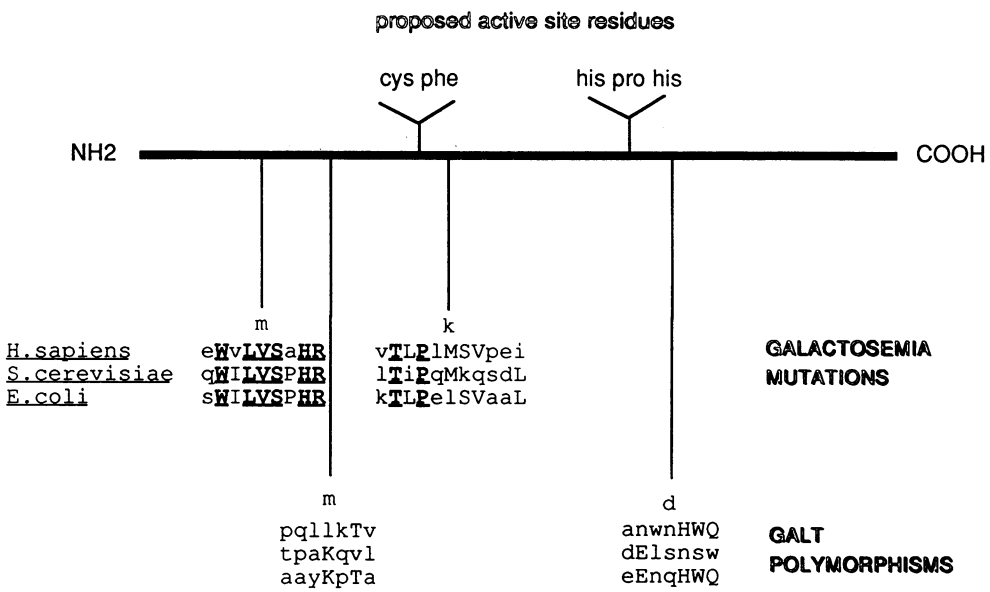

FIG. 3. Galactosemia mutations occur in conserved domains of GALT while the polymorphisms affect highly variable regions. The areas surrounding the four GALT mutations (single-letter amino acid code) are shown. The human sequence (21) is aligned with the homologous proteins GAL7 from *S. cerevisiae* (20) and galT from *E. coli* (19). Residues conserved in two or more species are indicated by uppercase letters and those identical in *E. coli*, *S. cerevisiae*, and *Homo sapiens* are in underlined boldface type. The proposed active site residues are those thought to be important for the function of the enzyme based on evolutionary considerations and chemical modification data (23).

aspartate-314 mutation in a normal control (Fig. 2*D*). Reconstruction and overexpression of these two GALT substitutions results in normal or slightly elevated levels of enzyme activity (Table 1), suggesting that they are polymorphisms. At present, we cannot identify which of the two galactosemia mutations, the methionine-44 one or the lysine-142 one, is linked to the methionine-62 polymorphism in GM2795.

To establish the frequencies of the galactosemia mutations and GALT polymorphisms, we hybridized allele-specific oligonucleotides to a panel consisting of four normal cell lines (eight normal alleles), seven cell lines from galactosemic patients (14 galactosemia alleles), and one Duarte/galactosemia compound heterozygote (one Duarte and one additional galactosemia allele). Our patient sample was obtained from different geographic areas of the United States and two racial backgrounds are represented. On 15 galactosemia alleles, the methionine-44 mutation and the lysine-142 substitution are present on 1 and 2 alleles, respectively (Table 2). Thus, both of these galactosemia mutations are rare, accounting for 3 of the 15 galactosemia alleles. Finally, galactosemia appears to be caused by several different mutations. This molecular heterogeneity might explain the diverse clinical outcome observed in patients.

Comparison of the regions surrounding our four GALT mutations with the homologous proteins GAL7 from *Saccharomyces cerevisiae* and galT from *E. coli* reveals an interesting trend. The methionine-44 mutation alters a valine that is central to a tripeptide conserved in *E. coli* (19), *S. cerevisiae* (20), and humans (21) (Fig. 3). Furthermore, the area surrounding the mutation contains several identical residues (tryptophan-41, histidine-47, and arginine-48) and a conservative substitution (valine-42 to isoleucine). Thus, the methionine-44 mutation occurs in an area of the protein that has been highly conserved throughout evolution from prokaryotes, such as *E. coli*, to eukaryotes. Adjacent to methionine-142 are two identical residues (threonine-138 and proline-140) and two conservative substitutions (leucine-139 to isoleucine and isoleucine-147 to leucine). Finally, methionine-142 is conserved in two other eukaryotes, *S. cerevisiae* (20) and *Kluyveromyces lactis* (22), and its mutation to lysine introduces a new positively charged side chain into the protein. It is noteworthy, that the lysine-142 galactosemia mutation has higher residual activity than the methionine-44 mutation. This is correlated with the lower degree of amino acid identity surrounding the mutated residue (Fig. 3). Neither of the two galactosemia mutations affects the proposed active site residues in GALT (23). In contrast, the two GALT polymorphisms, methionine-62 and aspartate-314, occur in regions with no substantial similarity to either the *S. cerevisiae* or the *E. coli* protein (Fig. 3). We note that the aspartate-314 polymorphism actually increases the specific activity of the GALT enzyme (Table 1). In this context, it is interesting that both *E. coli* and yeast have an acidic dipeptide (Asp-Glu in the case of *S. cerevisiae* and Glu-Glu in *E. coli*) two amino acids upstream of the polymorphic change. It seems plausible that acidic residues are beneficial for the function of the enzyme in this area and, therefore, the aspartate-314 polymorphism increases the specific activity of the GALT enzyme.

Finally, we note that none of the galactosemia mutations or the GALT polymorphisms reported here affect a CpG dinucleotide, the most common site for mutation in humans (24).

Thus far, no restriction fragment length polymorphisms or haplotypes have been reported for GALT (6), but studies on linkage disequilibrium between mutations and haplotypes would be of interest.

In conclusion, in this paper we report a detailed molecular characterization of two galactosemia missense mutations and two GALT polymorphisms. It appears that the disease-causing mutations affect evolutionarily conserved residues, while the GALT polymorphisms tend to alter highly variable residues. The low frequency of the two galactosemia mutations suggests that a large number of mutant alleles can cause galactosemia. This is interesting because the clinical outcome of galactosemia is somewhat variable and different galactosemia alleles might predispose patients to different clinical courses.

This paper is dedicated to Paul Berg on the occasion of his 65th birthday. We thank Drs. Louis Elsas, Harvey Levy, John McReynolds, and Seymour Packman for patient samples. J.K.V.R. is an Associate and S.L.C.W. is an Investigator of the Howard Hughes Medical Institute.

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