Am. J. Hum. Genet. 56:630–639, 1995

Galactosemia: A Strategy to Identify New Biochemical Phenotypes and Molecular Genotypes

L. J. Elsas,[1] S. Langley,[1] E. Steele,[1] J. Evinger,[1] J. L. Fridovich-Keil,[2] A. Brown,[1] R. Singh,[1] P. Fernhoff,[1] L. N. Hjelm,[1] and P. P. Dembure[1]

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

Summary

We describe a stratagem for identifying new mutations in the galactose-1-phosphate uridyl transferase (GALT) gene. GALT enzyme activity and isoforms were defined in erythrocytes from probands and their first-degree relatives. If the biochemical phenotypes segregated in an autosomal recessive pattern, we screened for common mutations by using multiplex PCR and restriction endonuclease digestions. If common mutant alleles were not present, the 11 exons of the GALT gene were amplified by PCR, and variations from the normal nucleotide sequences were identified by SSCP. The suspected region(s) was then analyzed by direct DNA sequencing. We identified 86 mutant GALT alleles that reduced erythrocyte GALT activity. Seventy-five of these GALT genomes had abnormal SSCP patterns, of which 41 were sequenced, yielding 12 new and 21 previously reported, rare mutations. Among the novel group of 12 new mutations, an unusual biochemical phenotype was found in a family whose newborn proband has classical galactosemia. He had inherited two mutations in *cis* (N314D-E203K) from his father, whose GALT activity was near normal, and an additional GALT mutation in the splice-acceptor site of intron C (IVSC) from his mother. The substitution of a positively charged E203K mutation created a unique isoform-banding pattern. An asymptomatic sister's GALT genes carries three mutations (E203K-N314D/N314D) with eight distinct isoform bands. Surprisingly, her erythrocytes have normal GALT activity. We conclude that the synergism of pedigree, biochemical, SSCP, and direct GALT gene analyses is an efficient protocol for identifying new mutations and speculate that E203K and N314D codon changes produce intraallelic complementation when in *cis*.

Received August 25, 1994; accepted for publication December 20, 1994.

Address for correspondence and reprints: Dr. Louis J. Elsas, Division of Medical Genetics, Emory University School of Medicine, 2040 Ridgewood Drive N.E., Atlanta, GA 30322.

0002-9297/95/5603-0011$02.00

Introduction

Galactose-1-phosphate uridyl transferase (GALT [E.C.2.7.7.12]) catalyzes the production of glucose-1-phosphate and uridyldiphosphate (UDP)-galactose from galactose-1-phosphate and UDP-glucose (Leloir 1957). This reaction is important in the conversion of galactose to energy in most organisms, including man, where impairment leads to the disease galactosemia (Segal 1989). In humans, GALT has been difficult to isolate and characterize but is thought to function as a dimer composed of subunits of 43 kD (Frey et al. 1982; Anderson et al. 1983). It has distinctive isoforms when analyzed by electrophoresis, and several variants have been defined (Ng et al. 1973; Kuhnl et al. 1974; Kelley et al. 1983; Shin et al. 1987; Sparkes et al. 1987; Kelley and Segal 1989). In classical galactosemia (G/G), the homozygote has no activity and the heterozygote (G/N) ~50% of GALT activity with distinct isoform bands by using isoelectric focusing (Elsas et al. 1994). In another biochemical phenotype, Duarte galactosemia, the homozygote (D/D) has ~50% of normal activity in red blood cells, and the heterozygote (D/N) has GALT activity that is ~75% of normal, with isoforms expressing stronger activity bands, which move toward the anode and lower pH (Beutler et al. 1965, 1966; Elsas et al. 1994).

The GALT gene was cloned and sequenced originally from *Escherichia coli* (Lemaire and Mueller-Hill 1986) and yeast (Tajima et al. 1985). By using islands of amino acid sequence identity among these species, a human GALT cDNA was cloned (Reichardt and Berg 1988) and correctly sequenced (Flach et al. 1990). More recently, the human GALT gene was cloned and fully sequenced (Leslie et al. 1992). With these normal sequences in hand, several groups began to use direct sequencing of PCR-amplified cDNA or genomic DNA to identify candidate mutations in the GALT genes of galactosemic patients. For example, the Q188R mutation, which substitutes an arginine for glutamine at codon 188 in exon 6, has a prevalence of 70% in Caucasians with galactosemia (Leslie et al. 1992; Elsas et al. 1993, and in press). The Q188R allele is associated with essentially no activity in human erythrocytes or lymphoblasts or in yeast engineered to express the human alleles (Fridovich-Keil and Jinks-Robertson 1993).

Another common mutant allele of the GALT genome is an A-to-G transition in bp 2744 of exon 10, which results in the substitution of an acidic aspartate (D), for an asparagine (N) at codon 314 (N314D) (Reichardt et al. 1992*a*). The N314D variation had extraordinary concordance with the biochemical phenotype of the Duarte allele (Leslie et al. 1992; Elsas et al. 1994). The N314D mutation is frequent and has an estimated population prevalence of 5.9%, which conforms with previous estimates of the population frequency of the Duarte allele's biochemical phenotype (Mellman et al. 1968; Beutler 1973; Fox 1987).

In the present study, we define an efficient strategy to identify and explore additional GALT biochemical phenotypes and candidate mutations for impaired enzyme activity in the GALT gene, in a retrospective study of known patients with classical and variant galactosemia. Probands were obtained from newborn-screening programs or from physicians caring for them. GALT activity and isoforms were analyzed in erythrocytes from the probands and their parents. If erythrocyte GALT activity was impaired in a Mendelian pattern in this nuclear pedigree, we screened the GALT gene for the common Q188R and N314D mutations by using a new multiplex PCR method. Using pedigree analysis together with biochemical and mutational screening, we identified 86 non-Q188R, enzyme-impairing, mutant alleles. We then amplified all 11 exons of the GALT gene with intron-specific, flanking oligonucleotide primers. We found 75 abnormal and 11 uninformative SSCPs. Of the 75 abnormal SSCPs, 41 have been sequenced, and altered nucleotide sequences were present. Twelve new mutations were found, while the rest had been previously described. One novel mutation replaced a negatively charged aspartate (E) in exon 7 at codon 203 with a positively charged lysine (K) (E203K). In heterozygotes it impaired GALT activity by 50%. This E203K mutation produced unique isoform-banding patterns on isoelectric focusing. One patient had normal GALT activity, had eight isoform bands, and carried the E203K/N314D mutation in *cis* with a third, N314D, codon change on her *trans* GALT allele. This observation supported the hypothesis that these oppositely charged codon changes may complement one another under certain phase conditions.

Patients, Material, and Methods

Patients

About ½ of the probands in this study were detected through Georgia's newborn-screening program, which assays uridyldiphosphoglucose consumption on samples of dried blood eluted from filter paper (Mellman and Tedesco 1965; Beutler et al. 1966).The other ½ were referred from physicians contacted directly by phone or mail.

Enzyme and molecular analyses were performed on 1–5 cc of heparinized blood. Genomic DNA was extracted from the buffy-coat layer of this sample. Patients and families gave informed consent to participate, and the research project was approved by the institutional review board at Emory University.

Enzyme and Biochemical Reagents

Restriction endonucleases *Ava*II, its isoschizomer, *Sin*I, and *Hpa*II were purchased from Promega. ^{14}C-galactose-1-phosphate was purchased from Dupont/New England Nuclear. Oligonucleotide primers were synthesized on an Applied Biosystems oligonucleotide synthesizer at the Emory microchemical facility. α[^{32}P]dCTP and gamma ^{32}P-ATP were from Amersham. Hydrolink MDE gel was purchased from J. T. Baker. Other restriction enzymes and materials for molecular and biochemical analyses were from Boehringer Mannheim.

Red Cell GALT Assay

Red cell GALT activity was measured by assaying the conversion of ^{14}C-galactose-1-phosphate and UDP-glucose to glucose-1-phosphate and ^{14}C-UDP-galactose at 37°C, as described elsewhere (Henry 1964; Mellman and Tedesco 1965; Ng et al. 1967; Lee and Ng 1982; Elsas et al. 1994).

GALT Isozyme Assay

GALT was semipurified from red cell lysates on a DEAE Sephacel column, was concentrated, was electrophoresed on a pH 5–6 isoelectric focusing gel, and was stained for enzyme activity as described elsewhere (Sparkes et al. 1987; Elsas et al. 1994).

Galactose-1-Phosphate

Galactose-1-phosphate was measured in deproteinized hemolysates of patients' erythrocytes by using alkaline phosphatase conversion to D-galactose and NADH production through galactose dehydrogenase (Bergmeyer 1974).

Multiplex, PCR-Based, Simultaneous Detection of Q188R and N314D

Genomic DNA was isolated from the buffy coat of 1–5 cc of heparinized blood with a QIAamp blood kit (Qiagen) as per the manufacturer's instructions. Two sets of PCR primers were designed that flanked exons 6 and 10: 1481-F 5′-GGGTCGACGTCGGATGTAACGCTGCCACTCA-3′; Int7-R 5′-GGGGACACAGGGCTTGGCTCTCTCCCA-3′; IntI-F2 5′-GGGTCGACGAGATGCTGGGACTGAGGGTGGAGCA-3′; IntJ-R 5′-GGGGTCGACGCCTGCACATACTGCATGTGA-3′. The resultant amplified DNA included constitutive *Hpa*II and *Sin*I sites to serve as positive controls for restriction endonuclease digestion.

PCR was carried out in a final volume of 50 μl, with

5 pmol of each primer, 100 ng genomic DNA, 200 μM dNTPs, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ and 1.25 U Amplitaq (Perkin Elmer), was overlayed with mineral oil, and was immediately placed at 95°C. The following cycling conditions were used (MJ programmable thermal controller PTC-100): cycle 1: 95° for 5 min, 65° for 1 min, and 72° for 1 min; cycles 2–34: 95° for 45 s, 65° for 1 min, and 72° for 1 min; cycle 35: 95° for 45 min 65° for 1 min, and 72° for 8 min.

PCR products were extracted once with chloroform and then ethanol-precipitated with 1/10 volume 3-M sodium acetate pH 5.2, were washed with 70% ethanol, were dried, and then were resuspended in 10 μl ddH_2O. The PCR products were then double-digested with restriction endonucleases *Hpa*II and *Sin*I, as recommended by the manufacturer (Promega), and were analyzed by electrophoresis through a 3% agarose gel.

Uncut amplification products were 637 bp and 430 bp in length, corresponding to the Q188R-containing and N314D-containing regions, respectively. Following restriction endonuclease digestion, these fragments were cut into a collection of smaller fragments—some constitutive and others diagnostic for either the presence or absence of each of the two mutations, Q188R and N314D.

Primer pairs were designed to flank 200–400-bp fragments of the GALT gene for each of the 11 exons and their intronic junctions as indicated in table 1. A total of 7.5 pmol of each of the above primer sets, 100 ng genomic DNA, 62 μmol of each dNTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 μl α-^{32}P-dCTP (3,000 Ci/mmol), 1 U *Taq* polymerase, and distilled water to 10 μl were overlayed with mineral oil and kept on ice prior to "hot start" PCR. The following cycling conditions were used: cycle 1: 95°C for 5 min, 60°C for 30 s, and 72°C for 30 s; cycles 2–29: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; cycle 30: 95°C for 30 s, 60°C for 30 s, and 72°C for 10 min.

PCR products were denatured in 95% formamide, 10 mM NaOH, were heated to 94°C for 2 min, and were placed on ice. The PCR products of all 11 exons were resolved by MDE gel electrophoresis at 6 W for 16 h. Gels were then transferred to Whatman 3MM paper, were dried, and were analyzed by autoradiography, after exposure to Kodak XAR film at −70°C for 3–4 h.

Sequencing of GALT Exons

DNA was amplified for sequencing with the same primer sets used for SSCP analysis (see above). Amplified double-stranded DNA was purified by low-melting-temperature agarose gel electrophoresis and was sequenced directly with one of the two end primers or an internal primer end-labeled with gamma-[^{32}P]ATP (Longo et al. 1992, 1993).

Table 1

SSCP Analysis of GALT Exons

Exon(s) Amplified and Primer	Sequence 5′-3′
1 and 2:	
F29	AGCGGATCCCCCGGTGGCCTC
R2-3′	CCGTGGCCCAGCCTGCAGATG
2:	
F2-5′	AGCTCTGAGGACTGATCTTGA
R2-3′	CCGTGGCCCAGCCTGCAGATG
2 and 3:	
F2-5′	AGCTCTGAGGACTGATCTTGA
R3-3′	AGCAGCAGTTGGAGCCAGGTT
3 and 4:	
FHGSP1	GTACGATAGCACCTTCCTGTT
R4-3′	GCTGAGTCTCCAACTCTGGTT
5:	
F5-5′	TTGGGGTTCGCCCTGCCCGTA
R5-3′	CAAAGCTTCATCACCCCCTCC
6:	
F6-5′	AGGAGGGAGTTGACTTGGTGT
R6-3′	CTGTTCCCATGTCCACAGTGC
7:	
F7-5′	TGGGACAGAGGAAATATGCCA
R7-3′	CCTTTACACACCTCTCTCATG
8:	
F8-5′	GGCTCCTATGTCACCTTGATG
R8-3′	CAACCTCCATCCAGTGCCTAG
9:	
F9-5′	GGTCAGCATCTGGACCCCAGG
R9-3′	AGGTTGCAGTTCACTAGGCTG
10:	
F10-5′	AGGTGCTAACCTGGATAACTG
R10-3′	CACATACTGCATGTGAGAGTC
11:	
F11-5′	TGGGCAACAGAGCAAGACCTC
R11-3′	CAGGCCAGGATTCAAGGCCCT

Results

Detection of New Mutations

We have developed an efficient approach to pedigree analysis, biochemical phenotyping, and molecular genotyping to identify and clarify the function of infrequent and new mutations in the GALT gene. Biochemical analyses of red blood cell GALT from probands and parents included assessment of enzymatic activity, phenotyping by isoelectric focusing, and measurement of RBC gal-1-P concentrations.

We developed a simple, robust, internally controlled method for rapid simultaneous detection of the known, common Q188R and N314D mutations in the human GALT gene (fig. 1). The method used multiplex, PCR-based amplification, restriction endonuclease digestion, and gel separation of fragments. The method was based on the previous observations that each of these two mutations

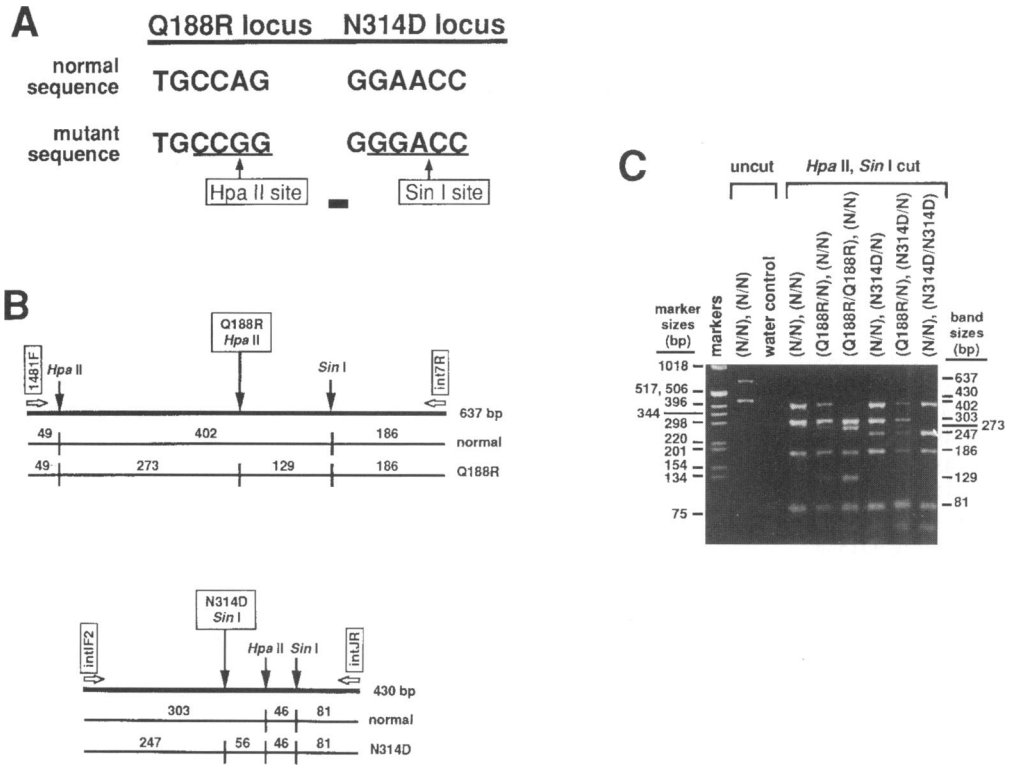

Figure 1 PCR amplification and restriction endonuclease double-digestion of genomic fragments containing the Q188R and N314D regions of human GALT. Nucleotide changes and consequent restriction sites gained are shown in panel A. In panel B, primers and restriction sites are indicated by the unblackened and blackened arrows, respectively. See Patients, Material, and Methods for primer sequences. Panel C shows analysis of DNA from individuals representing one normal control as well as five subjects carrying a variety of Q188R and N314D mutation combinations (as indicated). In each case, "N" refers to an allele that does not carry the mutation in question. Marker sizes are indicated to the left of the gel; band sizes representing both uncut and double-digested PCR products are indicated to the right.

created a different restriction site in a different exon; Q188R created a new *Hpa*II site in exon 6 and the N314D mutation, a new *Sin*I site in exon 10 (Leslie et al. 1992; Elsas et al. 1993, 1994) (fig. 1*A*). The primer sets and anticipated fragment sizes are shown in figure 1*B*. The nucleotide sequence for each primer is defined in Patients, Material, and Methods. As is shown in figure 1*C*, homozygotes for Q188R or N314D, and heterozygotes and compound heterozygotes for both mutations were easily distinguished using this method.

From Georgia's newborn-screening program and from 30 different physician referrals, we had identified 132 Q188R alleles in 107 (214 G alleles) patients with classic galactosemia. From this source we had 86 molecularly unclassified G alleles with DNA available for further analysis. Seventy-five of these 86 samples gave positive (or informative) migration patterns, while 11 were uninformative following SSCP analysis. From this pool of 75 unclassified G alleles with positive SSCP analyses, we have sequenced 41 GALT genomes, and all had nucleotide sequence changes. Sequence changes included 4 deletions, 2 intervening sequence (IVS) splice-acceptor site alterations, 2 "wobble" polymorphisms, and 33 codon changes. Of these codon changes, 21 had been previously reported and 12 had not previously been described (table 1; see also Elsas et al. 1994, and in press). The 12 new mutations include an A-to-C transversion at bp 956 (IVSC); T138M; R148Q; V151A; an A-to-G transversion at bp 1632 (IVSF); S192N; R201H; E203K; E291K; E308K; A320T; and Y323D. These are defined in table 2, which also summarizes a total of 32 highly probable and proved mutations in the GALT gene of classic galactosemic patients.

Identification of Phase and New Mutations in Families

The proband in one particularly interesting family is III-1 in figure 2 and table 3. He was detected in Georgia's population-based newborn screening at age 2 d and was diagnosed and placed on a lactose-restricted diet at age 10

Table 2

Sequence Changes in the Human GALT Gene

Amino Acid Change	Exon	Nucleotide Change	Conserved?	Reference
Val 44 Met (V44M)[a]	2	GTG→ATG	Yes	Reichardt et al. 1991*b*
Leu 62 Met (L62M)	2	CTG→ATG	No	Reichardt et al. 1991*b*
Leu 74 Pro (L74P)	2	CTG→CCG	Yes	Reichardt et al. 1992*b*
Ala 81 Thr (A81T)	2	GCC→ACC	No	Reichardt et al. 1992*a*
Del 38 (frameshift)[b]	3	bp 281→318	Yes	Ashino et al. 1993
IVSC	4	bp 956 A→C	Yes	Present study
Ser 135 Leu (S135L)	5	TCG→TTG	No	Reichardt et al. 1991*b*
Thr 138 Met (T138M)	5	ACG→ATG	Yes	Present study
Leu 139 Pro (L139P)	5	CTG→CCG	No	Reichardt et al. 1992*a*
Met 142 Lys (M142K)[a]	5	ATG→AAG	No	Reichardt et al. 1991*b*
Arg 148 Trp (R148W)[a]	5	CGG→TGG	No	Reichardt et al. 1992*a*
Arg 148 Gln (R148Q)	5	CGG→CAG	No	Present study
Val 151 Ala (V151A)	5	GTT→GCT	Yes	Present study
Phe 171 Ser (F171S)	6	TTT→TCT	Yes	Reichardt et al. 1992*b*
Gln 188 Arg (Q188R)[a,b]	6	CAG→CGG	Yes	Reichardt et al. 1991*a*
IVSF	7	bp 1632 A→G	Yes	Present study
Ser 192 Asn (S192N)	7	AGC→AAC	No	Present study
Leu 195 Pro (L195P)[a]	7	CTG→CCG	Yes	Leslie et al. 1992
Arg 201 His (R201H)	7	CGT→CAT	No	Present study
Glu 203 Lys (E203K)	7	GAG→AAG	No	Present study
Arg 231 His (R231H)[a]	8	CGT→CAT	Yes	Ashino et al. 1993
Lys 285 Asn (K285N)	9	AAG→AAT	No	Reichardt et al. 1992*a*
Glu 291 Lys (E291K)	9	GAG→AAG	No	Present study
Glu 308 Lys (E308K)	10	GAG→AAG	No	Present study
Asn 314 Asp (N314D)[c]	10	AAC→GAC	No	Reichardt et al. 1992*a*
His 319 Gln (H319Q)[a]	10	CAC→CAA	Yes	Reichardt et al. 1993
Ala 320 Thr (A320T)	10	GCT→ACT	No	Present study
Tyr 323 Asp (Y323D)	10	TAC→GAC	Yes	Present study
Arg 333 Trp (R333W)[a]	10	CGG→TGG	Yes	Reichardt et al. 1991
Arg 333 Gly (R333G)	10	CGG→GGG	Yes	Reichardt et al. 1992
Lys 334 Arg (K334R)	10	AAA→AGA	Yes	Reichardt et al. 1992
Gln 370 Stop (Q370X)	11	CAG→TAG	No	Reichardt 1993

[a] Reduced GALT activity in transfection experiments.
[b] Prevalent in G/G Caucasian population (Leslie et al. 1992; Elsas et al. 1993 and in press).
[c] Prevalent in Duarte galactosemia (Leslie et al. 1992; Elsas et al. 1994).

d. His GALT activity was 0.6 μmol/g Hb/h (normal range = 17–45), and his RBC gal-1-P at age 9 d was 46.4 mg/100 ml (normal < 1). Over a 2-mo period on lactose-free formula, his RBC gal-1-P fell to below 1 mg/100 ml. He is clinically normal at age 12 mo, and his RBC gal-1-P levels remain normal.

The biochemical phenotypes of GALT were unusual in this family (figs. 2 and 3 and table 3). The proband's mother (II-2) had impaired GALT activity of 5.7 μmol/g Hb/h, but his father (II-1) had almost normal activity at 16.9 μmol/g Hb/h, with an unusual pattern on isoelectric focusing (fig. 3). Red blood cells from the father (II-1), the proband (III-1), and the proband's asymptomatic sister (III-2), who had normal GALT activity, each displayed two unique GALT isoform bands migrating toward the cathode and higher pH. These novel bands are labeled as −1 and −2 in figure 3. In addition, the mother (II-2) and the asymptomatic sister (III-2) but neither the father (II-1) nor the proband (III-1) had biochemical evidence of the D-allele, which is usually the presence of bands 5 and 6, (fig. 3) (Elsas et al. 1994).

Multiplex PCR analysis of the proband's and parents' GALT DNA revealed the presence of the N314D mutation in all three. However, there was no Q188R mutation, indicating that the proband and mother had non-Q188R G alleles. The father's unusual GALT isoforms made his mutation of further interest, despite minimal impairment of erythrocyte GALT activity. The 11 exons of all three pa-

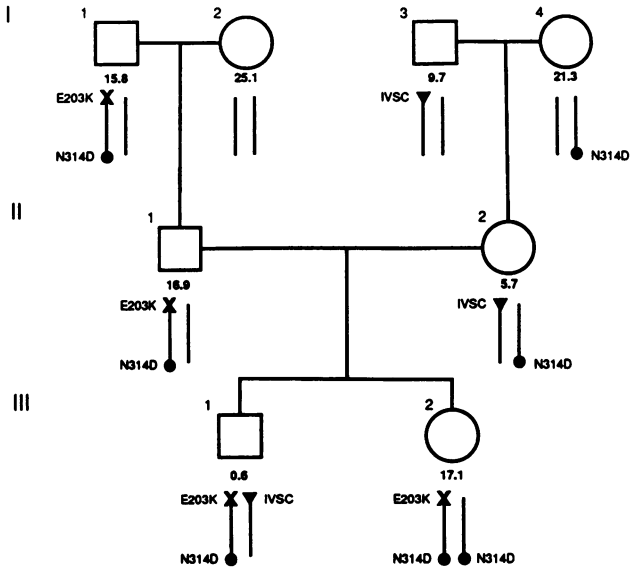

Figure 2 Summary of pedigree analysis, biochemical phenotypes and molecular genotypes in pedigree Bro. Phase of mutations is deduced from segregation of N314D and E203K mutations in informative meioses.

tients' GALT genes were then examined by SSCP analysis (fig. 4). The proband inherited a variation in nucleotide sequences around exon 4 from his mother (II-2) and within exon 7 from his father (II-1). All three demonstrated the SSCP variation in exon 10, which was known to be the N314D mutation (fig. 4). Therefore, the hypothesis that the proband had inherited three mutations, the N314D codon change and as yet unidentified changes in exons 4 and 7, was tested. Since both parents carried the N314D mutation and one unclassified G-allele, the proband's N314D mutation had to be transmitted in *cis* with one of these other two mutations.

By applying the same methods to his four grandparents (I-1, I-2, I-3, and I-4) and sister (III-2), both the phase of the N314D mutation and the putative effect of the exon 7 mutation on the N314D biochemical phenotype were resolved (fig. 2 and table 3). By direct sequencing, the mutation around exon 4 was determined to be an A-to-C transversion at bp 956, impacting the splice-acceptor site for intron C at the 5′ end of exon 4 (IVSC). This mutation was inherited from the heterozygous maternal grandfather (I-3), who had reduced GALT enzyme activity in a manner consistent with a G/N biochemical phenotype (fig. 2 and table 3). The presence of this IVSC mutation in *trans* with the N314D mutation in the mother (II-2) resulted in further loss of GALT activity and conformed to a D/G biochemical phenotype (figs. 2 and 3 and table 3). Of interest were the paternal grandparents, where the exon 7 SSCP was found in *cis* with the N314D mutation in the grandfather (I-1) with transmission to the father (II-1), proband (III-1), and sister (III-2). The paternal grandmother (I-2) was homozygous normal at the GALT locus and served as an intrafamilial control (figs. 2 and 3 and table 3).

The exon 7 mutation resulted from a G-to-A transition at bp 1675 of the GALT gene, resulting in the substitution of a lysine for an acidic glutamate at codon 203 (E203K). The effect of this mutation on GALT isoforms was striking (figs. 2 and 3 and table 3). When present in *cis* with a single N314D mutation alone, it acted to prevent the formation of the isoelectric focusing bands migrating toward the acidic pH that are characteristic of the Duarte allele (fig. 3, subjects II-1 and III-1). However, when two N314D mutations

Table 3

Biochemical Phenotypes and Molecular Genotypes in Pedigree Bro

	Biochemical Phenotype			Molecular Genotype	
Patient	RBC Gal-1-P[a]	GALT[b]	Isozyme[c]	Allele 1	Allele 2
III-1 (Proband)	46.4	.6	New (G/G)	E203K-N314D	IVSC
III-2	...	17.1	D/D	E203K-N314D	N314D
II-1	...	16.9	New (G/N)	E203K-N314D	Normal
II-2	.0	5.7	D/G	N314D	IVSC
I-1	...	15.8	New (G/N)	E203K-N314D	Normal
I-2	...	25.1	N/N	Normal	Normal
I-3	...	9.7	G/N	Normal	IVSC
I-4	...	21.3	D/N	N314D	Normal
Normal	<1	17–45	N/N	Normal	Normal

[a] Concentration of erythrocyte galactose-1-phosphate in mg/deciliter.
[b] Activity of erythrocyte galactose-1-P-uridyltransferase in μmol/g Hb/h.
[c] Biochemical phenotype based on pedigree, enzyme, and isoelectric focusing analyses.

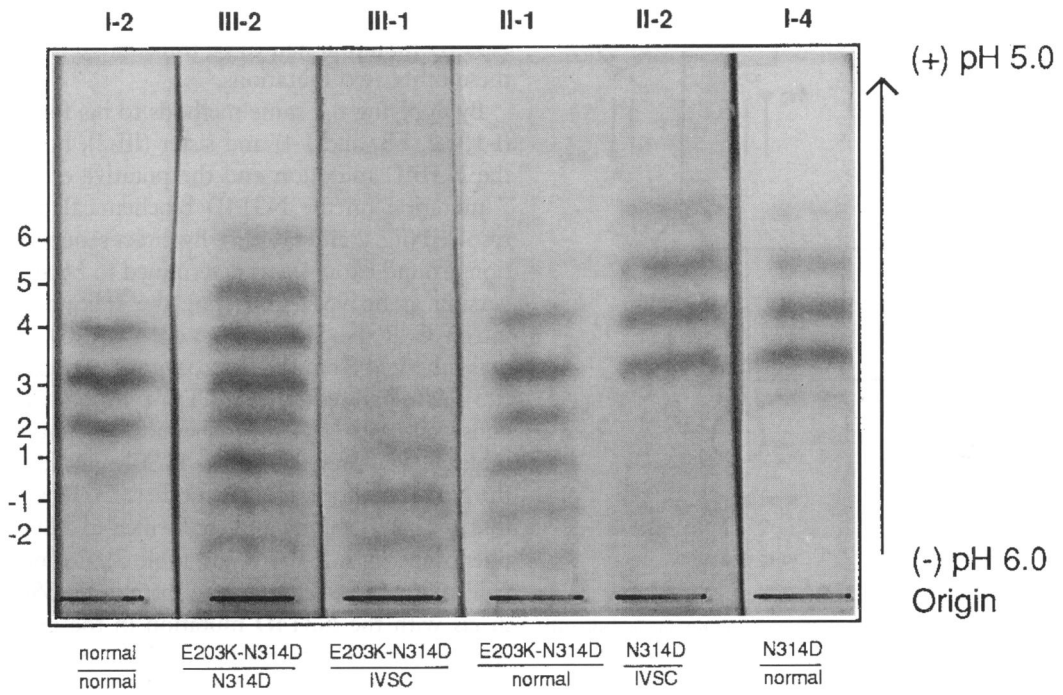

Figure 3 Isoelectric focusing gels of erythrocyte GALT from members of pedigree Bro. Members of the pedigree are indicated by Roman numerals for generation and Arabic numbers for position, as depicted in fig. 2. Origins of the gels are aligned with band number indicated to the left and the individuals' molecular genotypes below each lane.

were present, producing a homozygous state, as in subject III-2, the E203K mutation and the pair of N314D mutations produced a series of eight GALT isoform bands and unexpectedly, GALT activity was normal (17.1 μmol/g Hb/h) (fig. 3, subject III-2).

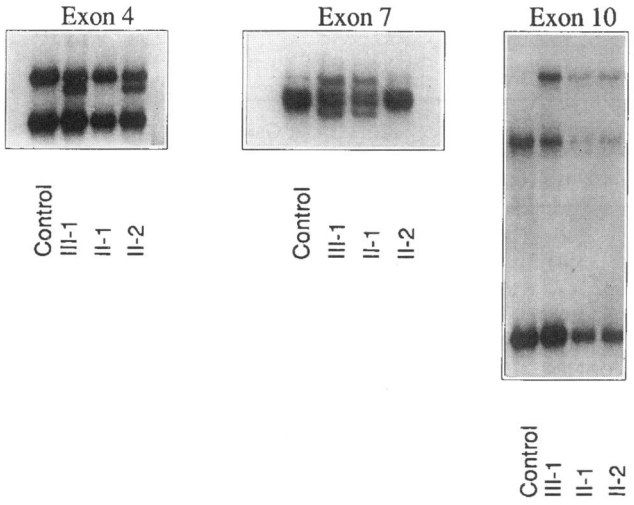

Figure 4 Autoradiographs of amplified exonic regions of interest and the SSCP patterns observed in members of pedigree Bro.

In a second unrelated family (pedigree Nay), a newborn proband was found to have GALT activity in erythrocytes of 5.4 μmol/g Hb/h and the eight isoform bands. She was a compound heterozygote for the E203K and N314D mutations in *trans*. Her father had an erythrocyte GALT activity of 14.1 and was heterozygous for E203K and a normal allele, while her mother had an erythrocyte GALT of 13.0 and was homozygous for the N314D, Duarte mutation. Thus the E203K allele when present in *trans* with the N314D mutation did not improve GALT function.

Discussion

The sequence of clinical, biochemical, and molecular techniques described herein was effective in identifying new mutations in the human GALT gene. There are several reasons why these approaches were effective when applied to galactosemia and the GALT gene. First, Georgia's newborn population is surveyed for galactosemia, with 0%–25% of control erythrocyte GALT activity used as the threshold for testing positive (Elsas et al., in press). In this population of >1.5 million screened newborns, ~1/40,000 had classic G/G galactosemia, and 1/11,500 had "variant" reduction in activity of GALT. Many of these patients were studied by the methods described. Second, simple enzy-

matic and electrophoretic analyses were used routinely to confirm screening and to phenotype biochemical variants. When these biochemical parameters were used as genetic discriminants in a pedigree analysis, the pattern of transmission of single genes producing impaired GALT activity became evident. Third, because the Q188R and N314D mutations account for the vast majority of G and D alleles in the U.S. population, their absence or presence, when defined by our multiplex PCR and restriction enzyme analyses, indicated the presence or absence of a rare or new mutation. Of course, the presence of one known mutation (e.g., N314D), even in the homozygous condition, does not rule out the presence of additional codon changes, as is illustrated by the family presented here (figs. 2 and 3 and table 3). In this family, a family with a G/G phenotype and the unique isoform-banding pattern seen in a sibling and parent led to the discovery of the E203K mutation. Fourth, SSCP screening and direct sequencing of abnormal exons were efficient in identifying the candidate mutation in question. When multiple biochemical phenotypes and molecular genotypes were present in the same pedigree, these combined genetic, biochemical, and molecular approaches could define phase of mutant alleles and the presence of one or more intragenic mutations in the same individual, as well as provide clues to their individual or combined effects on GALT enzyme activity (fig. 2 and table 3).

Screening the GALT gene for alterations in nucleotide sequence was quite efficient when using SSCP. Of the 86 rare or new "G alleles" studied, 75 were positive by SSCP screening. The GALT gene has only 11 exons enabling simultaneous screening of all of its exons. These exons are between 49 and 206 bp in length, which enabled robust amplification of the splice-donor and -acceptor sites as well as the coding regions, with simultaneous screening of several individuals per gel. Additionally, the same primer sets were effective for both SSCP screening and direct sequencing. Since the coding sequences are highly conserved throughout evolution (Leslie et al. 1992), polymorphisms are unusual, so that all of the altered SSCP patterns in our study had altered nucleotide sequences detected by direct DNA sequencing. Of these 41 changes identified by direct sequencing, 39 correlated with functional impairment of erythrocyte GALT enzyme. For example, in the Bro family, the nucleotide variations detected in exons 4 and 7 by SSCP and determined to impact the IVSC splice-acceptor site and produce the E203K codon change, respectively, are likely to be mutations. Impaired erythrocyte GALT activity was present at ~50% of control in the maternal grandfather (I-3) when the only codon change identified in his GALT gene was one IVSC-containing allele. When present in the proband's mother (II-2), the addition in *trans* of an N314D mutation with the IVSC allele further reduced GALT activity to ~20% of control. Further studies of transformed lymphoblasts from patients I-3 and II-2 will be needed to determine whether the mutation at this splice-acceptor site resulted in reduced mRNA production, frameshift with nonsense codons, or other altered splicing events leading to nonfunctional GALT.

By comparing two families with the new E203K codon change in *cis* and *trans,* we could judge its biochemical effect. The substitution of a lysine for glutamate in exon 7 produced a protein with visualized abnormal basic isoforms of GALT (fig. 3). Unusual bands migrating toward the higher pH indicated the presence of the abnormal GALT protein in the Bro proband (III-1), his father (II-1), and his sister (III-2). The effect of this E203K missense mutation could not be evaluated on GALT enzyme activity alone in the Bro family, because it was always transmitted together in *cis* with N314D. However, the father in the second, unrelated, pedigree Nay was heterozygous for the E203K variation and a normal allele and had partially impaired GALT at ~60% of control. His daughter was a compound heterozygote in *trans* for N314D and E203K and had GALT activity consistent with a D/G variant with 20% of control GALT. Therefore, the E203K codon change did impair erythrocyte GALT activity in the heterozygous genotype and did not return activity when present in *trans* with the N314D allele.

An intriguing observation made in the Bro family is the potentially complementary effect on GALT enzyme activity of the E203K mutation when present in *cis* or on an N314D homozygous background (individual III-2). There is continuing controversy over the structure of the active GALT enzyme. However, it is generally accepted that human erythrocyte and placental GALT is active as a dimer of molecular weight 88 kD, with each monomer ~43 kD (Tedesco et al. 1972; Anderson et al. 1983, 1984). We have determined that the N314D mutation, when present in the homozygous state, is associated with reduced erythrocyte GALT activity of ~43% of normal (Elsas et al. 1994). The mechanism for this reduction of activity remains unknown. One possibility is that the N314D substitution alters biological synthesis or degradation of the functional GALT dimer rather than creating a simple conformational change altering the catalytic properties of the mature protein. Evidence for this assumption is derived from Anderson et al. (1983), who observed ~50% reduction in immunoassayable GALT protein in a patient homozygous for the Duarte allele. It is possible that a destabilizing effect on protein turnover is caused by substitution of a negatively charged aspartic acid near the carboxyterminus of the GALT protein. By this reasoning, the addition of a positively charged lysine may stabilize the N314D-containing dimeric protein. Evidence for this hypothesis is given by the biochemical phenotype of III-2 in the Bro pedigree, who expresses large amounts of functioning isoforms characteristic of both the E203K and N314D proteins, suggesting that both proteins are present. She is homozygous

for N314D and heterozygous for E203K, and yet has normal erythrocyte GALT activity (figs. 2 and 3 and table 3).

Acknowledgments

We thank the following clinical scientists for submitting patient materials: R. Bachman, G. Berry, F. Bowyer, N. Buist, S. Cederbaum, J. Charrow, A. Drack, P. Ferreira, J. Gibson, R. Gitzelmann, C. Green, C. Greenburg, D. Jelley, A. Laszlo, C. Leonard, N. Leslie, C. Lozzio, E. Naughton, H. Northrup, M. Palmieri, S. Rarback, C. Riddell, L. Rutledge, R. Scott, C. Scriver, S. Segal, R. Steiner, M. Tuchman, L. Tyfield, and Y. Yasmin. We are grateful to Mr. B. Moore for his technical help in early stages of PCR and to Ms. Marcia Sternberg for her secretarial skills. This research was supported in part by Public Health Service grant M01-RR00039 from the General Clinical Research Centers Program, NIH, National Center for Research Resources, to the clinical research center of Emory University and by Health and Human Services grants from NICHD: PO-1 HD29847-01 (to L.J.E.) and NIDDK-1R29DK46403-02 (to J.L.F.-K.). Funds were also provided by the Emory University Research Committee and the Children's Research Center of the Henrietta Egleston Hospital and the Department of Pediatrics, Emory University School of Medicine.

References

Anderson MW, Williams VP, Helmer GR, Fried C, Popjak G (1983) Transferase-deficiency galactosemia: evidence for the lack of a transferase protein in galactosemic red cells. Arch Biochem Biophys 222:326–331

Anderson MW, Williams VP, Sparkes MC (1984) Transferase-deficiency galactosemia: immunochemical studies of the Duarte and Los Angeles variant. Hum Genet 65:287–291

Ashino J, Okano Y, Isshiki G, Lin H-C, Reichardt JKV (1993) Molecular characterization of two galactosemia mutations (type 1) in Orientals. Am J Hum Genet Suppl 53:887

Bergmeyer H (ed) (1974) Methods of enzymatic analysis. Academic Press, Chicago, pp 1291–1295

Beutler E (1973) Screening for galactosemia: studies of the gene frequencies for galactosemia and the Duarte allele. Isr J Med Sci 9:1323–1329

Beutler E, Baluda ML, Sturgeon P, Day RW (1965) A new genetic abnormality resulting in galactose-1-phosphate uridyltransferase deficiency. J Lab Clin Med 64:646–658

——— (1966) The genetics of galactose-1-phosphate uridyl transferase deficiency. J Lab Clin Med 68:646–658

Elsas LJ, Dembure PP, Langley S, Paulk EM, Hjelm LN Fridovich-Keil J (1994) A common mutation associated with the Duarte galactosemia allele. Am J Hum Genet 54:1030–1036

Elsas LJ, Fridovich-Keil JL, Leslie N (1993) Galactosemia: a molecular approach to the enigma. Int Pediatr 8:101–108

Elsas LJ, Langley SL, Paulk EM, Hjelm N and Dembure PP. A molecular approach to galactosemia. Eur J Pediatr (in press)

Flach JE, Reichardt JKV, Elsas LJ (1990) Sequence of a cDNA encoding human galactose-1-phosphate uridyl transferase. Mol Biol Med 7:365–369

Fox JG (1987) Experiences of the Manitoba perinatal screening program, 1965–1985. Can Med Assoc J 137:883–888

Frey PA, Wong LJ, Shey KF, Yang SL (1982) Galactose-1-phosphate uridyltransferase: detection, isolation and characterization of the uridyl enzyme. Methods Enzymol 87:20–36

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

Henry RJ (1964) Clinical chemistry. Harper & Row, Hoeber Medical Division, New York

Kelley RI, Harris H, Mellman WJ (1983) Characterization of normal and abnormal variants of galactose-1-phosphate uridyl transferase (EC 2.7.7.12). Hum Genet 63:274–279

Kelley RI, Segal S (1989) Evaluation of reduced activity galactose-1-phosphate uridyl transferase by combined radioisotopic assay and high-resolution isoelectric focusing. J Lab Clin Med 114:152–156

Kuhnl P, Nowicki L, Spielmann W (1974) Investigations on the polymorphism of galactose-1-phosphate uridyltransferase by means of agarose gel electrophoresis. Humangenetik 24:227–230

Lee JES, Ng WG (1982) Semi-micro techniques for the genotyping of galactokinase and galactose-1-phosphate uridyl transferase. Clin Chim Acta 124:351–356

Leloir LF (1957) Enzymatic transformation of uridine diphosphate glucose into galactose derivative. Arch Biochem Biophys 33:186– 194

Lemaire HG, Mueller-Hill B (1986) Nucleotide sequences of the GAL E gene and the GALT gene of E. coli. Nucleic Acids Res 14:7705–7711

Leslie ND, Immerman EB, Flach JE, Florez M, Fridovich-Keil JL, Elsas LJ (1992) The human galactose-1-phosphate uridyl transferase gene. Genomics 14:474–480

Longo N, Langley S, Griffin L, Elsas L (1993) Activation of glucose transport by a natural mutation in the human insulin receptor. Proc Natl Acad Sci USA 90:60–64

Longo N, Shuster R, Griffin L, Langley S, Elsas L (1992) Activation of insulin receptor signaling by a single amino acid substitution in the transmembrane domain. J Biol Chem 267:12416–12419

Mellman WJ, Tedesco TA (1965) An improved assay of erythrocyte and leukocyte galactose-1-phosphate uridyl transferase: stabilization of the enzyme by a thiol protective reagent. J Lab Clin Med 66:980–986

Mellman WJ, Tedesco TA, Feigl P (1968) Estimation of the gene frequency of the Duarte variant of galactose-1-phosphate uridyl transferase. Ann Hum Genet 32:1–8

Ng WG, Bergren WR, Donnell GN (1967) An improved procedure for the assay of hemolysate galactose-1-phosphate uridyl transferase activity by the use of ^{14}C-labeled galactose-1-phosphate uridyl transferase. Clin Chim Acta 15:489–492

——— (1973) A new variant of galactose-1-phosphate uridyltransferase in man: the Los Angeles variant. Ann Hum Genet 37:1–8

Reichardt J (1993) The molecular genetic basis of galactosemia. Int Ped 8:110–113

Reichardt J, Belmont J, Levy H, Woo S (1992*a*) Characterization of two missense mutations in human galactose-1-phosphate

uridyl transferase: different molecular mechanisms for galactosemia. Genomics 12:596–600

Reichardt JKV, Berg P (1988) Cloning and characterization of a cDNA encoding human galactose-1-phosphate uridyl transferase. Mol Biol Med 5:107–122

Reichardt JKV, Levy H, Woo S (1992*b*) Molecular characterization of two galactosemic mutations and one polymorphism: implications for structure-function analysis of human galactose-1-phosphate uridyl transferase. Biochemistry 31:5430–5433

Reichardt J, Novelli G, Dallapiccola B (1993) Molecular characterization of the H319Q galactosemia mutation. Hum Mol Genet 2:325–326

Reichardt JKV, Packman S, Woo SLC (1991*a*) Molecular characterization of two galactosemia mutations: correlation of mutations with highly conserved domains in galactose-1-phosphate uridyl transferase. Am J Hum Genet 49:860–867

Reichardt JKV, Woo SLC (1991b) Molecular basis of galactosemia: mutations and polymorphisms in the gene encoding human galactose-1-phosphate uridyl transferase. Proc Natl Acad Sci USA 88:2633–2637

Segal S (1989) Disorders of galactose metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease, 6th ed. McGraw-Hill, New York, pp 453–480

Shin YS, Niedermeier HP, Endres W, Schaub J, Weidinger S (1987) Agarose gel isoelectric focusing of UDP-Gal pyrophosphorylase and galactose-1-phosphate uridyl transferase: developmental aspect of UDP-galactose pyrophosphorylase. Clin Chim Acta 166:27–35

Sparkes MC, Crist M, Sparkes RS (1987) Improved technique for electrophoresis of human galactose-1-phosphate uridyl transferase (EC 2.7.7.712). Hum Genet 40:93–97

Tajima M, Nogi Y, Fukasawa T (1985) Primary structure of the Saccharomyces cerevisiae GAL 7 gene. Yeast 1:67–77

Tedesco TA (1972) Human galactose-1-phosphate uridyl transferase. J Biol Chem 247:6631–6635