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Molecular Basis for Duarte and Los Angeles Variant Galactosemia

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Summary

Human erythrocytes that are homozygous for the Duarte enzyme variant of galactosemia (D/D) have a characteristic isoform on isoelectric focusing and 50% reduction in galactose-1-phosphate uridyltransferase (GALT) enzyme activity. The Duarte biochemical phenotype has a molecular genotype of N314D/N314D. The characteristic Duarte isoform is also associated with a variant called the "Los Angeles (LA) phenotype," which has *increased* GALT enzyme activity. We evaluated GALT enzyme activity and screened the GALT genes of 145 patients with one or more N314D-containing alleles. We found seven with the LA biochemical phenotype, and all had a 1721C→T transition in exon 7 in *cis* with the N314D missense mutation. The 1721C→T transition is a neutral polymorphism for leucine at amino acid 218 (L218L). In pedigree analyses, this 1721C→T transition segregated with the LA phenotype of *increased* GALT activity in three different biochemical phenotypes (LA/N, LA/G, and LA/D). To determine the mechanism for increased activity of the LA variant, we compared GALT mRNA, protein abundance, and enzyme thermal stability in lymphoblast cell lines of D and LA phenotypes with comparable genotypes. GALT protein abundance was increased in LA compared to D alleles, but mRNA was similar among all genotypes. When LA/D and D/D GALT biochemical phenotypes were compared to N/N GALT phenotypes, both had 50%, as compared to 21%, reduction in GALT activity in the wild type (N/N) after exposure at identical initial enzyme activity to 50°C for 15 min. We conclude that the codon change N314D in *cis* with the base-pair transition 1721C→T produces the LA variant of galactosemia and that this nucleotide change increases GALT activity by increasing GALT protein abundance without increasing transcription or decreasing thermal lability. A favorable codon bias for the mutated codon with consequently increased translation rates is postulated as the mechanism.

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Introduction

Classic, or transferase-deficient, galactosemia results from impaired galactose-1-phosphate uridyltransferase enzyme activity to <2% of control and has an incidence of 1/41,545 in Georgia's Newborn Screening Program. Clinically significant variants with activity between 1% and 5% have an incidence of 1/11,118, for a total incidence of 1/8,771 (Elsas et al. 1995*a*). The biochemical phenotype of the most prevalent variant allele of galactosemia is called "Duarte" (D/D) (Beutler et al. 1965; Beutler and Baluda 1966; Mellman et al. 1968; Beutler 1973; Thomakos et al. 1977; Vaccaro et al. 1984). Red blood cells (RBCs) from patients homozygous for the D/D variant of galactosemia have ~50% of normal GALT enzyme activity, and their GALT enzyme is characterized by a shift in its isozyme-banding pattern toward the anode, on isoelectric focusing (Elsas et al. 1994). The molecular genotype of the D/D variant is N314D/N314D (Leslie et al. 1992; Elsas et al. 1994; Lin et al. 1994). The decreased GALT enzyme activity of D/D homozygotes is caused by increased biological degradation and reduced stability of the D/D GALT protein in human lymphoblasts (authors' unpublished data). This reduced stability is not seen when N314D-containing GALT protein is overexpressed by exogenous promoters in yeast (Fridovich-Keil et al. 1995) or monkey *COS* cells (Reichardt and Woo 1991). The D/D biochemical phenotype has also been associated with *increased* GALT enzyme activity, and this biochemical variant has been called the "Los Angeles (LA) variant" (Ng et al. 1973; Ibarra et al. 1979; Anderson et al. 1984; Greber et al. 1995; Strobl et al. 1995). We evaluated the biochemical phenotypes and molecular genotypes in cells from 145 patients with at least one N314D-GALT allele. Their GALT genomes were screened by SSCP for the presence of other nucleotide changes that might be associated with the LA variant. We found seven N314D alleles associated with the LA variant biochemical phenotype, and all seven had a C→T transition at bp 1721 in exon 7 (amino acid codon 218), producing the neutral polymorphism L218L. No other changes that segregate with the LA phenotype were found by direct sequencing. Herein, we define the effect of this 1721C→T transition in *cis* with N314D on mRNA abundance, protein abundance, thermal stability of the GALT protein, and segregation in pedigrees.

Patients, Material, and Methods

Patients

All patient and control cells were obtained though the Emory Genetics Clinic, except for one family's cells, which were graciously provided by Dr. Seymour Packman, University of California, San Francisco. Patients and families gave informed consent to participate, and the research project was approved by the institutional review board at Emory University.

GALT Biochemical Phenotyping and Mutation Detection

Erythrocytes from fresh peripheral blood were lysed, and their GALT enzymes were partially purified on DEAE-Sephacel columns, concentrated, electrophoresed on isoelectric focusing gels, pH 5.0–6.0, and stained for enzyme activity by previously defined methods (Elsas et al. 1994). GALT enzyme activity was quantitated per gram of hemoglobin from the peripheral blood prior to purification (Elsas et al. 1994). Molecular genotypes of GALT were ascertained from peripheral cells or genomic DNA of cultured lymphoblasts by SSCP and direct sequencing of PCR-amplified DNA on an ABI Prism 310 Genetic Analyzer, as reported by Elsas et al. (1995*b*).

MseI Digestion and Detection of 1721C→T

Genomic DNA from patients and controls was amplified using primers encompassing exon 7 of the GALT gene. Primers were designed to create a new *MseI* (Promega) site as control for restriction enzyme activity. The amplified DNA was 270 bp and contained the newly created *MseI* cut site. Two fragments of 244 bp and 26 bp were produced when the homozygous normal DNA was digested to completion with *MseI*. DNA homozygous for the C→T transition at bp 1721 (LA/LA) gained a second *MseI* site and produced three fragments of 170, 74, and 26 bp after digestion. DNA heterozygous for the 1721C→T transition (LA/N, LA/G, LA/D) had four fragments of 244, 170, 74, and 26 bp. Fragments were resolved on a 2% agarose gel. Forward primer 1 began 5′ at bp 1551 with the sequence TGGGACAGAGGAAATATGCCA. Reverse primer 2 was complimentary to bp 1821 at its 5′ end, with the sequence CACCTCTCTCATGGGATAAGAAAGTTAAGG. DNAs were amplified beginning with "hot-start" PCR and continuing for 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

RNase Protection

Total RNA was isolated from patient and control lymphoblasts by cell solubilization in Tri Reagent (Molecular Research Center; Chomczynski and Sacchi 1987). Radioactive antisense RNA probes were generated with the MAXIscript transcription kit of Ambion. Human GALT cDNA in pGEM3zf+ vector (Promega) was linearized with *NcoI* (Promega) and transcribed with Sp6 RNA polymerase in the presence of ^{32}P-UTP, to produce a 377-base antisense probe. Two other antisense probes that spanned the entire GALT cDNA were generated by subcloning fragment 1 (GALT cDNA bp 1–393) and fragment 2 (GALT cDNA bp 332–1089) into pGEM3zf+ and linearizing with *EcoRI* or *BglII*, respectively. One GALT probe and a 125-base human actin antisense probe (labeled to 0.1% of the specific activity of the GALT antisense probe) were gel purified and hybridized to total lymphoblast RNA (Ambion's RPA II). After RNase A/T1 digestion, the protected RNA was separated on a native 5% acrylamide gel. The dried acrylamide gels were scanned in a Packard Bell Instant Imager, and the radioactivity in the protected actin band was normalized between samples. The amount of GALT mRNA was expressed as counts per minute (CPMs) in the protected GALT band after normalizing to the amount of actin in each sample.

Western Blot Analysis

Protein extracts from patient and control lymphoblasts were obtained by harvesting 100 ml of cells by centrifugation, washing the cell pellets twice with PBS, resuspending the pellets in 200 μl of PBS and vortexing (3× each for 5 s) in the presence of Sigma G-8772 glass beads. Cell debris was removed by centrifugation. Protein concentration was determined by the Bradford assay (BioRad). One hundred micrograms of protein were then electrophoresed in a 0.1% SDS, 10% acrylamide gel, and blotted to Nytran membrane (Schleichee & Schuell). The blots were then hybridized to anti-GALT antibodies and visualized with anti-rabbit antibodies (Amersham's ECL system). The density of each band was quantitated by scanning with an Alpha Inotech IS 1000 Digital Imaging System.

Thermal Inactivation

Whole cell extracts from patient and control lymphoblasts were normalized to similar GALT enzyme activity by diluting the controls with cell extract from a homozygous GALT deletion cell line. Phenotypic N/N and D/D cell extracts were exposed to temperatures from 0°C to 95°C for 15 min, to establish the incubation temperature ($T_{1/2}$), which results in loss of half the enzyme activity, of GALT enzyme activity for the D/D extracts. The D/D $T_{1/2}$ (15 min at 50°C) was then used to compare thermal stability of different phenotypes. GALT enzyme activity was assayed as reported elsewhere (Elsas et al. 1994).

Results

The LA variant biochemical phenotype was defined elsewhere (Ng et al. 1973; Ibarra et al. 1979; Anderson

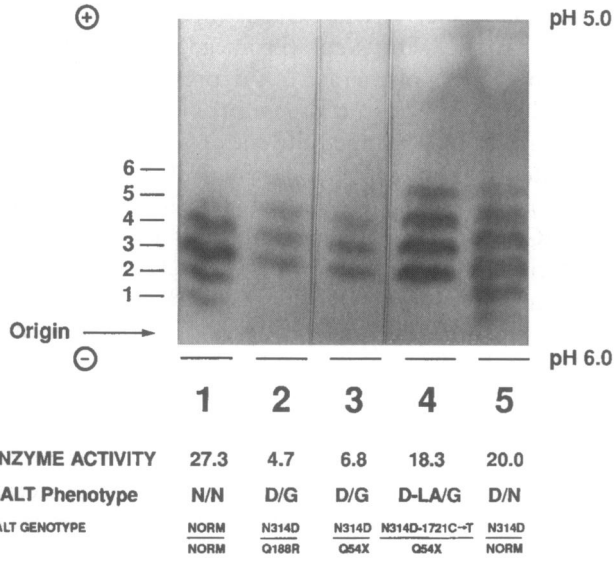

Figure 1 Isoelectric focusing gel, biochemical phenotypes, and molecular genotypes for D, LA, and G GALT alleles. GALT was partially purified from erythrocyte lysates. Ten milligram equivalents of hemoglobin were electrophoresed and stained for enzyme activity. The GALT biochemical phenotypes and molecular genotypes for individuals 1–5 are indicated below.

et al. 1984; Greber et al. 1995; Strobl et al. 1995), and our modification of this definition is displayed in figure 1. The D allele produces partial reduction in GALT enzyme activity with a shift in the isozyme-banding pattern toward the anode and lower pH on isoelectric focusing (fig. 1, lanes 2–5) (Elsas et al. 1994). In D/G, or classic, galactosemia, there is a 75% reduction in GALT enzyme activity with retention of this isoform shift (fig. 1, lanes 2 and 3). By contrast, the LA variant *increases* GALT enzyme activity over that expected from a given genotype but retains the classic D allele's isoelectric focusing pattern (fig. 1, compare lanes 2–3 with 4).

We analyzed GALT enzyme phenotypes and molecular genotypes among 196 patient and control samples. There were 91 patients with one N314D-containing GALT allele and one classic galactosemia (G) allele, as seen in table 1. Four of these patients had abnormal SSCP patterns for GALT exon 7, as well as N314D and a G allele. PCR amplification of exon 7, followed by *Mse*I digestion of the amplified DNA revealed that all four were heterozygous for a C→T transition at bp 1721 (1721C→T). This changed a CTA to a TTA codon and produced a neutral polymorphism, L218L in *cis* with the N314D mutation. The RBC GALT enzyme activity for these four patients was 258% greater than for the 87 N314D-only/G patients, with a *P* value of .0007. Since this dichotomy defines the LA variant, they were given the biochemical phenotype, LA/G. There were 14 N314D/N314D homozygotes, two of which had one N314D allele in *cis* with the 1721C→T transition. These two patients had 64% more RBC GALT enzyme activity than did the 12 homozygous for N314D-only, with a *P* value of .0283, and were defined as "LA/D." There was one N314D/N patient with the 1721C→T transition in *cis* with N314D (LA/N) and an RBC GALT activity 59% greater than the 39 N314D-only/N patients. Sequencing of the entire GALT genes from an LA/G, LA/D, and an LA/N patient revealed no other nucleotide changes associated with the LA alleles. There were, however, five intronic corrections to the original published GALT sequence (Leslie et al. 1992) found in all GALT genomes sequenced (two controls and two D/D homozygotes, as well as the LA heterozygotes): in intron A, delete the C at bp 157 and five Gs from bp 351–355 and change bp 217 from a C to G; in intron C, insert a G at bp 1071; and in intron I, insert a G at bp 2436.

In an exemplary pedigree with informative meioses, the D and LA alleles segregated independently (table 2). The proband was ascertained through Georgia's Newborn Screening Program for galactosemia and was found to have one D allele and one G allele. The G allele was a unique nonsense mutation at bp 468C→T (Q54X) (Elsas et al. 1995*b*). Pedigree analysis indicated that the Q54X mutation was maternal in origin and was transmitted in *trans* with the paternal N314D. The mother's other allele, N314D-1721C→T, was transmitted to the proband's two siblings. Biochemical phenotyping and molecular genotyping revealed elevated (above-expected) GALT activity as well as the expected isoform shift seen with the LA phenotype (fig. 1) segregating with the N314D-1721C→T allele. The mother (LA/G) had 172% more RBC GALT activity than the proband (D/G), while both displayed the anodal isoform shift. The 2 siblings (LA/D) had 64% more RBC GALT activity than did 12 D/D phenotypes.

We also evaluated the genomic DNA of 51 control patients who did not have an N314D-containing GALT allele for the presence of the 1721C→T transition alone. We divided these controls into groups whose GALT enzyme activities were 1 SD above and below the mean of control (N/N) erythrocyte GALT activity (table 3). Six of the controls had RBC GALT activities of >35.3 μmol UDPgal produced/h/g Hb, 33 of the controls had RBC GALT activities within the mean, and 12 had RBC GALT activities of <19.5 μmol UDPgal produced/h/g Hb. None of the controls exhibited the 1721C→T transition (table 3). Thus, the 1721C→T transition is a rare allele not found in 102 normal GALT alleles.

To determine whether the LA allele increased GALT protein abundance, we assessed western blots of lymphoblast GALT proteins from subjects with the D/N or LA/N phenotypes compared to a control with the N/N GALT phenotype (fig. 2). A histogram of the densitometry tracing from these immunoblots is presented below. Arbitrarily designating the N/N phenotype as

Table 1

RBC GALT Activity of Normal Controls, D and LA Galactosemia Variants

Biochemical Phenotype	Molecular Genotype[a]	RBC GALT Activity[b]	Percent of Control	*P* Values[c]
N/N	Normal/Normal	27.4 ± 8 (51)	100	
D/G	N314D/G	5.2 ± 2 (87)	19	.0007
LA/G	N314D-1721C→T/G	18.4 ± 5 (4)	67	
D/D	N314D/N314D	14.3 ± 5 (12)	52	.0283
LA/D	N314D-1721C→T/N314D	23.5 (2)	86	
D/N	N314D/Normal	18.7 ± 7 (39)	68	...
LA/N	N314D-1721C→T/Normal	29.7 (1)	108	

[a] A slash (/) separates alleles in *trans*, while a dash (-) signifies alleles in *cis*. The arrow (→) indicates nucleotide transition.

[b] RBC GALT activity is expressed as the mean μmol UDPgal produced/h/g Hb, ± 1 SD. The numbers in parentheses refer to the number of patients tested.

[c] *P* values were determined by Mann-Whitney *U* rank sum analysis and represent the significance of the increase in GALT activity produced by the 1721C→T transition in *cis* with N314D compared to its equivalent molecular genotype without this nucleotide change.

having 100% GALT protein, the D/N and LA/N phenotypes had 89% and 141% of the control GALT protein, respectively. All of the LA variant heterozygotes had a similar significant increase in 44-kD GALT monomer over their respective genotypic controls (D variant heterozygotes). No LA/LA homozygotes were identified among our galactosemia pedigrees, presumably because they would not be ascertained by population-based screening for impaired enzyme activity.

To determine whether the increased enzyme activity and protein abundance were due to increased GALT mRNA, we analyzed RNA among these GALT genotypes (fig. 3). The RNase protection histogram compared GALT mRNA isolated from lymphoblasts transformed from LA heterozygotes and appropriate controls. The amount of GALT mRNA from each genotype was compared with the amount of actin mRNA in the same extract, to correct for sampling variation. The normal control had 38.7 ± 4 CPM of protected GALT mRNA after normalizing to the protected actin. RNA from a lymphoblast cell line heterozygous for a GALT deletion and one normal allele had 15.1 ± 5 CPM (~50% of the control). As a negative control, we analyzed total RNA from a lymphoblast cell line homozygous for a GALT deletion that contained no GALT mRNA. All of the LA variant heterozygotes had a single GALT mRNA protected band within the normal control range of 38.7 ± 4 CPM with each of the three antisense GALT RNA probes used in this study. Thus, there was no evidence for increased transcription, decreased turnover, or alternative splicing of mRNA in the LA variant.

To determine whether the increased abundance of GALT protein was due to enhanced stability of the LA variant protein, studies of the thermal stability of the GALT protein were undertaken. A thermal inactivation profile (authors' unpublished data) revealed 21.4% inac-

Table 2

Family in Which the D and LA GALT Alleles Segregate

Biochemical Phenotype	Molecular Genotype	Family Member	RBC GALT Activity[a]	Percent of Control
N/N	Normal/Normal	...	27.4 ± 8 (51)	100
D/D	N314D/N314D	...	14.3 ± 5 (12)	52
D/N	N314D/Normal	Father	20.0	73
LA/G	N314D-1721C→T/Q54X	Mother	18.3	67
D/G	N314D/Q54X	Proband	6.8	25
LA/D	N314D-1721C→T/N314D	Sibling	23.6	86
LA/D	N314D-1721C→T/N314D	Sibling	23.4	85

[a] RBC GALT activity is expressed as μmol UDPgal produced/h/g Hb. Data for controls (N/N and D/D) are means ± 1 SD, with the number of individuals studied in parentheses.

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Table 3

Search for 1721C→T among GALT Genes from 51 Nongalactosemic Controls

Subjects[a]	RBC GALT Activity[b]	1721C→T
6	>35.4	0
33	27.4 ± 8	0
12	<19.4	0

[a] No. of N/N subjects. All were negative for N314D.

[b] RBC GALT activity is presented as μmol UDPgal produced/h/g Hb.

tivation of N/N and 55.3% inactivation of D/D lymphoblast GALT enzyme activity after 15 min incubation at 50°C. The percentage of residual GALT enzyme activity for N/N, D/D, and LA/D lymphoblast extracts heated to 50°C for 15 min is shown in table 4. The N/N, D/D, and LA/D GALT enzymes had 78.6%, 44.7%, and 53.4% residual GALT activity, respectively. The difference between the D/D and LA/D values was not statistically significant, indicating that the LA allele did not protect against the thermolability of the N314D mutation.

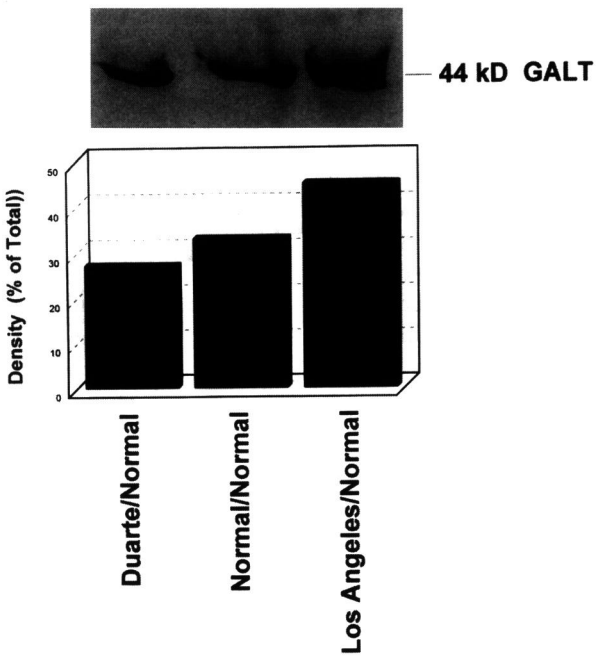

Figure 2 Western blot of whole cell extracts from lymphoblasts heterozygous for D/N and LA/N compared to an N/N phenotype. One hundred micrograms of extracted proteins were electrophoresed in a 0.1% SDS, 10% acrylamide gel. Proteins were transferred to a nylon filter and hybridized with anti-human erythrocyte GALT antibody. Protein that hybridized to the antibody was visualized with a labeled secondary antibody. The density of each band was quantitated.

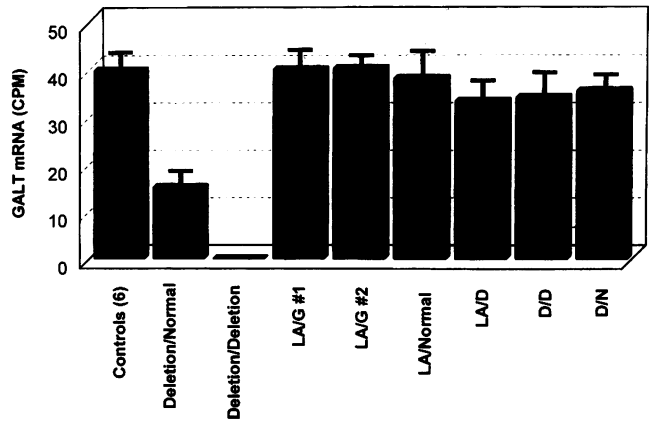

Figure 3 Histogram of RNase protection. Twenty-five micrograms of total RNA were extracted from lymphoblasts of defined GALT genotypes. The RNA samples were hybridized with radioactive antisense RNA probes for human actin and the 3′, 5′, or midportion of human GALT cDNA. Protected RNA was separated on a native 5% acrylamide gel and quantitated. Genotypes for the alleles are: D = N314D, LA = N314D-1721C→T, G1 = Q188R, and G2 = Q54X.

Discussion

Several observations indicate that the LA variant's biochemical phenotype is caused by an associated polymorphism in exon 7, which is a C→T transition at bp 1721. First, among 145 blood samples from families identified from probands with reduced GALT activity and the Duarte allele, 7 had higher-than-expected activity and the 1721C→T transition in *cis* with N314D (LA allele). Second, this 1721C→T transition was not found

Table 4

Percentage of Residual GALT Activity after Heating Cell Extracts

Biochemical Phenotype	$Time_0$ % of Control	$Time_{15}$ % of Control	*P* Value[b]
1. N/N	100	78.6 ± 13.6 (9)	...
2. D/D	100	44.7 ± 19.7 (11)	.001
3. LA/D	100	53.4 ± 14.6 (10)	.001

NOTE.—N/N and LA/D lymphoblast cell extracts were diluted with extract from a lymphoblast cell line homozygous for a GALT deletion to obtain the same GALT enzyme activity per μg of cell protein as was present in a D/D lymphoblast cell extract ($Time_0$). All three extracts were then heated to 50°C for 15 min, chilled to 4°C, and assayed for residual GALT enzyme activity ($Time_{15}$), which is presented as a percentage of the GALT activity at $Time_0$ ± SD. The number in parentheses refers to the number of replicates.

[a] *P* values were obtained by subjecting N/N and either D/D or LA/D to the student's *t*-test and determining that the *t*-values were higher (4.156 and 4.029, respectively) than the *t*-value for $P = .001$, and therefore significant. When LA/D and D/D were analyzed, the *t*-value obtained (.913) was smaller than *t* for $P = .001$, and therefore the difference between LA/D and D/D was not significant.

in any of the remaining 138 GALT genomes. Third, no other nucleotide changes were found in these seven LA variant GALT genomes. Finally, in families with the D and LA alleles segregating, the LA allele (N314D-1721C→T) was always associated with increased GALT activity when compared to the N314D allele and the "wild-type" cytosine at bp 1721. We conclude that the presence of the 1721C→T transition in *cis* with the N314D amino acid change produces the LA variant of "higher-than-expected" GALT activity while maintaining the anodal isoform shift. Two recent abstracts (Greber et al. 1995; Strobl et al. 1995) reported the presence of the 1721C→T transition in *cis* with N314D in patients with apparently normal GALT enzyme activity. They found two intronic substitutions, 1104G→C and 1391G→A, which were in *cis* with N314D alleles in their pedigrees with decreased GALT activity, and interpreted these results as indicating that N314D and 1721C→T were neutral polymorphisms and that the decreased GALT activity seen in the D/D variant was due to the intronic nucleotide changes or unidentified promoter mutations. We have analyzed the genomic DNA of D/D homozygotes, LA heterozygotes, and controls and do not find a correlation with the presence of 1104G→C or 1391G→A and decreased GALT activity. Furthermore, neither the A→G transition in exon 10 (N314D) nor the C→T transition in exon 7 (L218L) alters mRNA abundance (fig. 3).

By what mechanism could a midexonic nucleotide change increase GALT enzyme activity when no amino acid change is predicted? This increase in GALT enzyme activity could be due to increased translation, increased transcription, decreased mRNA turnover, or decreased enzyme turnover rates. We found an increased abundance of GALT protein in the lymphoblasts of patients with the LA phenotype (fig. 2), which agrees with earlier studies of RBC GALT protein in LA variants (Anderson et al. 1984) and is consistent with the presence of a neutral, polymorphic codon change. RNase protection results indicated that the amount of GALT mRNA in LA variants (fig. 3) is *not* elevated, eliminating the possibility that an unidentified promoter change linked to N314D-1721C→T resulted in increased transcription of the GALT gene or that this nucleotide transition increased mRNA abundance through other mechanisms such as reducing mRNA turnover rates.

Since the N314D (D) mutation produces protein instability (authors' unpublished data), we considered the possibility that the 1721C→T transition might reduce this effect. However, similar thermal inactivation profiles at 50°C indicated that the LA/D and D/D lymphoblast GALT enzymes had the same structural stability when compared to N/N (table 4). Thus, the L218L neutral polymorphism has no effect on the structural stability of the Duarte GALT protein. This is not surprising, since there is no charge or amino acid change associated with 1721C→T. We had previously found that another mutation in *cis* with N314D, which resulted in a charge change (N314D-E203K), did rescue GALT from biological instability and revert GALT activity to normal (Elsas et al. 1995*b*).

Since increased LA GALT activity is not due to increased mRNA or increased enzyme stability, could the increased abundance of protein be due to increased translation? The human GALT gene's translation start site is not preceded by the efficient Kozak consensus sequence for initiation of translation in vertebrates (Kozak et al. 1986; Flach et al. 1990; Leslie et al. 1992). Therefore, translation may be of significance in controlling enzyme abundance. A potential molecular mechanism for increased GALT translation of the LA variants is "codon bias" at codon 218, which might increase rates of protein translated from the new mRNA sequence (Kurland 1991). There are 38 leucine codons in human GALT mRNA, and only 1 is normally TTA. The 1721C→T transition would add a second TTA for leucine. Since TTA is a rarer leucine codon in human GALT mRNA, there may be an abundance of TTA tRNAs in the human cell that could produce more efficient translation when the mRNA contains this leucine codon. It is important to test this hypothesis of "codon bias" by translation of the altered LA mRNA in a mammalian translation system that would mimic the pool of tRNAs and amino acids normally available in human cells. It will be difficult to generalize from an in vitro translation system to differentiated human organs, because we have found that GALT mutations demonstrate organ specificity. The S135L mutation results in absent erythrocyte and lymphoblast GALT but normal total body galactose oxidation from other tissues with residual enzyme activity (Lai et al. 1996). However, the LA variant of GALT may well represent a human example of "codon bias," and this hypothesis will be further tested.

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