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Molecular analysis of 11 galactosemia patients

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ABSTRACT

Galactosemia is a human inborn error of galactose metabolism due to deficiency of galactose-1-phosphate uridyl transferase. In this paper, I describe the molecular analysis of genomic DNA, mRNA and protein from 11 different galactosemic patients by Southern, Northern and Western blotting. The results of these experiments lead me to conclude that galactosemia is caused mostly by missense mutations. The unusual preponderance of missense mutations in galactosemia led me to investigate its cause. I demonstrate that all 9 patients I investigated have detectable residual enzyme activity (ranging from 0.7–6.9% of normal). This finding is of potential importance in addressing the long-term complications of galactosemia.

INTRODUCTION

In humans, as in most other organisms, galactose is metabolized by the Leloir pathway (1, 2). Defects in this pathway result in a set of human diseases referred to as the galactosemias. The most frequent form of inherited galactosemia results from a deficiency in the enzyme galactose-1-phosphate uridyl transferase (EC 2.7.7.12; GALT; 1, 2). Transferase-deficiency galactosemia (McKusick 230400) is an autosomal, recessive disorder with a frequency of 1:60,000 liveborn infants (3). In the untreated infant, vomiting, diarrhea, failure to thrive and jaundice are commonly observed in the neonatal period (1). Later cataracts and *E.coli* sepsis are frequently diagnosed. These early onset symptoms can be eliminated by the institution of a galactose-restricted diet. Thus, many US states and Western countries have instituted newborn screening programs. Unfortunately, late-onset complications, such as neurologic abnormalities and ovarian failure, are commonly observed in managed patients (11).

A number of human genetic diseases have been examined at the molecular level and the general conclusion from these studies which include the hemoglobinopathies (15), Lesch-Nyhan syndrome (16), hemophilia A (18) and phenylketonuria (PKU, 21) is that any imaginable mutation can be found. Lesch-Nyhan syndrome, secondary to HPRT (hypoxanthine phosphoribosyl transferase) deficiency, is a good example in case: gene rearrangements such as deletions, insertions and duplications have been described (16). At the mRNA level aberrant transcripts have been detected as well as no detectable HPRT message. Finally, a high proportion (54%) of patients fail to produce CRM ((antigenically) cross-reacting material; 16). At present, the molecular basis of galactosemia is not well understood. Therefore, I examined the GALT gene in 11 galactosemic patients by Southern blotting, their mRNA by Northern blotting and their GALT protein by Western blotting. My results are consistent with the hypothesis that most galactosemia mutations are of the missense type.

This unusual situation led me to investigate possible causes for the preponderance of missense mutations in galactosemia. I wondered if the lack of GALT activity might be lethal. Accordingly, I used a sensitive radioactive assay to determine if galactosemic individuals retained some residual enzyme activity in their defective GALT proteins. I found that all 9 patients I analyzed had detectable levels of activity (ranging from 0.7 to 6.9% of normal).

MATERIALS AND METHODS

Molecular Techniques

All manipulations involving DNA, RNA and protein were as described in 5, except that Southern and Northern blots were sometimes transferred to Zetabind (Cuno, Meriden, CT). Autoradiograms were scanned with a Hoefer GS300 scanning densitometer connected to a BBC Servogor 120 strip chart recorder. The antigen for the anti-GALT serum was a trpE'-G-ALT fusion protein expressed from pATH2 (6). The GALT coding region from *Fnu* DII to *Xho* I (partial digest) was subcloned into the bacterial vector at *Sma* I and *Sal* I. Inclusion bodies were purified with minor modifications from *E.coli* HB101 bearing this construct after induction as described (6). At this stage the protein was about 30% pure and it was further purified on a preparative 10% SDS polyacrylamide gel (22). Rabbits were injected by BAbCo (Richmond, CA) with 500 μg for the immunization followed by two boosts in monthly intervals with 250 mg each. Antiserum diluted 1:20 was used on Western blots of 10% SDS polyacrylamide gels (7) and developed with goat-anti-rabbit serum conjugated to alkaline phosphatase (ICN, Irvine, CA).

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Tissue Culture

All cell lines prefixed 'GM' were obtained from the Corriell Institute for Medical Research (Camden, NJ), cell strains DP and JP were obtained from Dr. Seymour Packman (University of California, San Francisco, CA) and the CF20 fibroblasts were obtained from Dr. Thomas Tedesco (University of Southern Florida, Tampa, FL). Primary cells were grown in 20% horse serum (HyClone, Logan, UT) and transformed cells in 10% serum as described in 5.

Radioactive GALT Assay

Two published procedures were combined and modified (23, 24) resulting in a reaction mixture containing: 100 mM glycylglycine pH 8.5, 20 mM DTT (dithiothreitol), 10^{-4} M [^{14}C]-galactose-1-phospsphate (gal-1-p) (Amersham, Amersham, UK; 318 mCi/mmol), 10^{-4} M uridine diphospho glucose (UDPG) and 5–10 μl cell extract (1 μg/μl) in a final volume of 30 μl. The reaction mixtures were either incubated for 90 min at 37°C. After heat inactivation at 100°C for 5 min, the reaction mixes were incubated with 1 u calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C. 5–10 μl were then spotted on PEI-TLC plates (Brinkmann, Westbury, NY), air-dried and run for 3–5 cm in 1 N formic acid followed immediately by a run for another 15–17 cm in 2 N sodium formate pH 4.0. The position of UDPG was localized by quenching of the UV fluorescence, the plates were then autoradiographed and the relevant spots were counted in a scintillation counter. This assay is linear with respect to time and extract within the limits used in this paper. It is also totally dependent on the addition of UDPG (uridine diphospho glucose; 22). 1 unit is defined as the amount of enzyme required to convert 1 μmol gal-1-p in 1 minute. The results described in this paper are the average of two assays.

RESULTS

Patient Population

I investigated the status of the GALT gene, mRNA and protein in 13 cell lines representing 20 different galactosemic alleles (cf. Table 1) by appropriate blotting techniques. The galactosemic cell lines were obtained from patients from different geographic areas of the USA and they represent at least two racial backgrounds, Caucasian and African-American. Cell lines GM638 and GM639 are SV40-transformed fibroblasts of the same patients from which the EBV-transformed lymphoblastoid lines, GM 2795 and GM 2796, were established. Cell strains JP and DP are from two siblings and GM1996 is a compound heterozygote for galactosemia and the Duarte variant (D/G).

Southern Blotting

Genomic DNA from 13 patient cell lines was digested with three restriction endonucleases (*Bam* HI, *Eco* RI and *Xho* I), transferred to a membrane and then probed with the GALT cDNA. In no case did I detect any novel bands, which would be characteristic of insertions or gene deletions (Table 1). Furthermore, scanning of the autoradiograms did not reveal partial deletions within the accuracy of the scans. Thus, there are no gross changes in the structure of the GALT gene detectable by Southern blot analysis of patient DNAs.

Table 1. Blot Analysis of Galactosemic Patients

Cell Line	Genotype	Genomic DNA	mRNA	Protein
17 volunteers	+/+	normal	ND	ND
IM9	+/+	normal	full-length	full-length
VA13	+/+	normal	full-length	full-length
GM 148	G/G	normal	ND	ND
GM 422	G/G	normal	ND	full-length
GM 433	G/G	normal	ND	full-length
GM 638	G/G	normal	full-length	full-length
GM 639	G/G	normal	full-length	full-length
GM 1209	G/G	normal	ND	full-length
GM 1996	D/G	normal	full-length	full-length
GM 2412	G/G	normal	full-length	ND
GM 2795	G/G	normal	full-length	ND
GM 2796	G/G	normal	full-length	ND
DP	G/G	normal	full-length	full-length
JP	G/G	normal	full-length	full-length
CF20	G/G	normal	ND	full-length

ND: not done
Note: DP and JP are siblings and, therefore, presumably carry the same galactosemia mutation(s). GM 638 and 639 are SV40-transformed fibroblasts and GM 2795 and 2796 are EBV-transformed lymphoid cells derived from the same two patients.

Northern Blotting

I investigated the status of the mRNA in 8 galactosemic cell lines by Northern blotting (Table 1). In all cases was I able to detect full-length mRNA within the accuracy of my blots. It also appeared that the levels of the GALT RNA were essentially the same relative to a similarly sized control RNA, β-actin (22). These results suggest that the galactosemia mutation(s) do not affect metabolism of the GALT mRNA.

Western Blotting

Finally, I wanted to compare the mutant and normal GALT proteins to determine if aberrant size proteins were present in galactosemic patients. For example, nonsense mutations would result in a truncated protein while missense mutations in the coding region would lead to full-length protein.

I used an antiserum raised against a recombinant trpE'-GALT fusion protein (22) as a probe on Western blots of protein extracts obtained from 9 patients. While the preimmune serum did not recognize any proteins (Fig. 1B), the antiserum detected a band of 45 kD in all samples (Fig. 1A and Table 1). This is in good agreement with the predicted size of the GALT protein deduced from the cDNA sequence (43 kD; 25). No aberrant bands are seen in the patient samples when compared to normal control. Thus, all 9 galactosemia patients analyzed have full-length GALT protein within the detection limit of my blot.

Radioactive GALT Assay

In considering the question of why all patients analyzed retain intact genes, transcribe mRNA normally and translate protein in normal amount and size I wondered whether this was selected for, e.g. because some GALT enzyme activity is required for life. Accordingly I set out to determine if the GALT protein found in patients has some residual activity.

The assay I used is a combination of two published procedures designed to optimize the signal-to-noise ratio of the GALT enzyme assay (23, 24). When expressed as % of normal activity, cultured galactosemic cells have from 0.7 to 6.9% of the normal

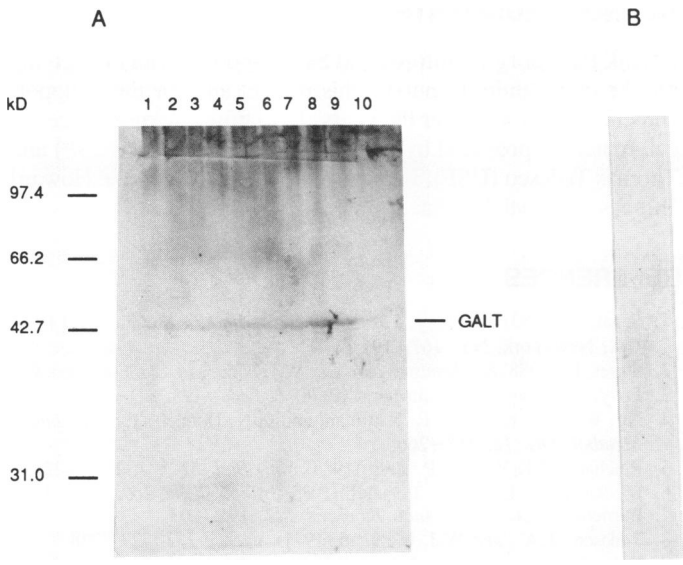

Figure 1. Western blot of 9 galactosemic cell lines. Panel A: Whole cell protein extracts from a control line, VA13 (lane 1), and 9 galactosemic cell lines (lanes 2–10): GM 422, GM 433, GM 638, GM 639, GM 1209, GM 1996, DP, JP and CF20 were prepared and blotted as described in Materials and Methods. This blot was decorated with anti-GALT serum, while in panel B the preimmune serum was used on normal extract alone.

level. GM 1996, a compound heterozygote(D/G), has significantly higher activity than classic galactosemic patients (24.9%) and is clinically unaffected.

DISCUSSION

The molecular basis of galactosemia is at present poorly understood. Thus, the data presented in this paper analyzes 11 galactosemic patients at the molecular level: their genomic DNA, mRNA and GALT protein were investigated. The patients I analyzed (Table 1) are from different geographic areas of the US and represent at least two different racial backgrounds, Caucasian and African-American. They are, therefore, presumably unrelated except for patients DP and JP who are siblings. Cell lines GM 638, GM 639, GM 2795 and GM 2796 are fibroblast or lymphoblastoid lines respectively derived from two patients.

Genomic DNA was analyzed by Southern blotting and the results obtained from 20 different galactosemia alleles (Table 1) show no sign of deletions or insertions because no novel bands diagnostic of such rearrangement events are seen. Furthermore, the relative intensities of bands on the blots are constant indicating that the patients analyzed bear no partial deletions. Analysis of the GALT message by Northern blots (Table 1) revealed normal size and levels of GALT mRNA. Therefore, it seems unlikely that the galactosemia mutation(s) affect transcription, splicing or mRNA stability. I infer from this data set that galactosemia is caused by point mutations in the GALT coding region and to further characterize the type of mutation I analyzed the state of the protein. Galactosemia could be caused by nonsense mutations which would result in truncated proteins or it could be the result of missense mutations that do not affect the size of the translation product but often alter its catalytic properties. In this study I used an antiserum raised against a trpE'-GALT translational fusion protein prepared from *E.coli* (22). The serum raised against this protein is specific and recognizes CRM of normal size in all 9 patients analyzed (Figure 1 and Table 1). Thus, galactosemia appears to be caused predominantly by missense mutations which substitute one amino acid for another in the polypeptide chain leaving the size of the protein unchanged but often affecting its catalytic properties.

Previous studies (8, 9, 10, 20) led to conflicting conclusions on the fate of the GALT protein in galactosemic patients. Two groups identified CRM in the 9 patients they investigated (8, 20), while the third group first reported the presence of inactive protein in one patient (9) and subsequently demonstrated the absence of CRM in another patient using a different antiserum (10). These discordant results are presumably due to the different antisera and the varying impurities they contain. My anti-GALT serum was raised against recombinant protein synthesized in *E.coli* (22). My experiments (Fig. 1, Table 1) are in agreement with those in the classic literature which demonstrated CRM which, however, was not always sized (8, 9, 20). Two other sets of experiments from the older literature also support my hypothesis: 1) reversion of galactosemic cells to $GALT^+$ has been observed (a result indicative of a point mutation; 12) and 2) interallelic complementation has been demonstrated in cell fusions of cells derived from different galactosemic patients (13). This result is to be expected because the enzyme is a dimer (14, 22) and the formation of an active heterodimer in a cell hybrid from two subunits containing different missense mutations would be possible.

It is important to consider the limitations of my blotting experiments. Based on my Western experiments, I conclude that each patient has to carry at least one missense allele. He or she, however, may also have a null allele resulting in no CRM. This second allele would be masked by the missense mutation on my Western blots. It is noteworthy, however, that I was unable to detect genetic rearrangements by Southern blotting or abnormalities in the GALT mRNA (Table 1). This bias towards missense mutations might be either a reflection of a limited sample size (even though I analyzed 20 different galactosemic alleles) or could be an indication of the absolute requirement of some GALT activity possibly intrinsic to each mutant protein.

These results raise an important question: why are only missense mutations seen in galactosemia and not mutations that are more severe, such as deletions? This situation is unusual since all kinds of imaginable mutations (point mutations, insertions, deletions and duplications) occur in most other genetic diseases (e.g. in the hemoglobinopathies (15), Lesch-Nyhan syndrome (16), hemophilia (18) and PKU (21)). Perhaps these more severe mutations are not tolerated in GALT and might be lethal *in utero*.

Consistent with the need for some GALT enzyme activity is the finding that all patients I analyzed have some detectable enzyme activity ranging from 0.7 to 6.9% of wild type (Table 2). I used a radioactive assay that combines features of two previously published procedures to detect these low levels of enzyme activity (23, 24). My results are in agreement with those of Russell and DeMars (24) who measured 2 to 5% of normal activity in 3 galactosemic cell strains. Diagnostic GALT assays in patients are performed on erythrocytes and generally no enzyme activty is detected (1). However, whole blood from a galactosemic patient was fractionated into reticulocytes, which actively synthesize protein, and red cells, which are incapable of *de novo* protein synthesis. It was found in this study that reticulocytes from galactosemic patients have residual GALT

Table 2. GALT activity in galactosemic cell lines.

cell line	genotype	specific activity (u/ng)	% (of normal)
VA13	+/+	124.7	100
GM422	G/G	3.2	2.5
GM433	G/G	7.0	5.6
GM638	G/G	2.4	2.0
GM639	G/G	6.8	5.4
GM1209	G/G	0.9	0.7
GM1996	D/G	31.0	24.9
CF20	G/G	8.7	6.9
DP	G/G	3.4	2.7
JP	G/G	5.1	4.1

1 unit is the amount of enzyme required to convert 1 μmol gal-1-p per minute. *Note:* DP and JP are siblings.

activity, while erythrocytes from the same patient do not (28). Furthermore, GALT activity has been found in cultured cells from patients (24), their livers (29) and intestinal mucosa (30). Thus, my data is in agreement with results obtained from cells and tissues with active protein synthetic capability. Finally, the Duarte/galactosemia (D/G) compound heterozygote GM1996 had about 25% of normal activity (Table 2). This value is comparable to the values traditionally measured in red cell assays (1).

In summary my data on the nature of the galactosemia mutation(s) suggests that this disease results from a preponderance of missense mutations. All 9 galactosemia patients analyzed by Western blotting are CRM^+ which is also of normal size. Thus, at least one allele in each one of these patients has to carry a missense mutation. Furthermore, both Southern and Northern blotting experiments reported here failed to detect other abnormalities in the GALT gene or its transcription unit. Molecular examination by sequencing PCR-amplified cDNA from two patients analyzed in this paper resulted in the identification and characterization of four galactosemia misense mutations, two GALT polymorphisms and one silent third-base pair change (26, 27). After reconstruction of each muation by *in vitro* mutagenesis of the GALT cDNA, it was introduced into mammalian cells to study each mutations' biochemical properties. All patients analyzed were found to bear at least one allele with residual activity in this system. Therefore, the hypothesis put forward in this paper, that galactosemia is caused predominantly by missense mutations, appears to be true in a small sample analyzed in detail at the molecular level. Thus, if galactosemia patients have indeed residual enzyme activity, then future therapies may be directed at boosting this residual level to overcome the long-term complications of this disease (11). Further molecular characterization of patients will be necessary to corroborate this hypothesis. Finally, the question remains why galactosemic patients have residual GALT activity. It is possible that GALT is an essential gene. One of its products, uridine diphospho galactose (UDP-gal), is the sole donor of the galactosyl moiety of glycoproteins and glycolipids (2). Therefore, residual GALT activity may be required to provide a minimum level of UDP-gal. In fact, it has been reported that galactosemic patients have reduced levels of this important metabolite (4). This finding has been correlated with ovarian failure in galactosemic patients (17). Future experiments in suitable animal model systems will address these questions.

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