Hum Genet (2001) 109 : 117–120
DOI 10.1007/s004390100540

ORIGINAL INVESTIGATION

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Galactosemia: deletion in the 5′ upstream region of the GALT gene reduces promoter efficiency

Received: 20 March 2001 / Accepted: 4 May 2001 / Published online: 14 June 2001

Abstract Galactosemia is a metabolic disorder caused by a defect in the galactose-1-phosphate uridyltransferase (GALT) enzyme. In previous studies, we have shown that the presence of a deletion in the 5′ upstream (promoter) region of the GALT gene is associated with the Duarte (D2) allele. In the present study, by using a promoter fusion assay we provide direct evidence that a GTCA deletion located in position –119/–116 of the GALT gene (considered in relation to the translational start site) decreases transcription of a reporter gene to about 55% compared with a normal "healthy" promoter transfected into human hepatocyte HepG2 cells. This result coincides well with previously published biochemical data showing 50% GALT-gene activity in Duarte (D2) galactosemia patients. By transfecting the same promoters (normal and deleted) into mouse NIH/3T3 cells, we show that the GTCA motif in the promoter region of the GALT gene was conserved throughout evolution. We conclude that the –119/–116delGTCA promoter mutation is a crucial factor in reduction of Duarte allele enzyme activity.

Introduction

Galactosemia (MIM 230 400) is an autosomal recessively inherited disorder of galactose metabolism. Absence or severe reduction of galactose-1-phosphate uridyltransferase (GALT) enzyme activity results in classical galactosemia (G/G), while 50% reduction of enzyme activity leads to the Duarte variant of galactosemia (D/D). Classical galactosemia is a potentially lethal disorder, which affects 1 in 30,000–60,000 live-born infants. Mutation Q188R was found to be the most common defect among classical galactosemia patients (Reichardt et al. 1991; Ng et al. 1994), whereas N314D was predominantly detected in Duarte galactosemia patients (Elsas et al. 1994; Lin et al. 1994).

The mechanism of partial GALT-activity impairment in the Duarte alleles is not fully understood. Several independent groups (Podskarbi et al. 1996; Greber-Platzer et al. 1997; Kozak et al. 1997; Langley et al. 1997) found that the Duarte (D2) alleles contain in cis the N314D mutation plus three intronic variants, IVS4nt-27g→c, IVS5nt+62g→a, and IVS5nt-24g→a, and the Los Angeles (D1) alleles (110–130% of normal GALT activity) carry in cis the same N314D mutation plus the silent mutation L218L. Podskarbi et al. (1996) speculated that two intronic mutations, IVS4nt-27g→c, IVS5nt+62g→a might be the regulatory ones, involved in regulation of GALT-gene expression. Later, Lai et al. (1998) reported that the N314D mutation reduces biological stability of the GALT dimeric protein in human lymphoblastoid cell lines. On the contrary, the results obtained from a yeast expression system (Fridovich-Keil et al. 1996) did not prove reduced stability of N314D containing GALT protein.

During mutation analysis of the GALT gene in galactosemia alleles and in the Duarte (D2) and Los Angeles (D1) alleles detected from population screening, a novel deletion of four nucleotides (GTCA) in the 5′promoter region was found only in Duarte (D2) but not in Los Angeles (D1) alleles (Kozak et al. 1999). A computer search for potential regulatory elements in the area of this deletion has shown that it could eliminate the binding sites for two activator proteins (AP1Q2 and AP1Q4). It is also known that the GTCA motif plays an important role in the regulation of transcription when it is present in cis upstream of a coding sequence (Cong et al. 1998; Ledo et al. 2000). Considering that, and also the reduced GALT-gene activity in Duarte galactosemia patients (Beutler et al. 1965), we decided to perform direct functional analysis of the deleted promoter.

In the present study, we show that there is a reduced level of reporter gene activity transcribed from the fused –119/–116delGTCA promoter compared with the normal

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GALT promoter when transfected into human hepatocyte (HepG2) cells. We also show here that the binding motif (sequence GTCA) is utilized in mouse (NIH/3T3) cells as well, indicating its evolutionary significance for transcription.

Material and methods

Cloning of human GALT promoters

The promoter of the human GALT gene was cloned from a normal homozygote and a –119/–116delGTCA homozygote by PCR using primers F (5′AAGCTTGATTGCAGCAAGCAAGTCCTG 3′) annealing to position –305/–285 and R (5′AAGCTTGATCCGCTGGAAAATCTGCAG 3′) annealing to position –14/–34. Primers were derived according to sequences in GenBank (Accession No. M96264). Both primers had Hind III cloning sites at their 5′-ends. The PCR protocol was the following: 94 °C for 2 min, then 30 cycles of 94 °C for 30 s, 55 °C for 40 s, 72 °C for 30 s, followed by 10 min final extension at 72 °C. The reaction mixture contained 10 ng of genomic DNA, 10 pmol of each primer, 200 μM dNTP, 1.5 mM $MgCl_2$, 1x reaction buffer and 0.2 U of Taq polymerase (Appligene). PCR was done in an MJ Research PTC-200 Thermal Cycler. An amplified PCR product was cut out of the gel, purified by a QIAquick gel extraction kit (Qiagen), digested overnight by 20 U of Hind III endonuclease (New England Biolabs) and ligated into a Hind III site of pGL3-Basic vector (Promega). The construct was transformed into *E. coli* JM 109 competent cells (Promega). Proper orientation of the GALT promoter in relation to the luc+ gene was verified by sequencing using the Sequenase Version 2.0 DNA sequencing kit (Amersham). The promoter sequence was as in GenBank with the correction published by Kozak et al. (1999).

Transfections and luciferase assay

The human hepatocyte HepG2 cell line was obtained from the European collection of cell cultures (ECACC, Great Britain). The mouse embryonic fibroblast NIH/3T3 cell line was obtained from University Hospital, Olomouc. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37 °C in 5% CO_2. Transfections were carried out in a 12-well plate by using a mixture of 0.48 μg DNA of GALT-promoter/pGL3 firefly luciferase constructs described above, together with 0.12 μg DNA (0.23 μg DNA for transfection into NIH/3T3 cells) of pRL-TK, an internal control plasmid in which *Renilla* luciferase coding sequences are fused to the *Herpes simplex* virus thymidine kinase promoter region. Plasmid DNA was mixed with the components of the Effectene transfection reagent kit (Promega) according to the manufacturer's instructions (8 μl of Enhancer DNA and 25 μl of Effectene reagent per 1 μg of plasmid DNA), diluted into 400 μl of DMEM and applied into each plate well where the cells were at approximately 60% confluency at the time of transfection, in 800 μl of fully supplemented DMEM. The time of transfection was 16 h for HepG2 cells and 9 h for NIH/3T3 cells (at 37 °C in 5% CO_2). At the end of the transfection the cells were washed in PBS and grown in 10% DMEM for an additional 72 h (or 36 h for NIH/3T3 cells). Then, the cells were lysed in 250 μl of passive lysis buffer (Promega) and briefly centrifuged to remove cell debris. Twenty microliters of lysates were sequentially measured for firefly and *Renilla* luciferase activities by using the Dual-Luciferase Reporter Assay system (Promega). Luminometry was performed on an LMO1-T luminometer (Immunotech Prague) with 2-s pre-measurement delay and 15-s measurement period.

Results and discussion

Normal and deleted GALT-gene promoters were cloned in a proper orientation into the pGL3-basic vector (Promega) lacking any regulatory sequences upstream to the reporter luc+ (luciferase) gene. All the transfections were done together with the second (cotransfected) plasmid (pRL-TK), coding for a different kind of luciferase and thus serving as an internal standard. By using the Dual-luciferase reporter assay system, we analyzed transcriptional efficiency of the normal human GALT-gene promoter in comparison with the –119/–116delGTCA Duarte (D2) allele promoter. We first used human hepatocyte cells for transfection, because liver is the organ primarily affected in galactosemia patients (Hsia 1968; Segal 1989).

Transcriptional activity of the deleted promoter reached about 55% of normal promoter activity when transfected as a fusion-promoter into the human hepatocyte HepG2 cell line. The results are summarized in Table 1.

The difference between normal and deleted-promoter transcriptional activity was highly significant, as indicated by a non-parametric Mann-Whitney test ($P<0.001$). These results strongly support the hypothesis that liver cells transcribe GALT gene less efficiently from the deleted promoter. The normal promoter showed approximately 1.8-times higher transcriptional activity in our model system. This finding coincides well with ~50% GALT-gene activity seen in Duarte galactosemia patients. It also fits with the results published by Shin et al. (1998), reporting that in competitive RT-PCR, the level of RNA from homozygous Duarte (D2) cultured human lymphocytes was lower than that obtained from control cultured human lymphocytes. Our current results strongly support the hypothesis that the –119/–116delGTCA mutation is the major factor contributing to the Duarte variant of galactosemia.

Moreover, in our previous study we also observed the –119/–116delGTCA mutation in two types of classical galactosemia alleles with unusual molecular genotypes (V151A plus N314D, -119/–116delGTCA, IVS4nt-27g→c, IVS5nt+62g→a, IVS5nt-24g→a and E340K plus N314D, -119/–116delGTCA, IVS4nt-27g→c, IVS5nt+62g→a, IVS5nt-24g→a) (Kozak et al. 2000). As proposed by Fridovich-Keil and Jinks-Robertson (1993), it is reason-

Table 1 Luciferase activity in human hepatocyte HepG2 cells. Results represent the average of three independent transfections, each performed in triplicate. Transfections were done with two independently isolated plasmid DNAs. Luminescent units are normalized according to internal standard

Subject analyzed	Luminescent units (absolute values) pGL3/pRL-TK	Luminescent units (normalized values) pGL3/pRL-TK
Normal GALT promoter	191,400/3,780	151,905/3,000
–119/–116delGTCA promoter	105,400/3,762	84,051/3,000
Control pGL3 (no promoter)	64/3,150	61/3,000

Table 2 Luciferase activity in mouse embryonic fibroblast NIH/3T3 cells. Results represent the average of three independent transfections, each performed in triplicate. Transfections were done with two independently isolated plasmid DNAs. Luminescent units are normalized according to internal standard

Subject analyzed	Luminescent units (absolute values) pGL3/pRL-TK	Luminescent units (normalized values) pGL3/pRL-TK
Normal GALT promoter	9,065/15,080	9,017/15,000
-119/–116delGTCA promoter	5,382/14,100	5,726/15,000
Control pGL3 (no promoter)	70/13,110	80/15,000

able to consider the final GALT-enzyme activity in a given cell to be a consequence of the sequence/structure/function relationship. We suggest now that the –119/–116delGTCA promoter mutation may also contribute to GALT phenotypes seen in some classical galactosemia patients.

Coding sequences of the GALT gene are very evolutionarily conserved among species (Flach et al. 1990; Leslie et al. 1992). Therefore, we also addressed the question of whether the transcriptional machinery utilizing the GTCA motif in the GALT-gene promoter is conserved throughout evolution. As other human genes may have this sequence motif in their promoters, we sought to determine the "relative importance" of that motif. For this reason, we also transfected the same promoters into mouse embryonic fibroblast NIH/3T3 cells. The results are summarized in Table 2.

The human GALT promoter functioned well in mouse cells, although absolute RLU values were not as high as in hepatocytes, indicating a somewhat lower transcription rate in mouse cells. Nevertheless, the difference between normal and deleted-promoter transcriptional activity was again highly significant according to a non-parametric Mann-Whitney test ($P<0.001$), although the difference between promoters was not as high as in human hepatocytes (~1.6-times higher activity of normal promoter). In conclusion, the results obtained from transfection into mouse cells support an important evolutionary role for a GTCA motif in transcriptional regulation.

After we completed our manuscript, Leslie and Bai (2001) published an article dealing with a transcriptional analysis of the mouse GALT promoter in vitro and in transgenic mice. A minimal promoter region of 145 bp was found to function in both human HepG2 cells and in mouse neuroblastoma cells. This minimal promoter contains two regions of homology to the corresponding rat and human GALT genes. One of these highly conserved elements overlaps with the critical GTCA motif discussed in our study. Taken together, these data suggest that the GTCA deletion is responsible for the partial GALT enzyme activity seen with the Duarte allele.

Note. After submission of this manuscript, Elsas et al. (2001) reported the functional analysis of the human GALT promoter in Duarte and LA variant galactosemia.

Acknowledgements. We thank Dr. Jan Bouchal from University Hospital Olomouc for providing NIH/3T3 cells, Dr. Antonin Lojek and Dr. Milan Ciz from the Institute of Biophysics (Brno) for help with luminometry and Dr. Karel Chroust from the Faculty of Sciences (Brno) for help with statistical evaluation. This work was supported by grant Interni grantova agentura (IGA) MZ No 4376-3 and by scientific project CEZ MZ/ 98001 from the Ministry of Health of the Czech Republic.

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