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Biochimica et Biophysica Acta 1740 (2005) 85–90

BIOCHIMICA ET BIOPHYSICA ACTA

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Promoter paper

Partial characterisation of the human GFAT promoter: Effect of single nucleotide polymorphisms on promoter function

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Received 30 April 2004; received in revised form 19 January 2005; accepted 25 January 2005
Available online 19 February 2005

Abstract

The 5′-flanking region of the human glutamine:fructose-6-phosphate amidotransferase (GFAT) gene was characterised as a functional active promoter and the GFAT gene contained multiple transcription start sites. A novel single nucleotide polymorphism identified at position −1412 (G to C) had a functional effect on promoter activity and EMSA revealed specific binding of nuclear proteins to this region.

Keywords: GFAT; Promoter; Hexosamine; Single nucleotide polymorphism; Mesangial cell; Transcriptional

Glutamine:fructose-6-phosphate amidotransferase (GFAT) is the rate-limiting enzyme of the hexosamine biosynthetic pathway (HBP) [1]. The HBP has been suggested to act as a glucose sensor and mediates decreased insulin sensitivity in vitro [1,2] and in vivo [3–6]. In addition, the flux of glucose through the HBP mediates glucose-induced upregulation of the renal expression of transforming growth factor β1 (TGFβ1) [7], a prosclerotic cytokine which is strongly involved in the increased production of extracellular matrix components leading to renal impairment in the diabetic kidney [8]. Thus, the HBP plays a role in the pathogenesis of insulin resistance and the late complication diabetic nephropathy and since the flux of glucose through the HBP is regulated by the amount of GFAT protein [9], it is of importance to study the regulation of GFAT expression. The enzymatic activity of GFAT is modulated by various factors including glucose, insulin [10], and forskolin [11,12] and is negatively regulated by UDP-*N*-acetylglucosamine, the major end product of the HBP [13]. However, there is little data on the transcriptional regulation of this enzyme and although the mouse GFAT promoter has been partially characterised [14], there is no information on the human GFAT promoter or the regulation of its activity. We therefore characterised the human promoter region of human GFAT1, the form present in most tissues, in C2C12 myotubes, a model for the study of insulin resistance and in mesangial cells (MC), which play a key role in the extracellular matrix deposition associated with diabetic nephropathy. In addition, we evaluated the potential regulation of human GFAT1 promoter activity, investigated the presence of potential polymorphisms and analysed the effect of the polymorphisms on the transcriptional activity of this gene.

The 5′-flanking region of human GFAT gene is shown (Fig. 1). To accurately define the promoter region of human GFAT, the 5′ end of the mRNA prepared from human myotubes [16] was precisely determined by the identification of transcription start site(s), using the RNA-ligase mediated-rapid amplification of cDNA ends (RLM-RACE) method (Ambion (Europe) Ltd, Cambridgeshire UK) following the manufacturer′s protocol and using the specific

Abbreviations: EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GFAT, glutamine:fructose-6-phosphate amidotransferase; HBP, hexosamine biosynthetic pathway; MC, mesangial cells; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends; SNP, single nucleotide polymorphism; TGFβ1, transforming growth factor β1

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doi:10.1016/j.bbadis.2005.01.007

Fig. 1. Schematic diagram of the 5′-flanking region of the human GFAT gene. The sequence of the 5′-flanking region of the human GFAT gene is shown with bases in upper case and in bold representing the primers used for the generation of the 5′ deletion luciferase constructs. The bases shown in lower case and in bold represent the primers used for the production of the 3′ deletion constructs. The polymorphisms at positions −1412 and −913 are shown in bold and in larger font.

antisense human GFAT primers 5′-CAGTCTTTG-TAGTTGGT-3′ and 5′-GTTCCATCTGGAGTGTTTGCA-CAGCT-3′. The resulting PCR product was cloned and separate clones were sequenced bidirectionally using a dye terminator cycle sequencing reaction kit (ABI PRISM 310, Applied Biosystems, Foster city, CA, USA) using the primers 5′-CTAGCAAAATAGGCTGTCCC-3′ and 5′-CTTTATGTTTTGGCGTCTTCC-3′. Multiple end points were detected at base pairs +1, +25, +34, +51, and +62 (numbering based on GenBank accession no. NT 033004.2

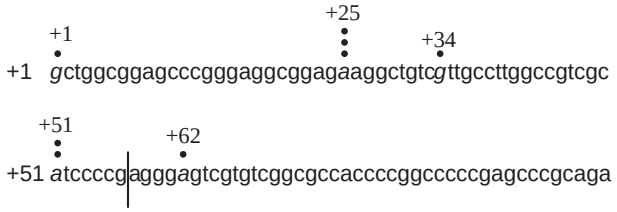

Fig. 2. Determination of the transcription start site of the human GFAT promoter with RLM-RACE. Sequence of human GFAT cDNA based on human GFAT accession number NT 033004.2. ● represents the 5′-terminus of 1 out of 8 clones. The vertical line in the sequence represents the boundary of the published human GFAT cDNA sequence.

(Fig. 2), suggesting the presence of multiple transcription start sites.

To then characterise the regions essential for the transcription of the human GFAT gene, GFAT promoter 5′ or 3′-deleted luciferase constructs, prepared by PCR using sequence specific primers and ligation into pGl3basic (shown schematically in Fig. 3A), were transfected into MC [7] or C2C12 myotubes [15] using FUGENE-6 (Roche) according to the manufacturer's instructions. The renilla luciferase gene-containing pRL-TK or pRL-CMV constructs (Promega, Madison, WI, USA) were co-transfected with the test plasmids to control for transfection efficiency and the cells were harvested 24 h later by the addition of passive lysis buffer (Promega). Firefly and renilla luciferase activities were determined in the cell lysate using the Promega dual luciferase reporter assay system and chemiluminescence was measured using a Magic Lite analyser (Ciba Corning, Fernwald, Germany). Results were normalised for transfection efficiency as relative light units per renilla luciferase activity. In the MC, the deletion of the human GFAT1 promoter in the 5′ region from −2037 to −1152 bp resulted in no change in promoter activity (Fig. 3B). Further deletion from −1152 to −656 bp increased GFAT promoter activity by 1.6 fold (P<0.05) compared to the −2037/+80 construct and the deletion of the region −656 to −283 bp increased transcriptional activity by 1.8 fold (P<0.05) compared to the 2037/+80 construct. Thus the −283 to +80 construct contains the core human GFAT1 promoter and the region from −1152 to −283 bp contains negative regulatory elements. In MC, the 3′ deletion of the human GFAT promoter construct, −283/+80 to −283/+55bp, decreased promoter activity by 1.4 fold and further deletion from +50 to +13 bp also decreased promoter activity by 1.3 fold (P<0.005) compared to the −283/+80 construct (Fig. 3B), suggesting that this region is important for transcriptional activity. In C2C12 myotubes only the 5′ deletion of the GFAT promoter was analysed with all constructs ending at +80 bp (Fig. 3C). The deletion of the −2037/+80 bp construct to −1152 resulted in a modest but significant diminution of luciferase activity (P<0.005). The deletion from −1152 to −656 bp significantly increased luciferase activity by 1.2 fold (P<0.05) compared to the −2037/+80 construct and further deletion of the −656/+80

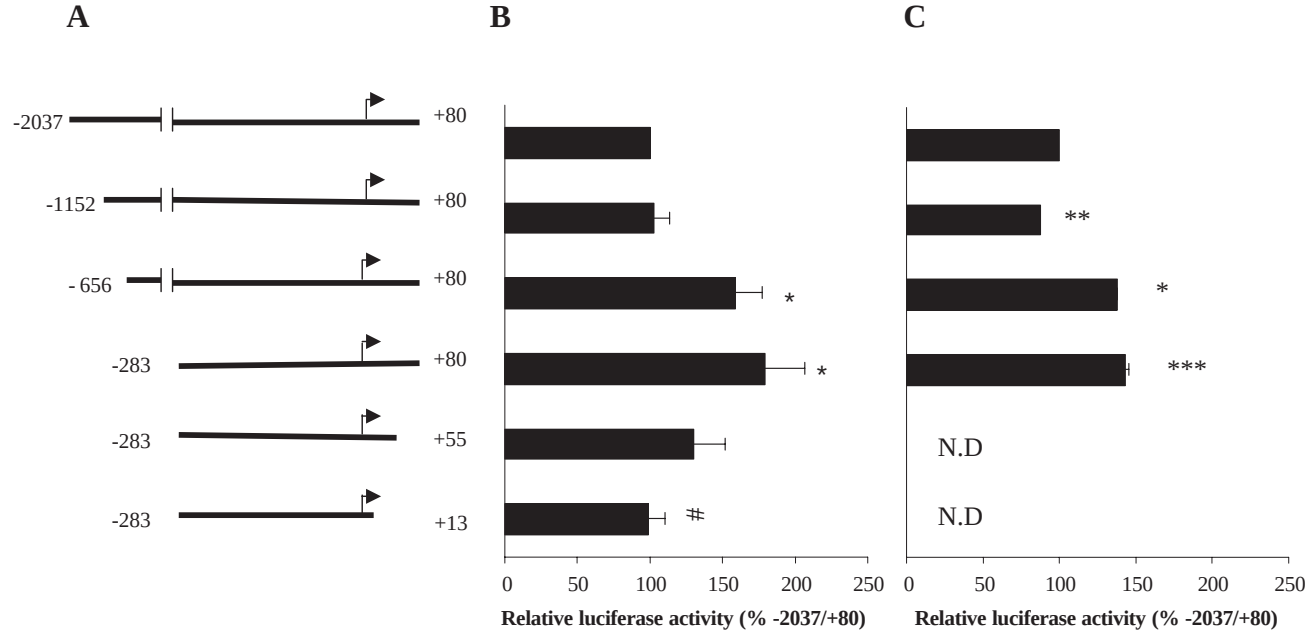

Fig. 3. Deletional analysis of the human GFAT promoter. Schematic representation of varying lengths of the 5′-flanking region of the human GFAT gene (A). The numbers shown next to the diagram represent the nucleotide positions for the 5′ and 3′ ends of each construct respectively. The arrow indicates the transcription start site designated as +1. Deletion constructs were prepared as described, placed upstream of the luciferase reporter gene and transfected into MC (B) or C2C12 myotubes (C) with the pRL-TK plasmid. Luciferase readings were taken 24 h post transfection. Values are normalised to the control vector and the mean values of the 5′ constructs are relative to the largest construct (−2037/+80). The mean values for the 3′ constructs are relative to the −283/+80 construct. Results are the mean±S.E. of three experiments each performed in triplicate. Student's t-test was used to compare differences between groups. Differences were considered significant at a value of P<0.05. (B) *P<0.05, # P<0.005. (C) * P<0.05, **P<0.005, ***P<0.0005.

construct to −283/+80 bp increased luciferase activity 1.4 fold compared to the −2037/+80 construct ($P<0.0005$) (Fig. 3C), again indicating regions of negative regulatory elements between −1182 and −283 bp. The profiles of the transcriptional activities of the deletion constructs in MC and in C2C12 myotubes was found to be generally similar. A different, cell type-specific regulation could be located in the −2037 to −1152 promoter region. Regulatory elements in this region could be activated in C2C12 cells, but not in MC. However, the impact of this region on gene activation is apparently minor and was not further studied.

We then wished to investigate in MC how factors involved in the pathogenesis of diabetic nephropathy might regulate human GFAT promoter activity. Cells transfected with the −2037/+80 construct were stimulated with glucose oxidase (1 mU/ml) (ICN, Eschwege, Germany), to increase the production of hydrogen peroxide in order to simulate oxidative stress, with PMA (0.1 μM) for 30 min to activate PKC, with TNF alpha (1 nM), with angiotensin II (100 nM), high glucose (25 mM), and glucosamine (5 mM) (all Sigma-Aldrich, Munich, Germany) to activate glucose flux through the HBP, with different concentrations of foetal calf serum (0%–2%), with the cAMP elevator forskolin (10 μM) (ICN) and the cAMP analogue 8-bromo-cAMP (1 mM) (Sigma) for 24 h. Increased cAMP accumulation has been shown to have diverging effects on GFAT enzyme activity [11,12]. Here, only the cAMP enhancers affected GFAT promoter activity with a significant 67.8% decrease after treatment with forskolin ($P<0.05$) and a significant 59% decrease with 8-Br-cAMP ($P<0.05$). However, there was no change in GFAT mRNA expression with forskolin treatment (data not shown). This might indicate that mRNA stability was increased by forskolin treatment or that under normal conditions GFAT transcription is not cAMP-regulated, but under experimental conditions, activity may be altered. Recently, we could demonstrate that GFAT mRNA expression is increased by palmitate treatment in human myotubes [16]. This induction was found with 0.25 mM of palmitate after a minimum incubation time of 16 h. However, we found no effect of 0.25 mM palmitate on GFAT1 promoter activity in MC and C2C12 cells using similar conditions (data not shown), perhaps due to the strong effects of palmitate on control vector expression. Another study demonstrates the upregulation of GFAT promoter activity and an increase in mRNA expression upon exposure to angiotensin II and PKC stimulation [17]. However, in that study the mouse GFAT promoter was used and it is possible that species specific differences exist. The data from the present study examining the potential regulation of human GFAT1 promoter activity suggests that the regulation of GFAT activity is not mainly at the transcriptional level. In support of this observation it has been shown that in diabetic rats GFAT activity was significantly decreased in tissues, but there was no effect of the diabetic state on GFAT mRNA expression [18].

GFAT has been demonstrated to mediate insulin resistance and growth factor expression, thus, a genetically determined variability of GFAT may be of importance in both the development of type 2 diabetes and diabetic complications. We therefore investigated the presence of potential polymorphisms in the human GFAT1 5′-flanking region in 100 patients with type 2 diabetes by PCR analysis followed by sequencing using specific primers according to the published GFAT sequence (GenBank accession number NT 033004.2). Two single base substitutions were identified by direct sequencing at position −1412 (C to G) and position −913 (A to G). The single nucleotide polymorphism database includes the −913 A/G SNP but not the −1412 base substitution or any other SNP from the 5′-flanking region of the human GFAT gene −1492/+80. In the group of 100 patients with type 2 diabetes, the CC and GG genotype frequencies of the −1412 SNP were 74% and 3% respectively and the GG and AA genotype frequencies of the −913 position were 12% and 36% respectively. Similar frequencies were found in a larger cohort of 412 non-diabetic subjects [19]. We next investigated the functional significance of the two SNPs by transfection experiments in MC and C2C12 cells using GFAT promoter luciferase constructs spanning −2037/+80 and containing either a C or a G at position −1412 and either a G or an A at position −913, prepared by oligonucleotide directed mutagenesis. In MC, the luciferase expression of the −1412/−913 G/A and −1412/−913 G/G containing constructs were 1.4 fold higher than −1412/−913 C/G and C/A ($P<0.05$) (Fig. 4A), whereas the point substitution from −913 (A to G) had no effect on promoter luciferase activity. In C2C12 myotubes (Fig. 4B), there were no significant differences in luciferase activity between the four SNP containing promoter–luciferase constructs. To further evaluate the putative functional relevance of the SNPs, we determined whether the SNPs affected the binding of nuclear factors to the promoter region. The SNP at −913 is in a GATA transcription binding site, which is abrogated by the G to A base change. Electrophoretic mobility shift assays (EMSAs) were performed with nuclear extracts prepared as described [16] and the complementary oligonucleotides 5′-CTTAATCATATAAC/GCAGCCATAGAA-3′ containing a C or G at position −1412 and 5′-CATGTACGTTTTTG/AATACTACTCTACTC-3′ containing a G or an A at position −913 (changed bases are underlined), which were annealed and end labeled as previously described [16]. EMSA revealed specific binding of MC nuclear proteins to each oligonucleotide, however, there was no detectable differences in the binding of nuclear factors to the point substitutions at either position (Fig. 4C,D). A difference in transcriptional activity between the different polymorphism-containing constructs may not be detected by EMSA, which is a largely qualitative test. Thus, the impact of the SNPs on DNA binding or promoter activation is at least minor or not detectable. However, we found recently an association of the −913 G allele with measures of

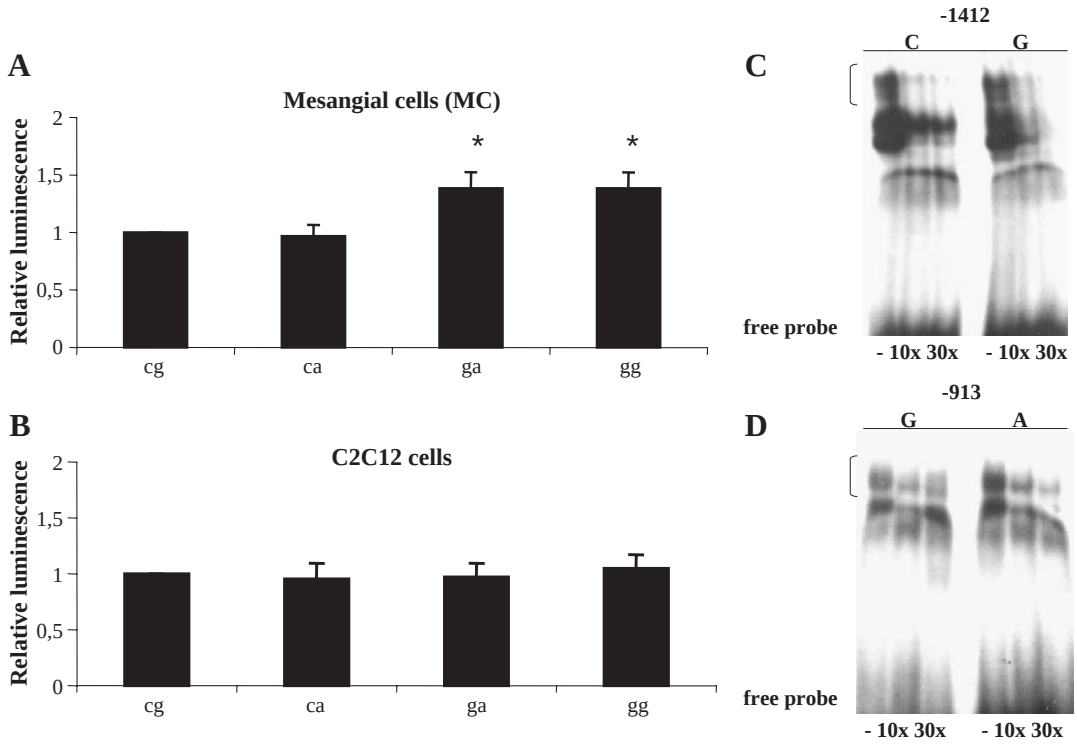

Fig. 4. Effect of the −2037/+80 region of the GFAT promoter on the luciferase expression for the two alleles at the −1412 and −913 positions. Luciferase levels in MC (A) and C2C12 myotubes (B) transfected with human GFAT promoter reporter gene constructs containing −1412C/−913G (CG), −1412C/−913A (CA), −1412G/−913A (GA), and −1412G/−913G (GG) base substitutions and with the control vector pRL-CMV. Luciferase measurements were taken 24 h following transfection. Results were normalised to control plasmid values. The data represents the mean±S.E. of the fold increase compared to the CG construct. *P<0.05 (n=3). ANOVA was used to compare differences between groups. Differences were considered significant at a value of P<0.05. (C,D) EMSA with nuclear extracts of MC incubated with oligonucleotide probes of the human GFAT promoter sequence with either (C) a C or a G at position −1412 or (D) a G or an A at position −913 as indicated. Competition was performed by preincubating nuclear extracts from MC with either a ×10 fold or a ×30 fold excess of unlabeled oligonucleotide. The brackets show specific binding.

obesity, e.g. higher body mass index and body fat content [19].

Therefore, we asked whether the −913 SNP might affect GFAT mRNA expression. We used human myotubes obtained from muscle biopsies of 8 genotyped non-diabetic subjects, 4 are −913 GG carriers, 4 are −913 AA carriers, all are homozygous for the −1412 C allele. We studied GFAT expression under basal conditions and after treatment with 0.5 mM of palmitate for 20 h. GFAT mRNA expression was measured by RT-PCR with the Lite Cycler System (Roche, Mannheim, Germany). We found no difference of basal GFAT mRNA levels (3.48±0.36 vs. 3.27±0.15 relative expression units, −913 G vs. −913 A). The stimulation of the myotubes with palmitate resulted in an approximately 2-fold increase of GFAT expression in the myotubes, both in cells obtained from −913 G carriers and from −913 A carriers (189±7% vs. 203±8%, respectively). The other stimuli tested, 30 mM glucose or 0.5 mM linoleate, did not regulate GFAT mRNA expression. Thus, no effect of the −913 SNP on GFAT mRNA expression levels was detectable. Similar results were obtained when GFAT mRNA levels were related to β-actin mRNA levels (data not shown). Since the −913 SNP is located in the promoter region, any impact on GFAT enzyme activity without an effect on GFAT expression levels appears unlikely. Therefore, an explanation for the observed association of the −913 SNP with measures of obesity remains open. When we tested the effect of the −1412 G allele on GFAT mRNA expression in the human myotubes, no different basal or palmitate-stimulated expression levels were found (data not shown). However, this does not exclude the possibility that a cell type-specific difference, e.g. in MC, might exist. In addition, the association of the novel SNP at −1412 and indices of DN is not currently known. Another recent report describes the screening of the functional regions of the GFAT1 gene and identification of SNPs, only one of which at position −1093, corresponding to the SNP at position −913 found in the present study, is in the promoter region. This report concludes that genetic variation in the GFAT1 gene is not responsible for genetic susceptibility to DN [20]. A possible association with insulin resistance or obesity was not tested in this study. In contrast, common variants in the GFAT2 gene have been shown to associate with type 2 diabetes and diabetic nephropathy [21].

In conclusion, the characterisation of the human GFAT promoter is described and we present evidence that the regulation of GFAT activity is most likely to be post-

transcriptional. The activity of promoter constructs containing a G at the novel −1412 SNP position were found to be higher than with a C at this position.

Acknowledgements

We gratefully acknowledge the support of the Fortüne grant F1284240 (D Burt).

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