Rapid Publication

A Novel Variant of Glutamine

Fructose-6-Phosphate Amidotransferase-1 (GFAT1) mRNA Is Selectively Expressed in Striated Muscle

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Glutamine:fructose-6-phosphate amidotransferase (GFAT) is the rate-limiting enzyme of the hexosamine synthesis pathway. Products of this pathway have been implicated in insulin resistance and glucose toxicity. GFAT1 is ubiquitous, whereas GFAT2 is expressed mainly in the central nervous system. In the course of developing a competitive reverse transcriptase–polymerase chain reaction assay, we noted that GFAT1 cDNA from muscle but not from other tissues migrated as a doublet. Subsequent cloning and sequencing revealed two GFAT1 mRNAs in both mouse and human skeletal muscles. The novel GFAT1 mRNA (GFAT1Alt [muscle selective variant of GFAT1]) is likely a splice variant. It is identical to GFAT1 except for a 48 or 54 bp insert in the mouse and human, respectively, at nucleotide position 686 of the coding sequence, resulting in a 16 or 18 amino acid insert at position 229 of the protein. GFAT1Alt is the predominant GFAT1 mRNA in mouse hindlimb muscle, is weakly expressed in the heart, and is undetectable in the brain, liver, kidney, lung, intestine, spleen, and 3T3-L1 adipocytes. In humans, it is strongly expressed in skeletal muscle but not in the brain. GFAT1 and GFAT1Alt expressed by recombinant adenovirus infection in COS-7 cells displayed robust enzyme activity and kinetic differences. The apparent K_m of GFAT1Alt for fructose-6-phosphate was approximately twofold higher than that of GFAT1, whereas K_i for UDP-*N*-acetylglucosamine was approximately fivefold lower. Muscle insulin resistance is a hallmark and predictor of type 2 diabetes. Variations in the expression of GFAT isoforms in muscle may contribute to predisposition to insulin resistance. *Diabetes* 50: 2419–2424, 2001

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AP, adapter primer; CMP, cytidine monophospho; F-6-P, fructose-6-phosphate; GFAT, glutamine:F-6-P amidotransferase; GFAT1Alt, muscle selective variant of GFAT1; GFP, green fluorescent protein; GlcN-6-P, glucosamine-6-phosphate; huGFAT1, human GFAT1; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; PCR, polymerase chain reaction; UDP-GlcNAc, uridine diphospho-*N*-acetylglucosamine

The hexosamine synthesis pathway is a minor contributor to overall glucose disposal by cells. It is, however, the obligatory source of essential building blocks for the glycosylation of proteins and lipids. Glucose entry into this pathway is regulated by its first and rate-limiting enzyme, glutamine:fructose-6-phosphate amidotransferase (GFAT), which catalyzes the conversion of fructose-6-phosphate (F-6-P) and glutamine into glucosamine-6-phosphate (GlcN-6-P) and glutamate. Subsequent steps yield the major products of the pathway, uridine diphospho-*N*-acetylglucosamine (UDP-GlcNAc), UDP-*N*-acetylgalactosamine, and cytidine monophospho (CMP)-sialic acid. UDP-GlcNAc is the most abundant product and acts as an allosteric feedback inhibitor of GFAT activity in eukaryotic cells (1–3).

GFAT is highly conserved among species. The deduced amino acid sequence of yeast and human GFAT1 exhibit ~55% identity, the *Escherichia coli* and human sequences ~35%, and the yeast and *E. coli* ~39% (3). In contrast to eukaryotic GFAT, the bacterial GFAT activity is not regulated by UDP-GlcNAc (3). Mouse and human GFAT1 amino acid sequences are ~99% identical (4). Recently, a second isoform, GFAT2, was identified in the mouse and human that exhibits ~75% identity at the amino acid level with GFAT1 in both species. The relative expression of GFAT1 and GFAT2 varies among tissues; GFAT2 is preferentially expressed in the central nervous system. GFAT1 and GFAT2 map to different chromosomes and are clearly separate genes (5,6).

Interest in GFAT expression and regulation has been stimulated by evidence suggesting that increased flux through the hexosamine synthesis pathway plays a role in glucose-induced insulin resistance (2,3,7–9). Because insulin resistance is a hallmark of type 2 diabetes, changes in GFAT expression or activity may be associated with the disease. Indeed, increased GFAT activity was observed in skeletal muscle biopsies of patients with poorly controlled type 2 diabetes (10). Transgenic mice, over-expressing GFAT1 in skeletal muscle and fat, develop insulin resistance (11,12). Skeletal muscle is the major site of postprandial insulin resistance in patients with diabetes (13). We identified a new variant of GFAT1 in the mouse and human that appears to be selectively expressed in striated muscle.

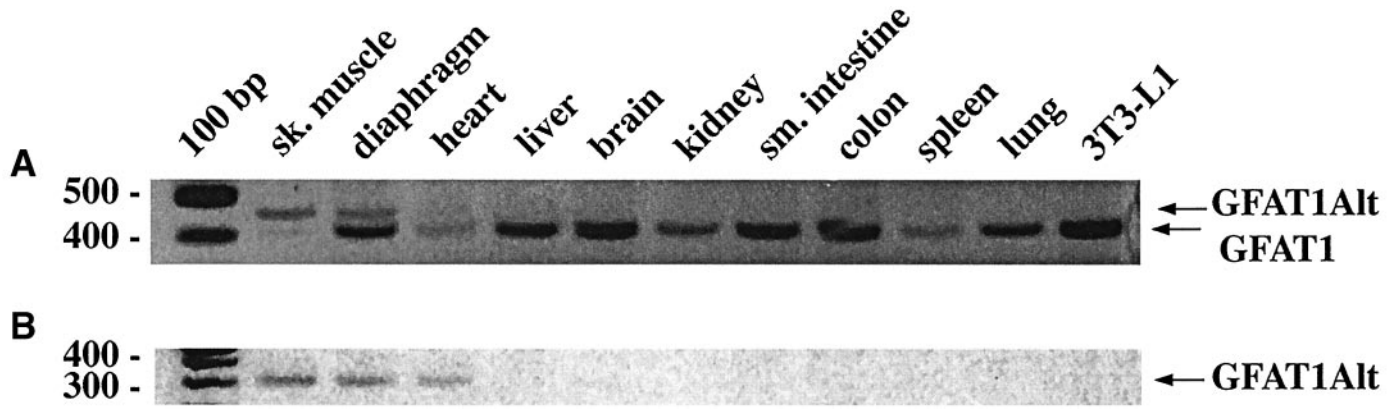

FIG. 1. Expression analysis of GFAT1 and GFAT1Alt in mouse tissues. Gel electrophoresis of RT-PCR products from various mouse total RNAs on a 2.5% Metaphor (FMC Biopolymer, Rockland, ME) gel containing ethidium bromide. *A*: A doublet of 464 and 416 bp representing the variant and GFAT1, respectively, was obtained using the primers mGFAT1-5′ and mGFAT1-3′. *B*: A single band of 306 bp representing the variant was obtained using the primers mGFAT1-5′ and altGFAT1-3′. *Lane 1* in *A* and *B* is a 100-bp ladder (Life Technologies). Documentation was done with a Kodak DC 120 (Rochester, NY), and the image was color-inverted in Adobe Photoshop 5.0.2 (San Jose, CA) for clarity.

RESEARCH DESIGN AND METHODS

RNA isolation. Mouse total RNA was isolated from the following tissues using TRIzol reagent (Life Technologies, Rockville, MD) and a Tissue-Tearor (Biospec Products, Bartlesville, OK): hind-limb skeletal muscle, diaphragm, heart, liver, brain, kidney, small intestine, colon, spleen, and lung. 3T3-L1 fibroblasts were cultured and differentiated into adipocyctes as previously described (14). Total RNA was isolated as above.

Human skeletal muscle total RNA (cat. no. 061015) and human whole brain total RNA (cat. no. 061002) was obtained from Biochain Institute (Hayward, CA).

Reverse transcriptase–polymerase chain reaction, 3′ rapid amplification of cDNA ends, and sequencing. Mouse total RNA (5 μg) was reverse transcribed with Superscript II (Life Technologies) and the GFAT1 specific primer RTGFAT (5′-GCACCTTGATTGTCCTTTTTGTATT-3′) or oligo dT. Polymerase chain reaction (PCR) was carried out using cDNA and AmpliTaq Gold (Applied Biosystems, Foster City, CA) and the following primer pairs (Sigma Genosys, Woodlands, TX):

1) UTRGF-5′ (5′-CGTGTCGCATCCCAGAGTCCT-3′) and mGFAT1-3′(5′-CTGCTGCAACATCATCATCTTCAA-3′);

2) mGFAT1-5′ (5′-TGAAACAGACACAGAAACCATTGCC-3′) and mGFAT1-3′ (Fig. 1*A*), with the following conditions: 95°C for 9 min, 95°C for 40 s, then 62°C for 40 s for 30 cycles followed by 62°C for 10 min (1.625 mmol/l Mg^{2+}, 1X GeneAmp PCR buffer II);

3) mGFAT1-5′ and ALTGF-3′ (5′-TCTTTGCCTCGTTCTGCCTGTGAT-3′) (Fig. 1*B*), with the following conditions: 95°C for 9 min, 95°C for 40 s, then 63°C for 40 s for 30 cycles followed by 63°C for 10 min (1.75 mmol/l Mg^{2+}, 1X GeneAmp PCR buffer II);

4) m617GF-5′ (5′-CTCTCTTGATTGGTGTGCGGAG-3′) and RTGFAT.

To obtain the COOH-terminal end, 3′ rapid amplification of cDNA ends (RACE) (Life Technologies) was conducted using the 3′ RACE adapter primer (AP) (5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′) in the first strand synthesis reaction followed by PCR with the following primer pair:

m1774GF-5′ (5′-ATCATCATGCGAGACCACACTTAT-3′) and (UAP) (5′-$(CUA)_4$GGCCACGCGTCGACTAGTAC-3′).

The 3′ end of GFAT1Alt was verified with the following primer pair:

mGFAT1-5′ and m2696GF-3′ (5′-TACTCTACTGTTACAGATTTGGCAAG-3′).

Human total RNA (5 μg) was reverse transcribed as above, except an oligo dT primer was used. PCR was carried out using the following primer pair (Sigma Genosys):

human GFAT1 (huGFAT1)-5′ (5′-GGAATCATCACCAACTACAAAGAC-3′) and huGFAT1-3′ (5′-AATACTCCACTGCTTTTTCTTCCAC-3′) (Fig. 2*A* and *B*) with the following conditions: 95°C for 9 min, 95°C for 40 s, then 67.7°C decreasing 0.3°C per cycle for 40 s for 20 cycles followed by 95°C for 40 s then 63°C for 40 s + 3 s/cycle for 20 cycles (2.5 mmol/l Mg^{2+}, 1X GeneAmp PCR Gold buffer).

PCR products were subcloned into pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced at the Biotechnology Resource Laboratory of the Medical University of South Carolina using the ABI 377 DNA sequencer (Applied Biosystems) (Figs. 3 and 4).

Genbank accession numbers for GFAT1Alt are AF33476 (mouse) and AF334737 (human).

Construction of adenoviruses. The adenoviral vectors and protocols described by He et al. (15) were used to generate murine GFAT1 and GFAT1Alt adenoviruses. Briefly, PCR was carried out on a plasmid containing the full-length murine GFAT1 (a generous gift from Dr. J.E. Kudlow) using the following primers:

5′-TGCGGCCGCCACCATGTGTGGTATATTTGC and 3′-TGATATCTCACT CTACAGTCACAGATTTTGCA.

A Kozak sequence was incorporated into the *Not*I site (underlined) in the 5′ primer, and an *Eco*RV site (underlined) was incorporated in the 3′ primer. The PCR product was ligated into pCR2.1 (Invitrogen) to generate pGFAT1nrv-2.1 and sequenced. A GFAT1Alt containing plasmid was digested with *Eco*RI and *Hin*dIII (New England Biolabs, Beverly, MA) to release the alternative splice region that was ligated into an *Eco*RI/*Hin*dIII digested pGFAT1nrv-2.1 to generate pAltGFnrv-2.1. These two GFAT1-containing plasmids were digested with *Not*I/*Eco*RV (New England Biolabs), and the GFAT1 clones were directionally ligated into pAdTrack-CMV. Homologous recombination with pAdeasy-1 in *E. coli* BJ5183 cells (Stratagene, La Jolla, CA) was carried out. Recombinants were selected, transformed into *E. coli* XL1-Blue MRF′ (Stratagene), and large-scale plasmid preps were done (Qiagen, Valencia, CA).

Adenovirus-mediated expression of GFAT1 and GFAT1Alt in COS-7 cells, GFAT activity assay. Adenoviruses expressing GFAT1, GFAT1Alt, and an empty adenovirus were generated and amplified in low-passage 293 cells (Microbix Biosystems, Toronto, ON) as described (15). After CsCl gradient purification, adenovirus concentration was determined by limiting-dilution plaque assay. COS-7 cells (ATCC, Manassas, VA) were grown to confluency on 100-mm dishes and were infected with 10^6 to 10^7 pfu/ml of adenovirus overnight at 37°C in DMEM:F12 (1:1), 10% fetal calf serum. Adenovirus was removed, and cells were incubated in fresh media for an additional 24 h before harvest. Cells were washed in phosphate-buffered saline and scraped into 25 mmol/l HEPES, pH 7.5; 5 mmol/l EDTA; 100 mmol/l KCl; 10 mg/ml *N*α-benzoyl-L-arginine ethyl ester, *N*α-p-Tosyl-L-arginine methyl ester, and benzamidine; 10 μg/ml leupeptin and aprotinin; and 1 mmol/l phenylmethylsulfonyl fluoride. Cells were lysed, and cytosolic extracts were prepared by centrifugation at 100,000*g* for 1 h. The resultant supernatants were partially purified by spin-filtration over 1.5–2.0 volumes of Sephadex G-25 and analyzed for GFAT

FIG. 2. Expression analysis of GFAT1 and GFAT1Alt in human skeletal muscle and brain. Gel electrophoresis of RT-PCR products from human skeletal muscle and whole brain total RNAs on a 2.5% Metaphor (FMC) gel containing ethidium bromide. *A*: A doublet of 456 and 402 bp in *lane 2* representing the variant and GFAT1, respectively, was obtained from skeletal muscle using the primers huGFAT1-5′ and huGFAT1-3′. *B*: A single band of 402 bp in *lane 1* from brain was obtained using the same primers and PCR conditions. *Lane 1* in *A* and lane *2* in *B* is a 100-bp ladder (Life Technologies). Documentation was done with a Kodak DC 120, and the image was color-inverted in Adobe Photoshop 5.0.2 for clarity.

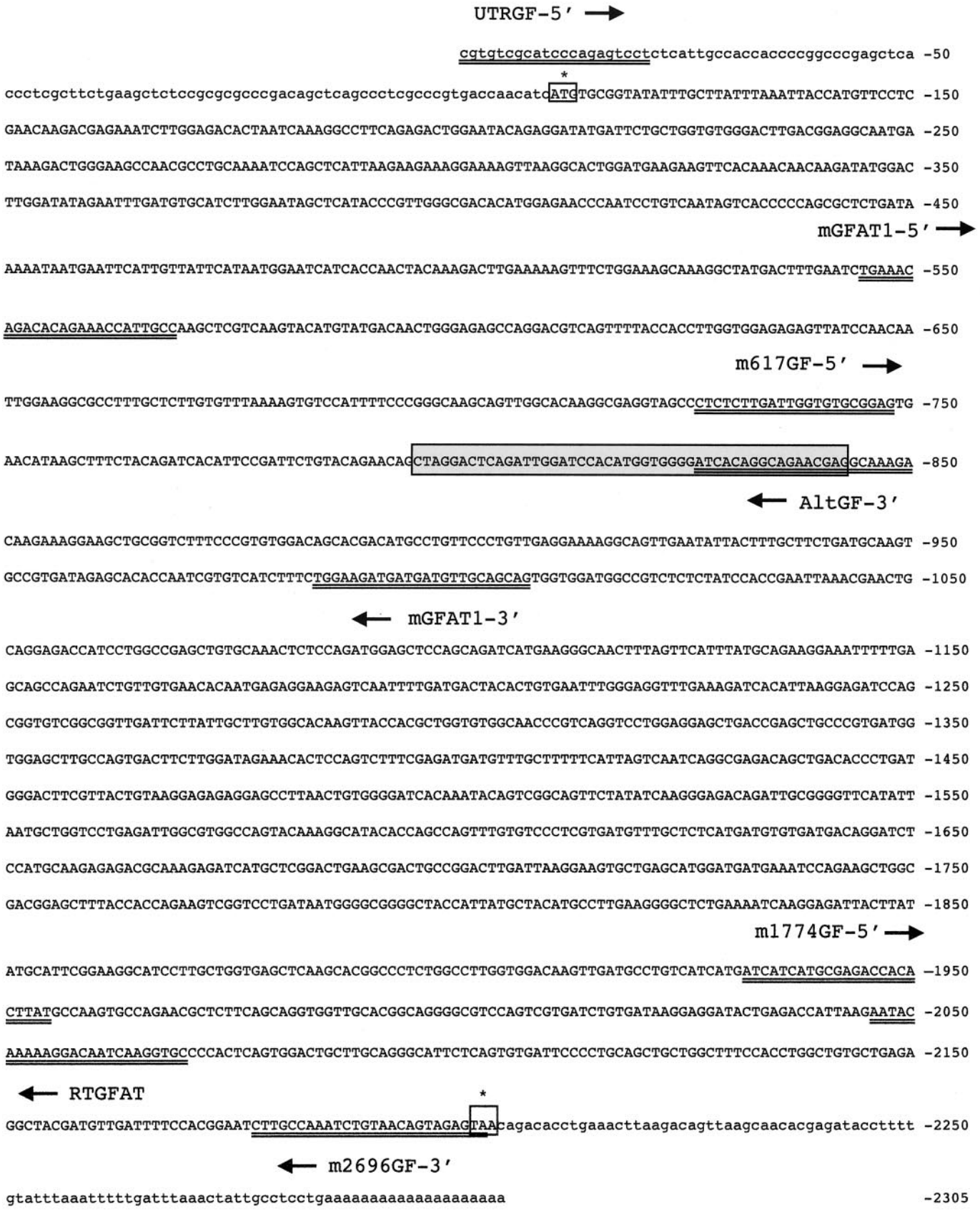

FIG. 3. Nucleic acid sequence of mouse GFAT1Alt. 5′ and 3′ untranslated regions (UTRs) are depicted in small letters. Start and stop codons are shown in boxes with an asterisk. Primer positions are indicated by double underlines with direction indicated by right and left arrows. The insert (48 bp) is shown as a gray box.

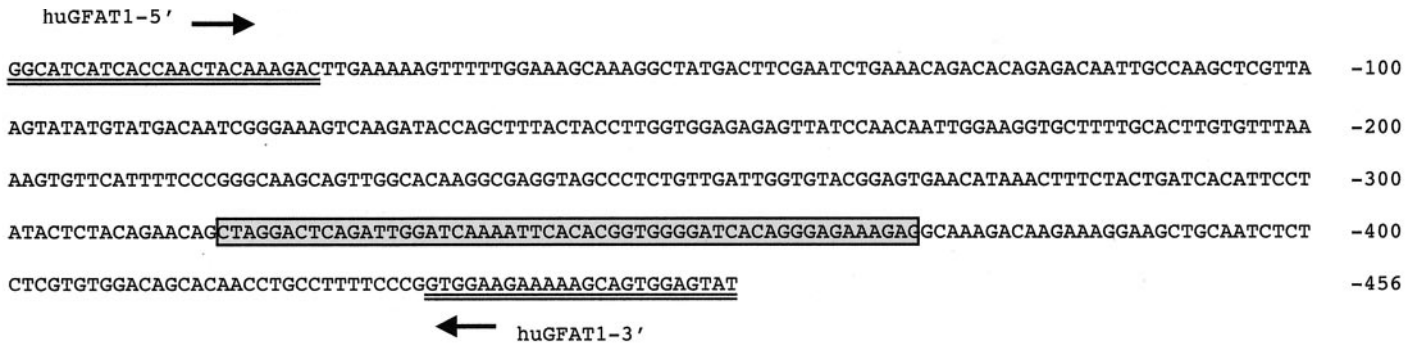

FIG. 4. Nucleic acid sequence of human GFAT1Alt. Primer positions are indicated by double underlines with direction indicated by right and left arrows. The insert (54 bp) is shown as a gray box.

activity as described (7). For kinetic analyses of GFAT1 and GFAT1Alt activities, cytosolic extracts were incubated with varying concentrations of substrates or of the allosteric feed-back inhibitor, UDP-GlcNAc. To assure that different cell extracts were assayed under optimal conditions (i.e., where product generation is lineal with time and enzyme concentration), cytosolic extracts were first assayed in a preliminary experiment for GFAT activity and diluted appropriately for the assays involving kinetic analyses, such that the maximal activity of all samples was similar. Protein concentration of extracts was determined by the Coomassie protein assay (Pierce, Rockford, IL). Green fluorescence was measured spectrofluorometrically by comparison to recombinant enhanced green fluorescent protein (GFP) standards (Clontech, Palo Alto, CA).

Statistical analysis. Means ± SE are shown. Where no SE appears on the graph, it falls within the symbol representing the mean. The significance of the differences between mean was analyzed by two-tailed Student's t test; $P < 0.05$ was considered significant.

RESULTS

RT-PCR. The primer pair mGFAT1-5′/mGFAT1-3′ was designed for use in competitive reverse transcriptase (RT)-PCR studies. In optimization experiments, the gel electrophoresis revealed a doublet when skeletal muscle RNA was used instead of 3T3-LI adipocyte RNA (data not shown). Subsequent sequencing showed the existence of two variants of GFAT1 in mouse skeletal muscle. Using this primer pair and the other various tissue, RNAs demonstrated that the variant (GFAT1Alt) is muscle-specific (Fig. 1*A*). The predominant GFAT1 mRNA in mouse hindlimb muscle is weakly expressed in the heart and is undetectable in the brain, liver, kidney, lung, intestine, spleen, and 3T3-L1 adipocytes. The primer pair mGFAT1-5′/ALTGF-3′ verified this expression (Fig. 1*B*). Likewise, the primer pair huGFAT1-5′/huGFAT1-3′, spanning the same region as in the mouse GFAT1, isolated a human GFAT1Alt mRNA from skeletal muscle that was not expressed in whole brain (Fig. 2*A* and *B*). Overlapping primer pairs were used to obtain the full-length mouse clone that was verified by sequence analysis.

Comparison of mouse and human GFAT1 muscle variants. Except for the insert sequences, the mouse and human GFAT1Alt clones share 100% amino acid sequence identity and 99% nucleotide identity with the previously reported GFAT1 isolates, respectively (data not shown) (mouse accession number U00932 and human accession number M90516). Both mouse and human GFAT1Alt have inserts (48 and 54 bp, respectively) at nucleotide position 686 of the coding sequence (Figs. 3 and 4) that results in a 16 or 18 amino acid insert at position 229 of the protein (data not shown). The mouse and human GFAT1Alt inserts share 94% identity and 11% gap at the nucleotide level and 89% identity and 11% gap at the amino acid level (Fig. 5).

Overexpression of GFAT1 and GFAT1Alt in COS-7 cells. To assess whether GFAT1Alt mRNA codes for a functioning GFAT protein, COS-7 cells were infected with adenoviruses expressing GFAT1 or GFAT1Alt, or "empty" adenovirus. Because the adenovirus contains a GFP expression cassette, examination of plates by fluorescent microscopy easily established that >90% of the cells expressed the adenovirus at the time of harvest. The GFAT activity of the cell extracts was quantified as previously described (7,8,21) under conditions in which both substrates of the enzyme, glutamine, and F-6-P are in excess, and the generation of the product, GlcN-6-P, is lineal with the time and enzyme concentrations. Naïve COS-7 cells or cells infected with the empty adenovirus yielded equal GFAT activity (1.26 ± 0.06 and 1.33 ± 0.12 nmol GlcN-6-P · mg protein^{-1} · min^{-1} in the assay, $n = 3$). Cells infected with the adenovirus expressing GFAT1 or GFAT1Alt expressed 40- to 100-fold greater GFAT activity than the naïve cells (48–137 nmol GlcN-6-P · mg protein^{-1} · min^{-1} in three independent infection experiments. There was no evidence of preferential expression or greater activity of GFAT1 or GFAT1Alt. When GFAT activity was normalized to the GFP concentration in the cell extracts, cells infected with the empty vector generated 10–24 nmol GlcN-6-P · mg GFP^{-1} · min^{-1}, whereas cells infected with adenovirus expressing GFAT1 or GFAT1Alt generated 400–1,400 nmol GlcN-6-P · mg GFP^{-1} · min^{-1} in three independent infections. Again, there were no apparent differences in effectiveness between the two GFAT1 species (data not shown).

Kinetic differences between GFAT1 and GFAT1Alt enzyme activities. The major kinetic differences between extracts from cells overexpressing GFAT1 or GFAT1Alt was a much greater susceptibility of GFAT1Alt to inhibition by UDP-GlcNAc (Fig. 6*A* and *D*). When cell extracts were incubated in the presence of saturating concentrations of F-6-P and glutamine, 50% inhibition of GFAT activity was observed in the presence of ~11 μmol/l UDP-

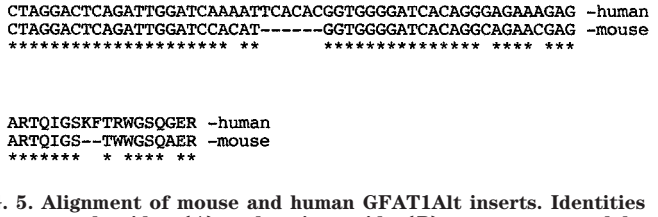

FIG. 5. Alignment of mouse and human GFAT1Alt inserts. Identities between nucleotides (*A*) and amino acids (*B*) are represented by asterisks. Gaps are shown as (–).

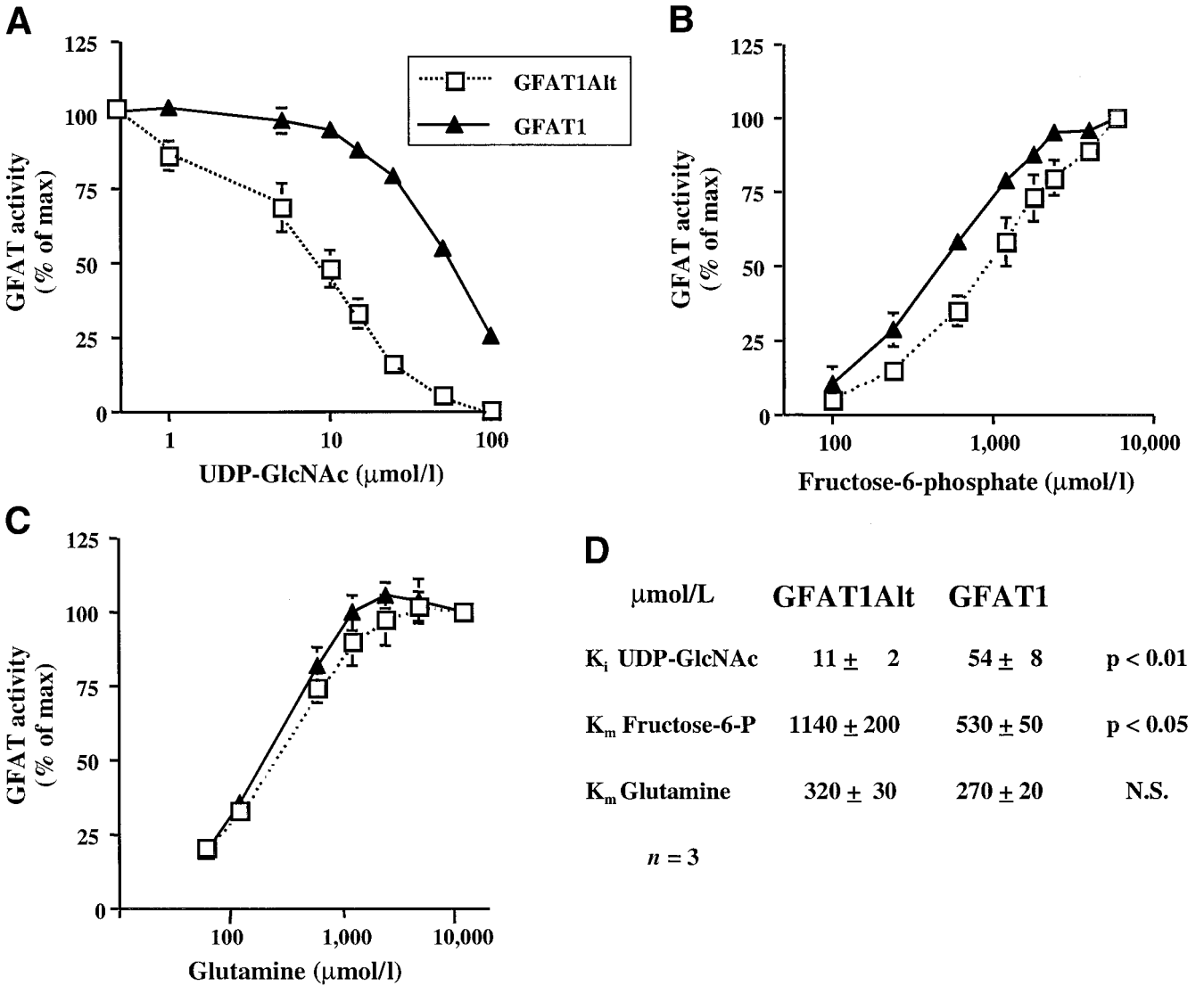

FIG. 6. Comparison of the enzyme kinetics of GFAT1Alt and GFAT1. COS-7 cells were infected with 10^6 to 10^7pfu/ml GFAT1Alt or GFAT1 expressing adenovirus, yielding approximately equal infection efficiencies. Cells were harvested 48 h after infection, and partially purified cytosolic extracts were prepared as described in RESEARCH DESIGN AND METHODS. For kinetic analyses, cell extracts were diluted to contain approximately equal maximal GFAT activity in each assay (161 ± 29 pmol GlcN-6-P · min^{-1} · 300 μl assay $mixture^{-1}$). GFAT activity was measured as previously described (7,8,21). *A*: Extracts were incubated with 2.4 mmol/l F-6-P, 12 mmol/l glutamine, and increasing concentrations of the allosteric inhibitor UDP-GlcNAc. *B*: The concentration of F-6-P was varied in the presence of 12 mmol/l glutamine. *C*: Glutamine concentrations were varied in the presence of 6 mmol/l F-6-P. The data represent means ± SE of three independent infections and analyses. Where no error bars are shown, they fall within the symbol. *D*: Summary of apparent kinetic constants estimated as half-maximal GFAT activity by graphic analyses of three separate experiments.

GlcNAc in extracts overexpressing GFAT1Alt vs. ~54 μmol/l UDP-GlcNAc for GFAT1 ($P < 0.01$, $n = 3$ independent experiments). When cell extracts were incubated with saturating concentrations of glutamine and the concentrations of F-6-P were varied, the affinity of GFAT1Alt for F-6-P appeared lower than that of GFAT1; half maximal activity of GFAT1Alt was observed at a F-6-P concentration of ~1.1 vs. 0.53 mmol/l for GFAT1 ($P < 0.05$, $n = 3$) (Fig. 6*B* and *D*). There was no apparent difference in glutamine kinetics between GFAT1 and GFAT1Alt (Fig. 6*C* and *D*).

DISCUSSION

The muscle-specific variant of GFAT1 mRNA described here differs from the previously reported GFAT1 mRNA only by the presence of a 48 or 54 bp insert in the mouse and human, respectively. Thus, the variant is most likely a result of alternative splicing of GFAT1 in muscle. The genomic sequence of GFAT1 has not yet been published. However, unpublished data from D. McClain's laboratory support the concept that GFAT1Alt is a splice variant. Based on the genomic structures of huGFAT1 proposed by this group (16), the insert in huGFAT1Alt mRNA is located between consensus splice sites separating the proposed boundaries of exons 7 and 8 (D. McClain, personal communication). Tissue-specific splice variants have been previously reported; a well-known example is the insulin receptor, where an alternative splice site around exon 11 results in two isoforms differing by 12 amino acids in the length of the α-chain (17). The relative expression of the two isoforms varies among tissues and affects the insulin-binding kinetics (18).

The crystal structure of GFAT has been determined only

from *E. coli*, and structure-function studies have been performed with GFAT purified from *E. coli* and yeast (19–21). Extrapolating from these findings to mammalian GFAT1, it appears that the insert in the muscle variant is located in the COOH-terminal portion of the glutamine-binding domain that is in close proximity to the F-6-P–binding domain. Kinetic studies of GFAT activity from partially purified tissue extracts revealed that GFAT from mouse and rat skeletal muscle differed from adipocytes, liver, fibroblasts, and numerous other cell lines (22) in that the apparent K_m of the enzyme for F-6-P was higher in skeletal muscle than in other tissues or cell lines. The approximate twofold increase in the apparent K_m for F-6-P observed with GFAT1Alt versus GFAT1 suggests that the selective expression of the former in muscle may contribute to these findings.

There is increasing evidence that accumulation of products of the hexosamine synthesis pathway may contribute to insulin resistance (2,3,7–12) and to the complications of diabetes (23). The earliest defect that can be demonstrated in subjects who are genetically predisposed to type 2 diabetes is insulin resistance of skeletal muscle (24). Increased glucose flux via GFAT increases the intracellular concentrations of UDP-GlcNAc (7–9), which in turn modulates the activity of UDP-GlcNAc:polypeptide β-*N*-acetylglucosaminyl transferase (25). Modification of serine and threonine residues of susceptible proteins by a single O-linked GlcNAc has been extensively demonstrated. The modification often involves known phosphorylation sites on proteins and may thus have functional implications (26). Numerous cytosolic and nuclear proteins can be modified in this manner, including numerous transcriptional regulators, such as *Sp1* (23,26,27), and this may in turn lead to altered gene expression. The markedly increased susceptibility of GFAT1Alt versus GFAT1 to feedback inhibition by UDP-GlcNAc suggests that the former isoform may protect against the development of insulin resistance, under conditions that promote glucose flux via the hexosamine synthesis pathway in muscle. Whether the expression of the GFAT1 isoforms in skeletal muscle is altered in patients who are genetically predisposed to type 2 diabetes warrants further study.

ACKNOWLEDGMENTS

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