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Cloning and characterization of the promoter region of the mouse glutamine:fructose-6-phosphate amidotransferase (GFAT) gene.

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CLONING AND CHARACTERIZATION OF THE PROMOTER REGION OF THE MOUSE GLUTAMINE: FRUCTOSE-6-PHOSPHATE AMIDOTRANSFERASE (GFAT) GENE

by

PETER P. SAYESKI

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biophysics in the Graduate School,The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

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Title Cloning and Characterization of the Promoter Region of the Mouse Glutamine:fructose-6-Phosphate Amidotransferase (GFAT) Gene

Glutamine:fructose-6-phosphate amidotransferase (GFAT) is the rate-limiting enzyme in the hexosamine biosynthetic pathway. This enzyme diverts 2-5% of the fructose-6-phosphate derived from glucose to glucosamine-6-phosphate, using glutamine as the nitrogen donor. Subsequently, glucosamine-6-phosphate is metabolized to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), which serves as the substrate for protein glycosylation. The hexosamine biosynthetic pathway regulates a diverse set of cellular events including glucose-induced-insulin desensitization in adipocytes, glycogen synthase activity, pyruvate kinase activity, and glucose-induced growth factor expression in vascular smooth muscle cells.

To better understand how this pathway mediates such events, we characterized the rate-limiting enzyme, GFAT. In our studies, we cloned the mouse GFAT cDNA and showed that the coding sequence is highly conserved when compared to human GFAT mRNA. However, the mRNA transcripts are processed differently from one species to the next. Further studies cloned the mouse GFAT gene and characterized the promoter region. We found the GFAT promoter lacking a TATA box, but was GC-rich. The transcription start site and 5'-UTR were identified. We found that the ubiquitous transcription factor, Sp-1, mediates basal expression of this gene and provides some evidence as to a more dynamic regulation.

Previous studies have shown that culturing vascular smooth muscle cells in either high glucose or glucosamine induces growth factor gene transcription. Since

GFAT is the rate-limiting enzyme in the conversion of glucose to glucosamine, we examined the ability of both glucose and glucosamine to increase growth factor transcription while GFAT was blocked. Our studies show that, when GFAT is blocked, the ability of glucose to increase growth factor transcription is greatly reduced. Conversely, the ability of glucosamine to increase growth factor transcription appears to act independent of GFAT blockage. These results suggest that the conversion of glucose to glucosamine is required for the glucose effect on growth factor transcription.

The data obtained from these investigations provide new information regarding the molecular characterization of the GFAT gene and its role in mediating glucose-induced growth factor gene transcription in vascular smooth muscle cells.

Abstract Approved by: Committee Chairman J G Cullen

Program Director Gilbert R. Hageman

Date 3/30/96 Dean of Graduate School

DEDICATION

This dissertation is dedicated to my parents, Aida and John Sayeski. Through their daily lives, both have impressed upon me the idea that I strive to be the very best in all that I attempt and never be complacent with anything less. Whenever I have failed, they were always there to show their love, support, and encouragement to stay the course. Any success that I may find is attributable to them, while my failures are of my own design. I hope that, through my own life, I am able to manifest the work ethic and ideas they have impressed upon me.

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Perhaps more than any other, Dr. Gilbert R. Hageman has demonstrated to me the significance of being an excellent scientist, lecturer, and administrator. As a friend and mentor, he challenged me in a wide variety of academic endeavors. He is a person from whom I have gained enormous knowledge and wish to emulate both inside and outside the laboratory.

Finally, I wish to thank my wife Eileen for the love and support she has given me during our years in Birmingham. Without her, life would not be as whole and rich as it is today. For this, I will forever be indebted.

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LIST OF ABBREVIATIONS

bFGF	basic fibroblast growth factor
bp	base pair
DMEM	Dulbecco's modified eagle's medium
DON	6-diazo-5-oxonorleucine
EGF	epidermal growth factor
GFAT	glutamine:fructose-6-phosphate amidotransferase
Glc	glucose
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
GSP	gene specific primer
HIV-1	human immunodeficiency virus-1
Kb	kilo-base
KD	kilo-dalton
L/S	linker scanning mutation
LUC	luciferase
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RASM	rat aortic smooth muscle cell
RLU	relative light units
TGF-α	transforming growth factor-alpha
UDP-GlcNAc	uridine diphosphate-N-acetylglucosamine
UTR	untranslated region (of mRNA)

INTRODUCTION

Glucose is a molecule that is fundamental to the existence of mammalian organisms. Once it is transported inside the cell, it is rapidly converted to glucose-6-phosphate. The metabolic pathways derived from glucose have been studied for nearly a century. The better understood pathways include glycolysis and the synthesis of glycogen. A lesser known pathway is the hexosamine biosynthetic pathway. Entry into the hexosamine biosynthetic pathway is controlled by its rate-limiting enzyme, glutamine:fructose-6-phosphate amidotransferase (GFAT) (Zubay, 1988). This enzyme diverts 2-5% of the fructose-6-phosphate derived from glucose to glucosamine-6-phosphate, using glutamine as the nitrogen donor (Hassell et al., 1986). Subsequently, glucosamine-6-phosphate is metabolized to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), which serves as a substrate for protein glycosylation. The glycosylation of proteins is a post-translational modification and it has been postulated that glycosylation alters protein function in a manner analogous to phosphorylation (Mitchell and Tjian, 1989).

Studies within the past 5 years have shown that the hexosamine biosynthetic pathway and specifically GFAT mediate a diverse set of cellular events. These include glucose-induced-insulin-desensitization in adipocytes (Marshall et al., 1991), glycogen synthase activity (Crook et al., 1993), pyruvate kinase activity (Traxinger and Marshall, 1992), and glucose-induced growth factor expression in vascular smooth muscle cells (McClain et al., 1992). Exactly which metabolites of the hexosamine biosynthetic pathway and the process of how they mediate these effects remains unknown.

Even though only a small fraction of intracellular glucose is shunted through the hexosamine pathway, this does not imply that GFAT is unimportant in cellular physiology. To the contrary, the cDNA sequence encoding GFAT has been conserved through evolution and is found in *Rhizobium leguminosarum* (Surin and Downie, 1988), *Escherichia coli* (Walker et al., 1984), *Saccharomyces cerevisae* (Watzele and Tanner, 1989), and *Homo sapiens* (McKnight et al., 1992). The degree of both nucleic acid and protein homology is striking (McKnight et al., 1992). However, much of the understanding of GFAT has come at the protein level. In eukaryotes for example, but not bacteria, GFAT protein activity is allosterically inhibited by the hexosamine product, UDP-GlcNAc (Kornfeld, 1967; McKnight et al., 1992). Analysis of hexosamine products and their effects on growth factor transcription was done by transiently over-expressing GFAT protein in vascular smooth muscle cells (Daniels et al., 1993). Furthermore, work that demonstrated a coordinated regulation of GFAT activity by insulin, glucose, and glutamine was done using crude cytosolic preparations with enzyme activity determined by spectrophotometric assays (Traxinger and Marshall, 1991). When looking at how GFAT is regulated at the transcriptional level, only several examples exist. These include the regulation of the yeast GFAT promoter by α-pheromones (Watzele and Tanner, 1989) and co-regulation of human GFAT mRNA by epidermal growth factor and glucose (Paterson and Kudlow, 1995).

Because the hexosamine pathway mediates such diverse cellular events and the knowledge of transcriptional regulation of GFAT is limited, we believe that cloning and characterizing the mouse GFAT promoter would be useful in understanding how one pathway mediates such different events.

As stated previously, the hexosamine biosynthetic pathway contributes to the regulation of various cellular events. One such example is glucose-induced growth factor transcription in vascular smooth muscle cells (McClain et al., 1992). Previous studies have localized transforming growth factor-α (TGF-α) and basic fibroblast

growth factor (bFGF) to vascular smooth muscle cells (Mueller et al., 1990; Klagsburn and Edelman, 1989; Lindner at al., 1991). These proteins are known to induce mitogenic stimulation of endothelial cells as well as have acute vasoactive effects in vascular smooth muscle cells. Furthermore, culturing these same cells in supra physiological concentrations of glucose leads to an increase in the transcription of these growth factors (McClain et al., 1992).

When the glucose metabolite, glucosamine, is added to the extracellular media of primary cultures of vascular smooth muscle cells, it mimics glucose in stimulating growth factor transcription, but at lower concentrations and with a greater effect on mRNA accumulation. Glucosamine, once it enters the cell, is converted to glucosamine-6-phosphate and enters the hexosamine pathway downstream of GFAT. In a similar manner, when the yeast GFAT cDNA was transiently over-expressed in rat aortic smooth muscle cells, the glucose induction of TGF-α expression was augmented two-fold when compared to controls (Daniels et al., 1993). Both extracellular glucosamine and transient over-expression of GFAT cDNA increase the intracellular pool of UDP-GlcNAc, the product of the hexosamine pathway (Bekesi and Winzler, 1969; Daniels et al., 1993). These data have lead some to hypothesize that the glucose-induced growth factor transcription in vascular smooth muscle cells is mediated through GFAT. However, since glucose has many metabolic fates, it remains possible that the phenomena is transduced through multiple pathways.

The critical question therefore becomes, is the conversion of glucose to glucosamine required for the transcriptional stimulation of TGF-α expression in vascular smooth muscle cells? To address this issue, we examined glucoregulation of TGF-α expression under conditions where GFAT is blocked.

The physiological relevance of these observations is to the diabetic state. It is known that diabetics suffer from a higher incidence of retinopathy, nephropathy, and vascular disease. Recent work has linked these pathologies to hyperglycemia per se

(Lorenzi, 1992). How glucose mediates these deleterious effects is believed to be multi-factorial and includes a combination of ischemia, hypoxia, lipidemia, growth factor expression, and abnormal Ca^{+2} regulation (Kohner et al., 1995). The ability to examine only the growth factor component in this equation may yield some understanding as to the overall pathology of vascular complications in diabetes.

Objective of Thesis-

The studies presented in this thesis are aimed at answering two separate issues: 1) how is the GFAT gene regulated at the transcriptional level and 2) does GFAT mediate the glucose-induced increase in growth factor transcription seen in vascular smooth muscle cells? To answer the first question, we present data that further characterize the GFAT gene. We cloned the murine GFAT cDNA and subsequently examined mammalian mRNA transcripts from multiple sources. In addition, we have cloned the murine GFAT promoter region and defined the transcription start site. We have characterized a basal promoter element within the GFAT gene and present some data that suggest a more dynamic transcriptional regulation mechanism for this gene. Furthermore, we confirm these promoter elements through functional assays. In addressing the second issue, we present data that demonstrate that, when GFAT is blocked, the ability of glucose to increase growth factor transcription is greatly reduced. In contrast, glucosamine is able to increase growth factor transcription to similar levels in a manner that is independent of GFAT activity. We present a novel strategy for generating high levels of anti-sense mRNA and we further characterize an antibody that serves as a useful tool in measuring products of the hexosamine biosynthetic pathway.

Collectively, these studies shed some light on the molecular regulation of the GFAT gene and in turn how this gene transduces carbohydrate induction of growth factor transcription in vascular smooth muscle cells.

MURINE GLUTAMINE:FRUCTOSE-6-PHOSPHATE AMIDOTRANSFERASE-ENCODING cDNA SEQUENCE

by

PETER P. SAYESKI, ANDREW J. PATERSON, and JEFFREY E. KUDLOW

Gene 140: 289-290, 1994

ABSTRACT

Glutamine:fructose-6-phosphate amidotransferase (GFAT) is the rate-limiting enzyme in hexosamine synthesis and has been implicated in the control of growth factor gene expression. We cloned a mouse cDNA that is 91% homologous to the human sequence. The deduced amino acid sequence shows 98.6% identity to human GFAT. The cDNA is derived from a 7-kb mRNA in the mouse, while there are multiple-sized human mRNAs.

MATERIALS AND METHODS

PCR Cloning-

The mouse GFAT cDNA was obtained in two fragments using a PCR cloning strategy. A 1.4-Kb fragment representing the 5'-end of the cDNA was generated using primer 1 (5'-ATGTGTGGTATATTTGCTTAC-3') and primer 3 (5'-TCAGGACCAGCATTAATATGAAC-3') with *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN). The final conditions were 25mM Taps-HCl, pH 9.6, 50mM KCl, 2mM $MgCl_2$, 1mM DTT, 0.2mM each dATP, dCTP, dGTP, and dTTP, and 2.5U *Taq* DNA polymerase. The temperature profile was 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min for 34 cycles. These primers were engineered with BamHI restriction sites for convenient subcloning. A unique HindIII site was found at +646 relative to the ATG translation codon. Primers 2 (5'-CTCTCTTGATTGGTGTGC-3') and 4 (5'-TCACTCTACAGTCACAGATTTGGC-3') were then designed to amplify the 3'-end of the cDNA through the HindIII site. The reaction conditions were the same, but the profile was 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min for 42 cycles. Primer 4 was also designed with a unique BamHI site and both PCR products were cloned separately in pT7T3-18U (Pharmacia, Piscataway, NJ), which had been cut at the HindIII and BamHI sites. After large scale preparations, the 5' fragment (0.65 Kb) was cut out with BamHI and HindIII while the 3' fragment (1.4 Kb) was cut out with HindIII and SmaI. Both fragments were gel purified and ligated at the HindIII

site as described by the manufacturer (Promega, Madison, WI) using T4 DNA ligase. The ligated DNA was ethanol precipitated and cut with BamHI. The products were separated on an agarose gel and the 2.05-Kb band was eluted and cloned into pT7T3-18U, which had been cut with BamHI and alkaline phosphatase treated.

Sequencing and Analysis-

DNA sequence was obtained using Sequenase 2.0 (USB, Cleveland, OH). Reactions were extended through points of overlap as indicated on the accompanying physical map. All unresolved regions were resequenced either in the opposite direction or with the use of dITP. Analysis was conducted on an IBM clone using the Microgenie software.

Construction of Physical Map-

The plasmid containing the full-length mouse GFAT cDNA insert was cut with various restriction endonucleases to determine a physical map. The principal restriction sites are indicated. Six fragments were subcloned into pT7T3 and sequenced on both strands using the appropriate plasmid primers.

Northern Blot Analyis-

Total RNA was extracted from the indicated cell lines or tissue using the guanidine thiocyanate protocol (1). A Northern blot containing 20ug of total RNA from each source was run on a 1% agarose-formaldehyde containing gel for 3 hours at 120 volts. The RNA was transferred onto a nylon membrane (Nytran, Schleicher & Schuell, Keene, NH) and subsequently cross-linked with exposure to UV 254nm for 4 minutes. Hybridizations were carried out using a 356bp mouse cDNA fragment (+1 to +356 relative to the ATG translation codon), which was ^{32}P labelled by the random hexamer method and filled with Klenow (2). The prehybridization solution contained 50% formamide, 5X SSC, 5X Denhardts, 0.1% SDS, and 0.2mg/ml herring sperm DNA. The hybridization solution was identical to prehybridization except for 2.5X Denhardts. The probe was at a concentration of 1.0ng/ml of hybridization solution.

After overnight hybridization at 42°C, the membrane was washed in low stringent solution several times and at high stringency with 0.2X SSC/ 1.0% SDS at 60°C for 60 minutes.

INTRODUCTION AND RESULTS

GFAT, an enzyme catalyzing the formation of glucosamine-6-phosphate, also plays a key role in the induction of insulin resistance by glucose (3) and the stimulation of growth factor gene transcription by glucose in vascular smooth muscle cells (4,5). The complete coding sequence for murine GFAT is shown in Figure 1.

cDNA was prepared from NIH-3T3 cell poly $(A)^+$ RNA and amplified by the polymerase chain reaction using primers 1 and 3 (Methods). These primer sequences were derived from the human GFAT sequence (6) and resulted in the amplification of a 1.4-Kb cDNA representing the 5'-end of the coding sequence. This segment was cloned into a plasmid, a restriction map was generated, and the resultant subcloned restriction fragments were sequenced. To obtain the 3'-end of the coding sequence, primer 2 (Methods) corresponding to the mouse GFAT sequence upstream of a unique, internal HindIII site located at nucleotide +646 was used in combination with primer 4 (Methods) which was based on the human sequence. We then cloned the 1.4 Kb 3'-end of the coding sequence by PCR. This strategy allowed reconstruction of the entire mouse coding sequence by ligation of the two PCR products at the unique HindIII site. The major restriction sites and primers used to sequence the product are shown in Figure 2.

The cDNA encodes a protein of 681 amino acids. Homology with the human sequence is 91% at the nucleotide level and 98.6% at the amino acid level. Those nine amino acids differing from the human sequence are in bold type.

Northern blot analysis of human and mouse mRNA was performed using the mouse GFAT cDNA as a probe (Figure 3). In the human cells, which were derived from tumors (1. melanoma; 2. hepatoma; 3. breast cancer), several hybridizing mRNA

Figure 1: cDNA of mouse GFAT and derived amino acid sequence. Nucleotide position is indicated by the numbers on the left side of the sequence and amino acid position is indicated by the right hand numbers. The bold type amino acids indicate those that differ from the human sequence. The underlined sequence corresponds to the translation initiation primer and the complementary sequence for the translation termination primer. Genbank accession number U00932.

Mouse GFAT

```
   1 ATG TGT GGT ATA TTT GCT TAC TTA AAT TAC CAT GTT CCT CGA ACA AGA CGA GAA ATC TTG GAG ACA CTA ATC AAA GGC CTT CAG AGA CTG GAA TAC AGA GGA
     M  C  G  I  F  A  Y  L  N  Y  H  V  P  R  T  R  R  E  I  L  E  T  L  I  K  G  L  Q  R  L  E  Y  R  G    34

 103 TAT GAT TCT GCT GGT GTG GGA CTT GAC GGA GGC AAT GAT AAA GAC TGG GAA GCC AAC GCC TGC AAA ATC CAG CTC ATT AAG AAG AAA GGA AAA GTT AAG GCA
     Y  D  S  A  G  V  G  L  D  G  G  N  D  X  D  W  E  A  N  A  C  K  I  Q  L  I  K  K  K  G  K  V  K  A    68

 205 CTG GAT GAA GAA GTT CAC AAA CAA CAA GAT ATG GAC TTG GAT ATA GAA TTT GAT GTG CAT CTT GGA ATA GCT CAT ACC CGT TGG GCG ACA CAT GGA GAA CCC
     L  D  E  E  V  H  K  Q  Q  D  M  D  L  D  I  E  F  D  V  H  L  G  I  A  H  T  R  W  A  T  H  G  E  P   102

 307 AAT CCT GTC AAT AGT CAC CCC CAG CGC TCT GAT AAA AAT AAT GAA TTC ATT GTT ATT CAT AAT GGA ATC ATC ACC AAC TAC AAA GAC TTG AAA AAG TTT CTG
     N  P  V  N  S  H  P  Q  R  S  D  K  N  N  E  F  I  V  I  H  N  G  I  I  T  N  Y  K  D  L  K  K  F  L   136

 409 GAA AGC AAA GGC TAT GAC TTT GAA TCT GAA ACA GAC ACA GAA ACC ATT GCC AAG CTC GTC AAG TAC ATG TAT GAC AAC TGG GAG AGC CAG GAC GTC AGT TTT
     E  S  K  G  Y  D  F  E  S  E  T  D  T  E  T  I  A  K  L  V  K  Y  M  Y  D  N  W  E  S  Q  D  V  S  F   170

 511 ACC ACC TTG GTG GAG AGA GTT ATC CAA CAA TTG GAA GGC GCC TTT GCT CTT GTG TTT AAA AGT GTC CAT TTC CCC GGG CAA GCA GTT GGC ACA AGG CGA GGT
     T  T  L  V  E  R  V  I  Q  Q  L  E  G  A  F  A  L  V  F  K  S  V  H  F  P  G  Q  A  V  G  T  R  R  G   204

 613 AGC CCT CTC TTG ATT GGT GTG CGG AGT GAA CAT AAG CTT TCT ACA GAT CAC ATT CCG ATT CTG TAC AGA ACA GGC AAA GAC AAG AAA GGA AGC TGC GGT CTT
     S  P  L  L  I  G  V  R  S  E  H  K  L  S  T  D  H  I  P  I  L  Y  R  T  G  K  D  K  K  G  S  C  G  L   238

 715 TCC CGT GTG GAC AGC ACG ACA TGC CTG TTC CCT GTT GAG GAA AAG GCA GTT GAA TAT TAC TTT GCT TCT GAT GCA AGT GCC GTG ATA GAG CAC ACC AAT CGT
     S  R  V  D  S  T  T  C  L  F  P  V  E  E  K  A  V  E  Y  Y  F  A  S  D  A  S  A  V  I  E  H  T  N  R   272

 817 GTC ATC TTT CTG GAA GAT GAT GAT GTT GCA GCA GTG GTG GAT GGC CGT CTG TCT ATC CAC CGA ATT AAA CGA ACT GCA GGA GAC CAT CCT GGC CGA GCT GTG
     V  I  F  L  E  D  D  D  V  A  A  V  V  D  G  R  L  S  I  H  R  I  K  R  T  A  G  D  H  P  G  R  A  V   306

 919 CAA ACT CTC CAG ATG GAG CTC CAG CAG ATC ATG AAG GGC AAC TTT AGT TCA TTT ATG CAG AAG GAA ATT TTT GAG CAG CCA GAA TCT GTT GTG AAC ACA ATG
     Q  T  L  Q  M  E  L  Q  Q  I  M  K  G  N  F  S  S  F  M  Q  K  E  I  F  E  Q  P  E  S  V  V  N  T  M   340

1021 AGA GGA AGA GTC AAT TTT GAT GAC TAC ACT GTG AAT TTG GGA GGT TTG AAG GAT CAC ATA AAG GAG ATC CAG CGT TGT CGG CGG TTG ATT CTT ATT GCT TGT
     R  G  R  V  N  F  D  D  Y  T  V  N  L  G  G  L  K  D  H  I  K  E  I  Q  R  C  R  R  L  I  L  I  A  C   374

1123 GGC ACA AGT TAC CAC GCT GGT GTG GCA ACC CGT CAG GTC CTG GAG GAG CTG ACC GAG CTG CCC GTG ATG GTG GAG CTT GCC AGT GAC TTC TTG GAT AGA AAC
     G  T  S  Y  H  A  G  V  A  T  R  Q  V  L  E  E  L  T  E  L  P  V  M  V  E  L  A  S  D  F  L  D  R  N   408

1225 ACT CCA GTC TTT CGA GAT GAT GTT TGC TTT TTC ATT AGT CAA TCA GGC GAG ACA GCT GAC ACC CTG ATG GGA CTT CGT TAC TGT AAG GAG AGA GGA GCC TTA
     T  P  V  F  R  D  D  V  C  F  F  I  S  Q  S  G  E  T  A  D  T  L  M  G  L  R  Y  C  K  E  R  G  A  L   442

1327 ACT GTG GGG ATC ACA AAT ACA GTC GGC AGT TCT ATA TCA AGG GAG ACA GAT TGC GGG GTT CAT ATT AAT GCT GGT CCT GAG ATT GGC GTG GCC AGT ACA AAG
     T  V  G  I  T  N  T  V  G  S  S  I  S  R  E  T  D  C  G  V  H  I  N  A  G  P  E  I  G  V  A  S  T  K   476

1429 GCA TAC ACC AGC CAG TTT GTG TCC CTC GTG ATG TTT GCT CTC ATG ATG TGT GAT GAC AGG ATC TCC ATG CAA GAG AGA CGC AAA GAG ATC ATG CTC GGA CTG
     A  Y  T  S  Q  F  V  S  L  V  M  F  A  L  M  M  C  D  D  R  I  S  M  Q  E  R  R  K  E  I  M  L  G  L   510

1531 AAG CGA CTG CCG GAC TTG ATT AAG GAG GTG CTG AGC ATG GAT GAT GAA ATC CAG AAG CTA GCA ACG GAG CTT TAC CAC CAG AAG TCG GTC CTG ATA ATG GGG
     K  R  L  P  D  L  I  K  E  V  L  S  M  D  D  E  I  Q  K  L  A  T  E  L  Y  H  Q  K  S  V  L  I  M  G   544

1633 CGG GGC TAC CAT TAT GCT ACA TGC CTT GAA GGG GCT CTG AAA ATC AAG GAG ATT ACT TAT ATG CAT TCG GAA GGC ATC CTT GCT GGT GAG CTC AAG CAC GGC
     R  G  Y  H  Y  A  T  C  L  E  G  A  L  K  I  K  E  I  T  Y  M  H  S  E  G  I  L  A  G  E  L  K  H  G   578

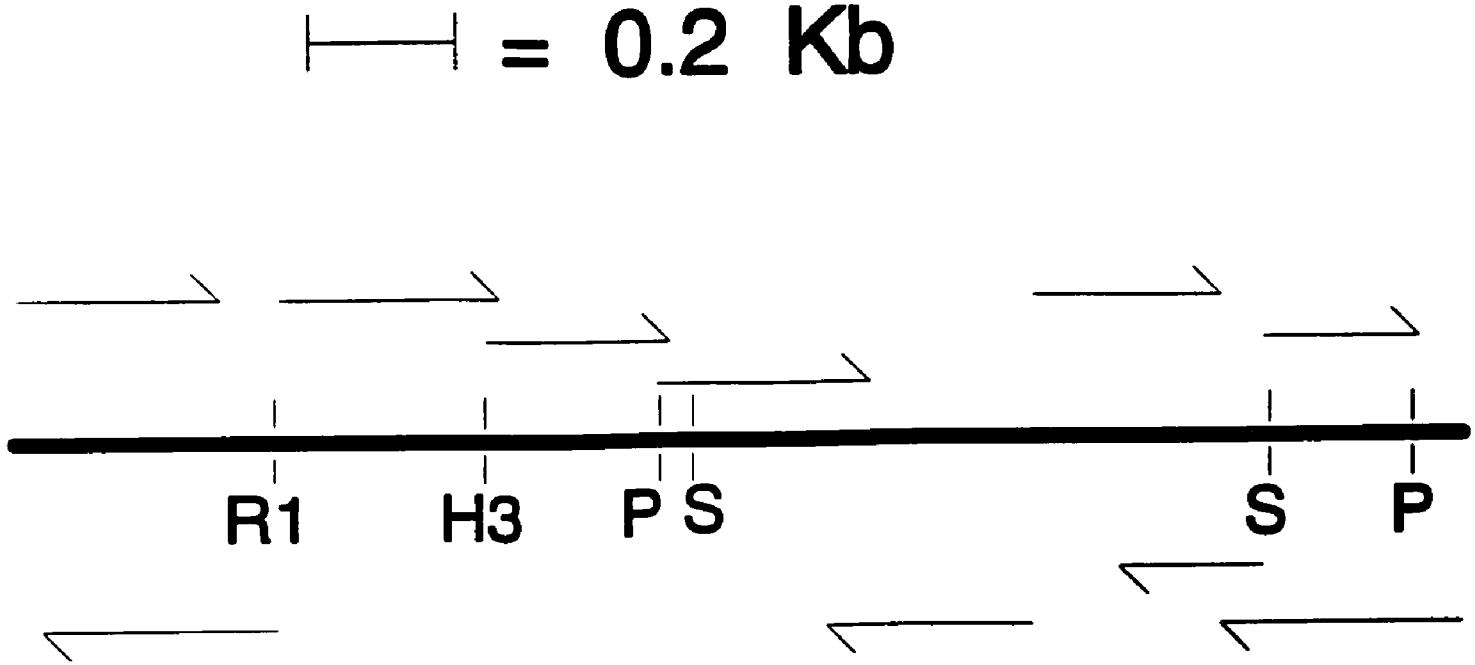
1735 CCT CTC GCT TTG GTG GAC AAG TTG ATG CCT GTC ATC ATG ATC ATC ATG CGA GAC CAC ACT TAT GCC AAG TGC CAG AAC GCT CTT CAG CAG GTG GTT GCA CGG
     P  L  A  L  V  D  K  L  M  P  V  I  M  I  I  M  R  D  H  T  Y  A  K  C  Q  N  A  L  Q  Q  V  V  A  R   612

1837 CAG GGG CGT CCA GTC GTG ATC TGT GAT AAG GAG GAT ACT GAG ACC ATT AAG AAT ACA AAA AGG ACA ATC AAG GTG CCC CAC TCA GTG GAC TGC TTG CAG GGC
     Q  G  R  P  V  V  I  C  D  K  E  D  T  E  T  I  K  N  T  K  R  T  I  K  V  P  H  S  V  D  C  L  Q  G   646

1939 ATT CTC AGT GTG ATT CCC CTG CAG CTG CTG GCT TTC CAC CTG GCT GTG CTG AGA GGC TAC GAT GTT GAT TTT CCA CGG AAT CTT GCC AAA TCT GTG ACT GTA
     I  L  S  V  I  P  L  Q  L  L  A  F  H  L  A  V  L  R  G  Y  D  V  D  F  P  R  N  L  A  K  S  V  T  V   680

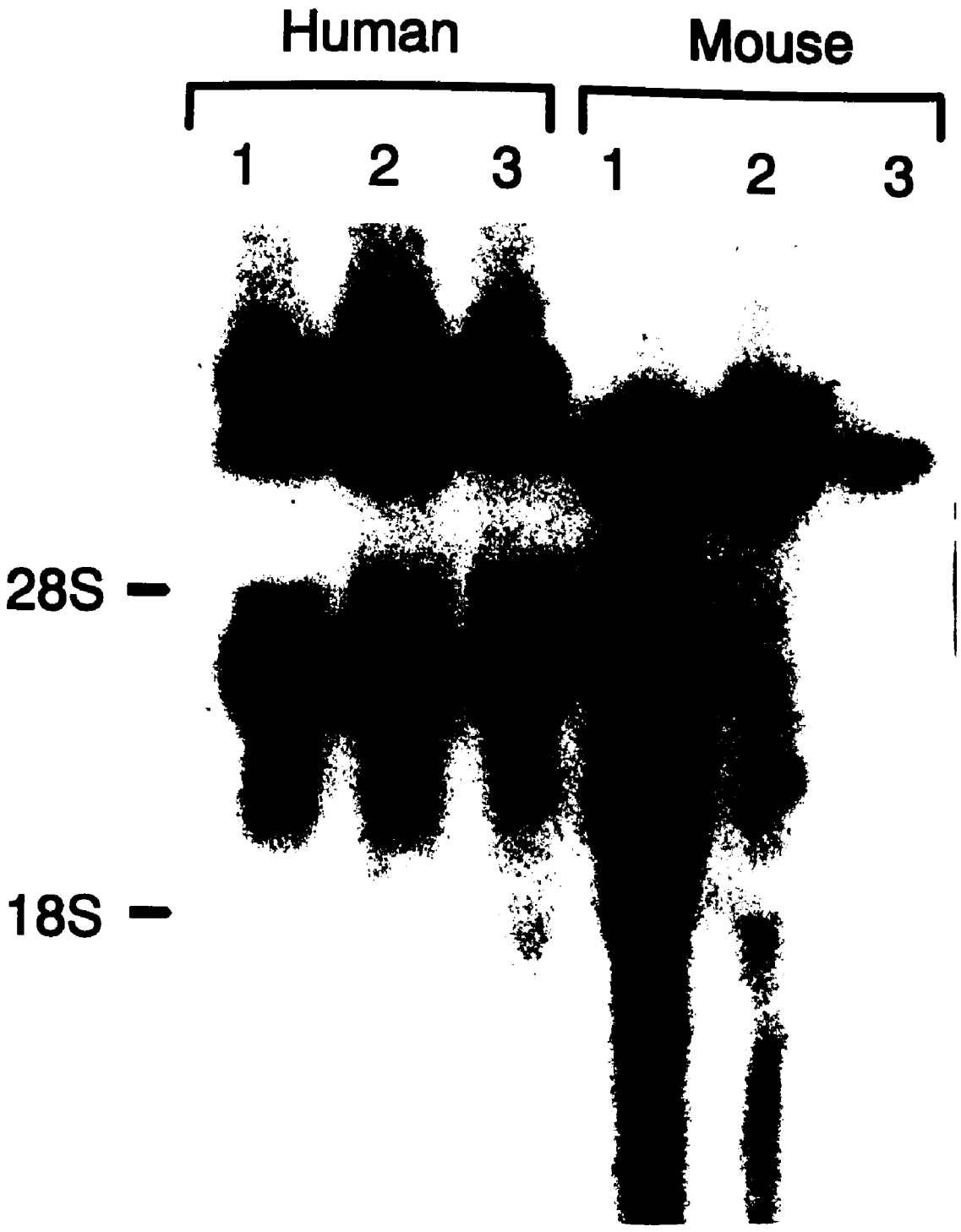
2041 GAG TGA
     E
```

Figure 2: Restriction map and sequencing strategy of the mouse GFAT cDNA. The major restriction sites of the mouse GFAT cDNA are shown and the primers used to obtain the sequence are indicated.



R1=EcoRI
H3=HindIII
P=PstI
S=SacI

Figure 3: Northern blot analysis of human and mouse mRNA using the mouse GFAT cDNA as a probe. The human cells are as follows: 1. HA-A melanoma cells; 2. HepG2 cells; 3. MDA-468 cells. The mouse cells are as follows: 1. 3T3 cells; 2. normal brain; 3. normal kidney.


Human
Mouse
1
2
3
1
2
3
28S
18S

species were evident ranging in size from about 2.5 to 9 Kb. However, in mouse cells (1. 3T3; 2. normal brain; 3. normal kidney), a predominant band of approximately 7 Kb was evident.

ACKNOWLEDGMENTS

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CLONING AND CHARACTERIZATION OF THE PROMOTER REGION OF THE MOUSE GLUTAMINE:FRUCTOSE-6-PHOSPHATE AMIDOTRANSFERASE (GFAT) GENE

by

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ABSTRACT

To better understand the molecular mechanisms that direct the expression of the gene encoding for the glutamine:fructose-6-phosphate amidotransferase (GFAT) enzyme, the 5' flanking region of the mouse GFAT gene was cloned and the promoter region was characterized. It lacks a canonical TATA box and has several GC boxes within a highly GC-rich region. Primer extension analysis by the 5'-rapid amplification of cDNA ends mapped the transcription initiation site to a guanine residue 149 bases upstream of the ATG translation initiation codon. The 30 base pair region immediately 5' to the start site showed a GC content of 84% and putative binding sites for the transcription factors Sp-1 and AP-2. Electrophoretic mobility shift assays of the region spanning from -31 to +13 found that Sp-1, but not AP-2, binds to this region. DNAse I footprint analysis localized Sp-1 protection to the -17 to -7 region with additional protection at the -90 to -84 region. Site directed mutagenesis of these two regions demonstrated functional significance as luciferase activity was greatly reduced when compared to controls. These results shed some understanding into the mechanisms regulating this gene.

INTRODUCTION

Glutamine:fructose-6-phosphate amidotransferase (GFAT) is the rate-limiting enzyme in the hexosamine biosynthetic pathway (1). This enzyme diverts 2-5% of the fructose-6-phosphate derived from glucose to glucosamine-6-phosphate, using glutamine as the nitrogen donor (2). Subsequently, glucosamine-6-phosphate is metabolized to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), which serves as a substrate for protein glycosylation. Previous studies have shown that the hexosamine biosynthetic pathway regulates a diverse set of cellular events, including glucose-induced-insulin-desensitization in adipocytes (3), glycogen synthase activity (4), pyruvate kinase activity (5), and glucose-induced growth factor expression in vascular smooth muscle cells (6,7).

Much of the understanding of the hexosamine pathway and specifically that of GFAT in mediating these effects has come at the protein level as opposed to transcriptional regulation. In eukaryotes, for example, but not bacteria, GFAT protein activity is allosterically inhibited by UDP-GlcNAc (8). The coordinated regulation of GFAT activity by insulin, glucose, and glutamine was done using crude cytosolic preparations with enzyme activity determined by spectrophotometric assays (9). Furthermore, analysis of hexosamine biosynthetic products and their effects on growth factor transcription was done by transiently over-expressing GFAT protein in vascular smooth muscle cells (10). Several exceptions include the regulation of the yeast GFAT promoter by α-pheromone (11) and co-regulation of human GFAT mRNA by epidermal growth factor and glucose (12).

Analysis of the hexosamine biosynthetic pathway at the molecular level might help elucidate answers as to how one pathway can mediate such a wide range of cellular events. To begin this understanding, we chose GFAT because it is the rate-limiting step in the hexosamine biosynthetic pathway. Here we report the cloning, sequence, and characterization of the 5' flanking region of this gene.

MATERIALS AND METHODS

Isolation of Genomic DNA Clones Containing the GFAT Promoter Region-

An amplified 129SV mouse genomic library (Stratagene, La Jolla, CA) was used to isolate the clones containing the 5'-region of the GFAT gene. The library was constructed from 9-22-Kb fragments of a partial Sau3A I digest of 129SV genomic DNA cloned into the Xho I site of the Lambda FIXII vector. Plaque lifts of 1 x 10^6 recombinants were hybridized with a ^{32}P labeled 356-bp fragment derived from the 5'-end of the mouse GFAT cDNA spanning from the ATG translation initiation codon to the internal EcoRI site (13). After several low stringency washes, membranes were washed in 0.2X SSC/1.0% SDS at 60° C for 60 minutes. Individual plaques were

identified by autoradiography and purified to homogeneity through subsequent rounds of screening.

5'- Rapid Amplification of cDNA Ends (5' RACE)-

5' RACE of the GFAT transcripts was performed using the Gibco/BRL 5' RACE kit (Grand Island, NY). Briefly, first strand cDNA was synthesized using 3ug poly (A^+) RNA isolated from NIH-3T3 cells and a GFAT gene-specific primer (GSP-1) that is complimentary to nucleotides +91 to +48 relative to the ATG translation initiation codon was used as a primer. After incubating with 8U of SuperScript Reverse Transcriptase for 30 minutes at 42° C, the RNA template was degraded with RNAse H and the cDNA was purified using the GlassMax DNA Isolation Spin Cartridge System. The 3'-ends of the cDNA were tailed using 10U of Terminal Deoxy Transferase and 2mM dCTP. The cDNA pool was then amplified by PCR using the Gibco/BRL poly dGTP anchor primer and an internal GFAT gene-specific-primer (GSP-2) that is complimentary to nucleotides +54 to +30 relative to the ATG translation initiation codon and engineered with an EcoRI restriction site. Amplification was carried out by 40 cycles of 94° C for 1 minute, 50° C for 2 minutes, and 72° C for 3 minutes. A single band of approximately 250-bp was seen when the product was electrophoresed through 1.2% agarose. The product was digested with EcoRI and Sal I, cloned into pT7T3 (Pharmacia, Piscataway, NJ), and independent clones were sequenced on both strands. 5' RACE of the chimeric GFAT/Luciferase gene was done by placing the GFAT promoter (-470/+88) upstream of the firefly luciferase cDNA (pGL-2 Basic, Promega, Madison, WI) and subsequently transfecting into NIH-3T3 cells. At 40 hours post-transfection, poly (A^+) RNA was isolated and luciferase gene-specific-primers were used to amplify the cDNA transcripts as follows: GSP-1 is 5'-AATGGCGCCGGGCCTTTCTTTATGTTTTTGGCGTCTTCCATT-3' and GSP-2 is 5'-CCATTTTACCAACAGTACCGGAATG-3'. After PCR amplification, the single

product was digested with Xho I and Sal I, cloned into pT7T3, and independent clones were sequenced on both strands.

DNA Sequencing-

DNA sequencing was performed by the dideoxy chain termination method (14) employing the Sequenase Version 2.0 sequencing kit (USB, Cleveland,OH). The SacI/SacI 1.9 Kb genomic DNA fragment was cloned into pT7T3 and sequenced using a series of overlapping nested primers. Sequence analysis was done with the GCG Version 7.2 Fragment Assembly System (FAS) and overlap was identified between the genomic sequence and the 5' RACE products.

Construction of GFAT/Luciferase Plasmids-

The SacI/SacI 1.9 Kb genomic DNA fragment (corresponding to -1822/+88 relative to the transcription initiation site) was cloned into pGL-2 Basic and orientation was confirmed by partial sequencing and the ability to generate luciferase. The -470/+88 construct was generated by cutting the vector with Kpn I and closing with ligase. The remaining deletant constructs were prepared by PCR amplification using the following oligonucleotides: 5'-CGGGGTACCTACAGCTTTTCTCTCTGTC-3' (for -275/+88), 5'-CATGGTACCCGCAGCTCTGCGTCTG-3' (for -120/+88), 5'-CATGGTACCCAATGGGAGAGCCG-3' (for -55/+88) with bottom strand primer being the luciferase GSP-2 listed above. The 5' UTR deletants were also generated by PCR with bottom strand oligonucleotides being 5'-CATGGATCCAGCGCTCGCTTCGCTCTC-3' (for -470/+19), and 5'-CATGGATCCGATGTTGGTCACGGGCGAG-3' (for -470/+149) with top strand primer being 5'-GTGCTGGGACTACTGAC-3'. For site directed mutagenesis, constructs were generated as previously described (15,16). Briefly, the GFAT/Luciferase vector (-120/+88) was used as template to generate ssDNA via R408 helper phage and annealed to oligonucleotides bearing different restriction sites for each

of the eight mutant constructs. After plasmid purification, all constructs were confirmed by both restriction digest and sequencing.

Tissue Culture and Electroporations-

NIH-3T3 cells were passed weekly in DMEM + 10% NCS supplemented with 100ug/ml penicillin and 50ug/ml gentamycin. For transfections, 6 x 10^6 cells were trypsinized, washed with cold PBS, and resuspended in DMEM + 10% NCS and 12ug of the appropriate luciferase plasmid was added in addition to 8ug of pCMV-βGal reporter plasmid to normalize for transfection efficiency. Cells were eletroporated at 400V and 500uF in a Gene Pulser (Bio-Rad, Richmond, CA) and plated at a density of 5 x 10^5 cells per well using 6-well plates (Fisher Scientific, Atlanta, GA).

Assays for Luciferase and β-Galactosidase Activity-

Luciferase activity was measured in detergent extracts of cells in the presence of ATP and luciferin as previously described (17) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Light output was integrated over a 10 second period and displayed as Relative Light Units (RLU). Background activity was found be less than 4% of all readings. The same extracts were assayed for β-galactosidase activity as previously described (18). All values were found to lie within the linear aspect of the absorbance curve.

Eukaryotic Expression of Recombinant Sp-1 and SpN Proteins-

The full-length human Sp-1 cDNA was cloned downstream of the cDNA encoding for the glutathione-S-transferase (GST) protein. The cassette was then cloned into the pTM3 vector and a recombinant vaccinia virus (vGST-Sp1) was generated using selection methods previously described (19,20). After co-infecting BSC-40 cells with vGST-Sp1 and vTF7-3 viruses, protein extracts were prepared as described (20). The fusion protein was affinity purified by incubating with glutathione covalently linked to sepharose beads (Pharmacia, Piscataway, NJ). After several washes, the beads were resuspended and the Sp-1 protein was released by cleaving with thrombin.

The Sp-1 containing supernatant was cleared of thrombin by incubation with sepharose beads covalently linked to benzamidine (Pharmacia, Piscataway, NJ). Concentration was determined by Coomassie blue staining with known standards. The SpN protein is from the same cDNA but lacks the initial 83 amino acids of the N terminus. The loss of molecular mass is approximately 10kD. The SpN cDNA was cloned directly into pTM3 generating the recombinant virus, vSpN. After co-infection with vTF7-3, protein extracts were prepared and the SpN protein was partially purified using wheat-germ-agglutinin chromatography (21). After elution from the column, protein concentrations were made using the Bio-Rad D_c Protein Assay (Richmond, CA).

Electromobility Shift Assay (EMSA)-

Nuclear extracts were prepared from MDA-468 cells as previously described (22). The GFAT oligonucleotide was made by annealing two complementary sequences spanning from -30 to +13 with respect to the transcription start site. The top strand sequence is 5'-CGCGTTGGCCGGGGGGGCGGGGCGGCAGTTGAGAGCGAAGCGA-3' with bottom strand being 5'-AGCTTCGCTTCGCTCTCAACTGCCGCCCCGCCCCCCCGGCCAA-3'. The DNA was labeled with ^{32}P using Klenow enzyme. Approximately, 20fmol of DNA was added to 2.5ug nuclear extract in a volume of 25ul containing 20mM Hepes (pH 7.9), 50mM KCl, 1mM DTT, 10% glycerol, and 1ug poly (dI:dC). Following a 20 minute incubation at room temperature, DNA-protein complexes were resolved on a 5.5% non-denaturing polyacrylamide gel with 90mM Tris borate/2mM EDTA buffer. Competitor oligonucleotides were purchased from Promega (Madison, WI). For antibody experiments, the radio labeled probe was added subsequent to a 25 minute pre-incubation of antibody and nuclear extract at 4° C. The human AP-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the human Sp-1 antibody is described elsewhere (23).

DNAse I Footprinting-

The vector containing the GFAT promoter (-470/+88) driving firefly luciferase was linearized with HindIII and end labeled with Klenow enzyme. After secondary digest with KpnI, the purified labeled 550-bp fragment was incubated with proteins in a final volume of 50ul with buffer containing 25mM Tris (pH=8.0), 6.25mM $MgCl_2$, 0.5mM EDTA, 0.5mM DTT, 7.5% glycerol, 10ug BSA, and 200 ng poly (dI:dC) for 25 min at 4° C. Varying concentrations of DNAse I were added and allowed to react for 30 to 120 seconds. The reactions were stopped with EDTA, phenol-chloroform extracted, and ethanol precipitated. The digested DNA was size fractioned on a 6% acrylamide/urea containing gel by electrophoresis and autoradiographed. The exact locations of the DNAse I footprints were determined by comparison to a sequence of known length electrophoresed concurrently.

RESULTS

Identification of Transcription Initiation Site(s)-

Although the entire open reading frame for the mouse GFAT cDNA is known (13), the 5'UTR has not been established. As a first step to identify the GFAT transcription start site(s), we used the 5' RACE method to obtain the 5'-end region of the GFAT cDNA. After first strand synthesis with GSP-1 and 3'-end tailing with dCTP, a single product was obtained by PCR using GSP-2 and the poly (G) anchor primer. After restriction digest, ligation, and transformation, four individual clones were selected and sequenced on both strands. The sequence was identical for all clones and each contained the known 50-bp of sequence 3' to the ATG translation initiation sequence identifying it as GFAT specific. Furthermore, all clones were found to initiate from the same guanine nucleotide 149 bases upstream of the ATG translation initiation codon (Figure 1).

Figure 1: Identification of the transcription start site of the mouse GFAT gene. The nucleotide sequence of the 5'-end of the GFAT cDNA. The ATG translation codon is in large font/bold-face print. GSP-2 was used as the GFAT specific primer in PCR amplification. The transcription start site is denoted as +1. The internal SacI site at +88 is underlined. Primer 642 was used localize the GFAT promoter region after SacI restriction digest of λ phage DNA.


primer 642
+1
GAGAGCGAAGCGAGCGCTGAGTCGGACTGTCGGGTCTGACGTGTCGCATCCCAGAGTCC
TCTCATTGCCACCACCCCGGCCCGAGCTCACCCTCGCTTCTGAAGCTCTCCGCGCGCCCG
SacI
ACAGCTCAGCCCTCGCCCGTGACCAACATCATGTGCGGTATATTTGCTTATTTAAATT
ACCATGTTCCTCGAACAAGACGAGA
GSP-2

Cloning and Characterization of the 5'-Flanking Region of the GFAT Gene-

Four clones were isolated after screening approximately 2 x 10^6 plaque forming units with the 5'-end of the mouse GFAT cDNA as described in "Methods". However, only two of these clones were positive when probed with primer 642. These two clones were digested with SacI and the fragments were separated on 1% agarose. After reprobing with primer 642, both clones produced positive bands of 1.9-Kb. This fragment was subcloned into pT7T3 and sequenced using a series of overlapping primers (Figure 2). Overlap was seen with the 3'-end of this fragment and with the 88 base pairs of the 5'-RACE product beginning with the transcription start site and continuing to the SacI site. To establish whether or not an intron existed in the sequence encoding the 5'UTR, the original λ phage genomic DNA was sequenced using primer 642. The result was continual sequence through the ATG translation initiation sequence identical to the 5'-RACE product thus establishing the lack of an intron in this region. Examination of the sequence immediately 5' to the transcription start site found it to be GC rich and lacking a TATA box. When the 1.9-Kb SacI/SacI fragment was placed upstream of firefly luciferase in the proper orientation, it was found to be transcriptionally active (data not shown). However, reverse orientation was found to be transcriptionally inactive. To further characterize this promoter, 5'-RACE was conducted on the chimeric GFAT/Luciferase mRNA transcripts. Five individual clones were sequenced on both strands. Relative to the +1 start site, these transcripts were found to initiate from -4, -3, -3, +1, and +3, suggesting that disruption of the endogenous promoter results in initiation from a small cluster of points as opposed to a single base.

Detection of DNA/Protein Interactions Using Electrophoetic Mobility Shift Assays-

In order to obtain an understanding as to the basal elements involved in the regulation of the GFAT promoter, the sequence surrounding the transcription start site was cross referenced with the transcription factor data base. DNA consensus sequence

Figure 2: Nucleotide sequence of the GFAT romoter. The sequence shown starts 1822 nucleotides 5' to the transcription start site and continues to the ATG translation codon. The transcription start site (+1) is in bold-face print. The ATG translation codon (+150) is in capital letters. The SacI sites at -1822 and +88 are underlined. The sequence has been deposited in the GenBank under accession number U39442.

```
-1822  gagctctaca cagaggaggg gcagtgtctg agctggcaca tggttacaaa
-1772  gcattctttg tatggtggtt tagtgagctt gttcaaggct gggctggaga
-1722  gcattggcct ttaaagtttc ctttcagctg gtgctgagca cctacccaga
-1672  cagcagtaga acggagatgg gtaagataag gaacctgtga gtcggtaggc
-1622  acagctgaac aaagtactgt agctgaaaca cttgctggta ctactgccat
-1572  ttcctttgtg gcatcgacag gtttgctgcc agcaaacaag ggaggcaagg
-1522  caccagaacc acacttcggt tcttgtgcag tatcattcaa gtcgccttcc
-1472  aggggagggt ctctaagagg tgctctaagg aacatgtcac tgtgttagct
-1422  ctgccaacgt aatctaggga cctgtgtaat ggcttggaat aatgaagagc
-1372  cttttagatg agaaatactt ccttgattac tttttctagc ctctatttga
-1322  gataggcgat aaaaaaactg aaaagccttt gcttgccttg cacatacaaa
-1272  gagcttagga caagcctata aggcttttat cactctttcc agccaccagg
-1222  acagttttaa tttgccacgt attgctctgg tctatgacta ggcaagtgtt
-1172  ggttattaaa aaaaaaaaac aaaaaacaaa aaaccaacca accaaacaaa
-1122  caaaaaaatc tcactctatc ataaggactt acagttagtg gggagaggaa
-1072  aaaaccacct ttaatacaga agtctgttat agccgggcgt ggtggcgcac
-1022  gctttaatcc cagcactcgg gaggcagagg caggcggatt tctgagttcg
 -972  aggccagcct ggtctacaga gtgagttcca ggacagccag ggctacacag
 -922  agaaaccctg tctcaaaaca aacaaacaaa aaaaaaacaa aacaaaacaa
 -872  aaaacaaaaa aacaaagaag tctgttattg tatgagacag atcaaaggat
 -822  gagatgttaa aaacatcttg gggagaccta aagatgtcac acttaaaagt
 -772  ggcaaggatg tgatgtggga ctctgtagaa taaccctggc gtgcagtttt
 -722  gaatgctggg gcagtaggaa ggggattctg gctgagagtg cttctgtggg
 -672  ccctactcat tcatttcgtt agagctatca agtgcgggct gataaccagc
 -622  tacttttgct ccaagtgatt atttaaacct tctgaaaggg aacgccgcta
 -572  actctctttt ctcaacggag agttcattcg tgacataagc gatcccgagt
 -522  acaacgtgcc cacagcttcc ctgagcgcag ggagtgctgg gactactgac
 -472  tagtggtacc ttaggcagag aacagtgagg aaggcaggga gtggggcgct
 -422  gagcaccttg cctacagctc aggtcgtcac cggaatggct gcccgagggc
 -372  ggcctgcagg ctcccgcacc tctgcxccag cagcgcagtt ccggccaccg
 -322  tggctctgca cactgacacg tctgtcccag gctctccctc ctgccctaca
 -272  gcttttctct ctgtctccct ccttcccgtt ccccttgaaa cacacaaact
 -222  gctgtgtaaa cattcccttc ctcctcctcc tccgaggctc ggctcgccct
 -172  cagcgggcgg ctccaggaac ctaaagcgga tgctggtcga gcccctgcca
 -122  cgtcgctacc cgcagctctg cgtctgctct ctgggcgggc gatgcctgcg
  -72  cgtgagcgcg cgggcgcggc ccaatgggag agccgcggcg cgcgcgttgg
  -22  ccgggggggc ggggcggcag ttggagagcga agcgagcgct gagtcggact
  +29  gtcgggtctg acgtgtcgca tcccagagtc ctctcattgc caccaccccg
  +79  gcccgagctc accctcgctt ctgaagctct ccgcgcgccc gacagctcag
 +129  ccctcgcccg tgaccaacat cATG
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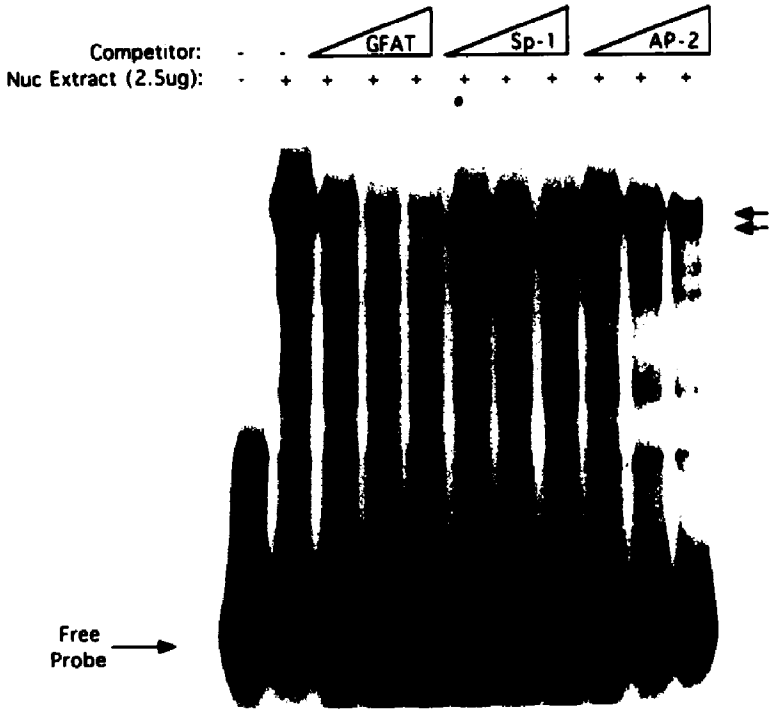
homology was found for Sp-1 (GGGGCGGGGC) and AP-2 (GGCCGGGGG). To assess the interaction of these elements on the GFAT promoter, a double stranded oligonucleotide was made spanning from -30 to +13 and gel shift assays were conducted (Figure 3A). The GFAT competitor demonstrated which bands were specific versus nonspecific binding. The Sp-1 oligo appeared to compete away the upper band entirely while the AP-2 oligo competed with both bands, but with much less affinity. This initial result seems to indicate that Sp-1 might be a better candidate for interaction with the GFAT promoter. To further test this hypothesis, specific and nonspecific competitions were done using the same labelled GFAT oligonucleotide (Figure 3B). Again, the GFAT oligo competed with both bands while the Sp-1 oligo competed away the dominant-upper band. The unrelated Oct-1 oligo was unable to compete with either band.

Interaction of Sp-1 with GFAT Promoter Sequence-

To confirm that Sp-1 was binding to the -30/+13 element of the GFAT promoter, an N terminal truncation of the Sp-1 protein (SpN) was produced through recombinant vaccinia expression (19). Although this deletant lacks 10 kilodaltons of mass, it still recognizes the Sp-1 DNA consensus sequence (24). As a result, it migrates slightly faster through an acrylamide gel when compared to native Sp-1. Gel mobility shift assays were conducted with nuclear extracts and the truncated protein as shown (Figure 4A). With nuclear extract, the dominant band and the ability of Sp-1 consensus sequence to compete away this band was reproduced. The SpN protein was also able to shift the labelled oligo, but to a position slightly lower than that of nuclear extract. This band could also be reduced by adding excess Sp-1 competitor. This result further identifies Sp-1 as being the protein that generates the dominant shift. Immunological confirmation was made by pre-incubating nuclear extract with an anti-Sp-1 antibody (Figure 4B). Treatment with pre-immune serum had no effect on the complex while the anti-Sp-1 polyclonal serum greatly reduced the dominant shift.

Figure 3: Electrophoretic mobility shift assays. A double stranded oligonucleotide corresponding to the -30/+13 region of the GFAT promoter was ^{32}P labeled and examined by gel shift analysis. A, the GC-rich competitors were added at 50-, 100-, and 150- fold molar excess. Arrowheads indicate the two discrete bands observed with the addition of nuclear extract. B, competitors were added at 75- and 150- fold molar excess. The Sp-1 oligo competes away the upper band of the doublet in addition to a lower, migrating band.

A


Competitor:
Nuc Extract (2.5ug):
GFAT
Sp-1
AP-2
Free
Probe

B

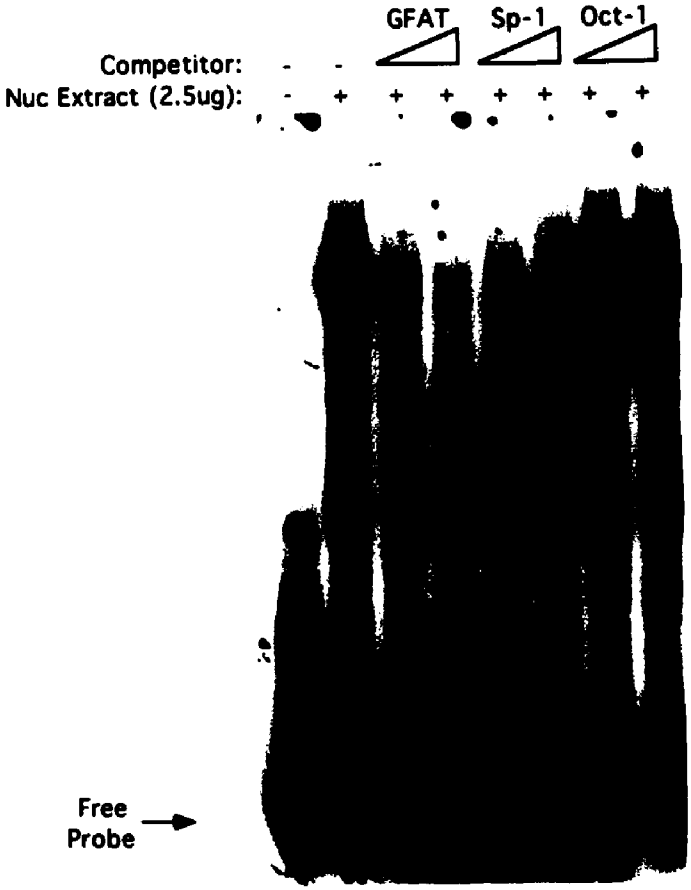
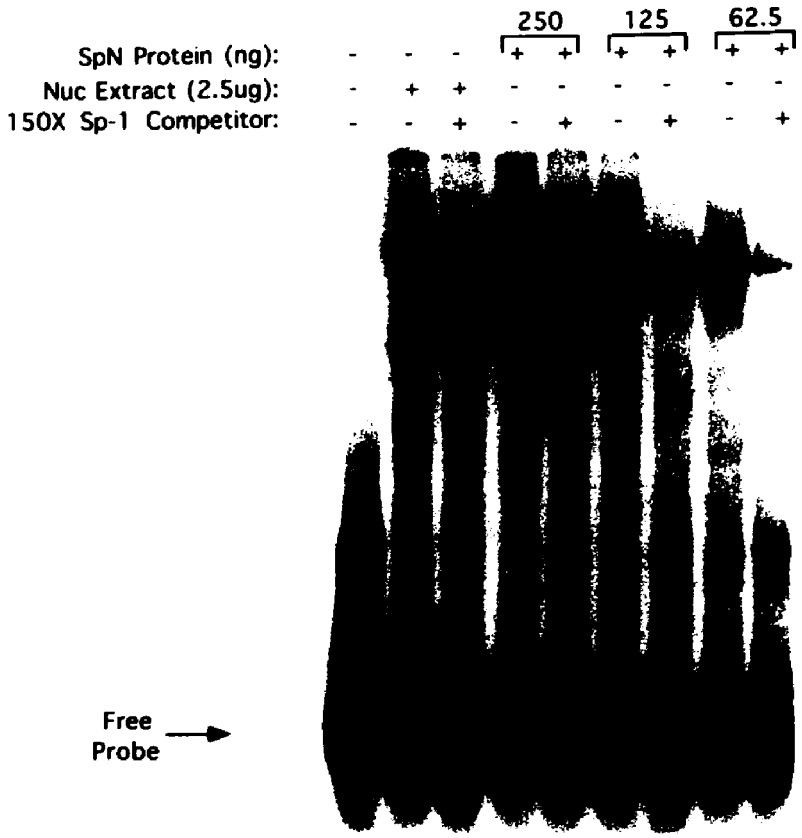
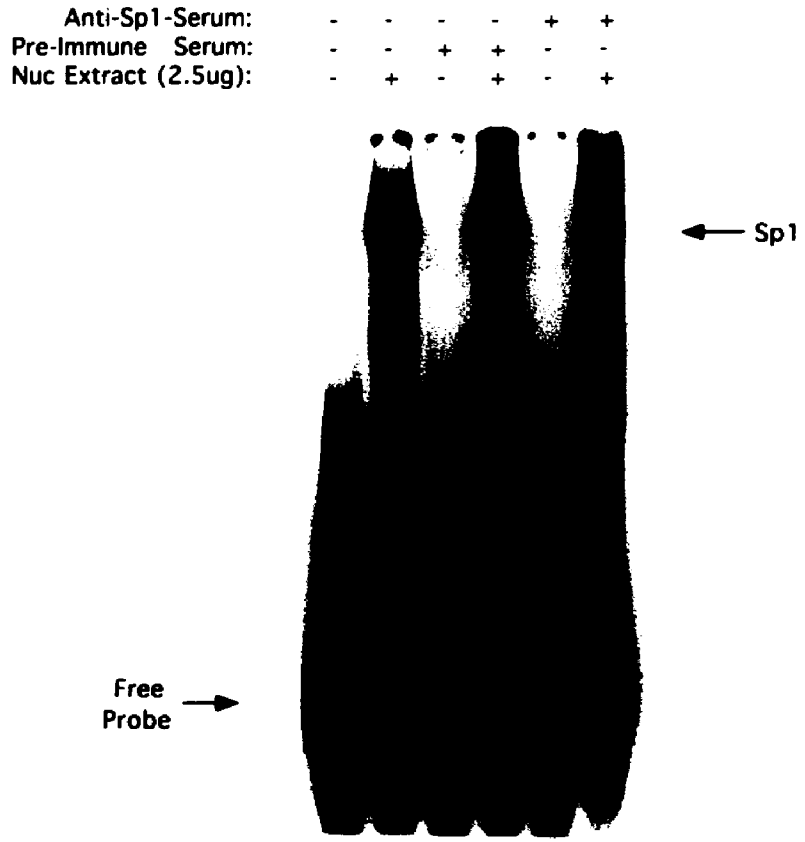

GFAT
Sp-1
Oct-1
Competitor:
Nuc Extract (2.5ug):
Free
Probe

Figure 4: Identification of Sp-1 in GFAT specific gel shifts.The same -30/+13 GFAT oligonucleotide was labeled and examined by gel shift analysis. A, nuclear extract and serial dilutions of recombinant SpN protein were added to the GFAT oligonucleotide in the presence and absence of 150-fold excess Sp-1 competitor oligonucleotide. B, Prior to the addition of the radiolabelled GFAT oligonucleotide, nuclear extracts were incubated with pre-immune or Sp-1 specific serum and subsequently electrophoresed through a non-denaturing acrylamide gel.

A


250
125
62.5
SpN Protein (ng):
Nuc Extract (2.5ug):
150X Sp-1 Competitor:
Free Probe

B


Anti-Sp1-Serum:
Pre-Immune Serum:
Nuc Extract (2.5ug):
Sp1
Free Probe

These results strongly indicate that Sp-1 is the protein producing the major band when nuclear extract is added to the GFAT oligonucleotide spanning from -30 to +13.

Exclusion of AP-2 in GFAT Promoter Oligonucleotide-

The gel shift shown in Figure 3A appears to indicate that the transcription factor AP-2 might have some, although weak, interaction with the putative AP-2 consensus sequence contained within the -30/+13 region of the GFAT promoter. To determine if the minor band was in fact AP-2, we used an anti-AP-2 antibody, which is known to interact with amino acids 420-437 mapping to the carboxy terminus of human AP-2 (25). We again labelled the GFAT oligo spanning -30/+13 and conducted super-shift assays as shown (Figure 5). The anti-AP-2 antibody was unable to super-shift the complex. When the Sp-1 band was removed by adding excess Sp-1 competitor, the minor band still remained with no apparent super-shift. As a positive control, an AP-2 oligonucleotide was labelled and incubated with the same nuclear extract. When the antibody was added, a specific super-shift was obtained and a 200-fold excess of Sp-1 oligonucleotide did not disrupt this interaction. These data seem to indicate that the AP-2 consensus sequence contained in this oligonucleotide is not functional when assayed with an immunoreactive anti-AP-2 antibody. Since both bands were competed equally with excess AP-2 oligonucleotide, shown in Figure 3A, it appears that this may reflect nonspecific competition by the GC-rich sequence. Identification of the minor band is under continued investigation.

DNAse I Footprint Analysis of the GFAT Promoter-

Further confirmation of Sp-1 interaction was made by labelling the top strand of the GFAT promoter and conducting DNAse I footprint analysis (Figure 6A). Recombinant Sp-1 protein was used as a positive control to localize the binding area within the promoter. Protection was observed in two areas that correspond to Sp-1 consensus binding sites: the -90/-84 and -17/-7 regions. With the addition of nuclear extract, protection was seen at the -17/-7 region, but the pattern of footprinting had

Figure 5: Exclusion of AP-2 from the GFAT promoter oligonucleotide. Supershift analysis with an anti-AP-2 antibody. The -30/+13 GFAT probe was labeled and examined for AP-2 specific supershifts. The minor band was unchanged with the addition of the antibody. As a positive control, an AP-2 oligonucleotide was ^{32}P labeled and examined for supershifts. The arrowheads mark the AP-2 specific shift and supershift with the antibody.

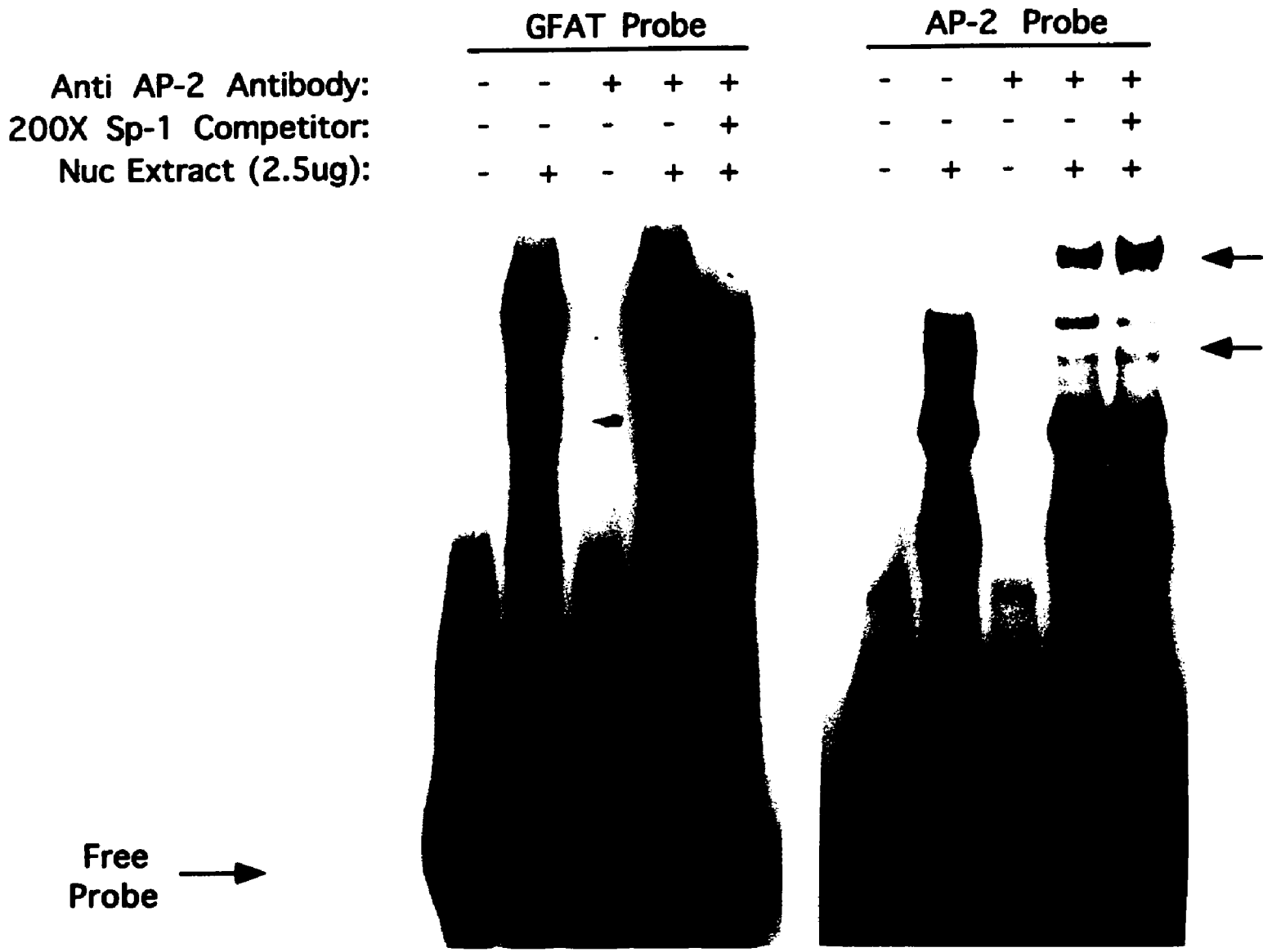
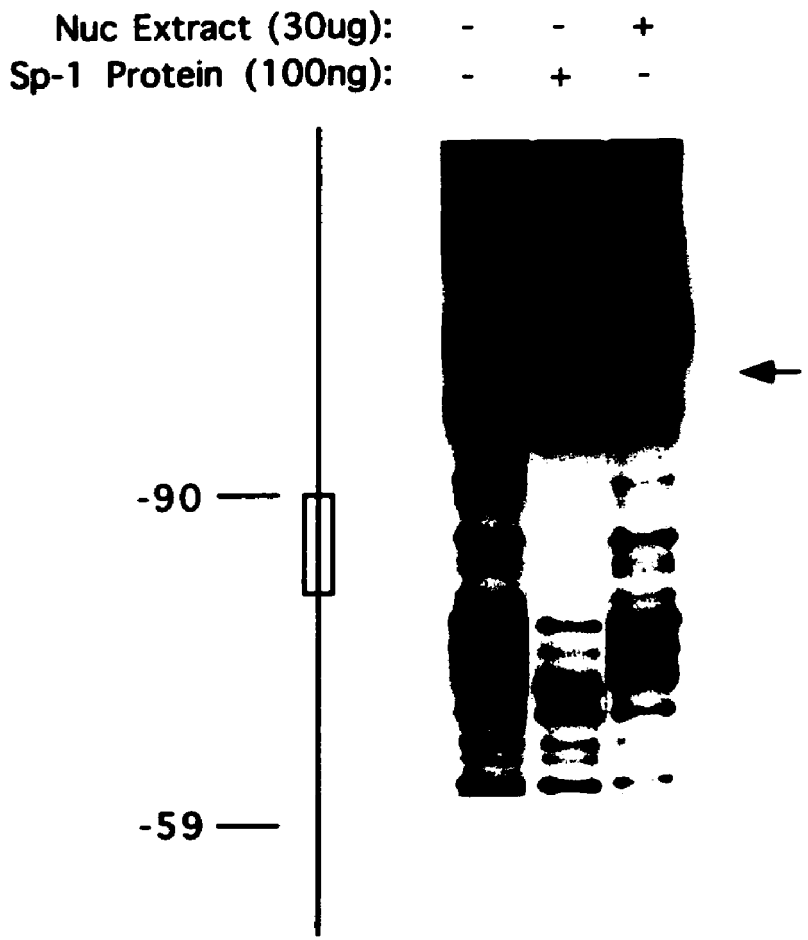

GFAT Probe
AP-2 Probe
Anti AP-2 Antibody: - - + + + - - + + +
200X Sp-1 Competitor: - - - - + - - - - +
Nuc Extract (2.5ug): - + - + + - + - + +
Free Probe

Figure 6: DNAse I footprint analysis of the GFAT promoter. The upper strand of the -470/+88 GFAT promoter region was ^{32}P labeled and incubated as described. A, footprint pattern for recombinant Sp-1 protein and nuclear extract. The numbers represent position relative to the transcription start site. The boxes indicate Sp-1 consensus DNA binding sites. B, footprint analysis of the -100 region of the GFAT promoter. With the addition of nuclear extract, the footprint pattern changed in that a hypersensitive site and weak area of protection were visible (arrowhead). The box indicates an Sp-1 consensus DNA binding site.

A


Nuc Extract (30ug): - - +
Sp-1 Protein (100ng): - + -
-90
-59
-23
+1

B


Nuc Extract (30ug): - - +
Sp-1 Protein (100ng): - + -
-90
-59

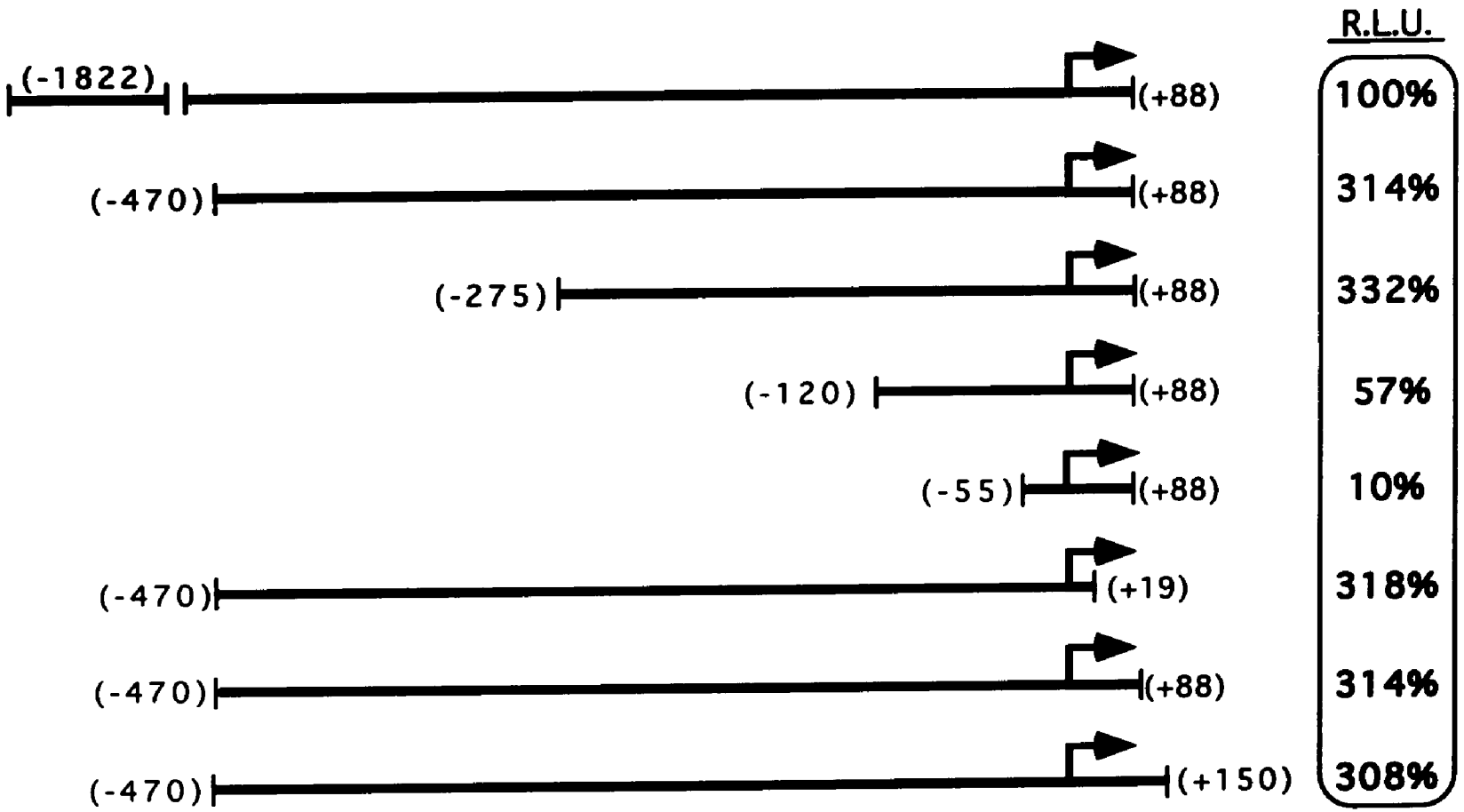
changed slightly. This is certainly a function of the complex internal milieu of proteins that exist in cellular extracts as opposed to the homogeneous recombinant protein. Of interest is the observation that the consensus AP-2 site (-24/-17) had no footprint. It appeared that the upper area of Sp-1 protection (-90/-84) had changed significantly with the addition of nuclear extract (Figure 6B). The footprint was reduced in size, but an additional area of protection was seen along with a hypersensitive site. Examination of this sequence revealed a putative consensus sequence for the "E" box element at -102/-97 (CGTCTG). These elements are known to regulate genes in muscle cell and B cell development (26,27,28). Recent work has demonstrated that the gene encoding for the glutamine hydrolyzing enzyme, carbamoyl-phosphate synthase/aspartate carbamoyltransferase/dihydroorotase (*cad*), is also regulated by "E" box elements (29).

Confirmation of Biochemical Protein/DNA Interactions Through Functional Assays-

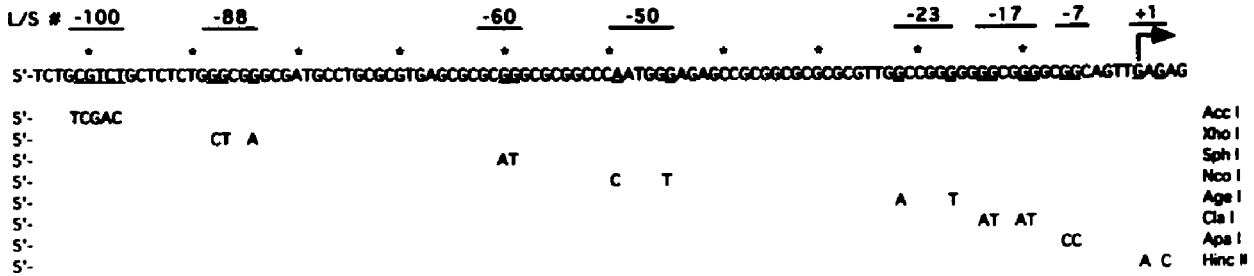
Having linked several transcription factors to the mouse GFAT promoter through protein/DNA biochemical analysis, we now wanted to test the functional significance of these interactions. Through the use of internal restriction sites and PCR, deletant constructs of the GFAT promoter were made and placed upstream of firefly luciferase. These plasmids were transfected into the mouse fibroblast cell line, NIH-3T3, and assayed for both luciferase and β-galactosidase activities (Figure 7A). The largest change observed was a 30-fold decrease from the -275/+88 vector to the -55/+88 construct. When the "E" box (-102/-97) and Sp-1 (-90/-84) sites were deleted by going from the -120/+88 to the -55/+88 construct, a 5.7-fold decrease in luciferase activity was observed. The formation of stable secondary RNA structures has been shown to have some effects on gene transcription (30). Although we found the 5'UTR to be very stable in forming a stem-loop structure (-46.1 kcal/mole), deletion of this region had no effect on luciferase activity. We continued by changing specific DNA sequences through linker-scanning (L/S) mutagenesis. Using the -120/+88 as the parent construct, eight mutations were placed along the promoter spanning from -100 to

Figure 7: Deletional analysis and site directed mutagenesis of the GFAT promoter. A, GFAT/Luciferase promoter deletants were made as described in "Methods." After transfection into NIH-3T3 cells, extracts were examined for luciferase and β-galactosidase activity. The largest construct, -1822/+88, was arbitrarily assigned the value of 100% and all other values are relative to it after normalizing for β-gal activity. Results are the means of two experiments, each performed in triplicate. All standard deviations were less than 10% of the means. RLU is Relative Light Units. B, sequence of the wild-type GFAT promoter and the base changes for each of the linker-scanning (L/S) mutants. At right is the restriction digest site introduced with each mutation. Asterisks represent every tenth base. Above each mutation is the corresponding linker-scanning construct number. Arrowhead represents the transcription start site. C, luciferase activity for GFAT promoter site directed mutagenesis. Each construct was transfected into NIH-3T3 cells and subsequently examined for β-gal and luciferase activities. Values are plotted as the percent of wild type after normalizing for β-gal activity. Results are the means +/- S.D. of two experiments, each assayed in triplicate.

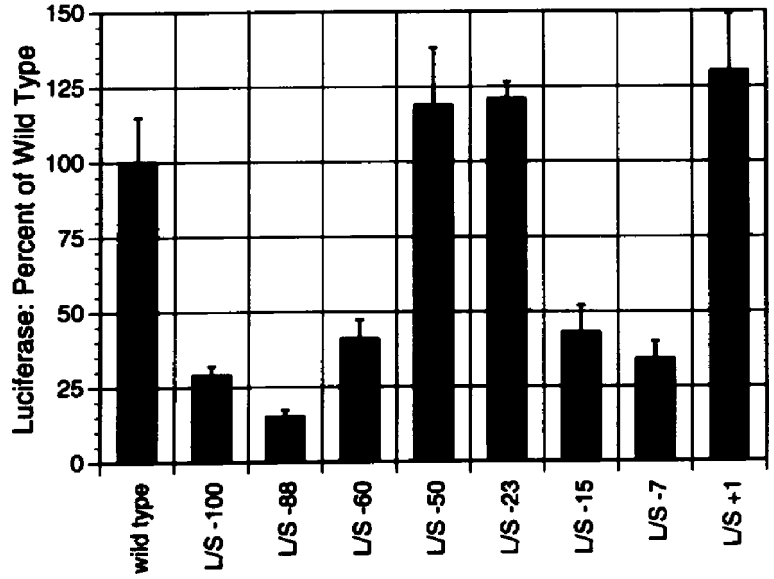
A


R.L.U.
(-1822)
(+88)
100%
(-470)
(+88)
314%
(-275)
(+88)
332%
(-120)
(+88)
57%
(-55)
(+88)
10%
(-470)
(+19)
318%
(-470)
(+88)
314%
(-470)
(+150)
308%

B


L/S # -100
-88
-60
-50
-23
-17
-7
+1
5'-TCTGCGTCTGCTCTCTGGGCGGGCGATGCCTGCGCGTGAGCGCGCGGGCGCGGCCCAATGGGAGAGCCGCGGCGCGCGCGTTGGCCGGGGGGGCGGGCGGCAGTTGAGAG
5'- TCGAC
CT A
AT
C T
A T
AT AT
CC
A C
Acc I
Xho I
Sph I
Nco I
Age I
Cla I
Apa I
Hinc II

C


Luciferase: Percent of Wild Type
150
125
100
75
50
25
0
wild type
L/S -100
L/S -88
L/S -60
L/S -50
L/S -23
L/S -15
L/S -7
L/S +1

+3 (Figure 7B). After transfection into NIH-3T3 cells, extracts were assayed for β-galactosidase and luciferase activities (Figure 7C). Disruption of the "E" box (L/S -100) and distal Sp-1 (L/S -88) sites resulted in a significant loss of promoter activity when compared to control. Furthermore, when mutations were placed in the Sp-1 binding sequence (L/S -17 and L/S -7) as determined by gel shift and DNase footprint assays, significant loss of activity was seen. When mutations were placed in a non-consensus sequence (L/S -50) and the nonfunctional AP-2 sequence (L/S -23), no significant change in activity was seen when compared to control. Interestingly, the L/S -60 sequence corresponds to a putative Krox-24 site (CGCGGGCG). This DNA binding element is known to regulate genes in a serum-response-dependent manner (31). The reduction in luciferase activity seen by mutating this site is of the same magnitude as the Sp-1 site in the -17/-7 region.

DISCUSSION

Glutamine:fructose-6-phosphate amidotransferase (GFAT) is the rate-limiting enzyme in the hexosamine biosynthetic pathway (1). The product of this pathway, uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), serves as a substrate for protein glycosylation. Glycosylation of proteins has been shown to be just as dynamic as phosphorylation in modulating cellular responses (32,33). Although only 2-5% of all fructose-6-phosphate is shunted through this pathway, it nonetheless has been shown to regulate diverse cellular events, such as the glucose-induced-desensitization of adipocytes (3), glycogen synthase activity (4), pyruvate kinase activity (5), and glucose-induced growth factor expression in vascular smooth muscle cells (6,7). Although GFAT plays a role in numerous cellular events and its expression is found in a diverse set of tissues (13), how it is regulated at the transcriptional level is poorly understood.

In this study, we reported the isolation and characterization of genomic clones containing the 5'-flanking region of the mouse GFAT gene. As determined by 5'-

RACE, the transcription initiation site of the endogenous GFAT mRNA was found to be 149 bases upstream of the ATG translation codon. However, when the region covering -470 to +88 was placed upstream of firefly luciferase, the luciferase transcripts were found to have initiated from a cluster of bases spanning from -4 to +3, suggesting the loss of point specific initiation. Two λ clones were identified and continuous promoter sequence was obtained from -1822 to the ATG translation codon. The first exon was continuous from the transcription start site through the ATG codon. Southern blot analysis was conducted on the λ clones and normal mouse genomic DNA using the 356 base pair probe described in "Methods." Identical banding patterns were seen, thus eliminating the possibility of genomic DNA rearrangement by the λ clones (data not shown). Furthermore, this would suggest a lack of gene duplication for the GFAT gene as the normal genomic DNA did not yield multiple band products. Examination of the promoter sequence immediately 5' to the transcription start site found it to be GC rich and lacking a TATA box.

In order to establish which transcription factor(s) might participate in the basal regulation of this gene, the sequence immediately 5' to the transcription start site was cross referenced with the transcription factor data base. Perfect matches were found for the transcription factors AP-2 and Sp-1. To determine if either of these proteins interacted with this sequence, a double stranded oligonucleotide spanning from -30 to +13 of the GFAT promoter was synthesized. Gel mobility shift assays were conducted and two specific shifts appeared. The dominant band was identified as Sp-1 because excess Sp-1 consensus oligonucleotide could specifically compete away the major band while an unrelated oligonucleotide could not. Furthermore, an anti-Sp-1 antibody could specifically disrupt this band. In addition, a truncated Sp-1 protein was able to shift the GFAT oligo to a position slightly lower than native Sp-1 shifts and this complex could also be specifically competed with excess Sp-1 consensus sequence. With an immunoreactive anti-AP-2 antibody, we were unable to detect AP-2 binding in this

complex. Previous studies indicate that AP-2 along with other transcription factors can interact with the Sp-1 consensus sequence (34).

DNAse I footprinting confirmed the presence of an Sp-1 binding site by localizing protection to the -17/-7 region of the GFAT promoter through both recombinant Sp-1 protection and nuclear extract protection. In addition, another Sp-1 footprint was identified in the -90/-84 region of the promoter. The presence of these Sp-1 binding sites is consistent with G/C boxes found in other TATA-less promoters. It has been shown that Sp-1 is critical for transcription initiation (35). Although these promoters do not directly bind the TFIID complex, the transcriptional component containing the TATA binding factor believed to be a key link between promoter-specific activators and the RNA Polymerase II initiation machinery, it has been postulated that DNA-bound Sp-1 acts through protein-protein interactions to recruit and anchor the TFIID protein to the TATA-less promoter (36). Such would be the case of the GFAT gene and its multiple G/C boxes.

Having established biochemical interactions between the GFAT promoter and specific DNA binding proteins, we assessed the functional impact of these sites on transcription. Promoter deletants were made and their ability to produce firefly luciferase was examined. Successive loss of DNA resulted in changes in activity over a 30-fold range. Even the smallest construct, -55/+88, was able to generate luciferase 25-fold higher than the empty vector control suggesting basal elements are contained in this region. Although the 5'UTR was predicted to form a stable stem-loop structure, deletion of the UTR resulted in no significant change in activity. We then examined the effects of point mutations on the promoter areas known to have biochemical interactions with specific proteins. Not surprisingly, mutations at Sp-1 sites (L/S -88, L/S -15, and L/S -7) were found to significantly reduce transcription from the GFAT promoter. Mutations in the putative "E" box element (L/S -100) also resulted in a significant loss of promoter activity. Recent work in NIH-3T3 cells demonstrated the significance of

"E" box regulation in conferring growth-dependent transcription of the *cad* gene (29). This gene is regulated in a cell-cycle dependent manner and is known to increase transcription 15-fold at the G_1/S-phase boundary in response to serum (37). It remains possible, although unlikely, that mutations in this element hinder the neighboring Sp-1 site at -90/-84, and it is the loss of Sp-1 that results in decreased activity. Similarly, loss of promoter activity was seen with the L/S -60 mutation. This mutation was centered in a Krox-24 binding element. Previous work has shown that the Krox-24 promoter is TATA-less and contains multiple copies of the serum response element (38). Activation of cell growth by serum therefore leads to induction of the Krox-24 gene. Like Sp-1, it binds GC-rich regions through Zn-fingers (34). It would seem plausible that, as cells enter a growth stage through serum stimulation, it would be important to increase macromolecular synthesis in preparation for mitosis. Such an increase would include the synthesis of cellular proteins and their subsequent glycosylation. The induction of the GFAT promoter by Krox-24 would therefore increase the pool of intracellular UDP-GlcNAC, allowing for this increased glycosylation.

Two linker-scanning mutations were found to show no change in promoter activity when compared to control: the negative control (L/S -50) and the nonfunctional AP-2 consensus sequence (L/S -23). When the transcription initiation sequence was changed (L/S +1), the activity was higher than control, although not statistically significant. Since the chimeric GFAT /Luciferase mRNA transcripts were found to initiate transcription from a cluster of bases, it is not surprising that mutating two bases near the initiation site did not lead to reduced transcriptional activity.

In summary, we have cloned, sequenced, and partially characterized the 5'-flanking region of the mouse GFAT gene. Although it lacks a TATA box, it is GC-rich and initiates transcription from a single point. The ubiquitous transcription factor, Sp-1, mediates the basal transcription from this gene and disruption of its binding sites results

in markedly reduced promoter activity. The GFAT gene product is known to regulate diverse cellular events. This may imply a more dynamic regulation of this gene than is currently understood. Nonetheless, this work may help to elucidate the molecular mechanisms that control its expression.

ACKNOWLEDGMENTS

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GLUCOSE METABOLISM TO GLUCOSAMINE IS NECESSARY FOR GLUCOSE STIMULATION OF THE TRANSFORMING GROWTH FACTOR-α GENE

by

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ABSTRACT

Transforming growth factor-α (TGF-α) gene transcription can be increased when arterial vascular smooth muscle cells are exposed to supra physiological concentrations of glucose. Previous studies have shown that products of the hexosamine pathway, such as glucosamine (GlcN), can also increase TGF-α transcription. To determine whether the metabolism of glucose to glucosamine is required for the glucose effect, we studied glucoregulation of TGF-α expression under conditions where the rate limiting step in glucose metabolism to GlcN through the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) was blocked. We found that blockage of the hexosamine pathway significantly blunted the glucose induced increase of TGF-α expression. However, this blockage of GFAT could be bypassed with GlcN. To confirm this blockage, we used Western blotting and indirect immunofluorescence with the RL-2 antibody that recognizes products of this pathway. Similarly, inhibition of GFAT severely reduced the glucose induced RL-2 signal while GlcN was able to bypass this inhibition. These results strongly indicate that, of all the pathways of glucose metabolism, it is the pathway to glucosamine that is necessary for the transcriptional stimulation of TGF-α expression in vascular smooth muscle cells by glucose.

INTRODUCTION

Diabetes is a disease that is known to have deleterious affects on several body tissues (1). Retinopathy, nephropathy, and a higher incidence of vascular disease are commonplace in uncontrolled diabetics. It is believed that hyperglycemia is the underlying cause for these maladies (2). Vascular smooth muscle cell proliferation is an early event in the pathogenesis of atherosclerosis, and growth factors have been hypothesized to play an important role in this process (3). Previous studies have localized transforming growth factor-α (TGF-α) and basic fibroblast growth factor (bFGF) to these same vascular smooth muscle cells (4,5,6). These proteins are know

to induce mitogenic stimulation of endothelial cells as well as have acute vasoactive effects in vascular smooth muscle. Furthermore, expression of these growth factors can be increased at the transcriptional level by placing these cells in supra physiological concentrations of glucose (7). A known metabolite of glucose and product of the hexosamine pathway, glucosamine, mimics glucose in stimulating growth factor gene expression, but at lower concentrations and with a greater effect on the transcription rate and mRNA accumulation (7). Recent data has implicated the hexosamine metabolic pathway and its rate-limiting enzyme, glutamine:fructose-6-phosphate amidotransferase (GFAT), with insulin resistance, a hallmark of Type II diabetes (8,9,10). When the yeast GFAT cDNA was transiently overexpressed in rat aortic vascular smooth muscle cells, the glucose induction of TGF-α expression was augmented 2-fold (11). The end product of this pathway, uridine diphosphate (UDP)-*N*-acetylglucosamine (UDP-GlcNAc), which serves as the substrate for protein glycosylation, was also found to be higher in GFAT expressing cells than in controls (11). Similarly, treatment of cells with extracellular glucosamine also appears to directly expand the UDP-GlcNAc pool (12).

Glycosylation of proteins is achieved by the transfer of the GlcNAc moiety from UDP-GlcNAc to proteins by cellular transferases (13,14,15). This attachment to proteins is both through *N*- and *O*-linkage. Recent work has shown that the GlcNAc content of proteins can be measured with the monoclonal antibody, RL-2 (16). Furthermore, when breast cancer epithelial cells are taken from glucose-free media to either glucose or glucosamine containing media, the RL-2 signal was increased (16).

In this report, we examined whether the conversion of glucose to glucosamine was required for the transcriptional stimulation of the TGF-α promoter by glucose in vascular smooth muscle cells. Using a pharmacological inhibitor and a novel anti-sense strategy, we show that blockage of GFAT inhibits the glucose, but not glucosamine, induced transcription of the TGF-α promoter. Furthermore, blockage of GFAT is

confirmed with the RL-2 antibody using glucose concentrations that are physiologically relevant.

MATERIALS AND METHODS

Cell Culture-

Rat aortic smooth muscle cells (RASM) were prepared from the aortas of 250- to 300-gram male Sprague-Dawley rats (17). Culture media was from GIBCO/BRL (Grand Island, NY) and supplies were purchased from Sigma Chemical (St. Louis, MO). The cells were cultured in DMEM + 10% FCS (5mM glucose) with penicillin G (100 ug/ml) and gentamicin (50 ug/ml). Cells were grown at 37°C in a humidified incubator with 7.5% CO_2. The cells were passed every 7 days and used for up to eight passages.

Plasmid Constructs and Electroporation-

The plasmid containing the 5'-flanking sequence of the TGFα gene cloned upstream of the firefly luciferase gene (pTGFα-LUC) has already been described (18). The pSV-TAT vector has been described elsewhere (19). The HIV/Anti-Sense GFAT vector was generated by cutting HIV-LUC (19) with Xba1 to drop out the 1.7-Kb Xba1/Xba1 luciferase cDNA and then closing with ligase. Still remaining was a small fragment of luciferase cDNA and the SV-40 splice intron. The full length murine GFAT cDNA (20) including 5' UTR was then directionally subcloned downstream of the HIV-LTR between the HindIII and Xba1 sites to generate pHIV/Anti-Sense. Orientation was confirmed by restriction digest of the internal and polylinker HindIII sites. The pHIV/Control plasmid is the same construct devoid of insert. Both constructs were tested and found to generate no luciferase activity. All plasmids were supercoiled and twice purified over CsCl gradients prior to transfections. Plasmids were used in the following amounts: 18ug pTGFα-LUC; 4ug pSV-TAT; and 20ug of either pHIV/Anti-Sense or pHIV/Control. Briefly, 6 x 10^6 cells were trypsinized, washed with PBS, and resuspended in DMEM + 10% FCS. After adding the appropriate DNA, cells were

electroporated at 400V and 500uF in a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA). Cells were plated in 6-well plates (Fisher Scientific, Atlanta, GA) with 4.5 x 10^5 cells per well in media described in each figure. For glucose and glucosamine dose response studies, cells were plated at 3.75 x 10^5 and 2.5 x 10^5 cells per well, respectively, using 12-well plates.

Assay of Luciferase Activity-

Luciferase activity was measured in detergent extracts of cells in the presence of ATP and luciferin as previously described (18) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Light output was integrated over a 10 second period and displayed as Relative Light Units (RLU). Background activity was found be less than 1% of all readings.

Northern Blot Analysis-

Briefly, 5 x 10^6 cells were electroporated with the appropriate DNA constructs and plated in 10-cm plates. At 18 hours post-transfection, cells were harvested and RNA was isolated by the acid guanidine thiocyanate-phenol-chloroform method of extraction (21). The RNA was quantitated and 20ug of total RNA from each condition was electrophoresed through 1% agarose - 6% formaldehyde, transferred, and UV-crosslinked onto GeneScreen nylon membranes (Dupont-New England Nuclear, Boston, MA). The membrane was probed overnight @ 42°C with cDNA, which was labeled on both strands to a specific activity of greater than 3 x 10^9 cpm/ug using the large fragment of DNA Polymerase I (22). The GFAT probe was a 1.0-Kb fragment of the mouse GFAT cDNA (20), and a 1.6-Kb fragment of β-actin was used as a control (23). Radioisotopes, ^{32}P-dATP (> 6,000 Ci/mmol) and ^{32}P-dCTP (> 6,000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL).

Western Blot Analysis-

Using 10-cm plates at 70% confluency, cells were pre-treated overnight with 40uM D.O.N. or vehicle control. The following morning, cells were stimulated with

the appropriate sugar and lysed 48 hours later. Briefly, each plate was scraped and cells were pelleted at 500g @ 4°C. After several washes with cold PBS, cellular pellets were lysed in 500ul of ice-cold 20mM HEPES (pH= 7.9), 500mM NaCl, 20% glycerol, 1mM dithiothreitol, and 1mM phenylmethanesulfonyl fluoride (PMSF). The extracts were placed through two cycles of freeze/thaw using liquid nitrogen and an ice bath. Cellular debris was pelleted for 1 hour at 135,000g @ 4°C in a TLA-100.3 rotor (Beckman, Palo Alto, CA). The supernatants were quantitated for protein concentration using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) and confirmed by Coomassie blue staining of SDS-PAGE gels. Equal amounts of protein from each sample were separated on an 8%- acrylamide-SDS-containing gel and transferred onto Nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The signals were detected using the Enhanced Chemiluminescence System (Amersham, Arlington Heights, IL) after incubation with the RL-2 (24,25) or Sp-1(26) antibodies.

Indirect Immunofluorescence and Confocal Microscopy-

Cells were either first transfected or directly plated onto 8-well chamber slides (Nunc, Naperville, IL). To terminate experiments, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% TX-100, washed, blocked with 5mg/ml BSA in TBST (100mM Tris pH=7.5; 0.9% NaCl; 0.05% Tween-20), and incubated with the mouse monoclonal RL-2 antibody and/or the rabbit polyclonal anti-TAT antibody (Agmed, Bedford, MA) diluted in the same BSA/TBST solution at 1:100 and 1:200, respectively. After washing with TBST alone, the RL-2 was detected with a goat-anti-mouse-Texas Red conjugated secondary antibody (1:500 dilution) (Cal Biochem, Cambridge, MA) while the TAT antibody was detected using biotinylated-Protein A (1:1000 dilution) followed by streptavidin-FITC (1:500 dilution). The Tat and RL-2 signals could be independently co-localized on the same cells because RL-2 is not detectable with protein A and the Tat antibody (rabbit IgG) is not detectable with the goat-anti-mouse conjugate. Omission of any regent resulted in the loss of specific

signal. Slides were dehydrated, mounted, viewed, and photographed using a Leica DMRB microscope. Same field images were compared with phase contrast photomicrographs for cellular localization. For fluorescent quantitation, cells were examined under a confocal microscope (Molecular Dynamics, Sunnyvale, CA) with arbitrary values assigned on a scale from 0 to 255 for each pixel. Area per pixel = 0.0143263 um^3.

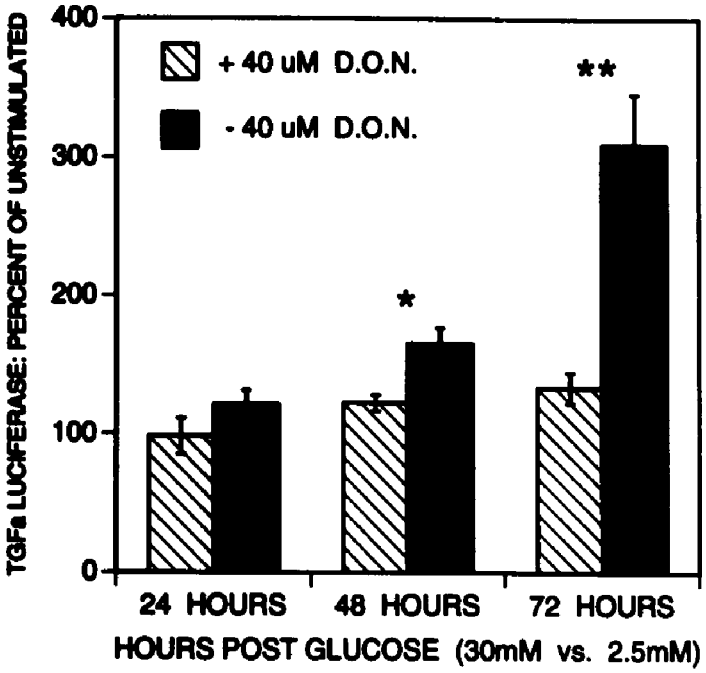
RESULTS

Effect of GFAT Inhibition on Glucose and Glucosamine Induced TGF-α Transcription-

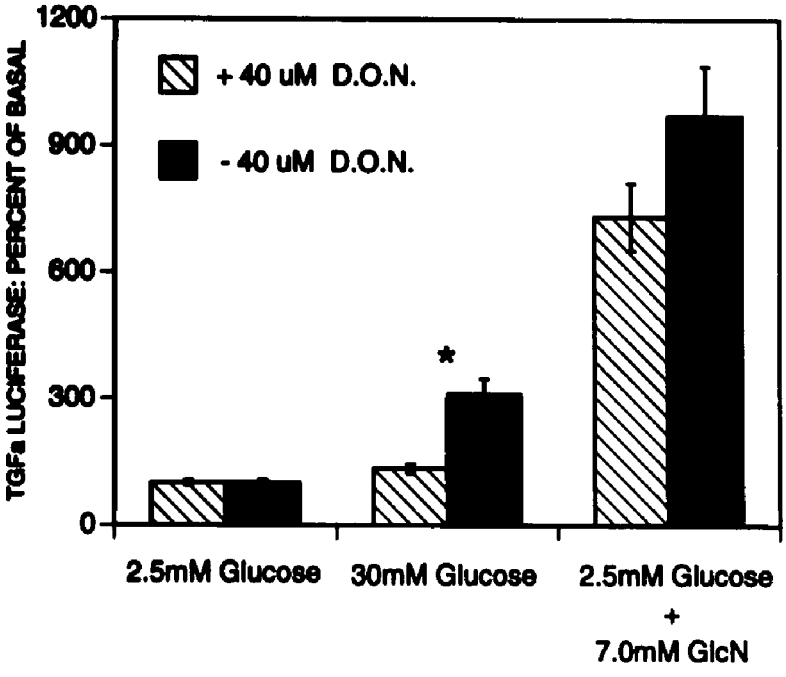
Glucose stimulates TGF-α transcription in vascular smooth muscle cells (7). Furthermore, this increase can reliably be reproduced when the TGF-α promoter is linked to firefly luciferase (7). Using this transient tranfection reporter system, we treated cells with a known inhibitor of GFAT enzymatic activity, 6-diazo-5-oxonorleucine (D.O.N.). This glutamine analog covalently binds to those enzymes that use glutamine in amidotransferase reactions (8). In the hexosamine synthetic pathway, GFAT is one such enzyme as it converts fructose-6-phosphate to glucosamine-6-phosphate. RASM cells, transfected with the TGFα-luciferase reporter gene, were pretreated for 16 hours with D.O.N. or vehicle alone. The following morning, glucose concentrations were left at 2.5mM or increased to 30mM and cells were assayed for luciferase at the indicated times (Figure 1A). In the absence of the inhibitor, glucose stimulated reporter function as usual. However, treatment with 40uM D.O.N. greatly reduced the glucose effect on TGF-α transcription. Glucosamine is known to enter the pathway downstream of GFAT. To determine whether glucosamine could bypass this block of GFAT, we again transfected RASM cells with pTGFα-luciferase. The pool of cells was pretreated for 16 hours with 40uM D.O.N. or vehicle alone and the following morning sugar concentrations were adjusted as indicated and cells were assayed for luciferase 72 hours later (Figure 1B). In the absence of the inhibitor, glucose and

Figure 1: Effect of D.O.N. on glucose and glucosamine induced TGF-α transcription. RASM cells were transfected with TGFα-luciferase, treated with DON or vehicle control, and stimulated 16 hours later with the appropriate sugar. Results are the means +/- SD of two experiments, each assayed in triplicate on independent cultures and normalized to the values observed at 2.5mM glucose at each point. A, cells were assayed for luciferase at the indicated times after stimulation with 30mM glucose. *$p<0.0025$; **$p<0.001$ (Student's *t* Distribution). B, cells were assayed for luciferase 72 hours after stimulation with high glucose or glucosamine. *$p<0.001$ (Student's *t* Distribution).

A


400
300
200
100
0
TGFa LUCIFERASE: PERCENT OF UNSTIMULATED
+ 40 uM D.O.N.
- 40 uM D.O.N.
*
**
24 HOURS
48 HOURS
72 HOURS
HOURS POST GLUCOSE (30mM vs. 2.5mM)

B


1200
900
600
300
0
TGFa LUCIFERASE: PERCENT OF BASAL
+ 40 uM D.O.N.
- 40 uM D.O.N.
*
2.5mM Glucose
30mM Glucose
2.5mM Glucose
+
7.0mM GlcN

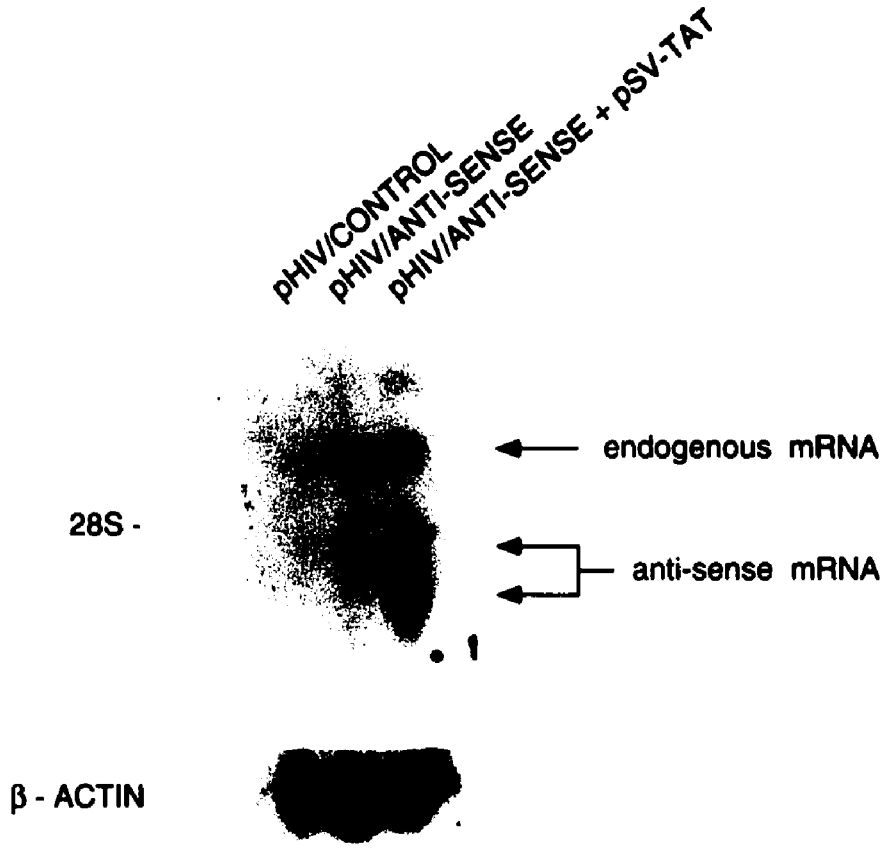
glucosamine stimulated the TGF-α reporter as usual; 3- fold with glucose and 7- to 12-fold with glucosamine (7). Although the glucosamine induced transcription was lower than control, it was near values reported in the literature (7,11).

Effect of Specific Anti-Sense GFAT mRNA on Glucose Induced TGF-α Transcription-

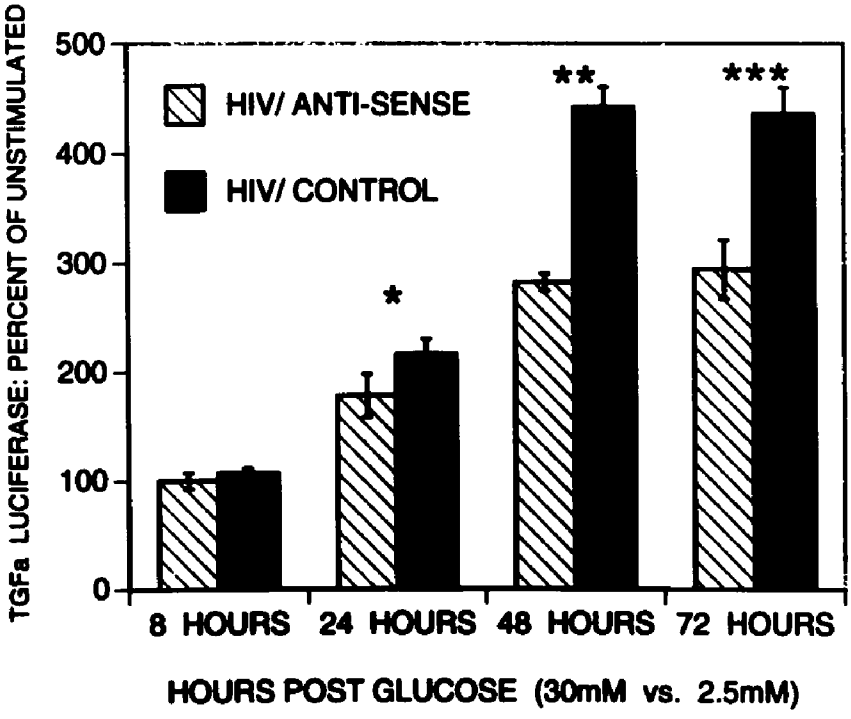
While D.O.N. has long been used as an inhibitor of GFAT activity, it is also known to have effects on other glutamine utilizing pathways. To address this issue, we elected to use a GFAT specific anti-sense mRNA strategy. The GFAT protein is known to be allosterically upregulated as the intracellular pool of UDP-GlcNAc decreases (27,28). Furthermore, GFAT mRNA transcripts are known to have a half-life on the order of 24-30 hours (29). To overcome this regulation, we placed the full-length GFAT cDNA downstream of the HIV-1 LTR in the anti-sense orientation (pHIV/Anti-Sense). While the HIV-LTR is a very strong viral promoter, its activity can be transactivated several 1000- fold with the introduction of the HIV-1 tat protein (19). We therefore tested the ability of the HIV-1 tat protein to transactivate the HIV-LTR in RASM cells. Using transient transfections, the tat cDNA under the control of the SV-40 viral promoter (pSV-TAT) was co-transfected along with the HIV promoter driving anti-sense GFAT mRNA and Northern blot analysis was conducted on the transfections (Figure 2A). Although all lanes are loaded roughly the same as determined by the β-actin message, the tat containing lane is generating large amounts of anti-sense GFAT mRNA transcripts. There are two bands for these transcripts because of the SV-40 splice-intron contained in the vector. Since our transfection efficiency is approximately 8% as determined by CMV-βgal staining (data not shown), the true message is roughly 10-fold higher. Having demonstrated the production of anti-sense GFAT mRNA in RASM cells, we performed a glucose induced time-course study on TGFα–luciferase in the presence and absence of anti-sense message. Cells were transfected with pTGFα-LUC, pSV-TAT, and pHIV/Anti-Sense. The control was the same three plasmids, but with pHIV devoid of the insert (pHIV/Control). Cells were allowed to

Figure 2: Synthesis of anti-sense GFAT mRNA and its effect on glucose induced TGF-α transcription. A, Northern blot of transfected RASM expressing anti-sense GFAT mRNA in the presence and absence of the HIV-1 tat protein. To maintain equal amounts of DNA for all transfections, the two non-TAT lanes were done with equal amounts of the SV-40 promoter vector devoid of the TAT insert. B, cells were transfected with pTGFα-LUC, pSV-TAT, and either pHIV/Anti-Sense or pHIV/Control. After overnight recovery, glucose concentrations were adjusted to 2.5mM or 30mM and assayed for luciferase at the indicated times after glucose stimulation. Results are the means +/- SD of two experiments, each assayed in triplicate on independent cultures and normalized to the values observed at 2.5mM glucose at each point. *$p<0.05$; **$p<0.0005$; ***$p<0.0025$ (Student's *t* Distribution).

A


pHIV/CONTROL
pHIV/ANTI-SENSE
pHIV/ANTI-SENSE + pSV-TAT
endogenous mRNA
anti-sense mRNA
28S -
β - ACTIN

B


TGFa LUCIFERASE: PERCENT OF UNSTIMULATED
500
400
300
200
100
0
HIV/ ANTI-SENSE
HIV/ CONTROL
*
**
***
8 HOURS
24 HOURS
48 HOURS
72 HOURS
HOURS POST GLUCOSE (30mM vs. 2.5mM)

recover overnight and the following morning glucose concentrations were adjusted to 2.5mM or 30mM. Cells were assayed for luciferase at the indicated times (Figure 2B). Although glucose stimulated TGFα–luciferase activity in the presence of anti-sense GFAT mRNA, it was significantly reduced when compared to HIV/Control.

Glucose and Glucosamine Dose-Response Curves in the Presence and Absence of Anti-Sense GFAT mRNA-

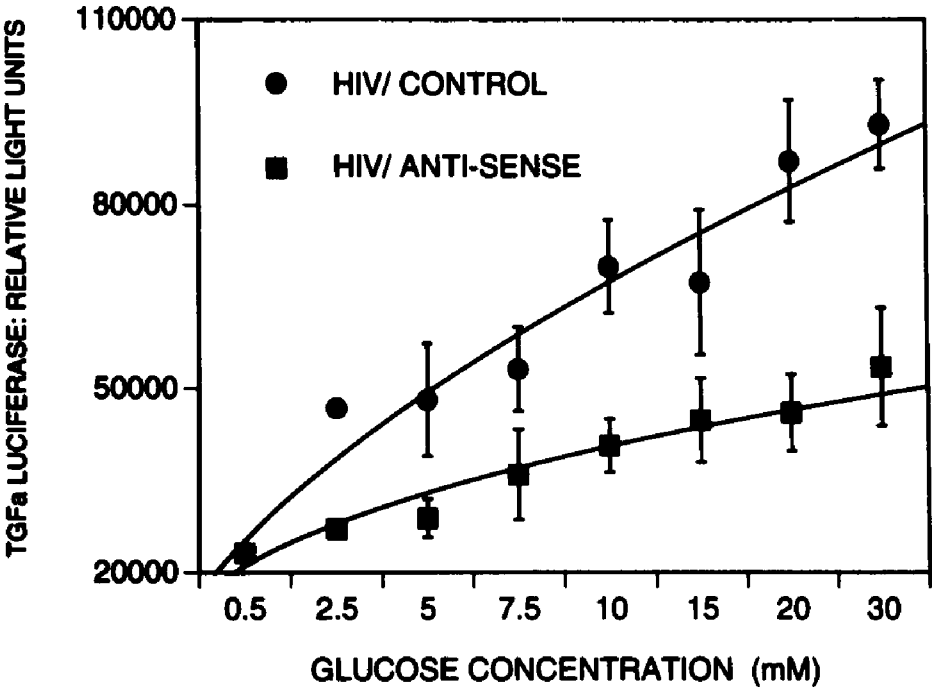
To further test the idea that anti-sense GFAT mRNA inhibits glucose induced TGF-α transcription, but not glucosamine induced transcription, we chose to generate both glucose and glucosamine dose-response curves in the presence and absence of anti-sense GFAT mRNA. If glucose does in fact increase TGFα transcription through the hexosamine pathway, one would predict that blockage of GFAT with pHIV/Anti-Sense would have some effect on glucose induced transcription of TGFα when compared to pHIV/Control. Furthermore, pHIV/Anti-Sense should have no effect on glucosamine induced transcription when compared to pHIV/Control. To test this idea, glucose and glucosamine dose-response curves were generated in transfected RASM containing either pHIV/Anti-Sense or pHIV/Control. Cells were transfected, allowed to recover, and stimulated with sugars as indicated (Figure 3). Analysis of the glucose dose-response curves revealed a significant difference between pHIV/Anti-Sense and pHIV/Control (Figure 3A). However, when the same two pools of transfected cells are stimulated with glucosamine, the dose-response curves are nearly identical (Figure 3B). These results strongly suggest that, when GFAT is blocked, the ability of glucose to increase TGF-α expression markedly reduced.

Analysis of Hexosamine Biosynthetic Products in the Presence and Absence of a GFAT Inhibitor-

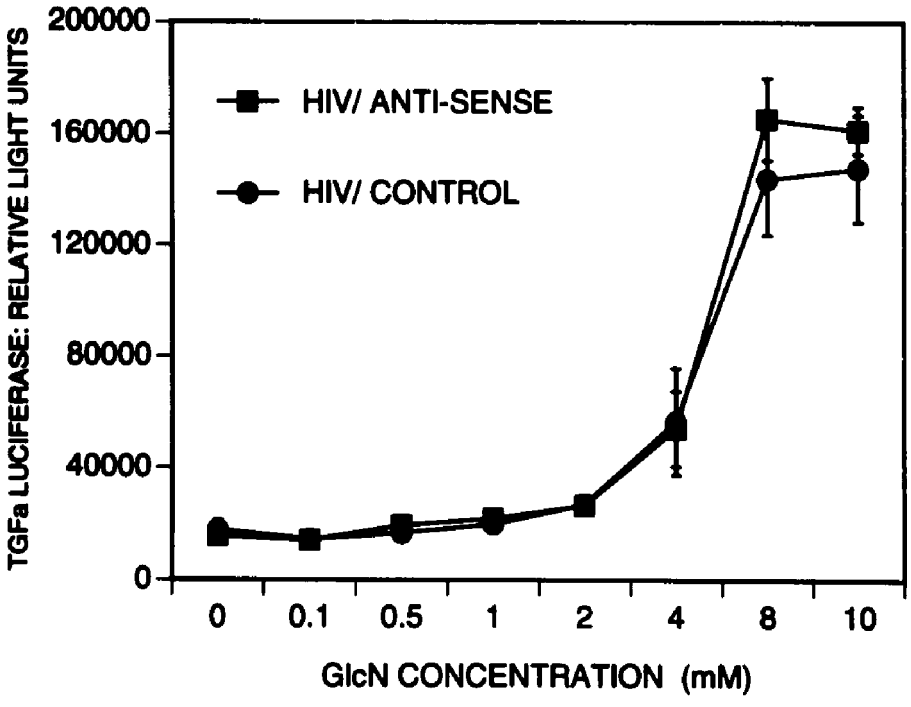
Previous studies have shown that either overexpression of the GFAT enzyme or treatment of cells with glucosamine increases intracellular levels of UDP-GlcNAc (11,12). Since UDP-GlcNAc serves as a substrate for protein glycosylation, we hypothesized that inhibition of GFAT by D.O.N. might decrease the level of these

Figure 3: Effect of anti-sense GFAT mRNA on glucose and glucosamine dose-response curves. Cells were transfected with pTGFα-LUC, pSV-TAT, and either pHIV/Anti-Sense or pHIV/Control. A, after overnight recovery, media was aspirated, refreshed with DMEM + 10% FCS (at the indicated glucose concentrations), and assayed for luciferase 48 hours later. Results are the means +/- SD of one experiment, assayed in triplicate at each time point. Differences between Anti-Sense and Control transfected cells are significant at all data points ($p<0.025$) using Student's *t* Test. B, the same two pools of transfected cells were allowed to recover, refreshed with DMEM + 10% FCS (2.5mM glucose) and supplemented with glucosamine (GlcN) at the indicated concentrations. Cells were assayed for luciferase 72 hours later. Results are the means +/- SD of one experiment, assayed in triplicate at each time point.

A


110000
80000
50000
20000
TGFa LUCIFERASE: RELATIVE LIGHT UNITS
HIV/ CONTROL
HIV/ ANTI-SENSE
0.5
2.5
5
7.5
10
15
20
30
GLUCOSE CONCENTRATION (mM)

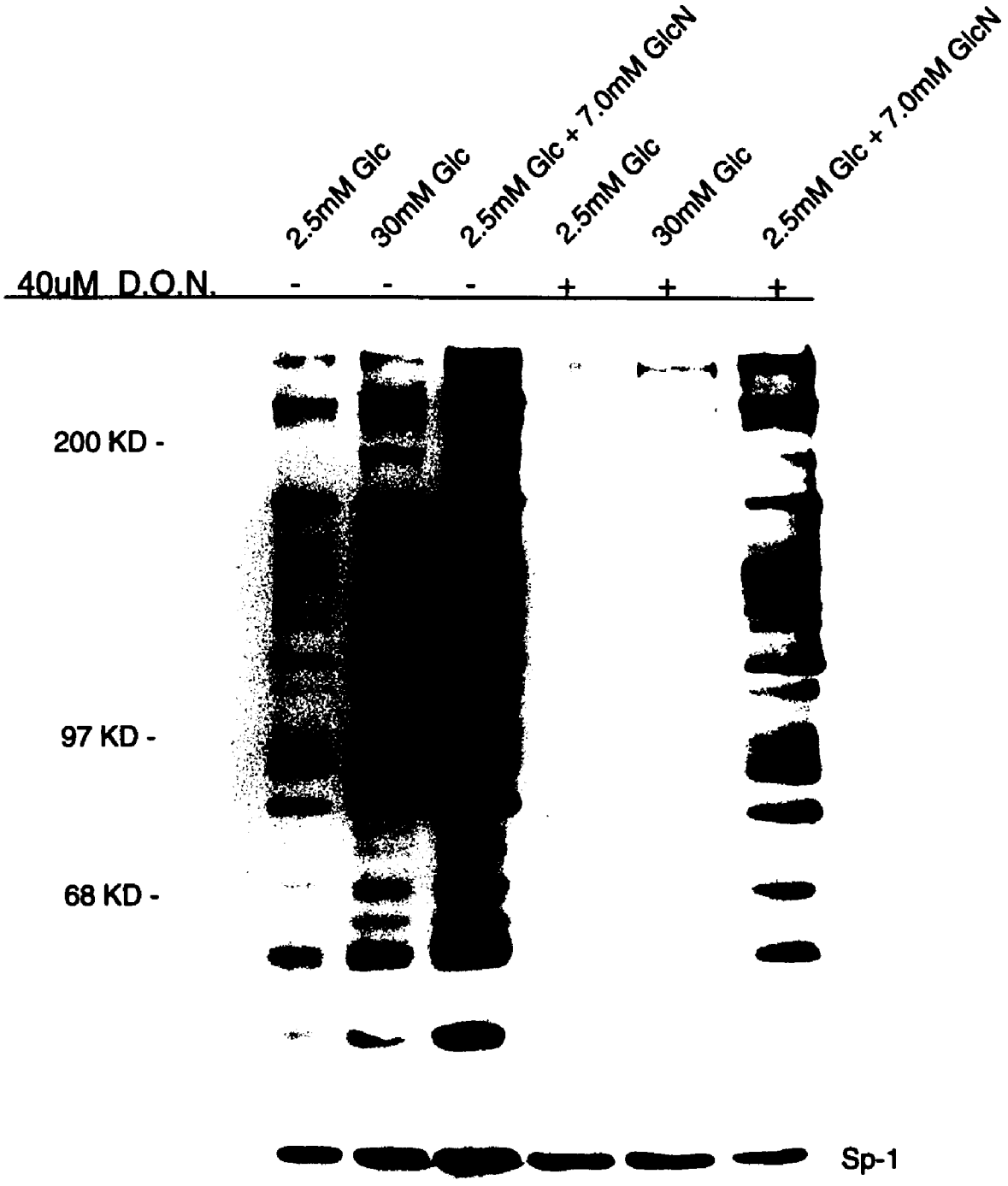
B


200000
160000
120000
80000
40000
0
TGFa LUCIFERASE: RELATIVE LIGHT UNITS
HIV/ ANTI-SENSE
HIV/ CONTROL
0
0.1
0.5
1
2
4
8
10
GlcN CONCENTRATION (mM)

glycosylated proteins. Recent work has demonstrated that the RL-2 monoclonal antibody can specifically detect the GlcNAc moiety on cellular proteins (16). The affinity is strong enough to recognize the *O*-linked GlcNAc moieties on proteins of low abundance, such as the transcription factor Sp-1 (Han and Kudlow, manuscript in preparation). It remains possible however, that the antibody also recognizes other glycoconjugates, such as *N*-linked GlcNAc. In addition, when breast cancer epithelial cells were transferred from glucose-free to glucose/glucosamine containing media, the RL-2 signal was found to be higher even in the presence of the protein synthesis inhibitor, cyclohexamide (16). To determine the effects of glucose and glucosamine on protein glycosylation in RASM cells in the presence and absence of D.O.N., detergent-free extracts were prepared in order to enrich for cytosolic and nuclear proteins and not those bearing *N*-linked GlcNAc saccharides. Equal amounts of protein were loaded onto the gel and the blot was probed with the RL-2 antibody (Figure 4). We found that multiple proteins were detected with RL-2 and that the intensity of the signal was augmented when the cells were cultured in high glucose. Exposure of cells to glucosamine showed a further increase in the signal. However, when cells were cultured in the presence of 40uM D.O.N., both the 2.5mM glucose and 30mM glucose RL-2 signals were greatly reduced. Glucosamine was able to restore the signal in the presence of 40uM D.O.N. though not to the levels seen in controls.

The blot was stripped and reprobed with an anti-Sp-1 antibody in order to confirm equal loading. The Sp-1 protein is known to harbor *O*-linked GlcNAc residues (30) and is present in all lanes.These results indicate that changes in ambient glucose and glucosamine concentrations can alter intracellular protein glycosylation. Furthermore, it appears that GFAT is involved in the glucose stimulation of intracellular protein glycosylation as it can be inhibited with D.O.N. Conversely, the glucosamine stimulation of protein glycosylation appears to occur independent of GFAT activity.

Figure 4: RL-2 Western blot to measure glycoproteins in the presence and absence of a GFAT inhibitor. Cells were grown in DMEM + 10% FCS (2.5mM glucose) and treated with D.O.N. or vehicle control for 16 hours. Cells were then stimulated with high glucose (Glc) or glucosamine (GlcN) and proteins were extracted 48 hours later. The blot was first probed with the RL-2 monoclonal antibody, stripped, and reprobed with an anti-Sp-1 polyclonal antibody to confirm protein content across all lanes. Size markers are in kilodaltons (KD).


2.5mM Glc
30mM Glc
2.5mM Glc + 7.0mM GlcN
2.5mM Glc
30mM Glc
2.5mM Glc + 7.0mM GlcN
40uM D.O.N.
- - - + + +
200 KD -
97 KD -
68 KD -
Sp-1

Cellular Localization and Quantification of the RL-2 Signal by Indirect Immunofluorescence-

In order to determine which cellular proteins are undergoing GlcNAc modification in response to glucose and glucosamine, we probed cells directly with the RL-2 antibody both in the presence and absence of D.O.N. Using a fluorescently labeled secondary antibody, we observed cells as shown (Figure 5). In the absence of D.O.N. and in low glucose, the RL-2 signal was predominately in the nucleus. However, some cytoplasmic staining was visible. Stimulation of these cells with 30mM glucose augmented the nuclear signal. Exposure to glucosamine had a dramatic effect on both the nuclear and cytoplasmic signals. In the presence of 40uM D.O.N., both the low and high glucose signals were significantly reduced. Similar to the Western blot, the glucosamine treated cells were able to restore much of the RL-2 signal even in the presence of the GFAT inhibitor. In order to quantitate the RL-2 fluorescence, the slides were examined with a confocal microscope using a 60X oil objective lens. At this setting, the area of one pixel of resolution is 0.0286526 um^2. Each section with the laser was at a thickness of 0.5um. Therefore, the volume of one pixel is 0.0143263 um^3. For each of the six conditions shown, 15 individual nuclei were chosen at random from five different fields. A line was drawn across each of the 90 nuclei and the relative fluorescence per pixel was recorded into a spread sheet for further analysis. A typical diameter for a nucleus was 95 pixels or roughly 16um. The relative fluorescent values per given area of nucleus are listed in Table 1. These data again indicate that glucose stimulation of intracellular glycosylation is GFAT dependent, whereas glucosamine induced stimulation is GFAT independent.With strong nuclear/cytoplasmic localization of the RL-2 antibody, we speculate that these GlcNAc modifications are most likely *O*-linked GlcNAc as this is where these modifications are known to reside (13,14,15).

RL-2 Immunofluorescence of Anti-Sense GFAT mRNA Transfected Cells-

Having demonstrated the ability to measure glycosylation products directly on cells, we wanted to determine if transfected cells expressing anti-sense GFAT mRNA

Figure 5: Cellular localization of the RL-2 signal by indirect immunofluorescence of cells in the presence and absence of a GFAT inhibitor. RASM cells were cultured on 8-well chamber slides and treated identical as described in figure 4. At 48 hours post sugars, cells were fixed and treated as described in "Methods." All conditions were processed together, as all samples were on the same slide. The RL-2 antibody was localized with a goat-anti-mouse-Texas Red conjugated secondary antibody. The calibration bar corresponds to 20um.

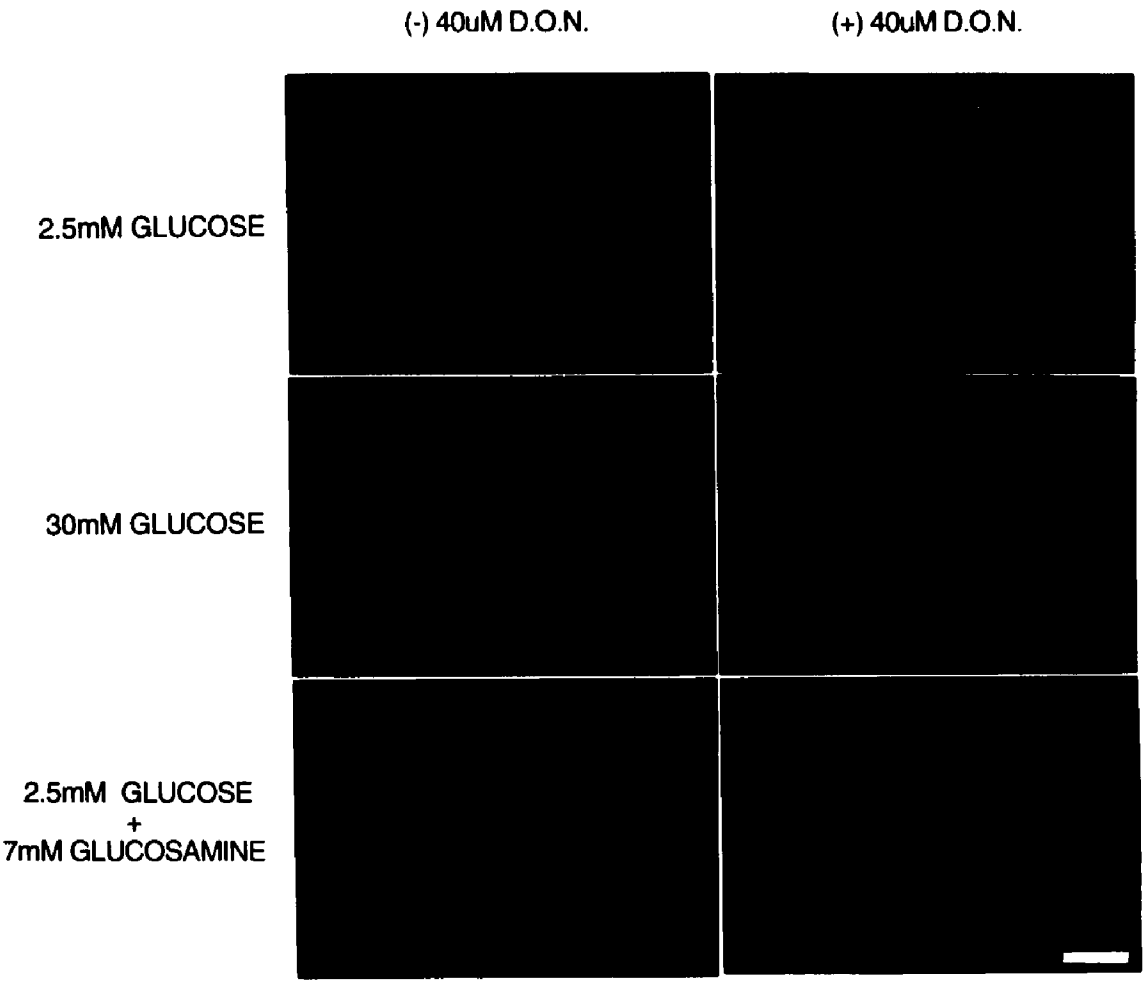

(-) 40uM D.O.N.
(+) 40uM D.O.N.
2.5mM GLUCOSE
30mM GLUCOSE
2.5mM GLUCOSE
+
7mM GLUCOSAMINE

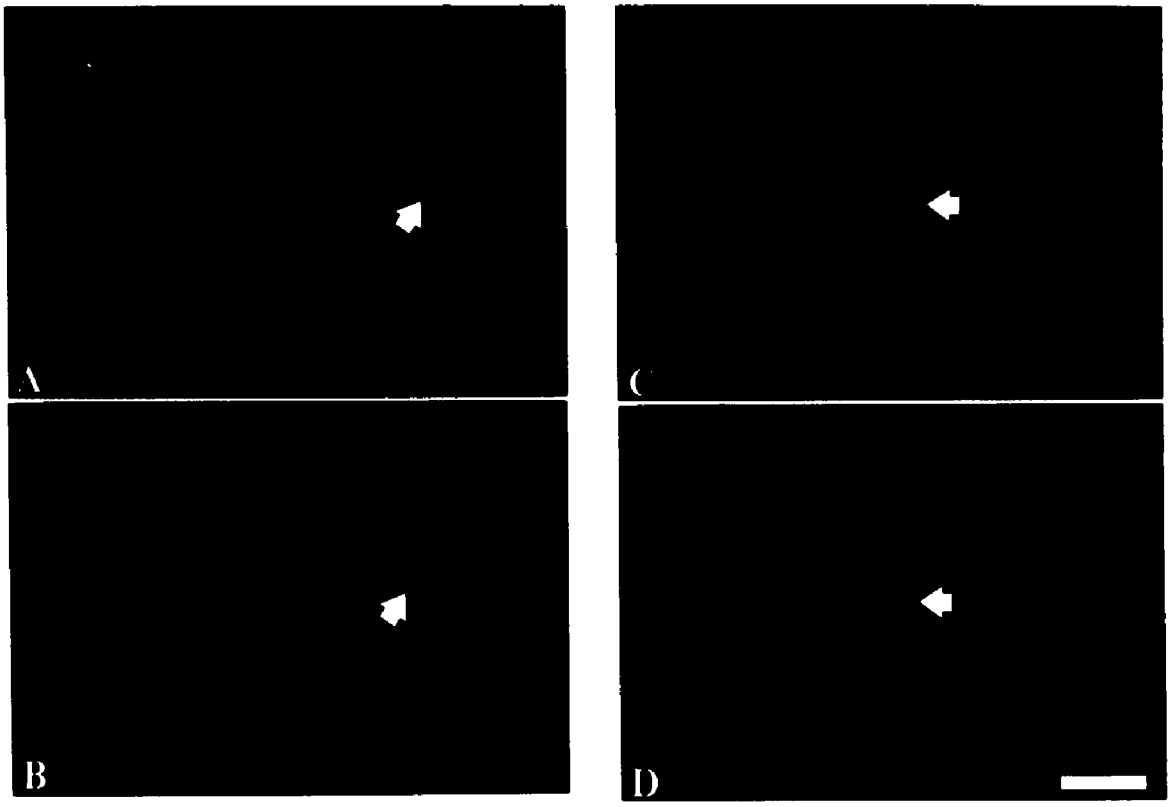
Table 1: Relative fluorescent intensities of the RL-2 signal of RASM cells shown in Figure 5.

CONDITION	40uM DON	MEAN +/- SEM	N
2.5mM Glc	-	30.85 +/- 0.67	1439
30mM Glc	-	46.59 +/- 0.81	1300
2.5mM Glc + 7mM GlcN	-	83.90 +/- 1.06	1410
2.5mM Glc	+	16.28 +/- 0.43	1534
30mM Glc	+	14.28 +/- 0.40	1461
2.5mM Glc + 7mM GlcN	+	58.81 +/- 1.29	1310

The slide containing the six conditions shown in Figure 5 was examined under a confocal fluorescent microscope to determine relative fluorescent intensities. Only nuclei were examined as this is where the signal was predominant. For each of the six conditions, 15 nuclei were chosen at random with the assistance of a blind observer and the intensity for each pixel was recorded on a scale from 0 to 255. The area per pixel of resolution was $0.0286526um^2$. Each section of the laser was at a thickness of 0.5um. The values are therefore displayed as the mean relative fluorescence per $0.0143263um^3$ of nuclear volume for each of the six conditions. N is the total number of pixels across all 15 nuclei for each condition. Glc = glucose; GlcN = glucosamine. Similar results were obtained in subsequent experiments.

also showed reductions in RL-2 signal as determined by indirect immunofluorescence. Cells were transfected with pHIV/Anti-Sense and pSV-TAT in a molar ratio of 5:1. Under these conditions, cells expressing TAT are predicted to have a 94% coincidence of expressing anti-sense GFAT mRNA (31). Cells were electroporated, plated directly onto chamber slides, and allowed to recover overnight. The following morning, cells were stimulated with either high glucose (30mM) or glucosamine (7mM) and fixed 48 hours later. Cells were simultaneously probed with the RL-2 and TAT antibodies. The RL-2 staining of cells grown in high glucose is shown in Figure 6A. A single cell (arrowhead) appears to have faint staining. When the same field is examined for TAT expression (Figure 6B), the same cell is TAT positive. These results were observed in an additional two experiments conducted independent of one another. When TAT positive cells were identified, their RL-2 staining was significantly lower than TAT

Figure 6: Double immunofluorescence for RL-2 and TAT in anti-sense GFAT mRNA transfected cells. RASM cells were transfected with pHIV/Anti-Sense and pSV-TAT. After overnight pre-treatment in DMEM + 10% FCS (2.5mM glucose), the cells were stimulated with either 30mM glucose (A and B) or 7mM glucosamine (C and D). At 48 hours post sugars, cells were fixed and treated as described in "Methods." A field of cells stimulated with high glucose was visualized for RL-2 (A) and the same field was examined for TAT expression (B). When similarly transfected cells were stimulated with glucosamine and examined for RL-2 (C), no loss of RL-2 signal was seen when the same field was visualized for TAT (D). Three independent experiments yielded 11 similar images for glucose stimulated cells and 9 images for glucosamine treated cells. The calibration bar corresponds to 20um.

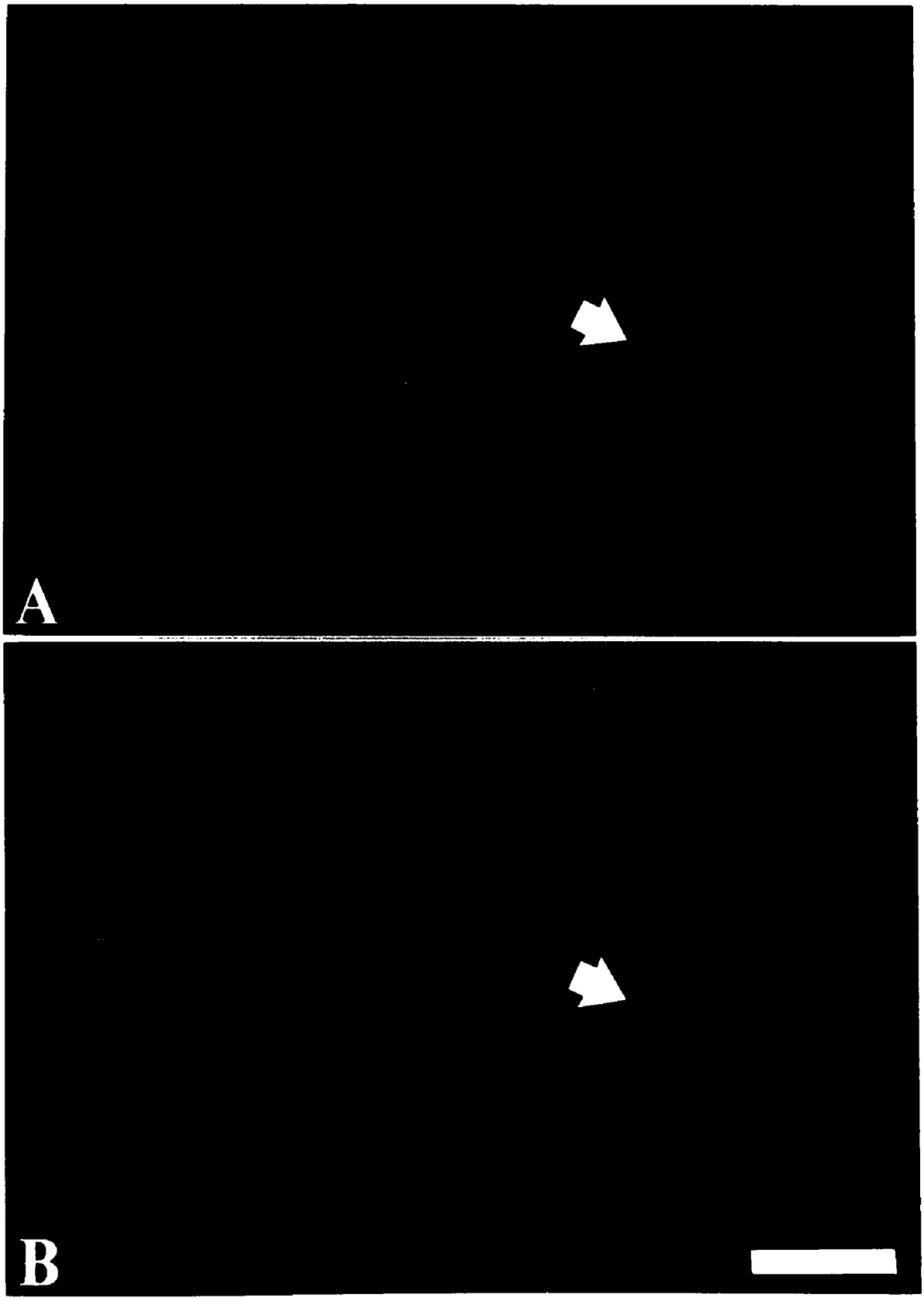

A
C
B
D

negative cells in the same fields. We then looked at cells that were stimulated with glucosamine, presumably bypassing the GFAT block. When cells were examined, no difference was seen in the RL-2 signal (Figure 6C) for a cell that stained TAT positive (Figure 6D) and TAT negative cells in the same field. These results were also reproduced in an additional two experiments. When cells were transfected with pHIV/Control instead of pHIV/Anti-Sense, the RL-2 staining was restored in TAT positive cells cultured in 30mM glucose (Figure 7). In contrast, if cells were stimulated with glucosamine, no difference was seen in the RL-2 signal between pHIV/Anti-Sense and pHIV/Control transfected cells (data not shown). These data further support the idea that glucose stimulation of intracellular protein glycosylation is GFAT dependent and that blockage of GFAT with anti-sense GFAT mRNA significantly reduces products of the hexosamine pathway even in the face of 30mM extracellular glucose. Furthermore, glucosamine stimulation of intracellular protein glycosylation is independent of GFAT activity.

DISCUSSION

TGF-α gene expression is seen in a variety of tissues and the level of expression can be modulated by exposure of cells to various environmental signals. In this paper, we examined the induction of TGF-α expression in vascular smooth muscle cells by glucose and one of its many metabolites, glucosamine. Previous studies have shown that both glucose and glucosamine increase TGF-α transcription in vascular smooth muscle cells (7). Furthermore, viral promoter-driven overexpression of the yeast GFAT cDNA, the rate-limiting enzyme in glucosamine synthesis, augments the effect of glucose on TGF-α transcription (11). While these studies demonstrate that glucose metabolism to glucosamine is capable of eliciting this response, it remains quite possible that glucose can signal through other pathways as well. These experiments were therefore designed to determine if the conversion of glucose to glucosamine was necessary for the glucose induced transcription of the TGF-α gene. The data from these

Figure 7: Double immunofluorescence for RL-2 and TAT in pHIV/Control transfected cells. RASM cells were transfected with pSV-TAT and pHIV/Control. The cells were allowed to recover as described in Figure 6 and stimulated with 30mM glucose the following morning. At 48 hours post stimulation, cells were fixed and probed for RL-2 and TAT as described in "Methods." The high glucose stimulated cells were examined for RL-2 (A) and the same field was then visualized for TAT expression (B). Image shown is representative of five different images produced by two independent transfections. The calibration bar corresponds to 20um.


A
B

experiments strongly suggest that the conversion of glucose to glucosamine is necessary for the glucose effect on TGF-α transcription.

With the TGF-α promoter linked to firefly luciferase cDNA, we used a transient transfection model to access the ability of ambient glucose to increase growth factor transcription. A known pharmacological inhibitor of GFAT, D.O.N., was able to significantly block the glucose effect. Glucosamine, which enters the pathway downstream of GFAT, was able to increase TGF-α transcription both in the presence and absence of D.O.N. In addressing the issue of D.O.N. being a general glutamine amidotransferase blocker, we expressed high levels of full length anti-sense GFAT mRNA using the HIV-LTR and the HIV-1 transactivator, tat. Because of the known allosteric regulation of the GFAT protein (27,28) and the very long half-life of GFAT mRNA (29), it was important to express very high levels of anti-sense message. We found that primary cultures of vascular smooth muscle cells would support tat transactivation of the HIV-LTR. Expression of high levels of anti-sense GFAT mRNA significantly reduced the glucose induced transcription of TGF-α over time. Furthermore, the anti-sense message was able to significantly blunt the glucose induced transcription of TGF-α in a dose-dependent manner when compared to control transfections. Examination of the glucose dose-response curves shows that the half-maximal stimulation for both control and anti-sense transfected cells occurs at about 5mM glucose. The difference in the curves is seen in the lower V_{max} value for the anti-sense transfected cells. Similarly, overexpression of GFAT in vascular smooth muscle cells does not change the concentration of glucose that results in increased TGF-α expression, rather it increases the maximal response (11). Collectively, these two results demonstrate that it is the relative levels of GFAT that are changing and not the kinetics of the enzyme. Of interest is the fact that the TGF-α promoter did respond to increasing amounts of glucose even in the face of anti-sense GFAT mRNA, although significantly lower than controls. Reasons for this may include either 1) not enough

anti-sense message was generated to block GFAT or 2) perhaps a small portion of the glucose effect on TGF-α is mediated through an alternate pathway, such as glycolysis. More likely, however, is the segregation of three different plasmids at the time of transfection. Previous studies have shown when multiple plasmids are transfected into cells, sub-populations of cells exist containing only one plasmid (31). If that were the case in this paradigm, it is quite possible that a small portion of these cells received the TGFα-luciferase plasmid while getting little or no pSV-TAT and/or pHIV/Anti-Sense. Such cells would respond to the higher levels of ambient glucose thus increasing their luciferase activity. This would not pose a problem for the glucosamine dose-response curve as it appears that glucosamine stimulation of TGF-α is independent of GFAT activity and all cells would respond accordingly. Such is the case as the two glucosamine dose-response curves are nearly identical.

It is assumed that high level expression of anti-sense GFAT mRNA results in a decreased flux through the hexosamine pathway. Typically, confirmation of this inhibition is made by direct measurement of UDP-GlcNAc levels via reverse phase HPLC (32). However, because of the large background of untransfected cells (approximately 92%), any specific change might not be detected. As these are low-passage primary cultures, we are unable to generate stable transfectants. To further confound the matter, direct measurements of GFAT protein with an anti-GFAT-antibody are not useful as diminishing UDP-GlcNAc levels will lead to higher enzymatic activity of the GFAT protein through allosteric regulation (27,28). Recent work has shown that the monoclonal antibody, RL-2, serves as a useful tool in assaying for downstream products of the hexosamine pathway (16). Characterization of the antibody demonstrated specificity for *N*-acetylglucosamine (GlcNAc), but not for glucosamine (GlcN) nor *N*-acetylgalactosamine (GalNAc). It was originally generated against nuclear pore proteins, which are known to be *O*-GlcNAc rich (24,25).

Previously however, it has been used for monitoring hexosamine products in cells that have been glucose starved.

When vascular smooth muscle cells were taken from physiological to supra physiological glucose concentrations, the RL-2 signal was found to increase both by Western blot and indirect immunofluorescence of the cells. Glucosamine was found to be more potent than glucose using these same two measurements. D.O.N. was able to block the glucose induced RL-2 signals. Although glucosamine was able to restore both the RL-2 Western blot signal and the fluorescent signal to values near controls, it was never a 100% recovery of signal. Although the cells did not appear distressed by phase contrast microscopy, it is known that higher doses of D.O.N. or extended exposure to the drug can be lethal. Furthermore, D.O.N., known to inhibit amidotransferase reactions, may also have effects on pyrophosphorylase reactions, which catalyze the entry of glucosamine into the hexosamine pathway.

With respect to the RL-2 indirect immunofluorescence of RASM cells, two observations of the staining pattern were noticed. The first was that, within the same treatment group, the staining intensity from one nucleus to the next was somewhat variable and could possibly be a consequence of the cell cycle. The second observation was that, regardless of the treatment, the staining intensity was also somewhat variable across each individual nucleus. For example, the intensity values for RL-2 staining in the nuclelous were near zero. The significance of these observations is not known.

Cells transfected with anti-sense GFAT mRNA were also examined for viability prior to fixation. Cellular morphology was largely intact and no selective loss of cells appeared to have taken place when compared to controls.

Similar to D.O.N. treated cells, anti-sense transfected cells cultured in 30mM glucose and staining TAT positive were found to have significantly lower fluorescence when compared to their TAT negative neighboring cells. More importantly, substitution of pHIV/Anti-Sense with pHIV/Control restored the RL-2 signal in TAT positive

cultured in 30mM glucose. Glucosamine induction of intracellular protein glycosylation, like that of TGF-α transcriptional induction, appears to occur independent of GFAT activity.

These data tend to suggest that the synthesis of glucosamine is vital for the glucose induced transcription of the TGF-α gene. With localization of the RL-2 signal largely to the nucleus, it is likely that the signal transduction involves *O*-linked GlcNAc modifications. Speculation as to the function of these modifications include 1) nuclear localization (33) 2) transcriptional activation (30) 3) transcriptional complex formation (34) and 4) modulating phosphorylation from competition for the same serine/threonine residues (35). It is quite likely however that the signal is mediated through nuclear pore elements as these proteins are known to be *O*-GlcNAc rich (36). The *O*-GlcNAc modification as a signaling molecule is not without precedent. Previous work has shown that lymphocyte activation results in rapidly decreased levels of *O*-GlcNAc in cytosolic proteins and a concomitant increase in the levels of *O*-GlcNAc modified proteins in the nuclear enriched fractions of the cell (33). We are currently examining several candidate proteins.

The fact that glucose-induced-insulin-desensitization in adipocytes and the glucose induced growth factor transcription in vascular smooth muscle cells are mediated by the same pathway may not be a coincidence. Like insulin resistance, the induction of growth factor transcription in vascular smooth muscle cells by supra physiological levels of glucose has relevance to the diabetic state. If this signal is mediated by a single pathway as we propose, the clinical implications could be significant.

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SUMMARY

The studies in this dissertation center on the hexosamine biosynthetic pathway and its rate-limiting enzyme, GFAT. The intentions of our studies were 2- fold: 1) to achieve a better understanding of the transcriptional regulation of the GFAT gene and 2) determine if the glucose induced growth factor transcription seen in vascular smooth muscle cells is mediated through the hexosamine pathway. The results contained herein shed some light on both questions.

We initially cloned and sequenced the mouse GFAT cDNA and subsequently deduced the amino acid sequence (Sayeski et al., 1994). A homology of 98.6% was seen between the human and mouse GFAT proteins. In spite of this homology, Northern blot analysis of human and mouse GFAT mRNA demonstrated a significant difference in the processing of RNA, as there were multiple messages for human GFAT, but a single 7-Kb message for the mouse. This would suggest a difference in the splicing and/or poly A signal of the GFAT message. The significance of this observation on the cell, however, is not known. It is worth noting, though, that the GFAT gene has been conserved through evolution as it can be traced from single cell organisms to humans (Walker et al., 1984; McKnight et al., 1992).

In continuing our examination of GFAT transcriptional regulation, we identified the 5'UTR of the mouse GFAT cDNA through 5'-RACE (Sayeski et al., 1995a). We found the GFAT specific transcript to initiate 149 bp upstream of the ATG translation initiation codon. Interestingly, the sequence homology was very poor when compared to the published sequence for the human GFAT 5'-UTR (McKnight et al., 1992). We continued and cloned the 5'-region of the mouse GFAT gene. Homology was found

between the genomic clone and the 5'-RACE product. We found the first exon to be continuous through the ATG translation codon. Examination of the area 5' to the transcription start site found it lacking a TATA box, but GC-rich and with multiple Sp-1 binding sites. Expression of Sp-1 itself is ubiquitous (Saffer et al., 1991) and some have hypothesized that these proximal sites serve to recruit elements associated with the RNA polymerase II transcription complex that would otherwise not bind because of the lack of a TATA box (Pugh and Tjian, 1991). Others have postulated that these proximal Sp-1 sites mediate interactions between general transcription factors bound near the initiation site and transcriptional activators that bind distal enhancer elements (Gerber et al., 1994). Regardless of which function these sites may serve, it is known that they are critical for proper transcription initiation of these genes (Pugh and Tjian, 1990).

Through the use of electrophoretic mobility shift assays and DNAse I footprint analysis, we mapped Sp-1 binding to the -90/-84 and -17/-7 regions of the mouse GFAT promoter. Loss of the -90/-84 region through deletional analysis resulted in a significant decrease in promoter activity as measured by luciferase reporter assays. To further link this loss of function to Sp-1, we employed site directed mutagenesis and found that mutations in both Sp-1 sites (-90/-98 and -17/-7 regions) resulted in a significant decrease in promoter activity when compared to controls. We further demonstrated that a minimal GFAT promoter (-55/+88) was able to generate luciferase to values 25-fold higher than empty vector controls, suggesting the existence of basal promoter elements within this segment of DNA. It is important to be mindful that, although Sp-1 has long been considered a mediator of basal transcription, recent work has implicated this ubiquitous DNA binding protein to such events as E1A-inducible Sp-1 repression (Suzuki et al., 1995) and E2F-mediated cell cycle transcription (Datta et al., 1995).

The data obtained from the site directed mutagenesis studies may have also alluded to a more dynamic regulation of the GFAT promoter. A mutation placed in the

-60 region of the promoter resulted in loss of activity of the same order as the distal Sp-1 mutation. Examination of this sequence revealed a putative krox-24 binding element. The krox-24 gene product is a transcription factor that binds GC-rich regions and is usually found adjacent to Sp-1 binding sites (Azizkhan et al., 1993). Like Sp-1, it binds GC-rich DNA sequence through Zinc-fingers (Azizkhzn et al., 1993). The gene that encodes krox-24 contains multiple copies of the serum-response element and activation of cells by serum leads to induction of this gene (Cao et al., 1990). It would therefore seem plausible that, as cells enter a growth stage through serum stimulation, it would be important to increase macromolecular synthesis in preparation for mitosis. Such an increase would include the synthesis of cellular proteins and their subsequent glycosylation. The induction of the GFAT promoter by krox-24 would therefore increase the pool of intracellular UDP-GlcNAc allowing for this increased glycosylation. Studies in different cell lines report that the induction of krox-24 transcription is within 30 minutes of serum stimulation (Bravo, 1990) while the induction of GFAT mRNA in response to epidermal growth factor (EGF) requires 12-16 hours (Paterson and Kudlow, 1995). Exactly how the kinetics of gene induction in the same cell would align with the cell cycle remains to be investigated.

In a manner analogous to the krox-24 observation, point mutations placed roughly 100 base pairs 5' to the GFAT transcription start site (L/S -100) may also hint to a more dynamic regulation of the GFAT promoter. Examination of the DNA sequence in the L/S -100 region indicates a putative "E" box binding element. Mutation of this region resulted in a significant decrease in luciferase activity when compared to controls. The best models of "E" box mediated gene transcription are in muscle cell differentiation (Funk et al., 1991) and B cell development (Jacobs et al., 1993; Genetta et al., 1994). Recent work in NIH-3T3 cells, however, has demonstrated that the gene encoding for the glutamine hydrolyzing enzyme, carbamoyl-phosphate synthase/asparate carbamoyltransferase/dihydroorotase (*cad*), is also regulated by "E"

box elements (Miltenberger et al., 1995). The *cad* gene is regulated in a cell-cycle dependent manner and is known to increase transcription 15-fold at the G_1/S-phase boundary in response to serum and part of this induction is mediated through "E" box elements (Miltenberger et al., 1993). Like cad, it is possible that the induction of GFAT by EGF (Paterson and Kudlow, 1995) could also be mediated in part by the "E" box consensus sequence. In order to further investigate this possibility, we have obtained an expression vector that has the "E" box binding protein, p47, under the control of the SV-40 viral promoter (Henthorn et al., 1990). Through co-transfections with the wild type and mutated "E" box GFAT promoters, we can assess the ability of p47 in mediating transcription from this element. Furthermore, gel shift analysis can be done on both mutant and wild type "E" box elements from cells that were transfected with the p47 expressing vector. Work underway in the laboratory will soon support or dismiss this type of transcriptional regulation in the mouse GFAT promoter.

A final mechanism of GFAT transcriptional regulation has come to light through studies that were initiated from work pertaining to this dissertation. Although the work is very preliminary and unpublished, a discussion of it here is perhaps warranted.

In examining Northern blots of GFAT mRNA, it appeared that normal tissues and primary cell cultures expressed far less GFAT message than transformed or immortalized cells (Sayeski et al., 1994; Paterson and Kudlow, 1995; Sayeski and Kudlow, 1995b). Knowing that tumor cells divide much faster than their nontransformed counterparts, it would seem likely that the rate of tumor growth would be dependent on de novo DNA and protein synthesis. We hypothesized that, as cells doubled their DNA and protein content, subsequent glycosylation of those proteins would be required. In knowing that GFAT is limiting in the synthesis of UDP-GlcNAc, a substrate for glycosylation, we were in a position to test this hypothesis.

The wild-type tumor suppressor protein p53 (wtp53) can both increase and decrease gene transcription. Its ability to increase transcription is mediated through

direct DNA binding elements and has been described in several genes including the muscle creatine kinase promoter (Zambetti et al., 1992) and the human transforming growth factor-α promoter (Shin et al., 1995). Paradoxically, wtp53 can suppress other promoters including *c-fos*, actin, and interleukin-6 (Ginsburg et al., 1991; Santhanam et al., 1991; Chin et al., 1992). It is believed that promoter inhibition is mediated indirectly through the waf-1/cyclin-dependent-kinase/E2F pathway (Hartwell and Kastan, 1994). The end result of this pathway is the suppression of those genes responsible for cell division and thus cell cycle arrest.

Our hypothesis therefore became: since tumor cells proliferate faster than primary cells, they must have higher levels of GFAT mRNA so that the glycosylation of proteins is not limiting in this process. Therefore, could over-expression of wtp53 inhibit GFAT transcription in a manner similar to the actin promoter? To test this hypothesis, we co-transfected a plasmid containing the wtp53 cDNA downstream of the SV-40 viral promoter along with a GFAT luciferase plasmid into NIH-3T3 cells. For controls, we used the same plasmid containing a single missense mutation of p53, W248 (mutp53). A recent examination of tumor-derived cells found this mutation of p53 to be the most frequent (Prives, 1994). Dose-response curves of both expression vectors found that the native tumor suppressor, wtp53, was able to inhibit GFAT/Luciferase activity when compared to the mutp53 control (data not shown). At maximal dose of expression plasmids (15ug), the wtp53 transfected cells had 10% the GFAT/Luciferase activity when compared to the mutp53 controls (data not shown). To demonstrate specificity of effector and target molecules, we transfected the same cells with a TGF-α/Luciferase vector and found, as previously reported, that increasing doses of wtp53 *increased* rather than decreased TGF-α/Luciferase expression when compared to mutp53 controls (data not shown).

The significance of this information is that it may demonstrate that GFAT mediates cellular mechanisms outside the realm of carbohydrate metabolism.

Specifically, it may have implications in tumorgenesis. It is worth noting that 25 years ago products of the hexosamine pathway were examined as potential chemotherapeutics because of their abilities to alter growth rates in the sarcoma 180 cell line (Bekesi and Winzler, 1969). Work continues in the laboratory in further characterizing the effects of p53 and other tumor suppressor proteins on GFAT promoter activity.

The second part of this dissertation dealt with the role of the hexosamine biosynthetic pathway in mediating the glucose-induced transcription of growth factors in vascular smooth muscle cells. As stated in the introduction, the hexosamine pathway has been implicated in mediating various cellular events including the glucose-induced-insulin-desensitization in adipocytes (Marshall et al., 1991), glycogen synthase activity (Crook et al., 1993), pyruvate kinase activity (Traxinger and Marshall, 1992), and glucose-induced growth factor expression in vascular smooth muscle cells (McClain et al., 1992). The induction of growth factors by glucose in vascular smooth muscle cells has been a subject of investigation in this laboratory for several years. We initially characterized the presence of transforming growth factor-α in vascular smooth muscle cells and the subsequent processing of the translated protein by neutrophil elastase (Mueller et al., 1990). We further demonstrated that treatment of these cells with glucose and glucosamine resulted in TGF-α mRNA accumulation, which was due to an increase in the rate of transcription as opposed to a decrease in the rate of decay (McClain et al., 1992). The question that remained was whether the conversion of glucose to glucosamine was required to mediate this effect or whether this signal could be transduced through multiple pathways. To answer this question, we examined glucoregulation of TGF-α expression under conditions where GFAT was blocked.

Using a transient transfection assay with the TGF-α promoter linked to firefly luciferase, we tested the ability of glucose to increase growth factor transcription in the presence and absence of a known pharmacological inhibitor of GFAT, 6-diazo-5-oxonorleucine (DON). Time-course studies demonstrated that DON was able to

significantly reduce the induction of TGF-α expression in response to glucose (Sayeski and Kudlow, 1995b). Furthermore, this block could be bypassed in large part by adding glucosamine to the extracellular media. In addressing the issue of nonspecific effects associated with inhibitors, we devised a novel anti-sense strategy, which allowed for high level expression of anti-sense GFAT mRNA. Using the HIV-1 transactivator, tat, we placed the full-length GFAT cDNA downstream of the HIV-1 LTR in the anti-sense orientation and produced high level expression by co-transfectioning the two plasmids into vascular smooth muscle cells. The same system is now being used in several laboratories to overexpress proteins at a level comparable to recombinant virus expression, but with much less labor. Time-course studies found that overexpression of the anti-sense GFAT mRNA significantly blunted the glucose induced TGF-α expression when compared to controls. Furthermore, dose-response curves were generated for glucose and glucosamine on the same set of transfected cells. Again we found that the ability of glucose to increase TGF-α expression in anti-sense transfected cells was significantly blunted when compared to controls. However, the presence of anti-sense GFAT mRNA had no effect on the glucosamine induced TGF-α expression. These results strongly suggest that the conversion of glucose to glucosamine in some part mediates the induction of TGF-α transcription in response to glucose.

In assaying for the biological activity of the hexosamine pathway for both DON treated and anti-sense GFAT transfected cells, we used the monoclonal antibody, RL-2, in both Western blot analysis and indirect immunofluorescence of vascular smooth muscle cells. The epitope is specific for the GlcNAc moiety and has previously been used to measure products of the hexosamine pathway, but only under conditions of glucose starvation (Roos et al., 1995). We demonstrated under glucose conditions which are physiologically relevant, that the flux through the hexosamine pathway is increased with high glucose and more so with glucosamine. We again observed that

DON and anti-sense GFAT mRNA could significantly reduce the glucose, but not glucosamine induced RL-2 signals.

Collectively, these data tend to suggest that GFAT, to some degree, does in fact mediate the induction of TGF-α mRNA by converting glucose to glucosamine. By coming to this conclusion, two new questions must be asked: 1) what is the effector molecule(s) in this process and 2) can this work on primary cultures of vascular smooth muscle cells be extrapolated to *in vivo* models?

In addressing the first question, the simplest answer would be that a new or existing transcription factor has been modified by glycosylation and can now interact with growth factor promoters where it could not previously. Many eukaryotic transcription factors including RNA polymerase II are known to be glycosylated (Berk, 1989). Speculation as to the function of these modifications include 1) nuclear localization, 2) transcriptional activation, 3) transcriptional complex formation, or 4) modulating phosphorylation through competition for the same serine/threonine residues (Jackson and Tjian, 1988). To further confound the problem, treatment of vascular smooth muscle cells with high glucose or glucosamine leads to the modification of dozens of proteins. We are currently examining several DNA binding proteins as potential mediators of this effect.

Perhaps of equal significance is the data obtained from the RL-2 immunofluorescence. While glucose and glucosamine did in fact increase the nuclear RL-2 signal, a similar increase in signal was seen along the nuclear membrane. The nuclear pore complex is known to be discriminating in terms of nuclear trafficking (Miller and Hanover, 1992). It remains possible that modifications of nuclear pore elements transduce this effect by allowing a cytoplasmic protein into the nucleus and thus interacting with growth factor promoters. Similar to the DNA binding proteins, several nuclear pore proteins are candidates for effector molecules and are currently being scrutinized in the laboratory.

The second issue to address is the ability of glucose and glucosamine to increase growth factor transcription in vascular smooth muscle cells, *in vivo*. To achieve this end, we have developed a transgenic mouse which has the human TGF-α promoter driving expression of the LacZ cDNA. Initial staining with X-gal has demonstrated blue staining for transgenic animals, but not controls. As carriers are identified, animals can be made diabetic by injecting them with streptozotocin. The toxin will lead to β-cell destruction and subsequent loss of insulin production. Blood vessels and other tissues associated with diabetic pathologies can be examined for X-gal staining. Glucosamine can be delivered either through I.P. injections or from the drinking water. The end result would be to identify glucose and glucosamine induced X-gal staining in transgenic animals, but not controls. Only time stands between these questions and their respective answers.

In terms of health care dollars, diabetes is one of the most expensive diseases in this country (Glasgow, 1995). The complications associated with diabetes can be debilitating and even lethal (DCCTRG, 1993). As noted earlier, these deleterious effects have been attributed to hyperglycemia per se (Lorenzi, 1992). The transduction pathways between hyperglycemia and the pathologies associated with diabetes are known to include such factors as ischemia, hypoxia, lipidemia, growth factor expression, and abnormal Ca^{+2} regulation (Kohner et al., 1995). Our goal has been to better understand the growth factor component within this equation by determining whether or not the hexosamine biosynthetic pathway mediates, to some degree, the ability of high blood glucose to turn on growth factor transcription. Our studies conclude that, to some extent, this is true. One hope would be that the next generation of GFAT specific inhibitors could reduce this glucose effect on TGF-α transcription. By doing so, this may change the equilibrium of the variables that lead to diabetic complications. The fact that glucose-induced-insulin-desensitization in adipocytes, a hallmark of Type II diabetes, and the glucose-induced growth factor transcription in

vascular smooth muscle cells are mediated by the same pathway may not be a coincidence. The clinical implications could be significant.

As stated in the introduction, the purpose of the studies contained in this dissertation was to address two specific questions: 1) how is the GFAT gene regulated at the transcriptional level and 2) does GFAT mediate the glucose-induced increase in growth factor transcription seen in vascular smooth muscle cells? To some degree, these questions have been answered. At the same time, however, new and perhaps even more important questions must now be addressed as a result of the findings contained in this thesis.

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