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Identification of an Insulin-Responsive Element in the Rat Glucose-6-Phosphate Dehydrogenase Gene

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IDENTIFICATION OF AN INSULIN-RESPONSIVE ELEMENT IN THE RAT
GLUCOSE-6-PHOSPHATE DEHYDROGENASE GENE

by

Sheri Lynn Holmen

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
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Sheri Lynn Holmen

IDENTIFICATION OF AN INSULIN-RESPONSIVE ELEMENT IN THE RAT GLUCOSE-6-PHOSPHATE DEHYDROGENASE GENE

Sheri Lynn Holmen, M.S.

Western Michigan University, 1995

The expression of several genes has been shown to be either positively or negatively regulated by insulin at the transcriptional level, however no single insulin responsive DNA element has yet been identified. The transcription factors responsible for stimulation or inhibition of gene expression by insulin also remain to be identified. In recent years, expression of the gene for glucose-6-phosphate dehydrogenase (G6PDH) in primary rat hepatocytes has been found to be influenced by hormones such as insulin, dexamethosone, and glucagon. The molecular mechanism(s) however, underlying hormonal regulation of G6PDH gene transcription remains to be elucidated. The focus of this study was to characterize the insulin responsive elements which exhibit positive regulation of the glucose-6-phosphate dehydrogenase gene by insulin.

In this study, a segment (+57 to -878) of the rat G6PDH promoter region was cloned into a plasmid containing the luciferase reporter gene. Serial 5' deletions were used to define minimal sequences required for insulin regulation. The results suggest that the insulin responsive element(s) within the G6PDH promoter may be located in the region from -130 to -50. Gel mobility shift assays using the sequence from -130 to -39 as a probe revealed nuclear factor(s) from primary rat hepatocytes that complexed with this sequence. Competition for the nuclear factor(s) using unlabeled probe, determined that this DNA-protein interaction is specific. Deoxyribonuclease I (DNase I) footprinting revealed a protected region spanning from -126 to -99.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
INTRODUCTION.....	1
Genetic Regulatory Mechanisms.....	1
Transcription	1
Initiation of Transcription.....	2
Regulation of Transcription.....	3
Hormonal Regulation of Transcription.....	3
Insulin.....	4
Insulin Action	5
Transcription Factors.....	6
LITERATURE REVIEW	8
Insulin Responsive Genes.....	8
Glucose-6-Phosphate Dehydrogenase.....	9
Objective of the Study.....	11
Significance of the Study	13
MATERIALS AND METHODS.....	14
PCR Amplification.....	14
Plasmid Construction.....	14
Plasmid DNA Preparation	16
Hepatocyte Isolation and Culture	16

Table of Contents-Continued

Transfection	17
Cell Processing.....	17
Luciferase Assay.....	18
G6PDH Assay	18
Nuclei Isolation	19
Radioactively Labeled DNA Probe	20
Gel Mobility Shift Assay.....	20
DNA Footprinting	21
RESULTS.....	23
Expression of G6PDH-Luciferase Fusion Genes by Transient Transfection of Primary Rat Hepatocytes.....	23
Insulin Stimulation of G6PDH-Luciferase Expression.....	24
Basal G6PDH Promoter Activity of 5' Deletion Constructs In Primary Rat Hepatocytes	25
Gel Mobility Shift Assays	26
Gel Mobility Shift Experiments Using Nuclear Extracts From Primary Rat Hepatocytes	26
Competition Analysis of Factors Binding to a 92 bp G6PDH Probe in Gel Mobility Shift Assays.....	28
Time Course of Insulin Action	29
DNA Footprint Analysis	31
DISCUSSION	33
BIBLIOGRAPHY	38

LIST OF TABLES

1. Biochemical and Biophysical Actions of Insulin.....	4
2. Sequence Homology in the Promoters of Insulin-Responsive Genes.....	8
3. Oligonucleotide Primers.....	15
4. Comparison of Reported DNA/Protein Binding Buffers.....	26

LIST OF FIGURES

1. Transcriptional Control Region for a Mammalian Gene.....	2
2. Insulin Signaling Cascade	5
3. Structure of Common DNA Binding Domains.....	7
4. Rat G6PDH Promoter DNA Sequence.....	12
5. Reaction of Luciferase.....	18
6. Reaction of G6PDH.....	19
7. p-GL2 Basic Vector With G6PDH Insert.....	23
8. Effect of Insulin on Constructs Containing Deletions of the G6PDH Promoter.....	24
9. Basal Activity of the G6PDH Promoter in Primary Rat Hepatocytes Transfected With G6PDH-LUC Fusion Genes	25
10. Gel Mobility Shift Assay Using Different Binding Buffers.....	27
11. Gel Mobility Shift Assay Using Primary Hepatic Nuclear Extracts.....	28
12. Gel Mobility Shift Assay to Determine the Time Course of Insulin Induction of G6PDH.....	30
13. DNase I Footprint Analysis	31

INTRODUCTION

Genetic Regulatory Mechanisms

Following the discovery and characterization of bacterial operons, many studies were initiated to locate similar regulatory systems in eukaryotes (Jacob et al., 1961). While a similar system has been found in yeast, no operons have yet been found in multicellular eukaryotes. Eukaryotic genetic systems appear to be much more complex as compared to the less advanced systems in prokaryotes. Eukaryotic cells contain a greater amount of DNA, the DNA complexes with histones and other proteins to form chromatin, the genetic information is carried on many chromosomes as opposed to one, and these chromosomes are surrounded by a nuclear membrane. The processes of transcription and translation are separated from one another spatially. The transcripts of eukaryotic genes are modified before transport to the cytoplasm, and many transcribed sequences never exit the nucleus. Therefore, regulation of gene expression may potentially occur at several levels (Klug et al., 1993).

Transcription

The primary level at which gene expression may be controlled is transcription, the initiation of mRNA synthesis (Latchman, D.S., 1993). Studies utilizing bacteria and viruses have begun to dissect how transcription is controlled in prokaryotes. This work has provided paradigms for understanding how transcription is regulated in multicellular organisms. It has been shown that only a small fraction of the entire genome of an organism is transcribed in any one cell at any one time. As little as 1 % of the total DNA may be transcribed in a differentiated cell (Mathews et al., 1990). The

control region in the immediate vicinity of a transcription start site is called the promoter. Variations in rates of transcription of different genes may be due to differences in promoter structure. This may direct RNA polymerase II to bind certain promoters and not others (Alberts et al., 1994).

Initiation of Transcription

In the eukaryotic cell, most of the structural genes (those encoding proteins) are transcribed by RNA polymerase II. Several accessory proteins to RNA polymerase II have been found. Some of these proteins identified so far include (TFII)D, A, B, F, E, H, and J (Roberts et al., 1993). These proteins have been termed basal transcription factors. The consensus sequence TATA is located 25 bp 5' of the start site and is thought to bind TFIID (Figure 1). A consensus sequence includes the nucleotides, cis-elements, which occur most frequently at each position when a series of sequences thought to have similar functions are compared (Mathews et al., 1990). The transcription factor TFIID is a complex of proteins which includes the TATA binding protein (TBP) and associated factors (TAF's). The minimal required complex for initiation of transcription appears to include only (TFII)D, B, F and RNA polymerase II (Buratowski, 1994).

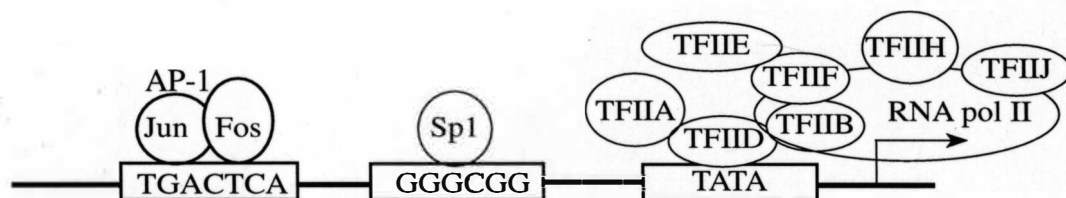


Figure 1. Transcriptional Control Region for a Mammalian Structural Gene.

Regulation of Transcription

Extracellular signals may cue the cells to regulate the extent of transcription of active genes or to turn certain genes on or off. The metabolic capabilities of a cell are governed by the mechanisms which determine which genes will be transcribed. The mechanisms and biochemical pathways by which cells regulate transcription are still unknown for many external stimuli. Ultimately, regulation of transcription by these signalling pathways depends on factors that interact with DNA in a highly site-specific manner (Mitchell et al., 1989).

Hormonal Regulation of Transcription

During the development of multicellular organisms, changes in gene expression are often influenced by external signals such as hormones. Hormones are defined as chemical messengers. They are produced by specialized cells and secreted into the circulation where they interact with specific receptors on target cells to mediate a response. Evidence that gene expression in eukaryotes is regulated by extracellular signals was first provided by Ulrich Clever and Peter Karlson in 1960. Clever and Karlson discovered that the steroid hormone ecdysone induced changes in the puffing pattern of salivary gland chromosomes of *Chironomus tentans* (Clever et al., 1960).

Both positive and negative regulation of gene expression by hormones is possible. Regions which increase transcription from a distance independent of orientation are called enhancers or positive regulatory elements. If transcription is decreased, the regions are called silencers or negative regulatory elements (O'Brien et al., 1991).

Insulin

Two of the hormones involved in regulation of metabolism include the peptides glucagon and insulin (Mathews et al., 1990). Insulin is a small peptide, 5.8-kDa, synthesized by the beta cells of the islets of Langerhans in the pancreas. The secretion of this hormone into the bloodstream is a tightly controlled process and is dependent on plasma glucose levels. After eating, plasma glucose levels rise. This results in increased secretion of insulin from the beta cells. When glucose levels are low, as in fasting, insulin levels are also low (Hargrove et al., 1993). Insulin is known to regulate the metabolism of most cells.

The functions of insulin in most tissues include the control of lipid, sugar, ion, amino acid, and nucleotide metabolism. There are three major sites of metabolic regulation in target tissues; in the plasma membrane the transport of glucose, ions, and other fuel substrates is increased, within the cell several enzymes are activated, and in the nucleus, gene expression is regulated. Table 1 summarizes the biochemical and physiological actions of insulin (Mathews et al., 1990).

Table 1

Biochemical and Physiological Actions of Insulin

Biochemical Action	Physiological Action
↑ Cell permeability to glucose and amino acids	Signals fed state
↑ Glycolysis	↑ Fuel Storage
↑ Glycogen synthesis	↓ Blood glucose
↓ Gluconeogenesis	↑ Cell growth and differentiation
↑ Triacylglycerol synthesis	
↓ Lipolysis	
↓ Protein degradation	
↑ Protein, DNA, and RNA synthesis	

Insulin Action

The actions of insulin are initiated by the hormone binding to its receptor, a transmembrane glycoprotein with intrinsic tyrosine kinase activity in its cytoplasmic domain. Autophosphorylation of the insulin receptor can result in phosphorylation of insulin receptor substrate (IRS-1) on multiple tyrosine residues. IRS-1 is a principle substrate of the insulin receptor, and when activated, binds to the SH2-domains of numerous signal transduction proteins (Kahn et al., 1993). SH2 domains refer to src homology regions. These regions are highly conserved noncatalytic domains which aid the docking of proteins to activated tyrosine kinases by recognizing phosphorylated tyrosine residues. These proteins, shown in Figure 2, include PI 3-Kinase, GRB2,

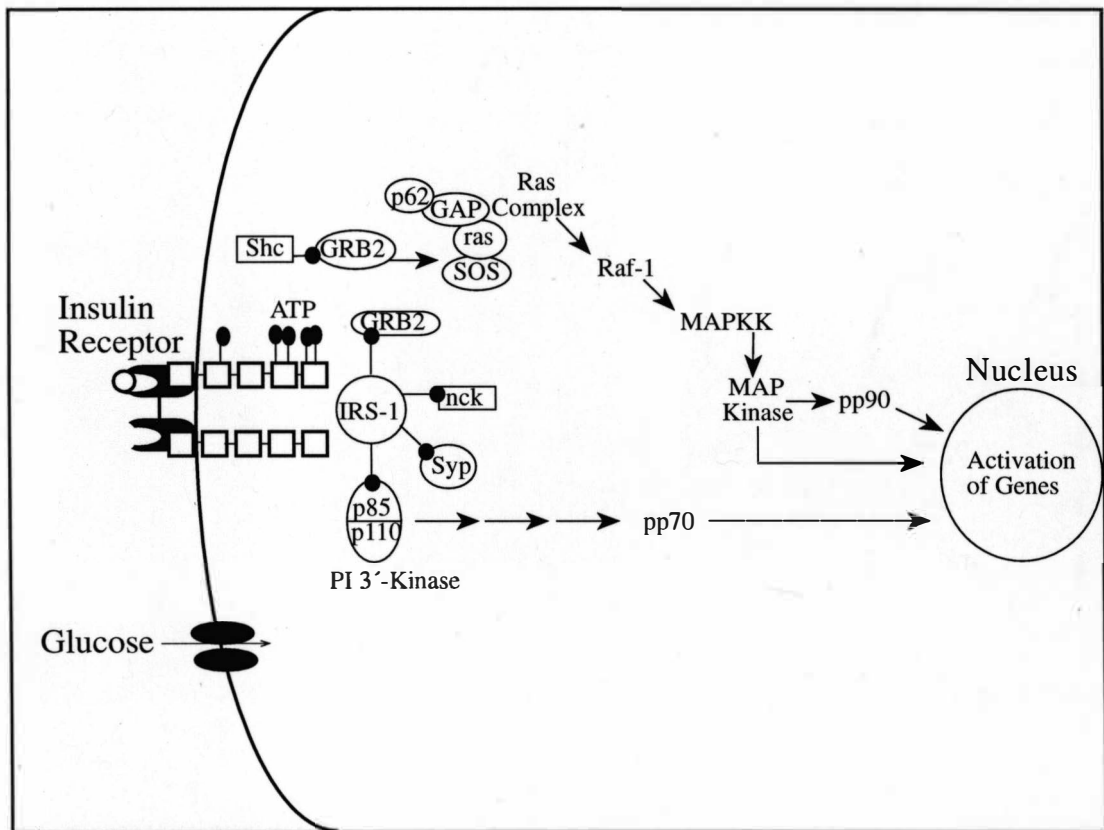


Figure 2. Insulin Signaling Cascade.

Syp, and nck. They couple with IRS-1 to mediate the insulin response which leads to the activation of genes (Cheatham et al., 1994). There are several proposed mechanisms which lead to gene activation: direct phosphorylation of a cytoplasmic transcription factor; phosphorylation of a cytoplasmic protein which results in the release of a bound transcription factor; activation of a transcription factor by ligand binding; removal of inhibitory proteins which block the entry of transcription factors into the nucleus and; activation of kinases which translocate to the nucleus where they change the DNA-binding activities of resident transcription factors enabling them to activate gene expression (Edwards, 1994; Alberts et al., 1994).

Transcription Factors

Transcription factors have been shown to bind to the DNA sequences in the promoter regions of structural genes. Several transcription factors have been isolated and identified. The tertiary structure of the DNA binding domain of a transcription factor suggests a mechanism by which the protein recognizes a specific DNA sequence and binds to it. DNA binding domains take on several forms. Some of the domains studied so far include zinc fingers, leucine zippers, and helix-turn-helix motifs (Klug et al., 1993) (Figure 3). The transcription factor specificity protein 1 (Sp1) is an example of a factor which contains three tandem zinc fingers at its carboxy terminus. Sp1 activates transcription by first selectively binding to GC box elements (Briggs et al., 1986) (Figure 1). Other transcription factors which contain zinc finger DNA binding domains have different DNA sequence specificities. Therefore, the structural framework of the binding motif is not the only determinant of binding specificity. Sp1 also contains a glutamine rich domain on its other end (Figure 3). This domain was found to be an activation domain which interacts with TAF_{II}110. This domain was found to be essential for Sp1 to stimulate transcription (Tjian, R. 1995).

The transcription factor AP-1 selectively binds cis-elements consisting of the sequence TGACTCA. The proteins c-jun and fos are capable of forming heterodimers that can bind to AP-1 sites. Homodimerization of c-jun can also bind to AP-1 sites (Mitchell, et al., 1989). These proteins are encoded by several genes including c-jun, junB, junD, fos, and fra-1, in mammals (Mitchell et al., 1989) (Figure 1). The similar DNA binding specificities of these proteins are due to their conserved leucine zipper DNA binding domains (Figure 3). This DNA-binding motif consists of a regularly spaced row of leucines on one side of an α -helix which interacts with another set of leucines on an α -helix to form dimers of the proteins.

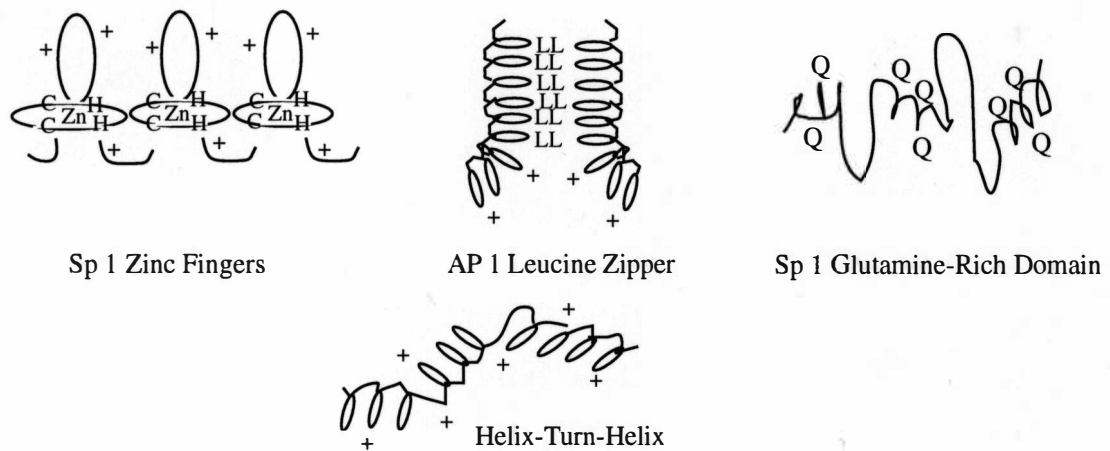


Figure 3. Structures of Common DNA-Binding Domains. Also included is the Sp1 glutamine rich protein binding activation domain.

LITERATURE REVIEW

Insulin Responsive Genes

Although much is known about the initiation of the signal transduction pathway for insulin, little is known about the proteins or DNA sequences directly involved in the expression of insulin responsive genes. There are currently over 50 genes known to respond to insulin (O'Brien et al., 1991). Positive cis-acting insulin responsive elements (IRE's) have been found in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Nasrin et al., 1990), amylase (Johnson et al., 1993), prolactin (PRL) (Stanley, 1992), human growth hormone (hGH)(Prager et al., 1990), glucokinase (Nouspikel et al., 1992), and fatty acid synthase (FAS) (Moustaid et al., 1994) genes (Table 2). Negative regulatory elements for insulin have been identified in the phosphoenolpyruvate carboxykinase (PEPCK) (O'Brien et al., 1990), insulin-like growth factor binding protein-1 (IGFBP-1) (Suwaniekul et al., 1993) and glucagon

Table 2

Sequence Homology in the Promoters of Insulin-Responsive Genes

Gene	5'	Sequence	3'
GAPDH	-461	CCC GCCT CTCAGCCTTTGAA	-453
Amylase	-168	<u>TCAGTTTATTTTGCGTGAGAGT</u>	-147
PRL	-106	TCTTAATGACGGAAATAGAT	-87
hGH	-287	ATGGCCTGCGG	-277
Glucokinase	-83	TGGTTCCTTG	-73
FAS	-71	<u>TCAGCCCATGTGGCGTGGCCGC</u>	-50
PEPCK	-416	TGGTGTTTGGACAAC	-403
IGFBP-1	-110	AGCAAAACAACTTATTTTGAACAC	-85
Glucagon	-268	<u>TAGTTTTTCACGCCTGACTGAGATTGAAGGG</u>	-238

genes (Philippe, 1991). Consensus cis-acting DNA sequences and trans-acting factors for insulin have not yet been identified. Table 2 compares the reported insulin responsive sequences for several of the genes listed above. There are many homologous regions found between the genes listed in Table 2 as indicated by the underline and boldface type. It remains to be determined if the same sequences are responsible for both positive and negative regulation of gene expression by insulin. Table 2 shows a common element with 4 consecutive thymidine residues, TTTTG, shared by the amylase, PEPCK, IGFBP-1, glucagon and glucokinase genes. The similarity appears to be the strongest between PEPCK and IGFB-1. However, studies which mutated the glucagon IRE to eliminate the insulin response did not involve this sequence (Phillippe, J., 1991). Another common element, TCAGCC, is shared between GAPDH and FAS. The glucagon gene contains a similar sequence TCACGCC.

Experiments done by Wolf et al. in 1994 studied the regulation of FAS by insulin. They proposed a 4 bp consensus sequence for insulin induction. The consensus sequence consists of the bases GCC^c/_T. The FAS, GAPDH, hGH, and glucagon genes all contain this consensus sequence. All of the genes except FAS contain the pattern GCCT. This consensus sequence implies that positive and negative regulation by insulin can be mediated similarly.

Glucose-6-Phosphate Dehydrogenase

Another gene known to be induced by insulin is glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49). G6PDH is an enzyme found in all mammalian cells and is responsible for catalyzing the conversion of glucose to 6-phosphogluconolactone with a concomitant formation of NADPH. This enzyme

controls the flow of carbon through the pentose phosphate pathway. The NADPH produced is important for reductive biosynthesis, such as the synthesis of fatty acids and steroids, and for maintaining the redox state of the cell. In growing cells, G6PDH is essential for the production of ribose-5-phosphate for nucleic acid synthesis (Molero et al., 1994).

G6PDH has been classified as a member of the lipogenic enzyme family. This family includes enzymes important for hepatic fatty acid synthesis such as fatty acid synthase, malic enzyme, and acetyl-CoA carboxylase. The process of fatty acid synthesis is increased by insulin and decreased by glucagon in rat liver. Since the regulation of these enzymes is coordinated with the rate of fatty acid synthesis, it has been hypothesized that the metabolic and hormonal factors, like insulin, which regulate this process also regulate the induction of G6PDH (Manos et al., 1986).

Studies utilizing intact animals have identified a variety of nutrients and hormones responsible for regulation of G6PDH in the liver. However, the use of intact animals makes it difficult to discern the differences between the direct and indirect effects of these nutrients and hormones on gene expression. The use of primary rat hepatocytes in culture provides a less complex system which retains the differentiated phenotype of the intact animal. By culturing these cells in chemically defined medium it is possible to add specific quantities of the nutrients and hormones, and therefore define the precise role these factors play in controlling G6PDH gene expression (Kleitzen et al., 1993).

The primary signal for induction of lipogenic enzyme gene expression, including G6PDH, is insulin. Insulin induction of G6PDH was first demonstrated in primary rat hepatocytes by Kurts and Wells in 1981. Subsequent studies showed that the increase in G6PDH activity was comparable to increases found in specific mRNA (Fritz et al., 1986; Manos, 1987). This data suggests that induction of G6PDH gene

expression by insulin is either the result of increased gene transcription or stabilization of the mRNA at the post-transcriptional level. Nuclear “run-on” assays employed on fasted-refed animals have demonstrated that there is a marked increase in G6PDH gene transcription (Prostko et al. 1989).

To fully investigate this regulation of G6PDH gene expression, a 935 bp segment of the rat G6PDH promoter region was isolated using the polymerase chain reaction (PCR). This fragment was sequenced and characterized revealing a high percentage of homology with both the human and mouse G6PDH sequences. Sequence comparison also revealed putative transcriptional regulatory elements (Rank et al., 1994). The promoter region is GC-rich, a characteristic typical of X-linked housekeeping genes (Figure 4). While the gene may be considered “housekeeping” in many tissues, expression of the gene for G6PDH in primary rat hepatocytes has been found to be influenced by hormones such as dexamethosone, glucagon, and insulin (Kleitzen et al., 1994).

Objective of the Study

Although insulin has been shown to regulate the expression of several genes at the transcription level, no single insulin responsive DNA sequence has yet been identified. The transcription factors responsible for stimulation or inhibition of gene expression by insulin also remain to be identified. Therefore, the molecular mechanism(s) underlying hormonal regulation of gene transcription remains to be elucidated. Since it is known that insulin stimulates G6PDH gene expression we were interested in defining the region(s) within the G6PDH promoter responsible for insulin regulation. Other objectives included determination of insulin kinetics of G6PDH induction and comparison of insulin response elements with other reported elements to generate a consensus sequence.

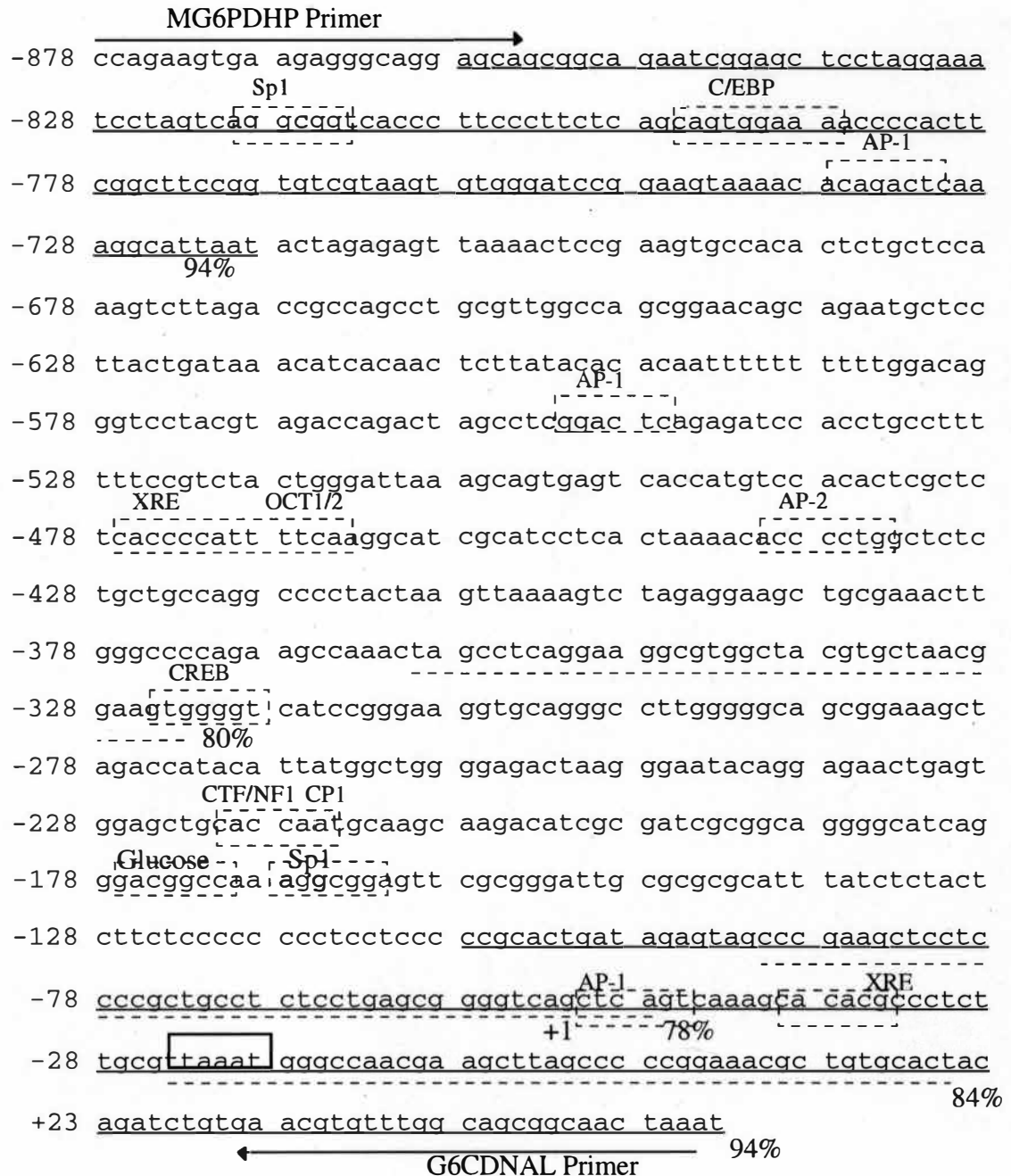


Figure 4. Rat G6PDH Promoter DNA Sequence. The transcription start site is denoted by a +1. Putative cis-elements are identified. Sequence homology to the human gene is shown by the dashed underline, and mouse homology is the solid underline.

Significance of the Study

Since insulin plays such an important role in the regulation of metabolism, it is essential to elucidate the mechanisms of insulin action at the molecular level. Elucidation of the insulin signaling cascade beginning outside of the cell and travelling deep within the nucleus, will give researchers better insight into the mechanisms of signal transduction and control of gene expression in multicellular organisms. Knowledge of these systems will aid in the development of treatments for diseases ranging from diabetes to cancer.

MATERIALS AND METHODS

PCR Amplification

The polymerase chain reaction (PCR) was utilized to amplify specific regions within the glucose-6-phosphate dehydrogenase (G6PDH) promoter region. The components and final concentration of the reaction mix consisted of 50 μ M of each nucleotide, 50 mM KCl, 10 mM Tris (pH 8.3), 0.15 mM $MgCl_2$, 1.0 μ M of each primer and 1.25 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in a total volume of 50 μ l. A hot start procedure was performed. This consisted of heating the samples to 95° C for 5.5 minutes, 85° C for 1 minute (at which time the Taq DNA polymerase was added), and 60° C for 4 minutes. The hot start was followed by 30 cycles of 94° C for 1 minute, 60° C for 2 minutes and 72° C for 3 minutes.

Plasmid Construction

The plasmids 935G6PDH-LUC, 627G6PDH-LUC, 187G6PDH-LUC, and 107G6PDH-LUC were constructed in the following way: Oligonucleotide primers (Eppendorf, Madison, WI) complementary to bases within the G6PDH promoter region were designed based on the rat G6PDH promoter region sequence previously reported (Rank et al., 1994). The primers PSH33 + Sma I and G6CDNAL + Sal I were used to clone the 935G6PDH-LUC plasmid. The primers contained 5' linkers specific for the restriction endonucleases Sma I and Sal I allowing for insertion of the

amplified PCR fragment in the proper orientation between Sma I and Xho I, 5' of the luciferase gene in the pGL2-Basic vector (Promega, Madison, WI). The 627G6PDH-LUC clone was also constructed using the primers PSH33 + Sma I and G6CDNAL + Sal I. The amplified 935 bp product was then cleaved with Sna B I and Sal I. This allowed for the proper insertion of a 627 bp fragment between Sma I and Xho I in the p-GL2 Basic Vector. (Sna B I and Sma I both generate blunt ends, while Sal I and Xho I have compatible cohesive ends). The primers FSP4 + Sma I and G6CDNAL + Sal I were used to amplify the 187 bp fragment. This region of DNA was also inserted between the Sma I and Xho I restriction sites in the p-GL2 Basic Vector. The 107 bp fragment was amplified using the primers G6107 + Mlu I and G6CDNAL + Sal I (Table 3). The 107 bp fragment was inserted into the p-GL2 Basic Vector between the sites Mlu I and Xho I. The ligation reactions were performed at 14° C overnight. The ligated vectors were then introduced by transformation into INVaF' competent *E. coli* cells (Invitrogen, San Diego, CA). The plasmid DNA from the *E. coli* bacteria was isolated and purified. Clones were verified by PCR and restriction digestion.

Table 3

Oligonucleotide Primers

Name	Sequence	Location
PSH 33 + Sma I	5'-ttccccggggccagaagtgaagagggcaggagc-3'	-878 to -856
G6CDNAL + Sal I	5'-tcgtcgacatttagtgccgctgccaacacgttcac-3'	+30 to +57
FSP4 + Sma I	5'-gtagcccgggctcttctccccccctctc-3'	-130 to -111
G6107 + Mlu I	5'-gtacgcgttcagtcaaagcacacgccct-3'	-50 to -30
G6AP1 + Sal I	5'-tcgtcgactgcttgactgagctgaccccg-3'	-61 to -39
FSP3 + Sma I	5'-gtagcccggggtggagctgcaccaatgcaa-3'	-230 to -210

Plasmid DNA Preparation

Luria-Bertani (LB) medium containing 50 µg/ml of the antibiotic ampicillin was inoculated with *E. coli* bacteria containing the plasmid DNA of interest and grown overnight at 37° C in a shaking incubator. Plasmid DNA was isolated from the bacterial culture by alkaline lysis (Sambrook et al., 1989) and purified using an ion exchange column (The Nest Group, Southboro, MA). The purity of the DNA was confirmed by agarose gel electrophoresis and A260/A280 absorption ratios.

Hepatocyte Isolation and Culture

Hepatocytes were isolated from 8-12 week old, male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) by in situ perfusion of the liver via the hepatic portal vein with 100 U/ml collagenase D (Boehringer Mannheim, Indianapolis, IN) and 93 U/ml type I-S hyaluronidase (Sigma, St. Louis, MO) in Mg²⁺-free Hepes balanced salt solution as previously described (Elliget et al. 1983). The resulting cell suspension was centrifuged at 50 x g for 2 min. The hepatocyte pellets were resuspended and washed twice in Waymouth's MB 752/1 medium (GIBCO BRL, Grand Island, NY) containing 0.5% bovine serum albumin (BSA) (Sigma, St. Louis, MO). A haemocytometer was used to count the hepatocytes and assess viability using the method of trypan blue dye exclusion. The hepatocytes were plated on 60 mm collagen-coated culture dishes to a confluency of approximately 90%. Culture substrata consisted of 5 µg type I collagen from rat tail (Collaborative Biomedical Products, Bedford, MA). Cultures were incubated in 4 ml Waymouth's MB 752/1 medium with 0.5% BSA at 37° C in a humidified environment of 5% CO₂, 95% air. The media was supplemented with 10 mg/ml gentamicin. After 6 hours, the media was changed to serum-free Waymouth's medium, and allowed to incubate overnight.

Transfection

The cells were transfected with a complex consisting of 5 μg of plasmid DNA and 15 μg of the cationic lipid (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) as previously described (Holmen et al., 1995). DOTAP vesicles (Avanti Polar Lipids, Alabaster, AL) were prepared by transferring 1.0 mg of a chloroform solution of DOTAP to a small glass vial and drying under vacuum for at least 2 hours at room temperature to create a film on the glass vial. One ml of double distilled water was added to the vial and mixed by vortexing at high speed for 15 seconds. The DNA lipid complex was diluted to a volume of 250 μl with serum-free medium, and allowed to sit for 15 minutes at room temperature. This complex was added to the cells in culture and incubated in 2 ml serum-free Waymouth's medium for 2 hours. After this time, the cells were washed and re-incubated in fresh serum-free medium for 36 hours from the start of transfection to allow for expression of the plasmids. Insulin treated cultures were dosed with 300 ng/ml porcine insulin (a gift from Eli Lilly) immediately after transfection, and allowed to incubate in serum-free medium for an additional 36 hours from the start of transfection.

Cell Processing

Primary rat hepatocytes in culture were processed by first removing the media and then washing the cells twice with sterile phosphate-buffered saline (PBS). The cells were lysed by adding 400 μl of 1X cell culture lysis reagent (Promega, Madison, WI), for 15 minutes. The cells were then scraped from the plastic culture dishes with a rubber policeman and transferred to a 1.7 ml eppendorf tube. The samples were centrifuged at 13,000 $\times g$ for 1 minute to pellet debris. The supernatant was transferred

to a new tube and assayed for protein content, luciferase activity, and G6PDH activity. A modified Lowry protein assay was utilized to assess protein content in each of the samples as previously described (Alam, 1992). Sample concentrations were determined by using a standard curve of BSA.

Luciferase Assay

A 20 μ l aliquot of cell lysate was added to 100 μ l of luciferase assay reagent (Promega, Madison, WI). The luciferase assay reagent consists of 20 mM Tricine, 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.67 mM MgSO_4 , 0.1 mM EDTA, 33.3 mM DTT, 270 μ M coenzyme A, 470 μ M luciferin, and 530 μ M ATP. The reaction was measured for two-one minute intervals using a scintillation counter. The reaction is illustrated in Figure 5.

luciferase + luciferin + ATP \longleftrightarrow luciferase · luciferyl-AMP + PPi

luciferase · luciferyl-AMP + $\text{O}_2 \longrightarrow$ luciferase + oxyluciferin + AMP + $\text{CO}_2 + h\nu$

Figure 5. Reaction of Luciferase.

The average of the two-one minute measurements was normalized to the amount of protein in the sample. Final values are expressed as counts per minute (CPM)/ μ g protein.

G6PDH Assay

G6PDH activity was measured as the rate of change of NADPH absorbance at 340 nm on a Gilford Response spectrophotometer as previously described (Dror et al., 1970). The reaction mix consisted of 0.38 mM NADP^+ , 7.1 mM MgCl_2 , and 81 mM triethanolamine (TEA) buffer pH 7.6, combined with 50 μ l of cell extract. The reaction was started by adding 1.2 mM glucose-6-phosphate. The change in absorbance was

measured over a period of 3 minutes. The specific activity of G6PDH was expressed as the change in absorbance per minute per mg protein. The reaction is shown in Figure 6.



Figure 6. Reaction of Glucose-6-Phosphate Dehydrogenase.

Nuclei Isolation

Nuclei were isolated from primary rat hepatocytes in culture using a modification of a protocol previously described (Schibler et al., 1983). Cells from 30 60-mm plates were pooled. After all the cells were harvested, the suspension was centrifuged at 900 rpm for 5 minutes in a sorval HG4L swinging bucket rotor. The pellets were collected and homogenized in 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β -mercaptoethanol, and 0.5% Nonidet P-40. The suspension was centrifuged at 2500 rpm for 10 minutes after which the pellets were collected and re-homogenized in a similar buffer as that mentioned above, except it contained 2.0 M sucrose and no detergent. The nuclei were purified by layering the homogenate over a cushion of 2.0 M sucrose buffer and centrifuging in a SW 40.1 rotor at 30,000 rpm for 1 hour. The isolated nuclei were resuspended in nuclei storage buffer consisting of 50% glycerol, 50 mM HEPES, pH 7.4, 150 mM NaCl, .1 mM EDTA, 10 mM dithiothreitol (DTT), 0.25 mM phenylmethylsulfonyl fluoride (PMSF). The nuclei were lysed by adjusting the final NaCl concentration to 300 mM. After a 30 minute incubation on ice, the nuclei were re-centrifuged at 24,000 x g for 20 minutes. The supernatant was transferred to a new tube, snap-frozen in dry ice/ethanol, and stored at -70° C until needed.

Radioactively Labeled DNA Probe

A 92 bp DNA fragment was amplified using the primers FSP4 +Sma I and G6AP1 + Sal I. The amplified fragments were separated from their primers on a 3% agarose gel in Tris-acetate (TAE) buffer, and extracted from the gel using DE81 ion exchange paper (Whatman International, Maidstone, England). The 92 bp DNA fragment was cut with the restriction enzyme Sal I overnight at 37° C to generate 5' overhangs. The 5' overhangs were filled in using the Klenow fragment of *E. coli* DNA polymerase I. The reaction mix contained 20 µCi [α -³²P]dCTP, 1 µl of 3 mM dNTP mix, and 1 U of Klenow in a total volume of 20 µl. The reaction was incubated at 37° C for 30 minutes, and stopped by adding 1 µl of 0.5 M EDTA. The Ap2 consensus oligonucleotide was 5' end-labeled using [γ -³²P]ATP. The reaction mix contained approximately 2 pmol of DNA, 10 µCi of [γ -³²P]ATP, 5 Units of T4 polynucleotide kinase and 1X T4 polynucleotide kinase buffer in a total volume of 10 µl. The reaction was incubated at 37° C for 10 minutes and stopped by adding 1 µl of 0.5 M EDTA. Unincorporated dNTP's were separated from the labeled oligonucleotides by gel filtration through a Bio-gel P6 column. The protocol used for filling in the 5' overhangs was modified from General Protocols in Molecular Biology.

Gel Mobility Shift Assay

Protein DNA binding reactions were performed at room temperature in 20 µl of 14 mM HEPES, 5.4 mM EDTA, 7% glycerol, 28.2 mM Tris-HCl, pH 7.5, 47 mM NaCl, 3.5 mM MgCl₂, 7 mM KCl, 0.8 mM β -mercaptoethanol, 0.05% Tween 20, and 2 µg of poly(dI-dC)poly(dI-dC) unless otherwise stated. The protein/DNA binding reactions of the positive controls from Promega's gel shift assay system were performed at room temperature in 10 µl of 2.5 mM EDTA, 20 % glycerol, 50 mM Tris-

HCl, pH 7.5, 250 mM NaCl, 5 mM MgCl₂, 2.5 mM DTT, and 0.25 mg/ml poly(dI-dC)·poly(dI-dC). Each reaction contained 20,000 cpm of labeled oligonucleotide and the indicated amount of nuclear extract. One hundred-fold molar excess unlabeled probe was added to the reaction mixture prior to the addition of labeled probe for competition experiments. The reactions were incubated at room temperature for 30 minutes unless otherwise stated. The samples were electrophoresed at 100 volts through a 4% non-reducing polyacrylamide gel in either high or low ionic strength buffer. The 1X high ionic strength tris-glycine buffer, pH 8.5, was diluted from a 5X stock consisting of 250 mM Tris-base, 1.9 M glycine, and 10.5 mM EDTA. The low ionic strength sodium acetate buffer consisted of 6.8 mM Tris-Cl pH 7.9, 3.3 mM sodium acetate pH 7.9, and 1.0 mM EDTA pH 8.0. The positive controls from Promega's gel shift assay system were electrophoresed through 0.5X TBE buffer. This was diluted from a 10X stock consisting of 890 mM Tris-HCl, pH 8.0, 890 mM boric acid, and 20 mM EDTA. After electrophoresis, the gels were dried and exposed to x-ray film. The total distance migrated by the free probe was divided by the distance migrated by the shifted probe to determine R_f values.

DNA Footprinting

DNase I footprinting was performed by the procedure described by Promega. The primers G6AP1 and FSP3 were used to amplify the region between -39 and -230 of the G6PDH promoter. This 192 bp DNA fragment was treated with calf intestinal alkaline phosphatase for 1 h at 37° C. The fragment was then isolated by agarose gel electrophoresis and purified by GeneClean (Bio 101, Vista, CA). The purified fragment was 5' end-labeled using T4 polynucleotide kinase (Promega, Madison, WI) and [γ^{32} P]ATP. The probe was cleaved with the restriction enzyme Sal I in order to

generate a probe which was 5' end-labeled on one strand only. Binding assays contained 50 μ l of 50 mM Tris-Cl, pH 8.0, 100 mM KCl, 12.5 mM $MgCl_2$, 1.0 mM EDTA, 20 % glycerol, 1.0 mM DTT, 0.5-1.0 ng of probe, and 30 μ g of nuclear extract. After a 10 minute incubation on ice, 50 μ l of 5 mM $CaCl_2$ and 10 mM $MgCl_2$ were added followed by the addition of 0.3 unit of RQ1 RNase-free DNase I (Promega, Madison, WI). The reaction was stopped after 1 minute by the addition of 90 μ l of 200 mM NaCl, 30 mM EDTA, pH 8.0, 1 % SDS, and 100 μ g/ml poly (dI-dC)-poly (dI-dC). The reaction was extracted with 200 μ l of phenol:chloroform:isoamyl alcohol (equilibrated with TEB buffer and 0.5 M NaCl) 25:24:1, followed by ethanol precipitation and resuspension in 4 μ l of 0.1 M NaOH:formamide (1:2 v/v), 0.1 % xylene cyanol, and 0.1 % bromophenol blue. The samples were denatured by incubation at 95°C for 2 min, after which they were loaded onto a 6 % polyacrylamide, 8 M urea sequencing gel and run at 1500 V, 60 watts in TBE 1X buffer for 1h 45 min. The gel was autoradiographed at -70°C.

RESULTS

Expression of G6PDH-Luciferase Fusion Genes by Transient Transfection of Primary Rat Hepatocytes

G6PDH-Luciferase fusion gene constructs were used in this study to identify putative insulin regulatory elements within the 5' flanking region of the G6PDH gene. Sequences between nucleotides -878 and +57 of the G6PDH gene were fused to the luciferase reporter gene such that transcription of the luciferase gene was directed by the G6PDH promoter (Figure 7). To examine how insulin exerts its effects on G6PDH gene expression, a series of 5' deletions of the G6PDH promoter were fused to the luciferase reporter gene and transiently transfected into primary rat hepatocytes incubated in chemically defined medium. The constructs containing smaller fragments of the 5' flanking region of G6PDH were named according to the number of nucleotide bases of the G6PDH promoter they included, i.e. 935G6PDH-LUC, 627G6PDH-LUC, 187G6PDH-LUC, and 107G6PDH-LUC. All of these deletions have a common 3' end at +57.

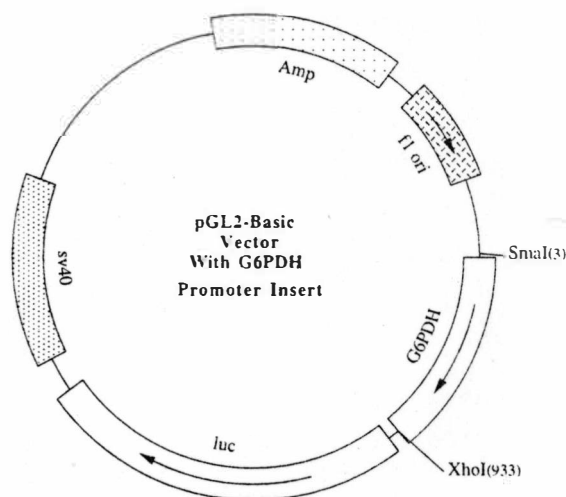


Figure 7. p-GL2 Basic Vector With G6PDH Insert.

Insulin Stimulation of G6PDH-Luciferase Expression

Insulin treatment increased luciferase activity 1.5-2-fold in constructs which contained sequences ranging from +57 to -130 (Figure 8). No effect of insulin was observed when plasmids containing G6PDH promoter sequences spanning from +57 to -50 were transfected into primary rat hepatocytes. These results suggest that the cis-acting DNA sequences that mediate insulin responsiveness in the G6PDH gene may be located in the proximal promoter between position -130 to -50 of the G6PDH promoter.

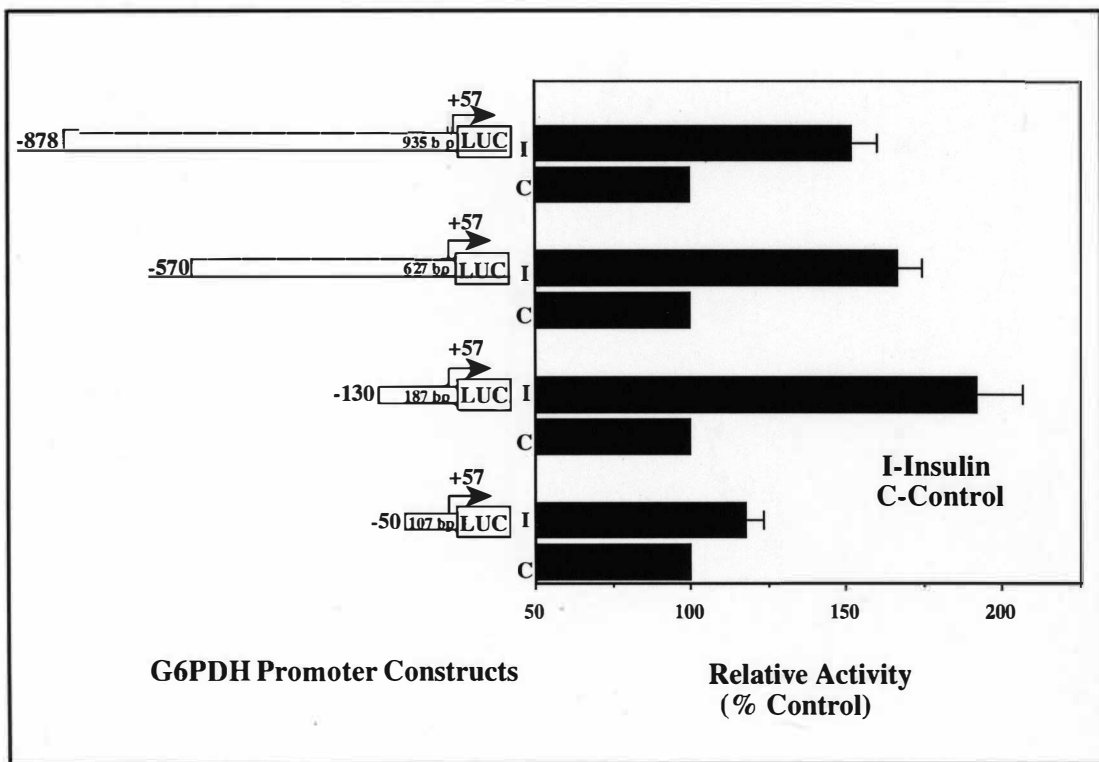


Figure 8. Effect of Insulin on Constructs Containing Deletions of the G6PDH Promoter. Hepatocytes were transfected for 2 h with the indicated G6PDH promoter construct in serum-free medium. Following transfection, the cells were incubated for 36 h either in the presence (I) or absence (C) of 300 ng/ml porcine insulin. The results are expressed as a percentage of the control. Error bars represent standard error of the mean. $n = 3$.

Basal G6PDH Promoter Activity of 5' Deletion Constructs in Primary Rat Hepatocytes

The basal promoter activities of the different constructs were determined by transient transfection into primary rat hepatocytes. Luciferase activities observed with the different deletions were compared to that of the 935G6PDH-LUC construct. Deletion of promoter sequences between -50 and -570 produced a significant decrease in the basal promoter activity of G6PDH (Figure 9). This decrease in basal activity suggests that elements important for basal G6PDH promoter activity are located within the -570/-50 region. Figure 4 identifies known sequences within this region which are likely to be binding sites for several important transcription factors; including Sp1 and AP-1.

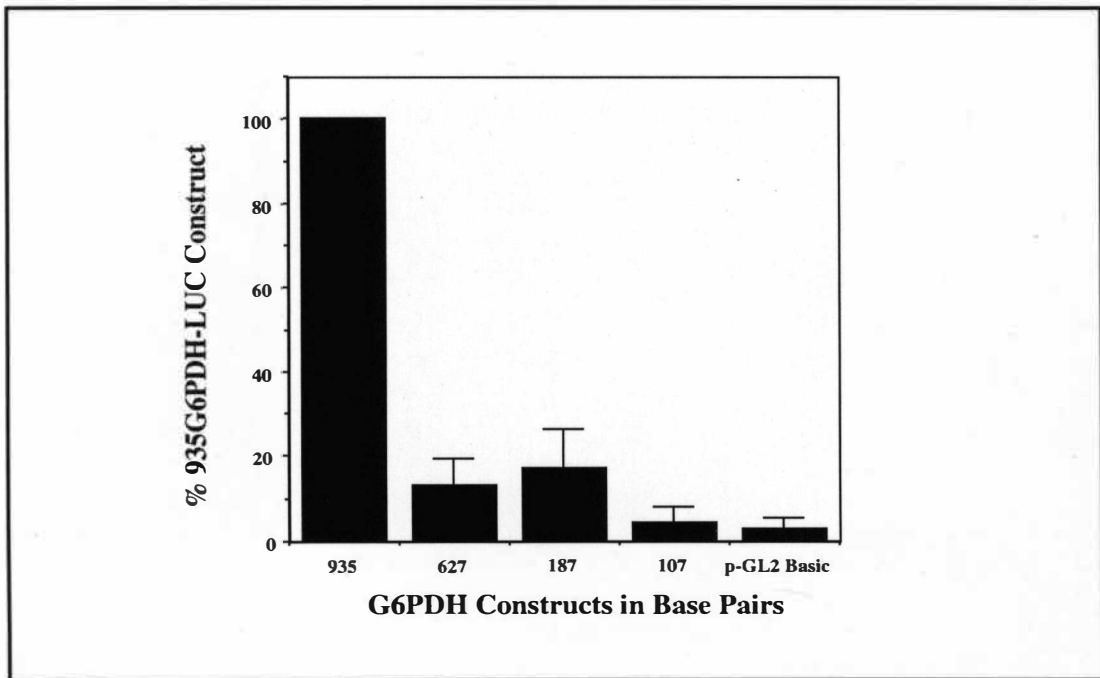


Figure 9. Basal Activity of the G6PDH Promoter in Primary Rat Hepatocytes Transfected With G6PDH-LUC Fusion Genes. Hepatocytes were transfected for 2 h with the indicated G6PDH promoter construct in serum-free medium. Following transfection the cells were cultured for 36 h. The results are expressed as a percentage of the 935G6PDH-LUC construct. Error bars represent standard error of the mean. $n = 3$.

Gel Mobility Shift Assays

The gel mobility shift assay was used to detect sequence-specific DNA-binding proteins. The gel shift assay core system was purchased from Promega and served as a positive control to aid in the optimization of this assay for G6PDH. This assay core system included pure AP2 extract and a consensus AP2 oligonucleotide which was 5' end-labeled with [γ - 32 P]ATP.

Gel Mobility Shift Experiments Using Nuclear Extracts From Primary Rat Hepatocytes

The gel mobility shift assays using the sequence from -130 to -39 of the G6PDH promoter as a probe allowed for the detection of sequence-specific DNA-binding proteins. Assay conditions were optimized by comparing several different previously reported DNA/protein binding buffers (Table 4).

Table 4
Comparison of Reported DNA/Protein Binding Buffers

	1	2	3	4	5	6
Hepes			12.5 mM	12.5 mM	14.0 mM	15.0 mM
EDTA	1.0 mM	2.0 mM	.10 mM	1.0 mM	5.4 mM	.50 mM
DTT	1.0 mM	2.0 mM	2.5 mM	1.0 mM		.75 mM
Glycerol	5%	20%	5%	13%	7%	15%
Tris-HCl	10 mM	40 mM		4.0 mM	28.2 mM	
NaCl	50 mM	100 mM			47 mM	
MgCl ₂	5.0 mM	4.0 mM			3.5 mM	5.0 mM
KCl	50 mM		10 mM	198 mM	7.0 mM	120 mM
BSA		100 μ g/ml				2 μ g/ml
β -ME					.8 mM	
Nonidet P-40		0.20%				
Tween 20					0.05%	
Spermidine						4 mM
Incubation Time	RT, 15'	Ice, 1 h	4 C, 20'	RT, 15'	RT, 30'	RT, 20'

Results from the gel mobility shift assay using the different DNA/protein binding buffers is shown in Figure 10. Buffers 3 and 5 appear to promote the best interactions between the DNA and the nuclear protein and therefore produce the clearest gel shift when incubated at the temperatures and times indicated in Table 4. Incubation of the protein/DNA complex on ice for longer periods of time seemed to produce more streaking in the lanes. Incubation at room temperature or at 4° C for 15-30 minutes appears to be optimum.

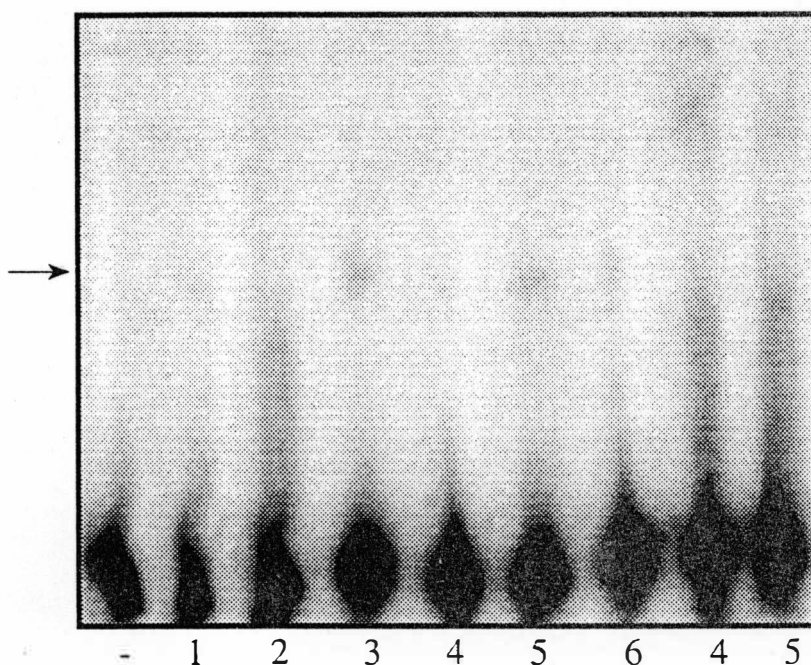


Figure 10. Gel Mobility Shift Assay Using Different Binding Buffers. Thirty μ g of nuclear protein from primary rat hepatocytes dosed for 24 h with 300 ng/ml insulin was incubated with the DNA probe in the indicated buffer. The DNA and the protein were incubated for the times and temperatures indicated in Table 3. The numbers indicate the buffer used. The first lane is the negative control (-). The last two lanes represent samples incubated in buffers 4 and 5 for 1 h on ice. Samples were run on a 6 cm x 8 cm, 4 % high ionic strength polyacrylamide gel at 100 Volts.

Competition Analysis of Factors Binding to a 92 bp G6PDH Probe
in Gel Mobility Shift Assays

Figure 11 shows that nuclear factor(s) from insulin treated primary rat hepatocytes bind within this sequence. Addition of 100-fold molar excess unlabeled probe, identical to the labeled probe, resulted in competition for the nuclear factors in insulin treated cells. Competition for the nuclear factor(s) indicates that this DNA-protein interaction is specific.

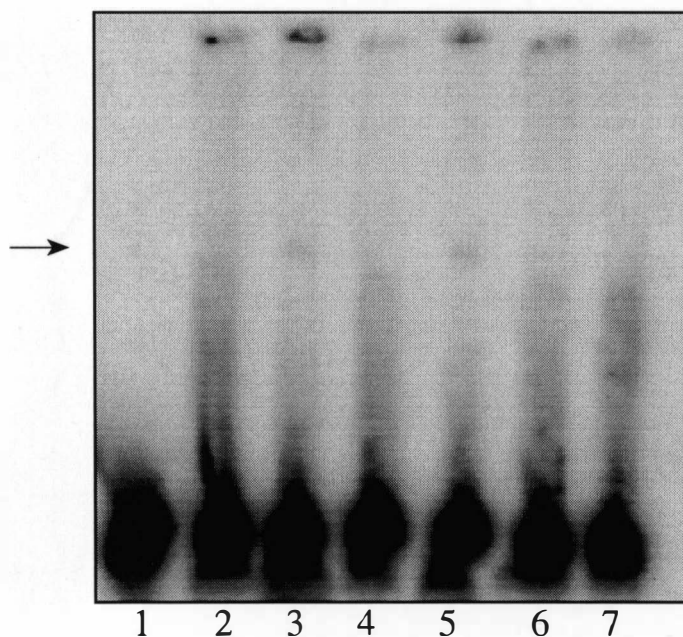


Figure 11. Gel Mobility Shift Assay Using Primary Hepatic Nuclear Extracts. Hepatocytes were treated with 300 ng/ml insulin for 24 h after which nuclei were isolated. Nuclear extracts were incubated with the 92 bp DNA probe in buffer 5 for 30 minutes at room temperature. The samples were then electrophoresed through a 6 cm x 8 cm 4 % polyacrylamide gel in high ionic strength tris-glycine buffer. Lane 1 is the negative control. Lanes 2, 4, and 6 contain nuclear extracts from hepatocytes not treated with insulin (control). Lanes 3, 5, and 7 contain nuclear extracts from hepatocytes treated for 24 h with insulin. Lanes 4 and 5 contain excess poly dI-dC to eliminate non-specific binding. Lanes 6 and 7 contain 100-fold molar excess competitor DNA.

Time Course of Insulin Action

Initial gel shift assays were done using nuclei from primary rat hepatocytes which had been treated with insulin for 24 h. Gel mobility shift assays of nuclear extracts from these cells showed a band with a relative mobility (R_f) of 2.3. Experiments were then performed to determine the time course of insulin induction of G6PDH. Primary rat hepatocytes were treated with 300 ng/ml porcine insulin for 1 h, 3 h, 6 h, 12 h, and 24 h. The extracted nuclei were allowed to complex with the labeled 92 bp DNA probe in buffer 5 for 30 minutes at room temperature. The samples were then electrophoresed on a 6 cm x 8 cm 4 % polyacrylamide gel in high ionic strength electrophoresis buffer. The results of this gel shift assay showed precipitates of the protein/DNA complexes in the wells of 1 h and 3 h time points. No gel shift was observed in the lanes of the other time points. Because of this precipitation, a larger gel apparatus (16 cm x 18 cm) was used to aid movement of the complex through the gel. Precipitates were still found within the wells, so a lower ionic strength sodium acetate electrophoresis buffer was used to alter the stringency of the binding. Nuclei extracted from cells dosed with insulin for 1 h, 6 h, and 24 h, as well as those not dosed at all (control) were complexed with the 92 bp DNA probe and subjected to electrophoresis through a 16 cm x 18 cm polyacrylamide gel. Figure 12 shows two shifted bands appearing in the 6 h sample. Band A has an R_f of 1.6, while band B has an R_f of 4.8. Both of these bands appear to be successfully competed for using unlabeled probe, and are therefore thought to be specific interactions. No bands are visible in the other time points, although a small precipitation does appear in the well of the 24 h sample. The relative mobilities of bands A and B in Figure 12 appear to be different than those observed for the 24 h samples run in high ionic strength electrophoresis buffer shown in Figure 11.

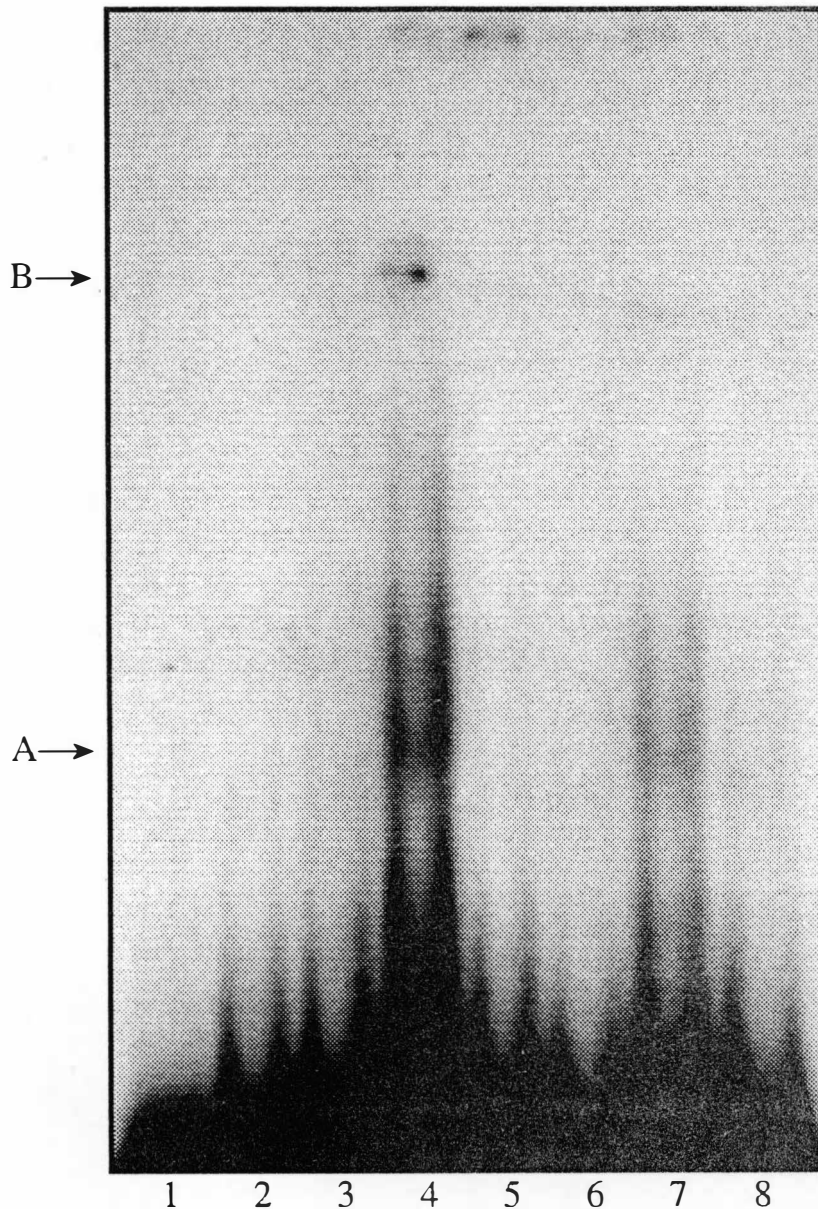


Figure 12. Gel Mobility Shift Assay to Determine the Time Course of Insulin Induction of G6PDH. 30 μ g of nuclear protein from primary rat hepatocytes was complexed with the DNA probe in buffer 5 for 30 minutes at room temperature. The samples were run on a 16 cm x 18 cm 4 % polyacrylamide gel in low ionic strength sodium acetate buffer at 100 V. Lane 1 is the negative control. Lane 2 contains samples of nuclear extract from cells not dosed with insulin (control). Lanes 3, 4, and 5 contain samples which were dosed for 1 h, 6 h, and 24 h respectively. Lanes 6, 7, and 8 are identical to lanes 3, 4, and 5 respectively except that they contain 100-fold molar excess competitor DNA.

DNA Footprint Analysis

DNase I footprinting was used to identify the region(s) within the G6PDH promoter to which nuclear factor(s) bind. A 192 bp DNA probe (-230 to -39) was 5'

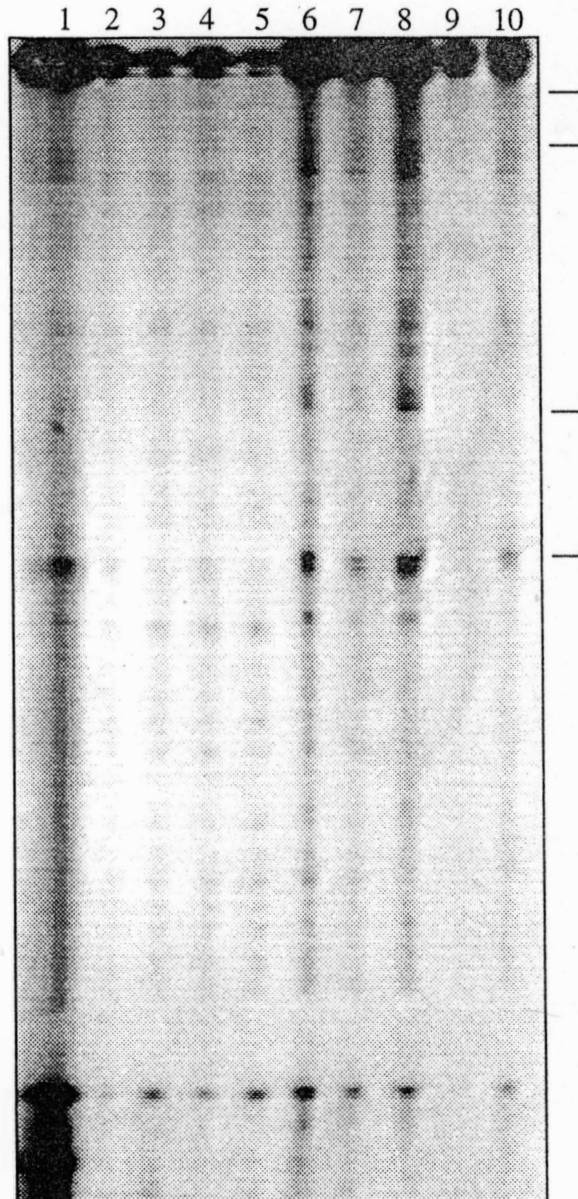


Figure 13. DNase I Footprint Analysis. Lanes 1-5 contain only the 192 bp DNA probe. Lane 1 was incubated with DNase for 1 min, Lane 2 for 2 min, Lane 3 for 3 min, Lane 4 for 4 min and Lane 5 for 5 min. Lanes 6-10 are the same as 1-5 only they also contain 60 µg of nuclear extract.

end-labeled with [^{32}P] γ ATP. Since the probe must be labeled on only one strand, the DNA was cut with the restriction enzyme Sal I such that the 5' label was removed from the anti-sense strand. This resulted in a radioactive phosphate on the sense strand only. A DNase activity curve was performed to determine the amount of time required to completely digest the 192 bp probe. Figure 13 shows the autoradiograph of this experiment. Two minutes appeared to be sufficient for the desired cleavage pattern. In addition, two protected areas were identified. The footprinted areas correspond to the regions -126/-99 and -54/-43 in the G6PDH promoter. Due to protein binding, hypersensitive regions are found on either side of these protected areas.

DISCUSSION

In primary rat hepatocytes in culture, insulin has been shown to increase the enzyme activity and mRNA levels of G6PDH 1.5-2 fold (Nakamura et al., 1982; Spence et al., 1982; Kleitzen et al., 1986; Manos et al., 1987). This increase in activity was shown to be due to increased levels of G6PDH protein. These results suggest that regulation by insulin is at the transcriptional level (Kleitzen et al., 1987; Manos et al., 1991). We set out to define the region(s) within the G6PDH gene responsible for insulin regulation.

Transfection studies using constructs containing serial 5' deletions of the G6PDH promoter linked to a luciferase reporter gene were used to localize insulin responsive elements within 935 bp of the G6PDH promoter region. It was important to ensure that insulin induction of our G6PDH reporter gene constructs was similar to that observed within the endogenous gene. In hepatocytes transfected with constructs containing the G6PDH promoter sequences ranging from -130 to +57, a 1.5-2 fold increase in luciferase activity was observed in samples treated with 300 ng/ml insulin (Figure 8). Therefore, this data is in agreement with previously reported insulin induced increases observed in endogenous G6PDH enzyme activities. Figure 8 also demonstrates that the insulin response is no longer present in hepatocytes transfected with the construct containing the promoter segment ranging from -50 to +57. These results define the region of insulin responsiveness in the G6PDH gene to be located between bases -130 and -50.

Using the gel mobility shift assay we identified an insulin-induced DNA-protein complex binding to the 92 bp DNA probe extending from -130 to -39 in the G6PDH

promoter region. Incubation of the nuclear extract with excess cold specific oligonucleotide sequences abolished binding confirming the specificity of the observed complex formation. Inclusion of excess non-specific DNA sequences (poly dI-dC) did not compete for the protein, providing further proof that the complex is indeed DNA sequence specific (Figure 11). Nuclear extracts from hepatocytes not treated with insulin were complexed with the 92 bp probe as well, but did not produce a gel shift similar to that observed with extracts from insulin treated cells. These results confirm that the gel shift is the result of insulin-induced protein binding. Insulin-induced protein binding has also been reported for the GAPDH, prolactin and hGH genes (Nasrin et al., 1990; Stanley, 1992; Prager et al., 1990). In contrast, no differences in DNA-protein complex formation were detected between non-treated and insulin treated cells for the FAS or PEPCCK genes (Moustaid, et al., 1994; O'Brien et al., 1990). Moustaid et al. suggest that the binding of proteins mediating insulin regulation of the FAS or PEPCCK genes are not under insulin control. They imply that the proteins responsible for increased gene expression are already present, and bound to the DNA, but are not active.

Initial gel shift assays involved the use of nuclear protein from primary rat hepatocytes which had been treated with insulin for 24 h. These samples were run on a polyacrylamide gel in high ionic strength electrophoresis buffer. The R_f value of the shifted band was 2.3 (Figure 11). Subsequent studies were done to determine a time course of insulin action. Hepatocytes were either not treated at all (control) or were treated with insulin for 1h, 3h, or 6h. Due to difficulties in complex formation and migration, a lower ionic strength polyacrylamide gel was used (see results). Two shifted bands were observed in the 6 h sample, with R_f values of 1.6 and 4.8, but no bands were observed in the 24 h sample (Figure 12). The gel shifts observed with the 6 h time point differ from that observed for the gel shift produced at the 24 h time point

run in high ionic strength buffer. From these results it was determined that the earlier time points only formed protein-DNA complexes in the low ionic strength buffer, whereas the 24 h time point only formed complexes in high ionic strength buffer. Since DNA-protein complex formation and migration is determined by pH, salt concentration (ionic strength), complex conformation, and the molecular mass of the proteins, the differences in band shifting observed in the gel mobility shift assays at 24 h and 6 h may be due simply to the different conditions under which they were run. However, if the same protein(s) is binding at both the early and late time points, this does not explain why samples from earlier time points precipitated in the high ionic strength gel. This data suggests that different proteins with different solubilities are interacting with the DNA. It may be that there is an early response and a late response. It is also possible that different proteins recognize an identical sequence. More proteins may be initially present to aid in the initiation of transcription, but disassociate after a given amount of time.

Kinetic analysis indicated that formation of the insulin induced complex required 6 h, and that a complex is still present at 24 h of insulin treatment. Since formation of an insulin induced complex required at least 6 h, *de novo* protein synthesis may be required. These results are in agreement with those reported for insulin induction of prolactin and hGH gene expression. Stanley et al. reported that induction of prolactin gene expression by insulin was observed at 5 h and reached its maximum at 24 h. Prager et al. reported that induction of hGH required 8 h and was maintained for 16 h. Using both cyclohexamide and puromycin as inhibitors of *de novo* protein synthesis, Prager et al. showed that *de novo* protein synthesis is required for increased gene expression of hGH by insulin. An *in vivo* study using streptozotocin induced diabetic mice reported that run on transcription analysis of FAS induced gene expression by insulin increased to 3.5-fold in 30 minutes. Increases in FAS gene

expression reached 7-fold by 2 h and were maintained at this level up to 6 h. Incubation of the cells with cyclohexamide determined that ongoing protein synthesis was required for increased gene expression of FAS by insulin (Paulauskis et al., 1989). Even though the protein which mediates increased gene expression by insulin appears to be already present (see above), another protein which functions to activate the bound protein may need to be synthesized. Paulauskis et al. 1989 compared their observations with those reported for the down-regulation of PEPCK gene expression by insulin. They stressed that this negative regulation of PEPCK by insulin was at the transcriptional level, but did not require ongoing protein synthesis in either rat liver *in vivo* or in hepatoma cells. Differences in the kinetics of genes either positively or negatively regulated by insulin may indicate different pathways of regulation.

To determine the location of nuclear factor binding to the G6PDH promoter region, DNase I footprinting was performed using a singly ^{32}P end-labeled 192 bp (-230 to -39) probe. Figure 13 shows an autoradiograph of a DNA footprint generated after different exposure times to DNase I. Two protected areas were observed. One protected region spans approximately 28 bp, the other approximately 12 bp. Also, binding of the hepatic nuclear extract to the DNA resulted in hypersensitive sites located adjacent to the protected regions. The distances migrated by fragments of known molecular size were used to generate an equation by which the sizes of other fragments could be determined based on their migration through the gel. Using this method, the nucleotide sequences of the protected areas were estimated to be between bases -126 and -99, and -43 and -54. The 12 bp protected region appears to correspond to a putative AP1 site located in that region. Competition with cold AP1 however did not abolish binding in gel shift assays.

The nucleotide sequence of the 28 bp protected region is as follows:

5' TCTCCCCCCCCTCCTCCCCCGCACTGAT 3'. Table 2 shows the sequences reported to be responsive to insulin in other genes. Comparison of this sequence to the other sequences in Table 1 reveals little sequence homology. The complimentary strand, however, does have some sequence homology to those sequences in Table 1. The complimentary strand is: 5' ATCAGTGCGGGGGAGGAGGGGGGGGAGA 3'. The underlined 4 bp sequence is found in the GAPDH, amylase, and FAS genes. However, only the sequence found in the GAPDH gene has been linked to insulin-inducible protein binding. Since a consensus sequence was not identified in all of the genes reported to be insulin responsive, different transcription factors may be involved in the regulation of these genes by insulin. It is possible that regulation of different genes by insulin occurs via different mechanisms which may be tissue and/or cell specific (Edwards, 1994). Further studies to identify the transcription factor(s) known to interact with this insulin responsive element could lead to a better understanding of the insulin signaling cascade.

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