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The Glucose and Insulin Responsiveness of the Rat Glucose-6-Phosphate Dehydrogenase Promoter

Daryl Arkwright-Keeler
Western Michigan University

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THE GLUCOSE AND INSULIN RESPONSIVENESS OF THE RAT GLUCOSE-6-PHOSPHATE DEHYDROGENASE PROMOTER

by

Daryl Arkwright-Keeler

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Submitted to the
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THE GLUCOSE AND INSULIN RESPONSIVENESS OF THE RAT GLUCOSE-6-PHOSPHATE DEHYDROGENASE PROMOTER

Daryl Arkwright-Keeler, Ph.D.
Western Michigan University, 2005

The regulation of expression of some enzymes involved in glucose homeostasis and fat metabolism requires both insulin and glucose to elicit an effect, while the regulation of others requires only one. Glucose-6-phosphate dehydrogenase (G6PDH), an important enzyme involved in glucose homeostasis, has been shown to be transcriptionally regulated by carbohydrate feeding in rat liver, a diet regime that increases the levels of both glucose and insulin. Our lab had previously shown that insulin induces expression of G6PDH, thus we wanted to determine if glucose also induced G6PDH expression and if so, are the glucose and insulin responses mediated through identical or separate mechanisms. Preliminary studies showed that G6PDH is regulated by glucose in primary hepatocyte cultures, however the mechanism responsible for this regulation is not clear.

Several key metabolic enzymes are regulated transcriptionally by glucose and possibly involve the transcription factors Upstream Stimulatory Factor, Sterol Regulatory Element Binding Protein or SP1. These factors may induce transcription through an E-box consisting of the sequence 5'-CACGTG-3', of which the first four bases appear to be the most critical for the glucose response. The G6PDH promoter
contains a single E-box, thus we investigated whether this E-box is involved in its glucose-regulated expression. Using primary hepatocytes transfected with G6PDH promoter constructs, we clearly established not only the regulation of G6PDH expression by insulin, but also by glucose. The two responses are additive, suggesting that they act though separate mechanisms. Mutation of the first four bases of the E-box in the G6PDH promoter resulted in not only significantly reduced glucose but also insulin responses. Insertion of E-box sequences into non-responsive constructs instilled modest, but significant insulin responsiveness, but not glucose responsiveness.

Mutation of a putative SP1 site near the E-box had no effect on the promoter response to insulin or glucose, indicating that this site is likely not involved in these responses.

Electrophoretic mobility shift assays showed a significant increase in specific hepatic nuclear protein binding to an E-box oligonucleotide in response to rats fed a high carbohydrate diet. Supershift studies revealed that USF is involved in this protein binding.

We also examined whether the promoter response to carbohydrate was specific for glucose. Hepatocytes were treated with pyruvate, which did not elicit a response. This result is in agreement with the hypothesis that glucose, or a metabolite of glucose, such as glucose-6-phosphate may actually be the carbohydrate signaling molecule.

In summary, our results suggest that the E-box is necessary, but probably not sufficient for the G6PDH glucose and insulin responses and that USF is involved in the hepatic nuclear protein binding to the E-box in response to a high carbohydrate diet.
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Daryl Arkwright-Keeler
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INTRODUCTION

Regulation of Gene Expression

Gene expression in eukaryotes is complex and requires the concerted action of many factors. To understand the general principles of gene regulation, one must first review the organization of eukaryotic genes. A typical eukaryote gene contains a promoter, a core promoter and a transcribed region (Figure 1). The transcribed region contains exons and introns. Introns are the non-coding regions which will not be included in the final messenger RNA (mRNA). Exons hold the genetic code for production of mRNA, which will eventually be translated into a protein. The core promoter contains the site to which RNA polymerase binds in order to begin decoding...

![Figure 1. Generalized Eukaryotic Gene Organization](image)

Eukaryote genes consist of a transcribed region, a core promoter and a promoter. The transcribed region contains the exons which serve as a code for production of mRNA. The core promoter contains the TATA box, where the general transcription factors and RNA polymerase bind in order to begin decoding the DNA sequence into mRNA. The promoter is the regulatory region of the gene. The promoter contains cis-elements, which are sites on the DNA where specific proteins can bind and interact with the core promoter. The gene is transcribed from the 5' to the 3' end. Cis: cis-element, TATA: the TATA box.
the DNA sequence into mRNA. The promoter is the regulatory region of the gene. It contains specific DNA sequences, termed cis-elements, to which proteins, called trans-factors or transcription factors, can bind. The binding of these transcription factors to the DNA cis-elements acts as a molecular switch for the activation (or repression) of gene transcription.

Low level, or basal, transcription requires the binding of general transcription factors (GTF) and RNA polymerase to the core promoter forming the core promoter complex (Figure 2A). The core promoter complex is essential for transcription, but it alone can not increase or decrease the rate of transcription.

The promoter region regulates the rate of gene transcription. Regulated gene transcription involves the assembly of many proteins bound to the cis-elements, forming a regulatory complex. The proteins (transcription factors) of the regulatory complex interact either directly or indirectly with the core promoter complex, resulting in the activation or repression of RNA polymerase and regulation of the transcription rate (Figure 2B). The assembly of these protein complexes is facilitated by protein-protein interactions between DNA-bound factors and protein-induced DNA bending (Chen, 1999). DNA bending allows for the interaction of proteins bound at distant sites. Since gene expression is frequently mediated by the binding of multiple proteins in a complex to the promoter, the availability of a particular combination of transcription factors ensures that a gene is transcribed at the proper time and in the appropriate cell type. This is termed combinational control. Thus, regulation of the transcription factors provides a means for regulating gene expression (Wolberger,
A. Basal Transcription

SP1 site: GGGCGG  
E-box site: CACGTG

B. Induced Transcription

SP1  
USF  
GGGCGG  CACGTG

Figure 2. Basal and Induced Transcription of a Eukaryote Gene

A. Basal transcription requires the binding of general transcription factors (GTF) and RNA polymerase to the TATA box of the core promoter to form the core promoter complex. SP1 and E-box sites are examples of many possible cis-elements in the promoter. Cis-elements are potential binding sites for specific transcription factors.

B. Induced transcription involves assembly of many proteins bound to the cis-elements to form a regulatory complex. The proteins of the regulatory complex interact either directly or indirectly with the core promoter complex. This occurs through protein-protein interactions and protein induced bending of the DNA. Interaction between the regulatory complex and the core promoter complex activates or represses RNA polymerase and regulates the transcription rate. SP1 and USF are examples of many possible proteins that can bind to specific sites in the promoter and regulate transcription.
1999). Two basic mechanisms by which transcription factors can be regulated involve either controlling the synthesis or the function of the existing protein. The majority of transcription factors are regulated by controlling their function, given that this type of regulation allows for the rapid response time required for inducible gene expression (Latchman, 2004).

**Eukaryotic Transcription Factors and Their Regulation**

Eukaryotic transcription factors are multifunctional proteins that are capable of DNA binding, interaction with other proteins and activation of transcription. They contain regions, called functional domains, which perform these specific functions. These functional domains may include the DNA binding domain, dimerization domain and activation domain.

Two of the more common DNA binding domains include zinc fingers and helix-loop-helix (HLH) motifs. Zinc fingers consist of sequences containing two cysteine and two histidine residues at repeating intervals. These cysteine and histidine residues collectively bind zinc molecules, which results in the amino acids folding into loops, known as zinc fingers. The amino acids within the loop bind to specific DNA sequences, especially to those that are G-C rich (Fairall and Schwabe, 2001). The HLH motif consists of two helices, separated by a loop of several amino acids. HLH motifs cannot function alone, but need to be part of a larger DNA binding domain. The amino acids of the HLH recognize the specific DNA sequences (Fairall and Schwabe, 2001; Garvie and Wolberger, 2001).
As the name implies, the dimerization domains provide a way for proteins to form dimers. These dimers may be homodimers, in which both proteins within the dimer are identical, or they can be heterodimers, in which both proteins are different. Whether a protein can form a homo- or a heterodimer depends on the structure and characteristics of the protein. There are various forms of dimerization domains, but leucine zippers (Zip) are a prevalent type. They consist of two helices, side by side, with specifically spaced leucine amino acids facing the same direction. There is bonding between the leucine residues of the two helices, which forms a “zipper” and holds the two helices together (Alber, 1992).

There are many types of activation domains and transcription factors often contain more than one. Three general activation domains consist of amino acid regions that are either acidic, glutamine-rich or proline-rich. These regions activate transcription by interacting directly with other transcription factors or RNA polymerase (Courey, 2001).

Transcription factors can be regulated by changing the operation of their functional domains. This can be accomplished by modification of the domain, for example, by phosphorylation/dephosphorylation, acetylation (Freiman and Tjian, 2003) methylation, ubiquination or a combination of these alterations (Latchman, 2004). DNA binding domains can be altered in this way, either enhancing the binding of the transcription factor or rendering it non-functional due its inability to bind to the DNA. The same is true for the other functional domains. Their modification could affect the proteins ability to dimerize, interact with other transcription factors or activate

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transcription. For example, heat shock factor (HSF) is regulated in two ways; through its tetramer formation and its DNA binding activity. The activation of HSF into a form that is capable of binding to DNA requires the conversion from a monomeric to a tetrameric form, which can bind to heat shock element (HSE) on DNA (Morimoto, 1998). The C-terminal region of HSF contains a leucine zipper, which is believed to interact with the N-terminal leucine zipper, resulting in the folding over of the protein and thus maintaining the monomeric form. Following heat shock, HSF unfolds, uncovering the DNA binding domain and allowing a DNA-binding trimer to form (Ahn and Thiele, 2003; Latchman, 2004).

Transcription factors can be regulated in other ways as well. They can be prevented from nuclear translocation, which is necessary in order for them to generate their effects (Karin and Hunter, 1992). Nuclear translocation control is employed with the transcription factor NFκB. NFκB exists in the cytoplasm in an inactive form which is bound to another protein known as IκB that inhibits its activity (Karin and Ben-Neriah, 2000). Treatment with substances which activate NFκB, such as lipopolysaccharides or phorbol esters, likely do so by producing the dissociation of NFκB from IκB, allowing NFκB to move into the nucleus where it can bind to DNA and activate gene expression. This is an example of preclusion of nuclear translocation and activation by association with an inhibitory protein (Latchman, 2004).

As mentioned previously, the synthesis of transcription factors can also be controlled at various stages, for example transcriptional or post-transcriptional control. Examples of post-transcriptional synthesis control include the regulation of mRNA
splicing and translation. Moreover, even after the transcription factor has been produced, the existing factor can also be regulated by targeted degradation. In many cases, ubiquitination serves to target the protein for degradation (Maniatis, 1999). An interesting example of a transcription factor under ubiquitin-mediated controlled degradation is hypoxia inducible factor, HIF-1. HIF-1 consists of two subunits, HIF-1α and HIF-1β and is activated when oxygen levels are low. After activation, it stimulates its target genes. In the presence of oxygen, HIF-1α is rapidly ubiquitinated and degraded. However, when oxygen levels fall, HIF-1α is no longer ubiquitinated and can associate with HIF-1β and activate gene transcription (Bruick and McKnight, 2002).

Ubiquitination may have a further role in transcriptional regulation beyond targeted degradation. It has been shown that ubiquitination may be necessary for transcription factor activation domains to stimulate transcription (Maniatis, 1999; Latchman, 2004). In fact, it has been suggested that these two roles may be related. Modification by ubiquitination may allow the factor to activate transcription but also targets it for destruction after activation has occurred (Tansey, 2001).

We have discussed how various regulatory processes can affect the activity of the transcription factor at a wide variety of stages. A single process, such as phosphorylation, for example, can alter the DNA binding ability of the factor, its location within the cell, transcription activation ability, association with other proteins or its degradation. In many cases, both regulated synthesis and regulated activity
allows the precise requirements of a particular response to be fulfilled rapidly but with minimum unnecessary waste of energy (Latchman, 2004).

Gene-specific Effects of Transcription Factors

If the same transcription factor(s) is involved in the regulation of many genes and these factors are regulated in similar ways, how do they assert gene-specific effects on transcription? It is most likely due to the fact that transcription factors rarely act independently. A single factor binding to a single site in a gene is almost never sufficient to result in the activation of that gene. Rather, the transcriptional activity of any given gene seems to be determined by combinations of factors acting together. This combinational control provides a means of integrating multiple inputs into the determination of whether a gene is turned on or off (Courey, 2001). For instance, changing one member of a protein complex can have very different results, as illustrated in the following example. Max is a widely expressed basic helix-loop-helix leucine zipper (bHLHZip) transcription factor that heterodimerizes with members of the Myc family of bHLHZip proteins, including Myc and Mad. Myc and Mad do not associate with each other, but the Myc/Max complex activates transcription while the Mad/Max complex represses it (Ayer, et al., 1993).

Combination control also determines tissue specificity. If a transcription factor that is required for the regulation of a specific gene is not expressed in a tissue, then that gene will not be expressed in that tissue. The reverse situation is true as well. The expression of particular transcription factor can lead to tissue specific expression. A
good example of tissue-specific regulation involves MyoD, a basic helix-loop-helix (bHLH) transcription factor that is expressed in skeletal muscle myoblasts and differentiated skeletal muscles (Asakura, et al., 1995) but is absent in all other tissues, including cardiac muscle. The regulated synthesis of MyoD results in the activation of muscle-specific gene expression and the production of skeletal muscle cells (Latchman, 2004). The forced expression of MyoD is sufficient to convert a large number of primary cells and cell lines into skeletal muscle myoblasts (Asakura, et al., 1995).

Now that the functional domains and regulation of transcription factors in general has been discussed, we will look at how diet and hormones regulate gene expression.

Dietary and Hormonal Regulation of Metabolic Genes

Since the early beginnings of man, we have been interested in the effects of nutrition, for such varied reasons as eliminating “evil spirits”, fasting and feasting for religious reasons, reducing anxiety and depression, and preventing and curing diseases. From these early practices, nutritional awareness evolved into a science. With the discovery of deficiency diseases and diabetes, glucose and fat metabolism became an important area of investigation (Berdanier and Hargrove, 1993).

Studies on the effect of nutrition and hormones on glucose utilization and fat production (lipogenesis) have been occurring for many decades. This is illustrated by a 1958 study of the effects of food intake on hepatic lipogenesis in rats. (Tepperman and Tepperman, 1958). Two years later, Dipietro and Weinhouse discovered hepatic
glucokinase (GK) (Figure 3) and demonstrated an increase in its activity in liver extracts from rats fed a high carbohydrate diet (Dipietro and Weinhouse, 1960). Later in the same decade, it was found that the concentration of pyruvate kinase (PK) (Figure 3) was decreased by starvation and increased by refeeding, especially when the diet is high in carbohydrate (Tanaka, et al., 1967) and that the activity of malic enzyme (ME) (Figure 3) is low in liver of prenatal chick embryos and increases dramatically when the birds are fed (Goodridge, 1968). At the time of these studies, the techniques available to study metabolism were limited to whole animal or tissue enzymatic activity studies. It was difficult to study metabolic mechanisms in whole animals, consequently cell culture systems were eventually developed. Through the use of primary hepatocytes cultures from prenatal and early postnatal chicks treated with serum and glucose, Goodridge et al., demonstrated that the activities of ME and fatty acid synthase (FAS) (Figure 3) were increased equivalently to the levels of newly hatched chicks that are fed a high carbohydrate diet. This study established primary hepatocytes as a suitable model system (Goodridge, et al., 1974).

By the early 1970’s, methods were available to determine the levels of mRNA for specific proteins (Hanson, 2005). Near the end of the decade, enzyme synthesis and degradation could also be determined by measuring the incorporation or loss of radioactively labeled substrate or protein. This method was used to show that fasting and refeeding a high carbohydrate diet increases the mRNA level of rat liver PK, which correlates with changes in the total PK enzyme activity, protein and rate of synthesis in vivo, suggesting that regulation may be at the transcriptional level.
(Cladaras and Cottam, 1980). A similar method was used to demonstrate that glucose increased the activity of FAS in rat primary hepatocytes and that this increase was due to an increase in the rate of enzyme synthesis (Giffhorn-Katz and Katz, 1986).

During this time, transcription rates could be measured using nuclear “run on” assays, which used $^{32}$P-UTP to measure the number of RNA polymerase molecules

![Diagram of metabolic pathways](Image)

Figure 3. Glycolytic and Lipogenic Pathways in the Liver

that were engaged in transcription at the time the treated tissues or cells were harvested (Goodridge, 1987). Nuclear “run on” assays were used to show that refeeding fasted rats a high-carbohydrate diet caused an increase in liver PK gene transcription and mRNA levels (Vaulont, et al., 1986).

Almost thirty years after the Tepperman and Tepperman study, it was discovered that diet and hormones regulate the expression of key enzymes involved in glycolysis and lipogenesis, including PK, GK, glucose-6-phosphate dehydrogenase (G6PDH), ME, acetyl CoA carboxylase (ACC), FAS (Figure 3) and S14. The expression of these genes was found to be reduced by starvation, diabetes or glucagon and significantly elevated by a high glucose diet or insulin (Kletzien, et al., 1985; Imamura, et al., 1986; Mariash, et al., 1986; Vaulont, et al., 1986; Goodridge, 1987; Pape, et al., 1988; Noguchi and Tanaka, 1993). In rat liver, increased ME mRNA levels caused by refeeding is accompanied by decreased degradation of the mRNA, with no change in the transcription rate of the gene (Dozin, et al., 1986). Contrary to ME, insulin and glucose regulate most of these key glycolytic and lipogenic enzymes at the transcriptional level. In hepatocytes in culture, the transcriptional activation of FAS (Giffhorn-Katz and Katz, 1986) and S14 (Shih and Towle, 1992) requires both insulin and glucose. In adipocytes, ACC induction also requires both insulin and glucose (Girard, et al., 1994).

Since glucose regulates the transcription of the insulin gene, it was unclear for many of these genes whether or not glucose itself or insulin regulates the transcription of these metabolic enzymes in vivo. A well-studied example of an insulin-regulated
gene is glucokinase (GK). GK is found in the liver and pancreas and catalyzes the reaction to convert glucose to glucose-6-phosphate (Figure 3). Insulin regulation of GK does not require glucose (Matsuda, et al., 1990). However, the insulin regulation of many other metabolic enzymes requires glucose. Therefore, a question remains as to whether insulin has only a permissive role in the regulation of these genes in the liver by allowing glucose phosphorylation to glucose-6-phosphate by GK or if it has another function in the glucose-dependent signaling pathway. Previous studies suggest that the role of insulin in the induction of these metabolic enzymes in hepatocytes is probably limited to the activation of GK, which is necessary for catalyzing the glucose phosphorylation (Vaulont and Kahn, 1994).

In hepatocytes, glucose entry is mediated through GLUT2 transporters, which are facilitative glucose transporters with a high $K_m$ for glucose (17-20 mM) (Gould and Holman, 1993). They have a high transport capacity which increases as a direct function of extracellular glucose concentration and are not saturated under most physiological conditions. Thus, glucose transport is never rate limiting for the entry of glucose into the liver (Rencurel and Girard, 1998). GLUT2 transporters are constitutively present on the cell membrane and are not regulated by insulin. Once glucose has entered the cell, it is phosphorylated by GK to glucose-6-phosphate. GK also has a high $K_m$ for glucose (10mM), which allows for immediate changes in the glucose phosphorylation rate depending on the plasma glucose concentration (Storer and Cornish-Bowden, 1976; Iynedjian, et al., 1988). GK is not feedback-inhibited by the product, glucose-6-phosphate (Mathews and van Holde, 1990). Glucokinase

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activity is low during fasting and increased after carbohydrate ingestion (Salas, et al., 1963; Iynedjian, et al., 1988). Glucokinase activity is determined by its synthesis rate, which is regulated by insulin (Weinhouse, 1976; Iynedjian, et al., 1988).

Conversely, in adipocytes, glucose enters the cell through GLUT4 transporters, which have a low $K_m$ for glucose (1-5 mM) (Gould and Holman, 1993). GLUT4 transporters are intracellular in the absence of insulin and translocate to the plasma membrane in the presence of insulin. Once glucose enters the cell, it is phosphorylated to glucose-6-phosphate by hexokinase (HK), an enzyme which can phosphorylate various hexose sugars and is feedback inhibited by the product. HK has a low $K_m$ (0.1 mM), therefore at normal blood glucose levels (5 mM), it is saturated (Mathews and van Holde, 1990). Unlike GK, HK is not regulated by insulin (Foufelle, et al., 1996).

Given that both liver and adipose tissue require insulin to regulate either glucose transport or glucose phosphorylation, it was difficult to distinguish the specific effects of insulin or glucose. The use of cells in culture allowed for the experimental separation of the insulin and glucose effects. The cell culture system led to the discovery that glucose metabolism is necessary for the induction of most of these metabolic enzyme genes. Thus it is likely that a glucose metabolite provides the necessary signal for the stimulation of these genes. However, provided that GK is expressed, glucose alone is capable of promoting the fasted-to-fed state in the absence of insulin (Doiron, et al., 1994; Ferre, et al., 1996; Scott, et al., 1998; Cournarie, et al., 1999; Collier and Scott, 2004). Furthermore, it was also discovered that insulin-
responsive genes contain an insulin-response element (IRE) which is a region in the promoter necessary for the insulin effect. And in parallel, glucose-responsive genes contain a glucose-response element (GRE) within the promoter which is required for the glucose response.

Glucose Regulation of Gene Transcription

The means by which glucose regulates transcription still remains largely unknown. However, it has been shown that carbohydrate-associated genes, such as PK and S14, contain a glucose-response element (GRE). All of these GRE's contain at least one E-box motif, which consists of the DNA sequence 5'-CACGTG-3'. The GREs of the rat PK and S14 genes consist of two E box motifs in an inverted orientation separated by 5 bp (Thompson and Towle, 1991; Shih and Towle, 1992) (Figure 4). The 5 bp separation is thought to be important for the full glucose response, exhibited by the fact that a 6 bp spacing between the E-boxes confers only a partial glucose induction, whereas a 4-bp separation does not respond to glucose (Shih, et al., 1995). Subsequent studies have also shown that only the first 4 bp of the E-boxes are critical for the glucose response of the rat PK and S14 GREs (Kaytor, et al., 1997).

The mouse S14 GRE has recently been characterized. It is different from the rat PK and S14 genes in that it contains two E-box half-sites in a direct orientation consisting of the sequence CACG, that are separated by 7 bp (Koo and Towle,
**Inverted orientation:**

\[
\text{rat S14} \quad \overrightarrow{\text{CACGTGGTGCCCTGTC}} \overleftarrow{\text{GTGpTGGCCCTGTC}} \\
\quad \overrightarrow{\text{[Shih et al, 1995]}} \overleftarrow{\text{[Bergot et al, 1992]}} \overrightarrow{\text{[Liu et al, 1993]}}
\]

\[
\text{rat PK} \quad \overrightarrow{\text{CACGGGGGACTCCGTG}} \overleftarrow{\text{CTCCGTG}}
\]

**Direct orientation:**

\[
\text{mouse S14} \quad \overrightarrow{\text{CACGC}} \overleftarrow{\text{GGAGTCAGCC}} \\
\quad \overrightarrow{\text{[Koo and Towle, 2000]}} \overleftarrow{\text{[Rufo et al, 2001]}}
\]

\[
\text{rat FAS} \quad \overrightarrow{\text{CATGTGACACAGCGT}} \overleftarrow{\text{GCGT}}
\]

\[
\text{rat ACC PI} \quad \overrightarrow{\text{CATGTGAAAAGCTCGT}} \overleftarrow{\text{GTG}}
\]

\[
\text{[O'Callaghan et al, 2001]}
\]

Figure 4. Carbohydrate Response Elements of the Pyruvate Kinase, S14, Fatty Acid Synthase and Acetyl-CoA Carboxylase Promoters

The boxed areas indicate the proposed E-box motifs. The bolded bases are those shown to be critical for the glucose response. Adapted from Foufelle and Ferre' (2002) and O'Callaghan, et al. (2001).

2000)(Figure 4). This finding led Koo and Towle to suggest a new model for the GRE. They propose that the GRE consists of two E-box half sites related to the CACG motif (Koo and Towle, 2000).

The GRE of the FAS gene is similar to that of the mouse S14 and rat ACC genes, containing two imperfect E-boxes oriented in the same direction and separated by 5 bp (Rufo, et al., 2001)(Figure 4).
Given that these key enzymes are transcriptionally regulated through the GRE and IRE, we will now review specific transcription factors that may be involved in their glucose and insulin responsiveness.

**Transcription Factors Involved in the Regulation of Glucose and Insulin Responsive Genes**

There are three transcription factors that have been implicated in the glucose responsiveness of PK, ACC, FAS and S14, through binding to the E-box region of the GRE. These include Upstream Stimulatory Factor (USF), Sterol Regulatory Element Binding Protein (SREBP) and Carbohydrate Response Element Binding Protein (ChREBP). SP1 is a transcription factor that does not bind to E-boxes, but has been shown to be involved in the glucose response of ACC (Daniel and Kim, 1996). SP1 opposes SREBP in the insulin regulation of phosphoenolpyruvate carboxykinase (PEPCK)(Figure 3), through competition for overlapping binding sites on the PEPCK promoter (Chakravarty, et al., 2004). SP1 has also been demonstrated to positively interact with SREBP in the sterol regulation of ACC (Lopez, et al., 1996) and FAS promoters (Bennett, et al., 1995; Dooley, et al., 1998). Sterol regulation of enzyme genes is of interest because when cellular sterol levels are low, genes involved in cholesterol synthesis and uptake are activated (Sanchez, et al., 1995). Genes involved in cholesterol and fatty acid synthesis are similarly regulated (Eberlé et al., 2004). Thus, factors involved in the sterol regulation could also be involved in insulin and glucose regulation.
The specific aspects of these four transcription factors, SP1, USF, SREBP and ChREBP will be discussed.

SP1

SP1 was first identified as a trans-acting sequence-specific DNA-binding protein required for the expression of the SV40 virus early promoter (Dyfan and Tjian, 1983). SP1 is ubiquitously expressed and is likely involved in the activation of a wide variety of promoters (Philipsen and Suske, 1999). SP1 binds with high affinity to a 10-base pair cis-element through the use of a zinc finger DNA binding domain (Kriwacki, et al 1992). It effects transcription by binding to TAF110, one of the general transcription factors involved in basal transcription (Chen, et al., 1994). SP1 interactions with other SP1 molecules may also be important in gene regulation (Courey, et al., 1989). SP1 is regulated by phosphorylation (Jackson, et al., 1990) and glycosylation (Jackson and Tjian, 1988). The phosphorylation state of SP1 regulates its inducible binding to GC-rich DNA regions (Merchant, et al., 1999). Glycosylation prevents its interactions with other SP1 molecules and TAF110, thus inhibiting its transcriptional capability (Yang, et al., 2001).

Many promoters, which contain SP1 sites, also contain other elements that confer regulated expression of the gene. These specific regulatory elements may function by influencing the activity of SP1 bound at a nearby site. Sterol Regulatory Element Binding Protein-1 (SREBP-1) and SP1 have been shown to synergistically activate sterol-induced expression of the low density lipoprotein (LDL) receptor
promoter when co-transfected in Drosophila tissue culture cells that lack endogenous SP1 (Sanchez, et al., 1995). The synergistic activation of the LDL receptor promoter by SREBP and SP1 occurs as a two step process. SREBP-1 first stimulates SP1 to bind to its adjacent site. Secondly, the binding of both proteins to the DNA activates transcription much more effectively than either protein alone (Athanikar, et al., 1997). The interaction between SREBP and SP1 appears to be a direct protein-protein binding, because their interaction can be specifically disrupted by the activator/repressor protein Yin Yang 1 (Bennett, et al., 1999).

The Drosophila co-transfection technique was also used to show that SP1 cooperates with SREBP in the sterol regulation of acetyl-CoA carboxylase (ACC) (Lopez, et al., 1996) and fatty acid synthase (FAS) promoters (Bennett, et al., 1995; Dooley, et al., 1998). However, the SP1 domains required to activate the LDL promoter are not required for the sterol activation of the ACC and FAS promoters. Thus, even though SREBP and SP1 are essential, the mechanism for activation of ACC and FAS promoters must be at least partially distinct from activation of the LDL receptor (Athanikar, et al., 1997). The specific mechanism by which SREBP and SP1 cooperate on the sterol regulation of ACC and FAS is not known. SREBP and SP1 have also been shown to cooperate in the induction of rat liver ACC in response to a high carbohydrate diet (Oh, et al., 2003).

The first reported involvement of SP1 in mediating the glucose response of a gene was published by Daniel and Kim (Daniel and Kim, 1996) who demonstrated that SP1 was involved in the glucose activation of ACC in mouse 30A5 preadipocytes.
SP1 was also recently shown to oppose SREBP in the insulin regulation of PEPCK, through competition for overlapping binding sites on the PEPCK promoter. This study proposed that insulin represses transcription of PEPCK by inducing SREBP-1c production in liver, which interferes with the stimulatory effect of SP1 (Chakravarty, et al., 2004).

**Upstream Stimulatory Factor (USF)**

Upstream Stimulatory Factors (USF) have been extensively explored as possible transcriptional factors that may be responsible for nutritional regulation of lipogenic enzymes such as FAS, S14, and pyruvate kinase (PK), in particular, through binding to the GRE or IRE. USF proteins belong to the Myc family of transcription factors characterized by a bHLHZip domain. USF proteins are encoded by two distinct genes, USF1 and USF2, and occur in three forms, USF1, USF2a and USF2b. USF proteins exist as homo- and heterodimers that are able to bind to E-boxes (Viollet, et al., 1996). The C-terminal region of USF contains the dimerization and DNA-binding domains (Sirito, et al., 1994). Interestingly, it has also been shown that USF is a tetramer prior to DNA binding. Tetramer formation requires the leucine zipper region. It is possible that USF may remain a tetramer and bind two distinct DNA sites simultaneously or dissociate into dimers upon DNA-binding (Ferré-D’Amaré, et al., 1994).

USF proteins were originally discovered because of their ability to interact and transactivate the adenovirus major late promoter. These genes are widely expressed in
mammals and in the liver, the USF-1/USF2α heterodimer accounts for 65% of the USF binding activity (Vallet, et al., 1997).

USF proteins have been shown to bind the GRE of glucose-responsive genes; however the binding of these proteins to the GRE of the PK gene is not modified by nutritional conditions, nor is it likely to be a primary carbohydrate-responsive transcription factor (Kahn, 1997). Evidence shows that USF binding is non-specific, since it lacks the ability to distinguish GREs that are glucose-responsive from glucose-unresponsive oligonucleotides either in electrophoretic mobility shift assays or in transfected cells (Kaytor, et al., 1997). In an additional study, overexpression of a dominant negative form of USF in hepatocytes failed to block the glucose response of the rat S14 and PK promoters (Kaytor, et al., 1997). Furthermore, a study by Koo and Towle (Koo and Towle, 2000) showed that protein binding to the GRE of the S14 and PK genes is not disrupted with anti-USF antibody, indicating that USF is not likely a critical component of the glucose-response complex of these genes. However, the diminished carbohydrate response of these genes observed in USF2 knockout mice or cells treated with dominant negative forms of USF for several days indicate that USF may play an indirect role in the process (Vallet, et al., 1997).

**Sterol Regulatory Element Binding Protein (SREBP)**

Sterol regulatory element binding proteins (SREBPs) are a family of transcription factors that are being studied as possible glucose-responsive factors since their expression is dramatically reduced by fasting and elevated upon refeeding, which
parallels the expression of lipogenic genes. SREBPs have been shown to bind to the promoter of some lipogenic genes, such as ACC, FAS and stearoyl-CoA desaturase (Shimano, et al., 1999).

SREBPs also belong to the large class of bHLHZip transcription factors. SREBPs were first identified because of their properties for binding to the sterol regulatory element and conferring sterol regulation to several genes involved in cholesterol synthesis. Unlike other members of the bHLHZip family, SREBPs are synthesized as precursor proteins that are inserted into the endoplasmic reticulum. When sterol levels fall, a proteolytic cascade cleaves the precursor protein and releases a transcriptionally active 68 kD N-terminal domain (the mature SREBP). The mature SREBP then translocates to the nucleus where it binds to the sterol regulatory element (SRE) present in the promoters of target genes (Brown and Goldstein, 1997). The mature SREBPs are rapidly degraded through the ubiquitin-proteasome system (Hirano, et al., 2001).

SREBPs have been shown to have dual binding specificity, not only binding to the SRE, but also to E-boxes (5'-CACGTG-3')(Kim, et al., 1995). To date, three SREBP isoforms, SREBP-1a, -1c, and -2 have been identified and characterized. SREBP-1a and 1-c are produced from a single gene through the use of alternate promoters. SREBP-2 is produced from a separate gene (Brown and Goldstein, 1997). SREBP-1a is the predominant isoform in continuously growing cells and in the spleen, where there is active turn over of cells in the immune system (Toth, et al., 2004). Most other adult animal organs, including the liver, mainly synthesize SREBP-1c and
SREBP-2 (Shimomura, et al., 1997). SREBP-2 is predominately involved in the regulation of cholesterol biosynthetic genes. SREBP-1 preferentially activates fatty acid metabolic genes. Recent studies have shown that SREBPs plays a crucial role in the induction of genes for lipogenic enzymes, including glucose-6-phosphate dehydrogenase (G6PDH), in the liver. In one study, the hepatic mRNA levels of various lipogenic enzymes were determined in SREBP-1 gene knockout mice after a fasting-refeeding treatment. The refeeding response of these lipogenic enzymes, including G6PDH, was completely abolished in these mice, suggesting that SREBP-1 plays a critical role in the nutritional induction of these genes, in particular by glucose.

A direct binding of SREBP-1 to the promoter of ACC, FAS, and stearoyl-CoA desaturase has been shown (Shimano, et al., 1999), however the G6PDH promoter has not been studied to determine if it is a direct target of SREBP-1. In fact, SREBP-1c has been shown to be necessary for the glucose response of the PK and FAS genes (Foretz, et al., 1999). SREBP-1c itself is rapidly induced in hepatocytes treated with insulin, which provides a way for insulin involvement in enhanced metabolic gene transcription (Horton, et al., 1998; Foretz, et al., 1999). Further evidence which suggests that SREBP regulates G6PDH expression was provided in a very recent study by Korczynska, et al, who showed that rats with chronic renal failure contain high levels of SREBP-1 in their white adipose tissue and this is associated with an upregulation of lipogenic genes, such as ACC, FAS, ME and G6PDH (Korczynska, et al., 2004). The increase in lipogenic gene expression in adipose tissue may not be due to changes in SREBP-1 mRNA levels given that a different study showed that levels of
SREBP-1c mRNA do not correspond with the changes in FAS, ME and G6PDH mRNA observed in fasting and refeeding (Bertile and Raclot, 2004).

SREBPs alone are inefficient transcriptional activators (Dooley, et al., 1998; Towle, 2003; Toth, et al., 2004). All SREBP-regulated promoters examined thus far require additional transcription factors for maximal activation in response to sterol deprivation. The required co-activator is not the same for all inducers and SREBP-regulated promoters. For instance, in the sterol regulation of the LDL receptor, ACC, and FAS promoters, the required co-regulator is SP1 (Sanchez, et al., 1995; Dooley, et al., 1998), but with the farnesyl diphosphate synthase promoter, it is CCAAT binding factor, also called nuclear factor Y (Dooley, et al., 1999). On the other hand, carbohydrate activation of the FAS promoter in primary hepatocytes is dependent upon SREBP and both the SP1 and CCAAT-binding factor/nuclear factor Y sites (Magaña, et al., 2000).

A study by Shimano, et al, examined the role of USFs in conjunction with deletion of the SREBP-1 gene by measuring the amount of USF-1 and -2 protein in liver nuclear exacts from SREBP-1 gene knock out mice and in wild type mice. They found no significant difference in the amount of USF-1 or -2 protein between the fasted or refed state in the knock out mice and in the wild-type mice. This suggests that USFs and SREBPs can not compensate for each other (Shimano, et al., 1999). Both USFs and SREBPs have been shown to be important in the activation of the FAS gene, although they have been shown to bind to the promoter independently, and there is no evidence of synergistic action (Wang and Sul, 1997).
Carbohydrate Response Element Binding Protein (ChREBP)

Carbohydrate response element binding protein (ChREBP) is a recently discovered hepatic bHLHZip transcription factor that binds to the GRE of the PK promoter (Yamashita, et al., 2001). It is activated by high glucose and inhibited by a high fat diet and cAMP. ChREBP is regulated at two levels, nuclear localization and DNA binding. This regulation occurs through a phosphorylation-dependent mechanism. ChREBP is located in the cytosol at low (fasting) glucose levels, but is found in the nucleus at high glucose levels. This cellular localization can be controlled by phosphorylation of a serine residue (S<sup>196</sup>), which is located next to the nuclear translocation sequence. Phosphorylation of this serine results in the retention of ChREBP in the cytosol where it remains inactive. Protein kinase A (PKA) is believed to be responsible for this phosphorylation. The DNA-binding activity also appears to be regulated by PKA phosphorylation of serine (S<sup>626</sup>) and threonine (T<sup>666</sup>) residues found next to the DNA binding domain. Phosphorylation of these residues prevents DNA binding to the PK GRE. Protein phosphatase 2A (PP2A) reverses both effects of phosphorylation on ChREBP, its cytoplasmic retention and inhibition of DNA binding (Kawaguchi, et al., 2001). An earlier study demonstrated that an isoform of PP2A in liver is activated by xylulose-5-phosphate (X-5-P), a glucose intermediate, in hepatocytes incubated in high-glucose conditions (Nishimura and Uyeda, 1995). It was subsequently discovered that, indeed, glucose, through X-5-P, activates ChREBP by stimulating cytoplasmic and nuclear PP2As, resulting in the dephosphorylation of key residues.
PKA phosphorylated sites on ChREBP, allowing its nuclear translocation and DNA binding (Kabashima, et al., 2003).

Stoeckman, et al. recently discovered that overexpression of ChREBP in primary rat hepatocytes activates PK and other carbohydrate responsive promoters in a manner similar to glucose itself. Aware that E-box binding proteins, such as ChREBP, must function as dimers, they sought a potential partner for ChREBP. Max-like protein X (Mix) was found to be a possible partner for ChREBP and the ChREBP-Mix dimer was able to dramatically enhance several lipogenic enzyme genes, such as PK, S14 and ACC (Stoeckman, et al., 2004).

Glucose and Insulin Regulation of Specific Metabolic Genes

**Pyruvate Kinase**

Pyruvate kinase (PK) has been known to be regulated by glucose for numerous years (Decaux et al., 1989). Some of the key components of its glucose-responsiveness have been elucidated. It contains a GRE with two E-boxes, as shown in Figure 4. SREBP-1c has been shown to be necessary for its glucose responsiveness (Foretz, et al., 1999) and ChREBP was found to bind to the GRE of the promoter (Yamashita, et al., 2001).

Transfection experiments in hepatocytes with GK and PK reporter constructs have shown its induction to be strongly stimulated by glucose independently of insulin (Vaulont and Kahn, 1994). Doiron, et al showed that in primary hepatocytes, 5 mM xylitol induced the accumulation of PK mRNA in the absence of insulin. The response...
to xylitol, as well as glucose, requires a functional GRE. These results suggest that the glucose signal for transcription of PK is mediated through X-5-P (Doiron, et al., 1996).

As previously mentioned, it was recently discovered that X-5-P activates PP2A, which then dephosphorylates ChREBP and allows it to translocate to the nucleus to activate PK gene transcription (Kabashima, et al., 2003). If X-5-P is required for nuclear translocation and DNA binding of ChREBP, then one would speculate that ChREBP is probably not involved in the glucose regulation of G6PDH. G6PDH is the rate limiting enzyme in the pentose phosphate pathway, leading to the production of X-5-P, along with other metabolites. Therefore, it is doubtful that a "downstream" product of this pathway would positively control an "upstream" enzyme. However, it can not be declared that ChREBP is not involved in the glucose regulation of G6PDH with further study.

S14

S14 is a gene that codes for a nuclear protein that is believed to play a regulatory role in lipogenesis. In cultured hepatocytes, the transcriptional activation of S14 requires both insulin and glucose. The S14 GRE contains two E box motifs separated by 5 bp in an inverted orientation (Shih and Towle, 1992)(Figure 4). USF was initially believed to play an integral part in the glucose-responsiveness of S14, but Koo and Towle (Koo and Towle, 2002) showed that protein binding to the GRE is not disrupted with anti-USF antibody, indicating that USF is not likely a
critical component of the glucose-response complex of S14. However, as presented earlier, there is evidence that USF may play an indirect role in this complex.

**Acetyl-CoA Carboxylase**

Acetyl-CoA carboxylase (ACC) is the enzyme that catalyzes the conversion of acetyl-CoA to malonyl-CoA (Figure 3). Glucose and insulin synergistically activate ACC expression in primary rat hepatocytes. Glucose induction of ACC is mediated, in part, by a glucose-regulated transcription factor, termed carbohydrate responsive factor (ChoRF) (O’Callaghan, et al., 2001). It is unknown if ChoRF is identical to ChREBP because both proteins were discovered simultaneously by separate research groups.

The glucose activation of ACC in mouse preadipocytes involves SP1 (Daniel and Kim, 1996). Additionally, SREBPs have also been shown to bind to the promoter of ACC (Shimano, et al., 1999), but full induction of ACC by SREBP also requires the co-activator SP1 (Lopez, et al., 1996; Dooley, et al., 1998; Oh, et al., 2003).

**Fatty Acid Synthase**

Fatty acid synthase (FAS) is a central enzyme in lipogenesis. It catalyzes the conversion of malonyl-CoA to palmitate (Figure 3). FAS is under strict hormonal and nutritional control in the liver.

FAS contains a GRE (Figure 4) and the glucose responsiveness provided by this GRE is independent of insulin. A unique carbohydrate responsive factor (ChoRF)
was shown to bind to the GRE (Rufo, et al., 2001). It is not know if this ChoRF is the same protein that binds to the ACC promoter.

In addition, using transgenic mice containing the chloramphenicol acetyltransferase (CAT) gene driven by various 5' deletions of the FAS promoter, Latasa, et al found that upon fasting-refeeding, there is induced binding of SREBP to an SRE in liver tissue and this SREBP binding and resulting FAS activation requires simultaneous binding of USF to an E-box. Therefore, SREBP and USF play vital roles in the transcriptional regulation of FAS gene by high carbohydrate feeding and insulin \textit{in vivo}. (Latasa, et al., 2003).

Glucose-6-phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PDH), an important enzyme involved in glucose homeostasis, has not been studied extensively in terms of glucose regulation. G6PDH is a key regulatory enzyme of the pentose phosphate pathway (Figure 3), which can be responsible for up to 50% of carbohydrate load. G6PDH controls not only the flow of carbon through this pathway, but also produces NADPH, which is necessary for fatty acid biosynthesis and maintaining the cellular redox state. Thus, G6PDH is important in both carbohydrate and fatty acid metabolism. The G6PDH gene had been considered to be a “housekeeping” gene, but several studies have shown its expression to be highly regulated. The expression of G6PDH has been shown to be increased by insulin in primary rat hepatocytes (Kurts and Wells, 1981; Stapleton, et al., 1993) and by oxidative stress in cells in culture (Ursini, et al., 1997;
Xu, et al., 2003). G6PDH is thought to also be regulated by glucose, however previous studies on glucose regulation of G6PDH expression or activity have yielded conflicting results. Glucose has been implicated in both an increase in G6PDH transcription and mRNA stability (Prostko, et al., 1989). A study by Salati, et al. showed that G6PDH is regulated exclusively at the posttranscriptional level and that changes in the rate of mature G6PDH mRNA are due to changes in the splicing efficiency of the G6PDH transcript (Salati, et al., 2004). We found that glucose, in a concentration dependent manner, does have a significant effect on G6PDH activity in primary rat hepatocytes. We have also shown insulin to directly affect not only the activity, but also the expression of G6PDH. Additionally, contrary to Salati, et al., we have recently demonstrated that G6PDH is regulated by glucose at the transcriptional level, although we can not exclude that it may also be regulated posttranscriptionally.

The mechanism in which glucose regulates G6PDH transcription is not known. As indicated previously, glucose may trigger the modification of a protein(s), possibly by phosphorylation/dephosphorylation, which then allows the movement of the protein to the nucleus where it binds to the GRE, resulting in a change in the transcription of the gene. This may involve an E-box containing GRE, as demonstrated in PK, S14, FAS and ACC genes.

The G6PDH promoter does contain a single imperfect E-box at position -539, consisting of the sequence 5’-CACCTG-3’. It is unknown whether this E-box is critical for the glucose responsiveness of the gene.
Significance of this Study

Many of the key metabolic genes in the liver were initially studied because of their response in starvation and refeeding. Over thirty years later, there is still contradictory information regarding the mechanisms that are responsible for the glucose regulation of these key metabolic enzymes. Not all glucose-regulated genes are controlled by the same cis-elements or transcription factors, illustrating the complexity of this research area. This complexity makes it important to investigate all glucose metabolic pathways. The genes coding for enzymes in the pentose phosphate pathway have not been studied in regard to glucose regulation. Knowledge of the effect of glucose on the regulation of these genes, such as G6PDH, will aid our understanding of the underlying mechanisms of starvation/refeeding and diseases such as diabetes and obesity. With the list of glucose-regulated genes continuously growing, there is an even greater need for research in this area.

Objectives of the Study

We have cloned the G6PDH gene and have previously shown that glucose increases G6DPH activity (Arkwright, 1998) and gene transcription. The goals of this study were to:

1. identify the general glucose-responsive region of the G6PDH promoter.
2. determine if the glucose and insulin responses are mediated through similar or distinct mechanisms.
3. establish if the E-box is necessary for the glucose and insulin responses.
4. if the E-box is found to be necessary, determine if nuclear proteins bind to this region in response to high carbohydrate.

5. determine if the transcription factors SREBP, USF and SP1 bind to the G6PDH promoter and whether or not they are involved in the glucose and insulin responses.
MATERIALS AND METHODS

Hepatocyte Isolation and Maintenance

Male Sprague-Dawley rats were obtained from Charles River, Portage, MI. Prior to use, rats weighing approximately 180-200 grams, maintained on Lab Diet 5001 standard rodent diet, were fasted for 48 hours (hrs). Drinking water was provided ad libitum. Animals were anesthetized with an intra-peritoneal injection of pentobarbital. Hepatocytes were isolated using the collagenase perfusion method (Stapleton, et al., 1993). The liver was excised and forced through three layers of gauze over a beaker containing perfusion solution, and then centrifuged in 50 ml tubes at 4°C for 3 minutes (mins) at 50xg. The supernatant was aspirated and the cell pellet was suspended, washed, and centrifuged twice with cold Waymouth’s MB 752/1 (Gibco, Grand Island, NY) medium containing 0.5% Bovine Serum Albumin (BSA). The cell pellet obtained after the final washing was gently resuspended in the medium and an aliquot was used to determine cell concentration and cell viability on a hemocytometer by trypan blue exclusion. Cells with a viability of greater than 80% were plated to 90% confluency on sterilized Falcon-3002 60 mm, collagen (rat-tail) coated plates. The cells were then incubated in 4 ml Waymouth’s MB 752/1 medium with BSA at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 4 hrs. After the 4 hr. attachment period, the media was aspirated and the cells were washed once with 1 ml of BSA free Waymouth’s medium. The wash was replaced with glucose-free
Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with gentamycin (2.2 μg/ml) and 5 mM glucose or 5 mM pyruvate. The cells were then incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 18 hrs.

**Transfection and Cell Processing**

Eighteen hours after attachment, the cells were transiently transfected with the various reporter constructs using lipofectin (Invitrogen, Carlsbad, CA) and maintained in 5 mM glucose or pyruvate DMEM media for 6 hrs. The media was aspirated and the cells were then treated with media containing either 5, 10, 20, or 30 mM glucose or pyruvate and when indicated, incubated with 250ng/ml (44nM) of insulin. Fourteen hours after treatment, the cells were washed twice with 1 ml of cold phosphate buffered saline (PSB). The last wash was aspirated and 400 μl of 1x reporter lysis buffer (Promega, Madison, WI) was added. After 15 min., the plates were scraped to remove the cells. The cell lysates were centrifuged at 9,300xg for 2 min. and the supernatant was transferred to a clean eppendorf tube. The luciferase activity of the cell lysates was measured using the luciferase assay system (Promega, Madison, WI) and a liquid scintillation counter. Ten microliters of cell lysate was added to a tube containing 100 μl of luciferase substrate. The luminescence was measured twice for 1 min. and the counts averaged. The luciferase activity of the cell lysates was normalized for total protein measured by the Lowry method (Lowry et al., 1951).
Plasmid Reporter Constructs

The 935, 635 and 187 G6PDH-Luc Reporter Constructs

The G6PDH promoter was isolated and sequenced by Rank, et al (Rank, et al., 1994). The 935 bp (-878 to +57), 635 bp (-578 to +57) and 187 bp (-130 to +57) regions of the rat G6PDH promoter were amplified by PCR. These PCR fragments were inserted between the Smal and Xhol restriction sites 5' of the luciferase gene in the p-GL2 Basic Vector (Promega, Madison, WI) to produce the 935, 635 and 187G6PDH-Luc constructs, respectively (Holmen, S.L., 1995).

The Emut1 G6PDH-Luc Reporter Construct

The Emut1 construct was created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutagenesis PCR reaction was performed using the 935G6PDH-Luc construct as a template and the following HPLC-purified, single-stranded oligonucleotide primers purchased from Invitrogen (Carlsbad, CA). A purine was exchanged for a purine, a pyrimidine for a pyrimidine. Similar substitution strategy was followed in subsequent mutation generated constructs. The underlined bases indicate the mutated bases of the G6PDH E-box sequence.

Emut1F(-557/-514):
5'-GCCTCGGACTCAGAGATCTGTTTGCCTTTTTCCGTCTACTGGG-3'

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EmulR(-560/-513):
5'-CCAGTAGACGGAAAAAAGGCAAACAGATCTCTGAGTCCGAGGCTAG-3'

The 4EBpGL2-Luc Basic Reporter Construct

The 4EBpGL2-Luc construct was created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Four copies on the G6PDH promoter E-box sequence 5’-CACCTG-3’ were inserted into the pGL2-Luc Basic Vector (Promega, Madison, WI), directly upstream to the +33 Xho I restriction site. The insertion was performed by PCR using the pGL2-Luc Basic Vector as a template and the following HPLC-purified, single-stranded oligonucleotide primers purchased from Invitrogen (Carlsbad, CA). The underlined bases indicate the four E-boxes that were inserted.

4EBPGL2F(+13/+54):
5' - CGAGCTCTTTACGCGTGCTAGCACCTGCACCTGCACCTGCACCTGCTCGA
GATCTAAGTAA-3'

4EBPGL2R(+54/+13):
5' - CCAAGCTTACTTAGATCTCGAGCAGGTGCAGGTGCAGGTGCAGGTGCT
AGCACGCGTAAG-3'

The 4EB187G6PDH-Luc Reporter Construct

The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to produce the 4EB187G6PDH-Luc construct. Four copies on the G6PDH promoter E-box sequence 5’-CACCTG-3’ were inserted into the 187G6PDH-Luc

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construct, directly upstream of the 187 bp G6PDH promoter sequence. The insertion was performed by PCR using the 187G6PDH-Luc construct as a template and the following single-stranded, HPLC-purified oligonucleotide primers purchased from Invitrogen (Carlsbad, CA). The underlined bases indicate the four E-boxes that were inserted.

4EB187F(-5580/-114):

\[
\begin{align*}
5' & - \text{GTAACTGAGCTACATAACCCGACCTGCACCTGCACCTGCACCTGGGGG} \\
& \quad G6PDH \rightarrow \\
& \quad \text{CTC-3'}
\end{align*}
\]

4EB187R(-114/-5580):

\[
\begin{align*}
5' & - \text{GGGGGGGAGAAGCCCCAGGTGCAGGTGCAGGTGCAGGTGGGGTTATGT-3'}
\end{align*}
\]

The 935(-728m) G6PDH-Luc Reporter Construct

The 935(-728m)G6PDH-Luc construct was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The PCR mutagenesis reaction was performed using the 935G6PDH-Luc construct as a template and the following HPLC-purified, single-stranded oligonucleotide primers purchased from Invitrogen (Carlsbad, CA). The underlined bases indicate the four mutated bases at the -728 site of the G6PDH promoter sequence.

\[
\begin{align*}
935(-728m)F (-759/-710):
5' & - \text{GTGTGGGATCCCGGAAGTAAAACACAGACTCAAGAATATTAATACCTAGA} \\
& \quad \text{GAG-3'}
\end{align*}
\]
The 935(SP1m) G6PDH-Luc Reporter Construct

The QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to produce the 935(SP1m) G6PDH-Luc construct. This construct contains a four base mutation in a putative -558 SP1 site of the G6PDH promoter sequence. The mutagenesis reaction was carried out by PCR using the 935G6PDH-Luc construct as a template and the single-stranded, HPLC-purified, oligonucleotide primers shown below (Invitrogen Carlsbad, CA). The putative SP1 site is shown as the boxed region. The four mutated bases are underlined.

935(SP1m)F (-579/-536):
5'- GGGTCCTACGTAGACCAGA CTGATTTCG GACTCAGAGATCCACC-3'

935(SP1m)R (-536/-579):
5'- GGTGGATCTCTGAGTTCGAAATCAGTCTGGTCTACGTAGGACCC-3'

Plasmid Reporter Construct Sequence Confirmation

The DNA sequence of all the reporter constructs was confirmed using a Beckman Coulter CEQ2000XL DNA analysis system.
Sterol Regulatory Element Binding Protein (SREBP) Cotransfection Studies

Sterol Regulatory Element Binding Protein -1a (SREBP-1a) or SREBP-1c expression vectors and the 935 G6PDH promoter-luc plasmid construct were cotransfected into primary rat hepatocytes. 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) Synthase SRE plasmid construct (pSYNSRE) was also cotransfected with SREBP-1a or SREBP-1c as a positive control.

Sterol Regulatory Element Binding Protein -1a and -1c Expression Vectors

The Sterol Regulatory Element Binding Protein -1a and -1c (SREBP-1a and SREBP-1c) expression vectors were obtained from T. F. Osborne (University of California, Irvine). DNA encoding amino acids 1-490 of the active form of SREBP -1a were inserted into the pCMV5 expression vector (Magaña and Osborne, 1996). A second set of expression vectors was produced in which a cDNA fragment encoding amino acids 1-403 of the active form of SREBP-1c was inserted into both the pCMV5 and pCMV4 vectors (Guan, et al., 1997).

pSYNSRE Control Plasmid

The pSYNSRE plasmid was obtained from T. F. Osborne (University of California, Irvine CA). The DNA sequence corresponding to -324 to -225 of the hamster HMG-CoA synthase promoter was inserted into the pGL2 Basic Vector. This region contains three sterol regulatory elements (SRE) (Dooley, et al., 1998).
Nuclear Extract Preparation

Male Sprague-Dawley rats were obtained from Charles Rivers, Portage, MI. Rats weighing approximately 180-200 grams, maintained on Lab Diet 5001 standard rodent diet, were weighed and fasted for 48 hrs. Drinking water was provided *ad libitum*. The rats were weighed before and after fasting, refeeding and sacrifice to account for weight loss and weight gain. The amount of high carbohydrate food consumed was also measured. Whole livers were removed from rats either fasted for 48 hrs or fasted and refed High Carbohydrate Rat Diet for 24 hrs. The High Carbohydrate Rat Diet (68% sucrose, 18% casein purified high nitrogen, 8% cottonseed oil, 2% brewers yeast, 4% salt mix) was purchased from ICN Biomedicals. The tissue for nuclei isolation was homogenized in buffer I (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 7.4, 2 mM ethylenediaminetetraacetate (EDTA), 0.5 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetate (EGTA), 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol (BME)). The suspension was layered over an aliquot of buffer II (similar to buffer I, but containing 0.88 M sucrose) and centrifuged. The pellets were collected and re-homogenized in buffer III (similar to buffer I, but containing 2.0 M sucrose). The nuclei were purified through a layer of buffer III by centrifugation for 1 hr at 210,000 x g at 4 °C. The isolated nuclei were resuspended in 50% glycerol, 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 10mM dithiothreitol (DTT), and 0.25 mM phenylmethylsulfonyl fluoride (PMSF) and stored at -80°C (Stapleton, et al, 1991). Nuclear protein extracts were prepared using the NE-PER Nuclear and Cytoplasmic
Extraction Reagents kit by Pierce. Nuclear extraction reagent, supplemented with aprotinin (10ng/µl), leupeptin (10ng/µl) and PMSF (2mM), was added to an equal amount of nuclei and vortexed for 15 sec. The sample was placed on ice and vortexed for 15 sec. every 10 min. for a total of 40 min. The sample was centrifuged at 16,000 x g for 10 min and the supernatant containing the nuclear proteins was collected. The excess salt was removed from the nuclear extract by concentration in a Micon3 microconcentrator to half the original volume and rediluted in buffer (20 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 0.5 M DTT, 0.2 mM PMSF, 25% glycerol, 70.8% H₂O)(Dalton, et al, 1997). Nuclear extract total protein was measured by the Lowry method (Lowry, et al, 1951). These nuclear extracts were used for the Electrophoretic Mobility Shift Assays and the DNAse I Footprinting Assays.

Electrophoretic Mobility Shift Assays (EMSA)

Assays were performed with the Gel Shift Assay System (Promega, Madison, WI). Double-stranded oligonucleotides were prepared by mixing equal amounts of complementary HPLC purified, single-stranded DNAs (Invitrogen, Carlsbad, CA) in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), heating at the indicated temperature for the designated time and cooling to room temperature. The annealing temperature used was 3 °C below the melting temperature (Tₘ) of the oligonucleotides. The annealed oligonucleotides were labeled with ³²P in the presence of [γ-³²P] ATP (Amersham Biosciences, Piscataway, NJ) and T4 polynucleotide kinase. Nuclear extracts (15 µg protein) were incubated with ³²P-labeled
oligonucleotide and binding buffer at room temperature for 30 min. In the competition assays, nuclear extracts were preincubated with unlabeled competitor oligonucleotide for 10 min. before the addition of $^{32}$P-labeled oligonucleotide. In the supershift assays, the specific antibody was added last and the reaction was incubated for an additional 30 min. at room temperature. The samples were electrophoresed through a 4.5% polyacrylamide gel in 0.5 X Tris-Borate-EDTA (TBE) buffer (44.5 mM Tris-base, 55 mM boric acid, 1 mM EDTA, pH 8.3). The gel was then incubated with a phospho-imaging screen for 4 hrs. The exposed imaging screen was scanned on a STORM 860 imager (Molecular Dynamics, Sunnyvale, CA). The density of the DNA-protein bands was determined using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA). The oligonucleotides and antibodies used in the assays are discussed below.

**Ebox Oligonucleotide**

The 21 bp Ebox oligonucleotide corresponds to the E-box region (-546/-526) of the G6PDH promoter. The complementary single-stranded, HPLC-purified DNAs were annealed at 42 °C for 2 min. The Ebox oligonucleotide consisted of the following sequence:

$$5'$$-AGAGATC[CACCTG]CCTTTTTT-3'
$$3'$$-TCTTAG[GTGGA]GGAAAAAA-5'

The E-box sequence is indicated by the boxed bases.
Emut1 Oligonucleotide

The 21 bp Emut1 oligonucleotide corresponds to the E-box region (-546/-526) of the G6PDH promoter except that the first four bases of the E-box sequence are mutated. The complementary single-stranded DNAs (HPLC-purified) were annealed at 38 °C for 2 min. The Emut1 oligonucleotide consisted of the following sequence:

\[
\begin{align*}
5' &- AGAGATC \underline{TGTTG}CCTTTTTT-3' \\
3' &- TCTCTAG \underline{ACAAACGGAAAAA}-5'
\end{align*}
\]

The E-box sequence is indicated by the boxed region and the four mutated bases are underlined.

Oct1 Oligonucleotide

A commercial double-stranded oligonucleotide containing an Oct1 binding site (Promega Madison, WI) was used:

\[
\begin{align*}
5' &- TGTCGAATGCAATCAGTAGAA -3' \\
3' &- ACAGCTTACGT TTAGTGATCTT - 5'
\end{align*}
\]

2USF Oligonucleotide

Oligonucleotides were designed corresponding to the USF consensus sequence reported on the TFFACTOR database (AC R02259, ID USFSCONS). The complementary single-stranded DNAs (HPLC-purified) were annealed at 72 °C for 5 min. The oligonucleotide sequence is given below:

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Oligonucleotides were designed corresponding to the SRE-1 consensus sequence from Koo, et al (Koo, et al., 2001). The complementary single-stranded DNAs (HPLC-purified) were annealed at 60 °C for 5 min. The oligonucleotide sequence is shown below:

5'- TGATCACCCCACTGAGGAG -3'
3'- ACTAGTGGGGTGACTCCTC - 5'

Specific Antibodies

Two μg of USF-1 (C-20) or SREBP-1 (H-160) antibody (sc-229 and sc-8984 respectively, Santa Cruz Biotechnology, Santa Cruz, CA) were added in each USF or SREBP supershift reaction.

DNAse I Footprinting

Footprinting analysis was done with the Core Footprinting System (Promega, Madison, WI). Single-stranded DNA oligonucleotides, purified by agarose gel electrophoresis (PAGE) were obtained from Invitrogen (Carlsbad, CA) Double-stranded oligonucleotides were prepared by mixing equal amounts of complementary, single-stranded DNAs in TE buffer, heating at the indicated temperature for the designated time and cooling to room temperature. The annealing temperature used
was 3 °C below the melting temperature (Tm) of the oligonucleotides. Tm is defined as the temperature at which half of the oligonucleotide strands are in the double-helical state and half are in the ‘random-coil’ single-stranded state (SantaLucia, 1998). Therefore, at the annealing temperature used, less than 50% of the oligonucleotides will be in the double-stranded state. However, 5 to 10 °C below the Tm is the typical temperature used for primer annealing in PCR and therefore 3 °C below the Tm was used in this protocol to improve dimer formation. The annealed oligonucleotides were labeled with 32P in the presence of [γ-32P] ATP (Amersham Biosciences, Piscataway, NJ) and T4 polynucleotide kinase. Following labeling, the oligonucleotides were digested with the restriction enzyme Hind III at 37°C for 1 hr. The DNA was extracted with phenol:chloroform:isoamyl alcohol (25:25:1, equilibrated with TEB buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 15 mM BME) and 0.5M NaCl). The extracted DNA was precipitated with 100% ethanol, dried and resuspended in 100 µl of TE buffer.

Nuclear extracts (15 µg protein), 32P-labeled oligonucleotide (0.75 pmol) and 25 µl binding buffer were combined and incubated on ice for 10 min. Calcium/magnesium solution (5mM CaCl2/10mM MgCl2) was added (to a final concentration of 2.5mM CaCl2/5mM MgCl2) and the reaction was incubated at room temperature for 1 min. RNase-free DNase (0.15 units) was added to the reaction and the sample remained at room temperature for 45 min. The DNase reaction was terminated by the addition of 90 µl Stop Solution (200mM NaCl, 30mM EDTA, 1% sodium dodecyl sulfate (SDS), 100 µg/ml yeast RNA). Each
reaction sample was extracted with phenol:chloroform:isoamyl alcohol (25:25:1, equilibrated with TEB buffer and 0.5M NaCl). The extracted DNA was precipitated with 100% ethanol and incubated on ice for 20 min. The samples were centrifuged at 14,000 x g for 5 min. The supernatant was removed and washed with 70% ethanol and dried under a vacuum. The resulting pellet was suspended in 10 μl of Loading Solution (1:2 0.1 M NaOH:formamide (v/v), 0.1% xylene cyanol, 0.1% bromophenol blue) by flicking the tube. The samples were heated at 95°C for 2 min. and quick chilled on ice for 2 min. Samples (5 μl) were loaded onto a 6% polyacrylamide sequencing gel (6% acrylamide:bis (19:1), 7 M urea, 89 mM Tris-base, 110 mM boric acid, 2 mM EDTA, 0.05% TEMED (v/v), 0.5% ammonium persulfate). The gel was run in a Model S2 Sequencing Gel Apparatus (Gibco, Grand Island, NY) at 1000V, 65 watts in 1X TBE buffer until the bromophenol blue band was at ~ ¾ the length of the gel. The gel was then incubated with a phospho-imaging screen for 5 h. The exposed imaging screen was scanned on a STORM 860 imager (Molecular Dynamics, Sunnyvale, CA). The oligonucleotides used in the assays are discussed below.

SV40 Positive Control

A commercial double-stranded 324 bp DNA fragment of the SV40 early promoter/enhancer region and bacterial extract containing human recombinant transcription factor AP2 was provided as a positive control with the Core Footprinting System (Promega, Madison, WI).
95 bp E-box Oligonucleotide

The 95 bp E-box oligonucleotide corresponds to the E-box region (-581/-488) of the G6PDH promoter. The oligonucleotide was designed to include a Hind III restriction enzyme site at the 3’ end. The complementary single-stranded, HPLC-purified DNAs were annealed at 88 °C for 5 min and allowed to cool to room temperature. The sequences of the single-stranded DNAs used to produce the 95 bp E-box oligonucleotide are shown below:

```
5'-CAGGGTCCTACGTAGACCAGACTAGCCTCGGACTCAGAGATCCTGAGCTACCATGTCCAAGCTTT-3'  
3'-AGCGAAAAAGGCAGGACATGGTGAATCGACTGTTTAATCCAGTACGCGGAAAAAGGAAGGCTGACTGCTGCTGCTGCTGGAAGGCTAGTCTGGTCTACGTAGGACCCTG-3'
```

The E-box sequence is indicated by the solid boxed bases. The dashed boxed bases indicate the Hind III restriction site.

95 bp E-mut Oligonucleotide

The 95 bp E-mut oligonucleotide corresponds to the E-box region (-581/-488) of the G6PDH promoter. The oligonucleotide was designed to include a Hind III restriction enzyme site at the 3’ end. The complementary single-stranded, HPLC-purified DNAs were annealed at 88 °C for 5 min and allowed to cool to room temperature. The sequences of the single-stranded DNAs used to produce the 95 bp E-box oligonucleotide are shown below:
The E-box sequence is indicated by the solid boxed bases and the four mutated bases are underlined. The dashed boxed bases indicate the Hind III restriction site.

Protein Assay

Total protein concentration of the cell lysate and nuclear extract samples was determined by the method of Lowry, et al (Lowry et al., 1951) using BSA as a standard. Protein values for all samples were expressed in μg/μl.

Statistical Analysis

All results are expressed as the mean ± standard error of the mean of five to eleven separate experiments from individual rats. The differences between the treatment groups were evaluated by one-tailed Students' t-test or Analysis of Variance (ANOVA). Significance was tested at P<0.05.
Acknowledgments

The SREBP-1a and SREBP-1c expression vectors and the HMG-CoA
Synthase SRE control plasmid were kindly donated by Dr. Timothy Osborne,
Department of Molecular Biology and Biochemistry, University of California, Irvine.
RESULTS

The Glucose-6-Phosphate Dehydrogenase Promoter Sequence

The 935 bp rat glucose-6-phosphate dehydrogenase (G6PDH) promoter region was isolated and sequenced by Rank, et al (Rank, et al., 1994). It contains many putative cis-elements, identified through the use of four separate databases (MatInspector, Genomatix, 2003; Tfsitescan, Ghosh, 2000; TFBind, Tsunoda and Takagi, 1999; and Transplorer, Biobase, 2004)(Appendix B). These cis-elements include a single E-box, possible USF, SREBP and SP1 binding sites. Although it contains an E-box, it is uncertain if the promoter contains a defined GRE. The 935 bp promoter sequence and a few of the putative cis-elements are shown in Figure 5.

Glucose and Insulin Responsiveness of the 935 Glucose-6-Phosphate Dehydrogenase Promoter Construct

To investigate the responsiveness of the G6PDH promoter to glucose, primary rat hepatocytes were transiently transfected with a luciferase reporter construct containing 935 bp of the G6PDH promoter (Holmen, 1995) and treated with 5, 10, 20, or 30 mM glucose for 14 hrs. The cells were processed and luciferase activity measured. There was a significant increase in expression of luciferase in the presence of 30 mM glucose when compared to that seen in 5 mM glucose (Figure 6). Our lab had previously shown that insulin induces expression of the 935 bp G6PDH promoter
Figure 5. Rat G6PDH Promoter DNA Sequence

The transcription start site is denoted by a +1. Some putative cis-elements are identified. Sequence homology to the human gene is shown by the dashed underline and mouse homology is shown by the solid underline. The 635 bp reporter construct consists of the sequence −578 to +57. The 187 bp reporter construct consists of the sequence −130 to +57. Adapted from Rank, et al. (Rank, et al., 1994).

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Primary rat hepatocytes were transiently transfected with the 935 G6PDH reporter construct, treated with 5, 10, 20, or 30 mM glucose and incubated for 14 hrs. The cells were washed with PBS and the luciferase activity was measured. * denotes a significant difference from 5 mM glucose by Factorial ANOVA followed by the Student-Newman-Keuls Test. Data is expressed as the mean ± S.E.M. from six to ten rats.

To determine if the glucose and insulin responsiveness of the G6PDH promoter are mediated through identical or separate mechanisms, primary rat hepatocytes were transiently transfected with the same construct and treated with 5, 10, 20, or 30 mM glucose for 14 hrs, and if indicated, treated with 250 ng/ml of insulin. The results show a significant additive effect in cells treated with the combination of insulin and glucose over glucose alone (Figure 7). This suggests that glucose and insulin may induce expression of G6PDH through separate mechanisms.
Figure 7. Glucose and Insulin Responsiveness of the 935 G6PDH Construct

Primary rat hepatocytes were transiently transfected with the 935 G6PDH reporter construct, treated with 5, 10, 20, or 30 mM glucose and, if indicated, treated with 250 ng/ml of insulin and incubated for 14 hrs. The cells were washed with PBS and the luciferase activity was measured. * denotes a significant difference from 5 mM glucose; ♦ denotes a significant difference from 30 mM glucose at p < 0.05 (by Factorial ANOVA followed by the Student-Newman-Keuls Test). Data is expressed as the mean ± S.E.M. from six to ten rats.

Deletion Analysis of the Glucose-6-phosphate Dehydrogenase Promoter

To identify a potential glucose-responsive region of the G6PDH promoter, we transfected primary rat hepatocytes with a luciferase reporter construct containing either 635 (-578 to +57) or 187 (-130 to +57) bp regions of the promoter (Figure 8). After transfection, cells were treated with either 5 or 30 mM glucose for 14 hrs. The reporter construct containing 635 bp of the G6PDH promoter responded similarly to
the full 935 bp promoter construct when incubated with different concentrations of glucose (Figure 8). The expression level of the reporter construct containing 187 bp of the promoter did not change when the glucose concentration was altered, which suggests that sequences between the 635 and 187 portions (-578 to -130) of the G6PDH promoter are required for the response to glucose. Within this critical segment (-578 to -130) of the G6PDH promoter, a unique E-box motif is found (Figure 5). This motif contains a single imperfect E-box at -539, consisting of the sequence 5'-CACCTG-3'.

Figure 8. Glucose Responsiveness of the 635 and 187 G6PDH Promoter Constructs

Primary rat hepatocytes were transiently transfected with the 635 or 187 G6PDH reporter construct. After transfection, cells were treated with either 5 or 30 mM glucose. The cells were washed with PBS 12 to 14 hrs after treatment and the luciferase activity was measured. * denotes a significant increase from 5 mM glucose mean at p < 0.05 (by the Students’ t-test). Data is expressed as the mean ± S.E.M. from five (the 635 construct) and seven (the 187 construct) rats.
Glucose and Insulin Responsiveness of a -539 E-box Mutant Construct of the Glucose-6-phosphate Dehydrogenase Promoter

The preliminary promoter deletion results, together with the fact that the E-box has been shown to be important for the glucose response in other genes, prompted us to test the importance of the E-box to the glucose responsiveness of G6PDH expression. An E-box construct was made containing the first four bases of the E-box mutated (Emut1). A purine was exchanged for a purine and a pyrimidine for a pyrimidine (Figure 9). Primary rat hepatocytes were transiently transfected with the Emut1 G6PDH reporter construct and treated as previously described. There was no significant difference between any of the glucose or combined glucose and insulin treatments for the Emut1 construct (Figure 10). The lack of response of the Emut1 construct to either glucose or insulin was clearly seen when it was compared to the 935 construct (Figure 11).

![Diagram of E-box Region in G6PDH Promoter Construct](image)

Figure 9. The E-box Region in the G6PDH Promoter Construct

This diagram represents the 935 bp of the G6PDH promoter. The wild-type E-box and the mutated E-box sequences are shown.
Figure 10. Glucose and Insulin Responsiveness of the Emul G6PDH Construct

Primary rat hepatocytes were transiently transfected with the Emul G6PDH reporter construct, treated with 5, 10, 20, or 30 mM glucose for 14 hrs and, if indicated, treated with 250 ng/ml of insulin. The cells were processed and the luciferase activity was measured. There was no significant difference between any treatments at p < 0.05 (by Factorial ANOVA followed by the Student-Newman-Keuls Test). Data is expressed as the mean ± S.E.M. from nine rats.

Glucose and Insulin Responsiveness of the 935(-728m) Glucose-6-phosphate Dehydrogenase Construct

To ensure that the site-directed mutagenesis procedure itself did not cause the lack of glucose and insulin responses observed in the Emul construct, we produced another 935 G6PDH mutant construct. Four bases at the -728 position were mutated to produce the 935(-728m) construct. This area of the G6PDH promoter did not appear to contain any putative cis-elements that could be involved in the glucose or insulin responses. This 935(-728m) construct was transfected into primary rat hepatocytes and the cells were treated as previously stated. The mutation of this area
Figure 11. Comparison of the 935 and Emutl G6PDH Constructs

* denotes significant difference from the 935 mean for the same glucose concentration at p < 0.05 (by the Students’ t-Test). Data is expressed as the mean ± S.E.M. from nine rats.

of the G6PDH promoter had no effect on its response to glucose or insulin (Figure 12). Comparison of the responses of the wild-type 935 and 935(-728m) G6PDH constructs demonstrates that they are virtually identical (compare Figures 7 and 12). Therefore, it was not the mutagenesis procedure per se that caused the loss of glucose and insulin responsiveness of the Emutl construct.

Electrophoretic Mobility Shift Assays (EMSA) of the Glucose-6-phosphate Dehydrogenase Promoter E-box Region

To examine the potential effect of glucose and insulin on the binding of nuclear proteins to the E-box region of the promoter, we prepared nuclear extracts from whole livers of rats that were fasted for 48 hrs or fasted and refed a high carbohydrate
Figure 12. Glucose and Insulin Responsiveness of the 935(-728M) G6PDH Construct

Primary rat hepatocytes were transiently transfected with the 935(-728M) G6PDH reporter construct, treated with 5, 10, 20, or 30 mM glucose for 14 hrs and, if indicated, treated with 250 ng/ml of insulin. The cells were processed and the luciferase activity was measured. * denotes significant difference from 5 mM glucose according to T-test at p < 0.01, ♦ denotes significant difference from 30 mM glucose and ♦ denotes significant difference from 5 mM glucose + I according to Students’ t-test at p < 0.05 Data is expressed as the mean ± S.E.M. from nine to eleven separate rats.

diet for 24 hrs. A high carbohydrate diet increases the levels of both glucose and insulin in the liver. Protein extracts from whole livers were used as opposed to cells in culture to provide a larger pool of proteins for the assays. These nuclear extracts were used in electrophoretic mobility shift assays (EMSA) along with the following oligonucleotides: a 21 bp oligonucleotide (-546 to -526) that contains the E-box, an Emut1 oligonucleotide (-546 to -526) in which the first four bases of the E-box have been mutated, and a commercial Oct1 oligonucleotide. Hepatic nuclear extracts from rats fasted or fasted and refed a high carbohydrate diet were incubated with 32P-labeled

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E-box oligonucleotide. For the competition assay, unlabeled competitor oligonucleotide was added before the labeled oligonucleotide (Figure 13A). A graph of the $^{32}$P band densities from several mobility shift assays shows a significant increase in protein binding to the E-box oligonucleotide in response to high carbohydrate (Figure 13B). To assure specificity of the EMSA, competition assays were run (Figures 13A and 14). Unlabeled E-box oligonucleotide competes with itself, while an unlabeled Oct1 non-specific oligonucleotide and unlabeled Emut1 oligonucleotide do not compete with the E-box oligonucleotide. This demonstrates specificity of the proteins binding in response to high carbohydrate.

To determine if there is reduced nuclear protein binding to the mutated E-box region in response to high carbohydrate, EMSAs were performed using $^{32}$P labeled Emut1 oligonucleotide (Figure 15A). A graph of the $^{32}$P band densities from several Emut1 EMSAs show significant increased protein binding to the Emut1 oligonucleotide in response to high carbohydrate (Figure 15B). However, in the competition assays, unlabeled Emut1 oligonucleotide competes with itself and the unlabeled Oct1 oligonucleotide also competes, suggesting the effect is due to non-specific binding. Unlabeled E-box oligonucleotide does not compete with the Emut1 oligonucleotide (Figure 16). Therefore, it appears that an intact E-box sequence is necessary for specific protein binding.
Figure 13. Electrophoretic Mobility Shift Assay Using the E-box Oligonucleotide

Hepatic nuclear extracts (15 μg protein) were incubated with 32P-labeled Ebox oligonucleotide at room temperature for 30 mins. The samples were electrophoresed through a 4.5% polyacrylamide gel in 0.5 X TBE buffer and incubated with a phospho-imaging screen for 4 hrs. The exposed imaging screen was scanned on a STORM imager and a representative blot is shown in A. NP: No nuclear protein, Fast: Fasted nuclear extract; Refed Hi Carb: Refed high carbohydrate nuclear extract, 100 Fold Ebox, 100 Fold Oct1, 100 Fold Emut1: 100 fold molar excess of unlabeled Ebox, Oct-1, or Emut1 oligonucleotide was added. B. Graph of 2 to 6 replicates from 6 rats expressed as the mean ± S.E.M. * denotes a significant difference from the fasted extract at p < 0.001 by Students’ t-test.
Figure 14. Electrophoretic Mobility Shift E-box Oligonucleotide Competition Assay

Data in the graph is expressed as the mean ± S.E.M. (N = 13 replicates from 5 rats). * denotes a significant difference from the fasted extract by Students’ t-test (p=0.05)

DNase I Footprinting Analysis of the Glucose-6-phosphate Dehydrogenase E-box Region

After determining specific increased nuclear protein binding to the E-box region of the G6PDH promoter in response to high carbohydrate, the next step was to ascertain the specific DNA nucleotides to which the proteins were binding. Nuclear extracts were prepared from whole livers of rats that were fasted for 48 hrs or fasted and refed a high carbohydrate diet for 24 hrs. These nuclear extracts were used in DNAse footprint analyses along with the following oligonucleotides: a 95 bp oligonucleotide (-581 to -488) that contains the E-box and a 95 bp Emul1 oligonucleotide (-581 to -488) in which the first four bases of the E-box have been
Hepatic nuclear extracts (15 μg protein) were incubated with 32P-labeled Emul oligonucleotide at room temperature for 30 mins. The samples were electrophoresed through a 4.5% polyacrylamide gel in 0.5 X TBE buffer and incubated with a phosho-imaging screen for 4 hrs. The exposed imaging screen was scanned on a STORM imager and a representative blot is shown in A. NP: No nuclear protein, Fast: Fasted nuclear extract; Refed Hi Carb: Refed high carbohydrate nuclear extract; 100 Fold Emul, 100 Fold Oct1, 100 Fold Ebox: 100 fold molar excess of unlabeled Emul, Oct-1, or Ebox oligonucleotide was added. B. Graph of 17 replicates from 6 rats. * denotes a significant difference from the fasted extract at p < 0.05 by Students’ t-test.
Figure 16. Electrophoretic Mobility Shift Emut1 Oligonucleotide Competition Assay

Data in the graph is expressed as the mean ± S.E.M. (N = 12 replicates from 3 rats). * denotes a significant difference from the Refed Hi Carb extract by Students’ t-test (p=0.05).

mutated. Hepatic nuclear extracts from rats fasted or fasted and refed a high carbohydrate diet were incubated with $^{32}$P-labeled oligonucleotide. A 324 bp DNA fragment of the SV40 early promoter/enhancer region, combined with AP2 extract, was used as a positive control. The samples were treated with DNAse, electrophoresed and the gel was incubated with a phospho-imaging screen. A representative image is shown in Figure 17.

The DNA bands on the footprint analysis are not as distinct as they could be, therefore only an estimate of possible footprints can be determined. Three possible footprints are shown in the numbered brackets (Figure 17). Footprint 1 is near the 3'
Hepatic nuclear extracts (15 μg protein) were incubated with $^{32}$P-labeled SV40, 95 bp Ebox or Emut oligonucleotide. DNase (0.15 units) was added to the sample and the reaction was maintained at room temperature for 45 min. The samples were electrophoresed through a 6% polyacrylamide sequencing gel in 1X TBE buffer and incubated with a phospho-imaging screen for 5 hrs. The exposed imaging screen was scanned and a representative image is shown. L: DNA ladder, B: Blank, SV40: SV40 positive control oligonucleotide, Ebox: Ebox oligonucleotide, Emut: Emut oligonucleotide, NP: No nuclear protein, AP2: AP2 extract, F: Fasted nuclear extract; R: Refed high carbohydrate nuclear extract. Numbered brackets indicate possible footprints.

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end of the oligonucleotide and shows increased binding to the E-box oligonucleotide in the refed extract. This region also appears to be bound by protein on the Emut oligonucleotide, although no difference between the fasted and refed extracts is seen. It is possible that footprint 1 may be an API binding site. A putative API binding site is located near the 3' end of the Ebox oligonucleotide (Figure 18) and we have shown increased non-specific protein binding to this site is response to high carbohydrate (data not shown). The identity of footprints 2 and 3 are unknown. One would like to speculate that footprint 2 is the Ebox, which seems to show greater protein binding to the E-box oligonucleotide in the refed extract when compared to the fasted extract. This would correlate with the binding pattern found in the E-box EMSA studies. There also appears to be protein binding Emut oligonucleotide in this same region. Non-specific binding was shown with the Emut EMSA studies. I theorize that footprint 3 is an SP1 binding site. We have seen SP1 binding activity in our nuclear extracts when using an SP1 oligonucleotide (data not shown). If one assumes that footprint 1 is an API binding site, this would be located at 13 bps from the 3' end of the oligonucleotide, 27 bp from the E-box and consist of an 11 bp binding region. If

![DNA sequence diagram](image)

Figure 18. Putative Transcription Factor Binding Sites on the 95 bp E-box Oligonucleotide

NF1: nuclear factor 1; H4TF2: histone H4 transcription factor 2

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footprint 2 is indeed the E-box, it would be located 52 bp from the 3' end or near the middle of the oligonucleotide and consist of a 6 bp binding site. Provided footprint 3 is indeed an SP1 binding site, it would be located 12 bps from the E-box and consist of a 9 bp binding site. Therefore, footprint 1, the putative AP1 binding site, would be larger than the other two footprints and footprint 2, the possible E-box, would be the smallest of the footprints. This correlates with the possible footprints observed on the analysis. However, this is purely hypothetical for there is no concrete evidence to support this conjecture.

Electrophoretic Mobility Supershift Assays of the Glucose-6-phosphate Dehydrogenase Promoter E-box Region

There is evidence to suggest that USF proteins may be involved in the glucose induction of some metabolic genes. USF proteins have been shown to bind the GRE of glucose-responsive genes, however the binding of these proteins to the GRE of the PK gene is not modified by nutritional conditions. Therefore, USF proteins may interact with other proteins bound near the GRE to form a glucose response complex, but are not likely the main protein responsible for glucose induction (Kahn, 1997). However, the diminished glucose response of PK and S14 expression observed in USF2 knockout mice (Vallet, et al., 1997) and the repressed glucose response of PK in hepatoma cells treated with dominant negative forms of USF for several days (Lefrancois-Martinez, et al., 1995) indicate that USF may play an indirect role in the process (Kaytor, et al., 1997; Koo and Towle, 2000). USF can bind to E-box sequences (Sawadogo and Roeder, 1985) and given that the E-box is necessary for the
glucose and insulin response of the G6PDH promoter, we investigated whether USF is involved in the binding of proteins to the E-box. Supershift EMSAs were performed using $^{32}$P labeled E-box oligonucleotide and anti-USF-1 antibody (Figure 19). An oligonucleotide was synthesized (purchased) containing two consensus USF binding

![Electrophoretic Supershift Assay of the E-box Oligonucleotide with Anti-USF Antibody](image)

<table>
<thead>
<tr>
<th>Ebox Oligo</th>
<th>2USF Oligo</th>
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<tr>
<td>Fasted</td>
<td>+</td>
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<tr>
<td>Refed Hi Carb</td>
<td>-  +  +</td>
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<tr>
<td>Anti-USF</td>
<td>-  -  +</td>
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Figure 19. Electrophoretic Supershift Assay of the E-box Oligonucleotide with Anti-USF Antibody

Hepatic nuclear extracts (15 µg protein) were incubated with $^{32}$P-labeled oligonucleotide at room temperature for 20 mins. After this time, anti-USF antibody was added and the reaction was incubated for an additional 30 mins at room temperature. The samples were electrophoresed and a representative blot is shown. Fast: Fasted nuclear extract; Refed Hi Carb: Refed high carbohydrate nuclear extract; Anti-USF: anti-USF antibody was added, B: blank.
sites, called “2USF oligonucleotide”, and used as a positive control. It can be seen from the EMSA that there is increased protein binding to the E-box oligonucleotide in response to high carbohydrate, as previously shown. However, when anti-USF-1 antibody is added, there is a substantial disruption of protein binding to the E-box. The anti-USF-1 antibody produces a supershift band when added to the 2USF oligonucleotide. This demonstrates that when the same nuclear extracts are used, the antibody is able to bind to the USF protein and cause a supershift band with the 2USF oligonucleotide, but reacts in such a way with the E-box oligonucleotide to disrupt the complex. A graph of the quantified $^{32}$P band densities from several anti-USF-1 E-box supershift EMSAs show a significant complex disruption when anti-USF-1 is added, without any observable supershift band (Figure 20). The protein binding disruption

![Graph of Electrophoretic Supershift Assays of E-box Oligonucleotide with Anti-USF Antibody](image)

**Figure 20.** Graph of Electrophoretic Supershift Assays of E-box Oligonucleotide with Anti-USF Antibody

Data in the graph is expressed as the mean ± S.E.M. (N = 8 replicates from 3 rats). * denotes a significant decrease from the Refed Hi Carb extract by Students’ $t$-test ($p=0.001$).
that we observed with the use of anti-USF-1 antibody has also been reported by other research groups. Tabuchi, et al observed a completely diminished protein-DNA band when exactly the same anti-USF-1 antibody (C-20) was added to a putative USF cis-element EMSA (Tabuchi, et al., 2002). In another study, DNA-protein complex formation was inhibited in an E-box EMSA when anti-USF antibodies directed to either the C-terminus (C-20, sc-229) or the mid-region (H86, sc-8983) of USF were added (Bidder, et al., 2002). The DNA-protein complex disruption observed in our studies indicates that USF-1 protein is involved in the binding of proteins to the E-box in response to high carbohydrate.

EMSA was used in an attempt to determine if SREBP is also involved in the promoter response to glucose and insulin. An SRE-1 consensus sequence oligonucleotide was used as a positive control. SREBP binding to this control oligonucleotide could not be detected in the nuclear extracts, even though several attempts were made (data not shown). Therefore, it remains unknown if SREBP is binding to the E-box region of G6PDH in response to insulin and glucose. It is not surprising that we had difficulty in detecting SREBP in rat liver nuclear extracts, given that other research groups have had the same problem due to the low abundance of SREBP (Briggs, et al., 1993; Koo, et al., 2001; Towle, 2003; Latasa, et al., 2003). The best method found thus far to study SREBP DNA binding is through overexpression of the protein. Thus, we attempted SREBP overexpression studies.
Sterol Regulatory Element Binding Protein (SREBP) Cotransfection Studies

Sterol Regulatory Element Binding Protein -la (SREBP-la) or SREBP-1c expression vectors and the 935 G6PDH promoter-luc plasmid construct were cotransfected into primary rat hepatocytes. 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) Synthase SRE plasmid construct (pSYNSRE), which contains three sterol regulatory elements (SRE), was also cotransfected with SREBP-la or SREBP-1c as a positive control.

Several different experimental conditions were performed in an attempt to achieve SREBP-la and -1c induction of the pSYNSRE (SRE) control plasmid. Initially, cotransfections were performed in 30 mM glucose Waymouth’s media, with 6 µg of plasmid DNA (Figure 21A), which did not result in significant SREBP-la or -1c induction of the control plasmid. The 935 G6PDH construct did have a high insulin response under these conditions, but it was not statistically significant. The amount of plasmid DNA was reduced to 3 µg in the subsequent cotransfections, which did produce larger SREBP-la and -1c inductions of the SRE control plasmid. However, reducing the amount of plasmid DNA resulted in no insulin induction and an even greater SREBP-la and -1c inhibition of G6PDH 935 construct than with 6 µg of plasmid DNA. Since it was possible that the high glucose was inducing the plasmid, masking the effects of SREBP-la and -1c, together with the fact that Towle’s group had shown that the SREBP-1c vector driven by the CMV4 promoter (SREBP-1c/CMV4) was more effective than the SREBP-1c/CMV5 plasmid (Towle, 2000),
The 935 G6PDH construct was cotransfected with SREBP-1a or SREBP-1c expression vector into primary rat hepatocytes. 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA)Synthase SRE plasmid construct (SRE) was also cotransfected with SREBP-1a or SREBP-1c as a positive control. A. Transfection was performed in 30 mM glucose Waymouth’s MB 752/1 media using 3 and 6 μg of plasmid DNA. The SREBP-1c/CMV5 expression vector was used. After 6 hrs of transfection time, the cells were maintained in 30 mM glucose media and, if indicated, were treated with 250 ng/ml of insulin. They were processed 14 hrs later and the luciferase activity was measured. N = 3 (6 μg) and 1 (3 μg). B. Transfection was performed in 5 mM glucose DMEM media. The SREBP-1c/CMV4 expression vector was used. After 6 hrs of transfection time, the cells were maintained in 5 mM glucose media and, if indicated, were dosed with 250 ng/ml of insulin. They were processed 14 hrs later and the luciferase activity was measured. N = 2.
cotransfections were performed in 5 mM glucose media with 3 μg of the SREBP-1c/CMV4 expression vector (Figure 2IB). Lowering the glucose concentration and using the SREBP-1c/CMV4 vector had little effect on the response of the SRE control construct. However, there was an insulin induction and no SREBP-1a or -1c repression of 935 G6PDH construct under these circumstances. Perhaps the SRE construct requires high glucose along with insulin or SREBP for induction (compare the left graphs of Figure 2A and B). The insulin induction of the SRE construct was much lower in low glucose than in high glucose.

Unfortunately, the conditions which produced a significant induction of the SRE control plasmid did not coincide with the conditions necessary to induce the 935 G6PDH construct, therefore we can not draw any conclusions from this data. Additionally, because we were not successful in detecting SREBP binding in the EMSA, we can not conclude whether or not SREBP is involved in the induction of the G6PDH promoter.

Glucose and Insulin Responsiveness of the pGL2 and 4EBpGL2 Constructs

The basic pGL2 plasmid was used to produce all of the G6PDH constructs used in these studies and was previously shown to be glucose and insulin non-responsive. We repeated these previous experiments to verify that it is indeed non-responsive. Primary rat hepatocytes were transiently transfected with the pGL2 plasmid and treated as indicated. The cells were processed and luciferase activity was measured. There is no significant difference between any of the glucose or combined
glucose and insulin treatments for the pGL2 plasmid (Figure 22), confirming earlier work.

Since the E-box was demonstrated to be necessary for both the insulin and glucose responses in the G6PDH promoter construct, we wanted to know if insertion of E-box sequences into the pGL2 plasmid would generate insulin and glucose responsiveness in the plasmid. Four copies on the G6PDH promoter E-box sequence 5'-CACCTG-3' were inserted into the pGL2 Basic Vector to form the construct 4EBpGL2. This construct was transiently transfected into primary rat hepatocytes and

![Figure 22](image)

**Figure 22. Glucose and Insulin Responsiveness of the pGL2 Basic Plasmid**

Primary rat hepatocytes were transiently transfected with the pGL2 Basic reporter plasmid, treated with 5, 10, 20, or 30 mM glucose for 14 hrs and, if indicated, treated with 250 ng/ml of insulin. The cells were processed and the luciferase activity was measured. There was no significant difference between any treatments at p < 0.05 by Factorial ANOVA. Data is expressed as the mean ± S.E.M. from nine rats.

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cells were treated as indicated. As can be seen in Figure 23, there was no significant difference between any of the treatments for the 4EBpGL2 plasmid. This suggests that the E-box alone is not sufficient to instill glucose and insulin responsiveness.

Figure 23. Glucose and Insulin Responsiveness of the 4EBpGL2 Basic Plasmid

Primary rat hepatocytes were transiently transfected with the 4EBpGL2 plasmid construct, treated with 5, 10, 20, or 30 mM glucose for 14 hrs and, when indicated, treated with 250 ng/ml of insulin. The cells were processed and the luciferase activity was measured. There was no significant difference between any treatments at p < 0.05 by Factorial ANOVA. Data is expressed as the mean ± S.E.M. from eight to ten rats.

Glucose and Insulin Responsiveness of the 4EB187 Glucose-6-phosphate Dehydrogenase Construct

To test whether the insertion of E-box sequences into the 187 G6PDH construct would beget insulin and glucose responsiveness in this construct, four copies of the promoter E-box were inserted into the 187 construct to produce the 4EB187 G6PDH construct. This 4EB187 construct was transiently transfected into primary rat
hepatocytes and the cells were treated as indicated. The glucose and insulin responsiveness of the 187 and 4EB187 constructs are compared in Figure 24. The 187 G6PDH construct does not respond to glucose or insulin, however the addition of four E-boxes into the construct, 4EB187, instills a significant 30 mM glucose + insulin response. Interestingly, this significant response is only observed with the high 30 mM glucose concentration. The high glucose alone is equal between both constructs. Because the high glucose alone is not significantly different, the 30 mM glucose + insulin response is likely due to insulin. However, due to the fact that the 4EB187 5 mM glucose + insulin response is not significantly different between the two

Figure 24. Comparison of the 187 and 4EB187 G6PDH Constructs

Primary rat hepatocytes were transiently transfected with the 4EB187 G6PDH construct, treated with either 5 or 30 mM glucose for 14 hrs and, if indicated, treated with 250ng/ml of insulin. The cells were process and the luciferase activity was measured. I denotes a significant difference between 187 and 4EB187 30mM glucose + insulin means. 2 denotes a significant difference between 4EB187 30 mM glucose and 30mM glucose + insulin means by Students’ t-test (p= 0.05). N = 6 to 10.
constructs, renders one to speculate if high glucose is, in some way, influencing the insulin response or if the 30 mM glucose + insulin response is really a true insulin response.

**Glucose and Insulin Responsiveness of the 935(SP1m) Glucose-6-phosphate Dehydrogenase Construct**

SP1 has been shown to be necessary for the glucose response of ACC (Daniel and Kim, 1996) and to cooperate with SREBP in FAS regulation (Dooley, et al., 1998). There are five putative SP1 sites in the G6PDH promoter. Three of these SP1 sites are within the region of the 187 G6PDH construct, which does not respond to glucose or insulin. This does not rule out the possible involvement of these SP1 sites in these responses, however, we concentrated on other areas of the promoter. The other two possible SP1 sites are at -820 to -815 and -560 to -552. The -560 to -552 site is very near to the E-box (-539 to -534), therefore we mutated four bases in this putative SP1 site to observe the effect it had on the 935 G6PDH glucose and insulin responses. This SP1 mutated construct, 935(SP1m), was transfected into primary rat hepatocytes and these cells were treated as previously stated. The mutation of the SP1 site had no effect on the 935 G6PDH glucose and insulin responses (Figure 25, compare to Figure 7). These results indicate that this site is likely not involved in these responses.
Figure 25. Glucose and Insulin Responsiveness of the 935(SP1m) G6PDH Construct
Primary rat hepatocytes were transiently transfected with the 935(SP1m) G6PDH reporter construct, treated with 5 or 30 mM glucose for 14 hrs and, if indicated, treated with 250 ng/ml of insulin. The cells were processed and the luciferase activity was measured. Data is expressed as the mean ± S.E.M. from six to seven separate rats. * denotes a significant difference from 5 mM glucose; ♦ denotes a significant difference from 30 mM glucose at p < 0.05 by Student’s t-Test.

Pyruvate and Insulin Responsiveness of the 935 Glucose-6-phosphate Dehydrogenase Promoter Construct

The mechanism by which transcription is activated by glucose is poorly understood. It is believed that glucose itself is not the signaling molecule, however there is conflicting evidence as to which glucose metabolite actually regulates the glucose-responsive transcription factor(s). It has been suggested that either glucose-6-phosphate (Prip-Buus, at al., 1995), a metabolite of pyruvate, such as phosphoenolpyruvate (PEP) (Kang, et al., 1996) or xylulose-5-phosphate (Doiron, et
al., 1996) could be the glucose-signaling molecule. Therefore, we tested whether the G6PDH promoter response to carbohydrate was specific for glucose. We chose pyruvate as an alternative carbon source because it is the end product of glycolysis and can be converted to acetyl-CoA to enter the citric acid cycle. Pyruvate is not utilized by the pentose phosphate pathway and therefore, is not converted to xylulose-5-phosphate. Consequently, the use of pyruvate could indicate if the signaling molecule is an intermediate of glycolysis or the pentose phosphate pathway. Primary rat hepatocytes were transfected with the 935 G6PDH construct, treated with either glucose or pyruvate for 14 hrs, and if specified, exposed to insulin. A comparison of the promoter response to glucose and pyruvate is shown in Figure 26. There was no significant difference between the promoter responses to glucose and pyruvate.

**Figure 26. Comparison of the Glucose and Pyruvate Responsiveness of the 935 G6PDH Construct**

Primary rat hepatocytes were transiently transfected with the 935 G6PDH reporter construct, treated with 5 or 30 mM glucose or pyruvate and, if indicated, treated with 250 ng/ml of insulin and incubated for 14 hrs. The cells were washed with PBS and the luciferase activity was measured. * denotes a significant difference from the 5 mM glucose or pyruvate mean; ♦ denotes a significant difference from the 30 mM glucose or pyruvate mean. ♦ denotes a significant difference from the 5 mM glucose + I mean. Statistical analysis was performed using Students’ t-test (p=0.05). N = 6 to 10.
significant difference between responses of the cells treated with 5 mM and 30 mM pyruvate. However, these pyruvate treated cells still maintain a significant insulin response. These results are in agreement with the hypotheses that glucose itself, or a metabolite of glucose, such as glucose-6-phosphate or xylose-5-phosphate may actually be the carbohydrate-signaling molecule. Additionally, the fact that the insulin response is still retained in the pyruvate treated cells also reinforces our hypothesis that glucose and insulin act through separate mechanisms.
DISCUSSION

Glucose is the major source of energy for all mammalian cells and is the sole energy source for the brain. Glucose must continuously be provided to the cells and tissues even during such diverse situations as following the ingestion of a high carbohydrate diet to periods of fasting. An intricate mechanism exists to maintain blood glucose within a fairly narrow range (Pilkis and Granner, 1992), so that high concentrations are not susceptible to oxidation and adequate levels are available to tissues. This is largely accomplished by the liver, through glucose metabolism as well as glucose synthesis (gluconeogenesis). Consequently, the liver was the tissue used in our studies so this discussion will focus on glucose metabolism in the liver.

During the fed state, blood glucose levels are high, which triggers the beta cells (β-cells) of the pancreas to release insulin. Concomitantly, glucose enters the hepatocyte through the GLUT2 transporters, which do not require insulin (Gould and Holman, 1993), however the glucose must be phosphorylated by glucokinase, an enzyme whose expression level is regulated by insulin (Weinhouse, 1976; Lynedjian, et al., 1988). The resulting glucose-6-phosphate can enter glycolysis or the pentose phosphate pathway, resulting in various metabolic intermediates, reducing equivalents, energy or energy stored as glycogen and fatty acids. The expression of the enzymes involved in these pathways is also stimulated by insulin. For example, pyruvate kinase (PK) in glycolysis, malic enzyme (ME) in the production of reducing equivalents for fatty acid production, acetyl-coA carboxylase (ACC) and fatty acid synthase (FAS) in...
fatty acid production, and glucose-6-phosphate dehydrogenase (G6PDH)(Figure 3) in the pentose phosphate pathway, which also provides reducing equivalents, are all positively regulated by insulin (Kletzien, et al., 1985; Imamura, et al., 1986; Mariash, et al., 1986; Vaulont, et al., 1986; Goodridge, 1987; Pape, et al., 1988; Noguchi and Tanaka, 1993).

The increase of insulin inhibits the expression of gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK)(Figure 3) and inhibits the activity of glycogenolytic enzymes, such as phosphorylase $\alpha$ (Alston, et al., 2003). These enzymes are involved in the breakdown of glycogen and the release of glucose into the blood. Therefore, insulin stimulates the phosphorylation, metabolism and storage of glucose and inhibits the production of glucose from glycogen, pyruvate or lactate (Pilkis and Granner, 1992).

Insulin inhibits glucagon secretion as well as glucagon gene transcription (Philippe, 1989). Insulin inhibition of glucagon gene transcription depends on the transcription factor Pax6, which binds to the G1 and G3 element within the glucagon promoter (Grzeskowiak, et al., 2000). A very recent study by Schinner, et al. demonstrated that the inhibition of glucagon gene transcription by insulin is mediated thorough the activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB or Akt) in a glucagon-producing pancreatic islet cell line. They also showed that the activation of Akt is sufficient to mimic the effect of insulin on the glucagon promoter and propose that the effect of insulin and Akt on the glucagon promoter requires the transcriptional coactivator CREB-binding protein (CBP) and the
transcription factor Pax6 (Schinner, et al., 2005). The insulin suppression of glucagon transcription can be altered in diabetes. Lefebvre described a hyperglucagonemia in diabetic patients that contributed to hyperglycemia (Lefebvre, 1995). The elevated glucagon levels are likely due to the loss of insulin-mediated inhibition of glucagon synthesis and secretion (Schinner, et al., 2005).

Normally, when insulin levels are high, the amount of the counter-regulatory hormone, glucagon, is low. Insulin, high glucose and free fatty acids suppress glucagon secretion and low glucose and amino acids (especially alanine) stimulate glucagon secretion (Unger, et al., 1978; Philippe, 1989). Glucagon is secreted by the alpha cells of the pancreas in response to low blood glucose levels. Glucagon has the opposite effects of insulin, stimulating the breakdown of glycogen and lipids (glycogenolysis and lipolysis) (Randall, et al., 1997). Glucagon binds to hepatic receptors linked to the adenosine 3',5'-cyclic monophosphate (cAMP) second-messenger pathway, therefore cAMP serves as the intracellular messenger for glucagon (Pilkis and Granner, 1992; Randall, et al., 1997). cAMP stimulates the expression of the gluconeogenesis enzymes, such as PEPCK (Lamers, et al., 1982), and inhibits the expression of the enzymes of glycolysis (GK, PK and others) (Hers and Hue, 1983; Vaulont, et al., 1984; Iynedjian, et al., 1987), the pentose phosphate pathway (G6PDH) (Garcia and Holten, 1975) and fatty acid synthesis (FAS and ACC) (Lakshmanan, et al., 1972; Volpe and Marasa, 1975; Towle, et al., 1997). At times, both insulin and glucagon levels are increased. A high protein, low carbohydrate diet results in a substantial rise in glucagon as well as a rise in insulin levels. This
prevents the hypoglycemia which would occur due to the protein-induced increase in insulin secretion by the counteraction with glucagon-induced gluconeogenesis (Unger, et al., 1978). The antagonistic actions of insulin and glucagon are vital for maintaining an appropriate blood glucose level, as well as proper metabolism of glucose in tissue.

Glucagon and insulin were believed to be the main dietary regulators of gene expression. It is now known that other hormones are involved as well. Leptin, a hormone mainly secreted by adipocytes, was discovered in 1994 as the product of the ob gene. In normal individuals, leptin communicates the body’s nutritional status to the hypothalamus, the center of appetite control, through leptin receptors (Tartaglia, et al., 1995). There are long and short forms of the leptin receptor. The long form is expressed in the various areas of the brain, including the hypothalamus. In other tissues, such as the liver, the short receptors are the main form expressed (Zhao, et al., 2000).

Leptin suppresses food intake (Zhang, et al, 1994) and increases physical activity, heat production (thermogenesis) (Campfield, et al., 1995) and metabolic rate (Levin, et al., 1996). Leptin also acts as a signal of nutritional deprivation, with low levels initiating a response to conserve energy (Cohen and Friedman, 2004). Rodents which are defective in leptin synthesis, ob/ob mice, or leptin receptor function, db/db mice, are obese and develop hyperinsulinemia and insulin resistance similar to metabolic abnormalities related to type 2 diabetes (Zhang, et al., 1994; Tartaglia, et al., 1995). Conversely, leptin levels are usually increased in diabetes, possibly due to the frequent presence of obesity (Fischer, et al., 2002; Faraj, et al., 2004).
Leptin synthesis and secretion is regulated by nutritional signals, such as fat (Havel, et al., 1999), fasting (Weigle, et al., 1997), feeding and insulin. Insulin stimulates expression, synthesis and secretion of leptin (Saladin, et al., 1995; Bradley and Cheatham, 1999), an effect which seems to depend on insulin-stimulated glucose uptake and utilization (Mueller, et al., 1998; Faraj, et al., 2004). A high carbohydrate, low fat diet increases circulating leptin levels over 24 hrs in women compared to a low carbohydrate, high fat diet (Havel, et al., 1999). Refeeding, weight gain and obesity increase circulating leptin levels while short-term fasting, energy restriction, weight loss and dietary fat decrease plasma leptin concentration (Havel, et al., 1996; Weigle, et al., 1997; Havel, 2004).

The effects of leptin are not only mediated through the hypothalamic appetite center. Important physiological actions of the hormone act directly on target tissues as well (Zhao, et al., 2000). Cohen et al. demonstrated that leptin may lower insulin activity in isolated hepatocytes (Cohen, et al., 1996). Leptin causes a depletion of lipid from the liver and other peripheral tissues by stimulating β-oxidation of fatty acids. The leptin-induced increase in fatty acid oxidation may be mediated by its activation of AMP-activated protein kinase (AMPK) (Minokoshi, et al., 2002) or by its suppression of stearoyl-CoA desaturase-1 (SCD-1). Suppression of SCD-1 increases fatty acid levels, which inhibits ACC activity, resulting in decreased malonyl CoA levels. Low malonyl CoA levels signal carnitine acyltransferase to transport fatty acids into the mitochondria for oxidation (Cohen and Friedman, 2004; Mathews and van Holde, 1990). Interestingly, a high carbohydrate, low fat diet increases plasma leptin levels.
and also increases FAS and ACC in the liver, although leptin has been show to suppress FAS transcription (Fukuda and Iritani, 1999) and ACC mRNA levels (Iritani, et al., 2000). Therefore, hepatic gene regulation must be achieved by a balance between the induction by glucose and insulin and the suppression by leptin and glucagon. Rat hepatocytes cultured in William’s E media (11 mM glucose) and treated with physiological concentrations of leptin (1 - 5 nM), induced signal proteins of the insulin signaling pathway, such as insulin receptor substrate 1 and 2 (IRS-1 and -2), PI3K and phophodiesterase 3B (PDE3B). PDE3B reduces cellular cAMP levels. The leptin activation of PI3K appears to occur through Janus kinase (JAK). Leptin activation of PI3K is not sufficient to activate Akt. Since both insulin and leptin activate PI3K, there may be cross-talk between the insulin and leptin signal pathways. (Zhao, et al., 2000).

Leptin also exerts effects on the pancreas as well. Under physiological conditions, leptin significantly decreases the release of insulin from the β-cells through at least two known actions – activation of ATP-dependent K⁺ (K_{ATP}) channels and reduction of protein phosphatase 1 (PP1). Briefly, insulin secretion from the β-cells partially depends on the activity of K_{ATP} channels. Closure (inactivation) of the K_{ATP} channels in response to glucose depolarizes the cell, which activates Ca^{2+} channels, raises the cytosolic calcium concentration and results in an increase in insulin secretion (Keiffer, et al., 1997; Ashcroft, 2000). Insulin secretion is further increased by hormone-mediated rise of second messengers cAMP/protein kinase A (PKA) and phospholipase C/protein kinase C (PKC). Leptin causes the opening (activation) of the

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K<sub>ATP</sub> channels, which reduces the cytosolic calcium concentration and inhibits insulin secretion (Havel, 2004; Seufert, 2004). Thus, leptin acts directly on the K<sub>ATP</sub> channels. The molecular mechanism by which leptin activates these channels is not fully understood, but a phosphorylation/dephosphorylation mechanism appears to be involved (Harvey and Ashford, 1998). The inhibitory effects of leptin on insulin secretion appear to be mediated through PI3K activation of PDE3B, and a resultant decrease of intracellular cAMP (Havel, 2004; Seufert, 2004).

The second mechanism by which leptin reduces insulin secretion is through its regulation of the PP1 gene. PP1 is a serine-threonine phosphatase in the insulin-signaling cascade in the liver. Upon leptin stimulation, the leptin receptor activates the receptor-associated JAK2 kinase by transphosphorylation, which phosphorylates the leptin receptor. Transcription factors of the signal transducers and activators of transcription (STAT) family, STAT3 and STAT5, are recruited to the leptin receptor, where they are tyrosine-phosphorylated by JAK2. The phosphorylated STATs dimerize and translocate to the nucleus, where they regulate gene transcription, such as the inhibition of the expression of the catalytic subunit of PP1. The reduced PP1 activity lowers intracellular calcium concentration and inhibits insulin secretion. Leptin reduction of PP1 mRNA and protein leads to dramatic inhibition of both glucose and glucagon-induced insulin secretion (Seufert, 2004).

Leptin not only inhibits insulin secretion, it also represses insulin gene transcription through the JAK/STAT pathway, thereby suppressing insulin production (Seufert, et al., 1999). High glucose (25mM) may be necessary for the inhibitory
actions of leptin on the insulin promoter, illustrated by the finding that leptin inhibited a rat insulin 1 promoter reporter plasmid in INS-1 cells at stimulatory concentration of 25 mM glucose, but not at 5 mM glucose (Seufert, 2004).

In summary, the effects of leptin in the pancreatic β-cells occurs through at least three mechanisms; suppression of insulin production, inhibition of insulin secretion through suppressed PP1 mRNA and activity and inhibition of insulin secretion via direct interaction with the K\textsubscript{ATP} channels.

Leptin inhibits insulin production and secretion, thereby inhibiting insulin's lipogenic effects. In turn, insulin stimulates leptin secretion from adipose tissues, producing a feedback loop between leptin and insulin called the “adipo-insular axis”.

In most overweight individuals, the regulation of leptin is disturbed, resulting in a state of “leptin resistance”, which disrupts the adipo-insular axis, causing hypersecretion of insulin (hyperinsulinemia) and the development of insulin resistance. This vicious cycle of hyperinsulinemia and stimulating hyperleptinemia results in insulin resistance and leptin-resistance and eventually manifests in type 2 diabetes. It is important to point out that leptin suppression of insulin secretion does not appear to interfere with the short-term stimulatory actions of glucose-dependent insulin secretion, however, leptin still interferes with insulin and PP1 regulation (Seufert, 2004).

Moreno-Aliaga, et al., through the use of glucose metabolic inhibitors, showed that a leptin promoter reporter gene was induced by insulin-stimulated glucose metabolism and not insulin \textit{per se} in 3T3-L1 adipocytes (Moreno-Aliaga, et al., 2001). The induction by glucose metabolism raised the possibility that the leptin gene may be
regulated by glucose through a putative GRE. The leptin promoter contains two possible E-boxes, separated only by two bps. However, mutation of either E-box did not affect promoter activity in rat adipocytes (Mason, et al., 1998). The spacing between the E-boxes in the leptin promoter is not the usual motif shown to confer glucose-responsiveness. The E-boxes in most GREs, such as in the rat hepatic PK and S14 genes, are spaced 5 bp apart and a 4-bp separation is not glucose-responsive (Shih, et al., 1995). Also, a single E-box is not sufficient for glucose responsiveness of the leptin promoter, as we have demonstrated in the G6PDH promoter. In fact, the E-boxes appear to not be involved in the glucose-induced activation of leptin, because mutation of either E-box had no effect on promoter activity. The glucose/insulin stimulation of the leptin promoter is suppressed by PUFA, leptin or cotransfection of an SP1 expression vector in rat hepatocytes and adipocytes (Fukuda and Iritani, 1999).

Insulin, glucagon, and leptin are not the only hormones regulated by nutritional status. Thyroid hormone triiodothyronine (T3) is also nutritionally regulated. T3 levels are decreased during starvation and increased in the fed state in rats. Thyroidectomy of fed rats decreases the activities of ME, FAS, PK and G6PDH. T3 replacement therapy increases the activity of these enzymes (Hillgartner, et al., 1995). T3 can also stimulate transcription, as illustrated by the T3-induced increase in FAS transcription in chick hepatocytes (Stapleton, et al., 1990). Lastly, the thyroid state also indirectly influences circulating leptin levels, though the regulation of fat mass (Syed, et al., 1999).
For many years, the effects of dietary carbohydrate were attributed to insulin (Foretz, et al., 1999). But now it is known that glucose has direct effects on gene expression as well. Glucose positively regulates PK, S14, ACC, and FAS (Koo, et al., 2001; O’Callaghan, et al., 2001; Rufo, et al., 2001). Our studies have shown that glucose also regulates G6PDH in primary rat hepatocytes.

Primary rat hepatocyte cultures were used for all of our in vitro studies since genes, such as GLUT2, PK (and possible others), are not expressed or regulated in hepatoma cell lines (Meienhofer, et al, 1987; Rencurel and Girard, 1998). In primary rat hepatocytes, we demonstrated that glucose transcriptionally regulates G6PDH. A reporter construct containing 935 bp of the G6PDH promoter responds to both glucose and insulin in a concentration dependent manner.

Previous studies on glucose-induced G6PDH gene expression have shown that it may be under transcriptional and posttranscriptional control. Glucose has been implicated in the increase in G6PDH transcription and mRNA stability in the liver of fasted and refeed rats (Prostko, et al., 1989). Hodge and Salati showed that in liver tissue of mice starved and refeed a high carbohydrate diet, the regulation of G6PDH expression was mediated by a nuclear posttranscriptional mechanism (Hodge and Salati, 1997). A recent study by the same group clarified this nutritional posttranslational regulation, showing that it was due to changes in the efficiency of splicing of new G6PDH transcript. They further state that the nutritional regulation of G6PDH synthesis involves steps exclusively at a posttranscriptional level (Salati, et al., 2004). Our results are in contrast with those of Salati, et al., for we demonstrate that
glucose regulation of G6PDH does occur at the transcriptional level in primary rat hepatocytes. We have also observed a glucose-induced increase in G6PDH activity, which may be due to an increase in mRNA. The difference may be that the mRNA is increased more than the two to three fold increase that we see in transcription, however, measured changes in activity of G6PDH parallel those seen transcriptionally. Our study did not examine glucose-induced G6PDH mRNA levels; therefore we do not know if G6PDH mRNA is actually increased by glucose at a posttranscriptional level in addition to the effects we see transcriptionally. Those studies are currently underway. However, our lab has previously shown that a combined high glucose (30 mM) and insulin treatment increased G6PDH transcription and mRNA levels (Wagle, et al., 1998). We also can not rule out that G6PDH may be regulated by posttranslational modification. The discrepancy observed between the two studies may be explained by differential regulation between species. Our studies, as well as those by Prostko, et al. (Prostko, et al., 1989), were performed in rat liver while the studies by Salati, et al., were done in mice. The Salati group has also studied G6PDH regulation in primary rat hepatocytes in which they found that the transcriptional activity of G6PDH did not change in nuclei from hepatocytes treated with glucose or insulin (Stabile, et al., 1998). These hepatocytes were isolated and maintained for at least 24 hrs in media containing 27 mM glucose before G6PDH transcription was measured. It is plausible that a change in transcription was not detected because the rate was already increased due to the high glucose in the media from the time of cell culture, although it does not explain why they did not see an insulin effect. In our
studies, the hepatocytes were maintained in 5 mM glucose for 18 hrs before treatment. This glucose concentration is the normal physiological glucose level. Following 18 hrs, the culture media was changed to glucose concentrations of 5 to 30 mM, which is equivalent to a range of normal to the hyperglycemic glucose levels observed in diabetes. Using this treatment regime, we did observe increased transcription of the G6PDH reporter gene. Therefore, the disparity between the two primary rat hepatocyte studies may be due to the difference in cell culture conditions.

The additive responsiveness of the 935 G9PDH construct to glucose and insulin suggests that they act through separate mechanisms. Interestingly, Stabile, et al. found that arachidonic acid, a polyunsaturated fatty acid shown to inhibit G6PDH expression, had no effect on the increased G6PDH mRNA in hepatocytes treated with 27 mM glucose alone, but did decrease G6PDH mRNA in cells treated with 27 mM glucose and insulin (Stabile, et al., 1998). This suggests that either arachidonic acid inhibits insulin signaling at a step downstream from that which regulates glucose utilization or that insulin and glucose operate through separate pathways. The theory of separate induction processes for glucose and insulin in hepatocytes has also been proposed for S14 and PK in a study by Koo, et al. They cotransfected an SRE containing reporter construct and an SREBP-1c expression vector into primary hepatocytes which were incubated with either low (5.5 mM) or high (27.5 mM) glucose and showed that there were no significant difference in luciferase activity between the glucose concentrations. This suggested that the transactivation capability of SREBP was not affected by glucose. They also demonstrated that reporter
constructs containing carbohydrate-responsive elements (ChoRE) were significantly induced by glucose, but an SRE containing construct was not. In addition, overexpression of SREBP in these same constructs only caused an induction of the SRE construct, but did not significantly induce the carbohydrate-responsive constructs. Electrophoretic mobility shift assays (EMSA) showed that SREBP and ChoRF have different binding specificities. Lastly, a mutation of the S14 ChoRE resulted in significant reduction of the glucose response, but not the insulin response. Mutation of the SRE dramatically reduced the response to insulin and a combined ChoRE/SRE mutant possessed no glucose or insulin responsiveness. Consequently, SREBP and ChoRF were established as the factors mediating the insulin and glucose responses, respectively (Koo, et al., 2001). It would be interesting to investigate if these transcription factors also mediate the insulin and glucose responses of G6PDH. The glucose and insulin responsive region of G6PDH promoter was shown to be between -578 and -130. Within this critical segment, there is a putative SRE at position -324 and a single imperfect E-box consisting of the sequence 5'-CACCTG-3'. This E-box is necessary for both the insulin and glucose responsiveness of G6PDH, confirmed by the fact that mutation of this E-box abolishes both responses in cultured hepatocytes. Using similar approaches as Koo, et al., one would speculate that SREBP probably mediates G6PDH insulin responsiveness through the putative SRE and/or the E-box. However, it is unlikely that ChoRF mediates the glucose responsiveness of G6PDH. ChoRF binding and function requires two E-box half-sites similar to CACG, separated by 7 to 9 bases. ChoRF does not bind directly to a single CACGTG motif.
(Koo and Towle, 2000). The G6PDH promoter contains a single imperfect E-box, therefore probably would not bind ChoRF.

If ChoRF is found to be the same protein as ChREBP, which also requires two E-box motifs, then it is even more unlikely that it is involved in the glucose regulation of G6PDH. As discussed earlier, X-5-P is required for nuclear translocation and DNA binding of ChREBP, and G6PDH is the rate limiting enzyme in the pentose phosphate pathway, leading to the production of X-5-P. Therefore, it is doubtful that a product of this pathway would control G6PDH.

We found that the E-box alone is not sufficient to elicit a response to glucose or insulin. The insertion of four E-box sequences into the basic pGL2 plasmid (4EBpGL2) did not instill glucose or insulin responsiveness in the plasmid. However, the insertion of four E-boxes into the non-responsive 187 G6PDH (4EB187) construct did impart significant insulin responsiveness at 30 mM glucose. This insulin responsiveness was not observed at 5 mM glucose. Why the insulin response was not observed at the lower glucose concentration is not clear. Intriguingly, the arrangement of four tandem E-boxes results in two sets of E-boxes, separated by 6 bp, as shown in Figure 27. These double E-boxes may act as a GRE, similar to that found in PK and S14. In these two genes, two E-boxes separated by 6 bp confers partial glucose induction. Both S14 and PK also require insulin, thus the insertion of four E-boxes into the 187 construct could act as a S14 or PK-like GRE and thus require both insulin and glucose to illicit a response. This may explain why the insulin response was only observed at the high glucose concentration in the 4EB187 construct.
Two possible GREs consisting of two E-boxes separated by six bases may be formed from four tandem E-boxes. The two possible GREs are shown as set 1 and set 2.

The fact that the 4EBpGL2 construct does not respond to glucose or insulin, but the 4EB187 construct does respond provides further evidence that additional sequences within the G6PDH promoter are necessary for the glucose and insulin responses. The 187 promoter sequence contains putative cis-elements, which may interact with the inserted E-boxes to confer insulin responsiveness. Within this 187 bp sequence, there are three possible SP1 elements and a potential E-box “half-site” (5'-CACGCC-3') at position -38. SP-1 has been shown to cooperate with SREBP, which can bind to E-boxes. SREBP has been implicated in the insulin response of the S14 and PK genes. Thus, it is possible that the four E-boxes can bind SREBP and, with the cooperation of the three SP1 sites, instill insulin responsiveness to the 4EB187 construct.

This potential second E-box “half-site” at position -38 is the same sequence seen in the rat FAS promoter (Figure 4), however in that promoter, as in PK, S14 and ACC genes, it is only five bases away from the second E-box. The pair of E-boxes, with a five bp separation, has been shown to be necessary for the binding of ChREBP to the PK promoter (Kawaguchi, et al., 2001). E-box binding proteins, such as
ChREBP, must function as dimers and Max-like protein X (Mlx) was found to be a possible binding partner for ChREBP. The ability of ChREBP/Mlx to activate PK, S14 and ACC may depend on the formation of a heterotetramer (two ChREBP and two Mlx proteins) between the two five bp-spaced E-boxes (Stoeckman, et al., 2004). The potential second E-box in the G6PDH promoter is 500 bases away from the -539 E-box, which was shown to be critical to the glucose and insulin responses. It is not known if the element at -38 is a functional E-box, nevertheless, even if it is, the G6PDH GRE would remain unlike any other reported to date. It would be interesting to mutate the -38 E-box “half-site” within the 935 G6PDH construct to see if it has a role in the G6PDH glucose and insulin responses.

In our studies, we also found specific, increased liver nuclear protein binding to an E-box oligonucleotide in response to a high carbohydrate. Only non-specific binding could be detected in the mutated E-box oligonucleotide. One of the proteins bound to the wild-type E-box oligonucleotide was identified by supershift assay as USF. USF is a necessary component of this protein complex, because anti-USF antibody disrupts the DNA binding. It is not known if USF is bound to the E-box in the fasted state as well. As discussed earlier, previous studies by other groups suggest that USF is probably not the glucose or insulin responsive factor; therefore it is likely that USF would also be found in the fasted extract. USF may be bound to the E-box in both conditions; therefore it is probably the combination of proteins in the complex that changes between the fasted and fed states. In fact, using transgenic mice containing a reporter gene driven by various 5' deletions of the FAS promoter, Latasa,
et al. established that USF was bound to two separate E-boxes (at -332 and -65) both in the fasted and refed state. USF bound to the -65 E-box was required for the induced binding of SREBP to an SRE in the refed state (Latasa, et al., 2003). Other studies showed that USF2 knockout mice have a diminished PK and S14 glucose responsiveness (Vallet, et al., 1997) and Lefrancois-Martinez, et al. found that the glucose induction of PK was repressed in hepatoma cells treated with dominant negative forms of USF for several days (Lefrancois-Martinez, et al., 1995). Together, these studies suggest that USF is likely an important member of a protein complex involved in glucose induced gene expression, but is not the key factor.

The -539 E-box was shown to be necessary, but not sufficient for the G6PDH glucose and insulin responsiveness, therefore other elements must be involved. These additional elements are likely different for glucose and insulin signaling. These additional factors may or may not lie with the -578 and -130 region. Potential accessory promoter elements include five putative SP1 sites (-820, -560, -126, -118 and -87), and one possible SREBP site at -324. In an attempt to identify if one of these elements could bind an accessory factor, we began with SP1 because it was demonstrated to be necessary for the glucose responses of ACC in preadipocytes (Daniel and Kim, 1996). Moreover, nuclear extracts from glucose-treated preadipoctyes exhibit increased SP1 binding activity (Daniel, et al, 1996). SP1 was also found to be necessary for the glucose/insulin activation of FAS and the glucose induction of the insulin receptor gene in primary hepatocytes (Magaña, et al., 2000; Fukuda, et al., 2001).
We chose to mutate the putative SP1 site closest to the E-box because accessory or cofactor binding sites that interact with SREBP are usually found within the neighboring 15 bp of the SRE or E-box (Wang, et al., 1993; Shimano, 2001). A -560 SP1 mutant construct was made and tested for its response to glucose and insulin. The mutation of this putative SP1 site had no effect. This does not rule out SP1 as a potential accessory factor, because there are other SP1 sites that could be involved in the glucose and insulin responses. But if SP1 is involved, it is not likely through the putative -560 SP1 site.

The role of SP1 in the glucose activation of gene expression remains controversial. SP1 has been shown to both positively and negatively effect glucose mediated gene expression. SP1 expression vectors suppressed the transcription of FAS and ATP citrate-lyase (ACL, a lipogenic gene) reporter genes when cotransfected into primary hepatocytes. In fact, SP1 binding to both promoters was also found to be decreased in rats fed a high-carbohydrate diet (Fukuda, et al, 1999). The glucose/insulin transcriptional activities of FAS and leptin were also suppressed in rat hepatocytes and adipocytes cotransfected with SP1 (Fukuda and Iritani, 1999). Therefore, although SP1 appears to be involved in the glucose activation of ACC, it seems to suppress glucose/insulin activation of FAS, although both enzymes are involved in fatty acid synthesis. It may be the other factors which interact with SP1 that actually confer the promoter responsiveness. Conversely, a study by Magaña, et al. found that SP1 was necessary for the glucose/insulin activation of FAS in primary hepatocytes. Unfortunately, the effect of glucose alone was not examined (Magaña, et
al., 2000). Consequently, it is unknown if SPI is involved in the insulin or glucose effects on FAS. It is conceivable that excess SPI, as observed in cotransfection studies, could have a negative regulatory effect on gene expression, which may explain the contradiction between the two studies. A recent investigation by the Sul laboratory, using transgenic mice containing reporter genes with various deletions of the FAS promoter, did not detect significant changes in the binding of SPI between the fasted and fed states. Therefore, they concluded that SPI may not be involved in the glucose regulation of FAS \textit{in vivo} (Latasa, et al, 2003). However, SPI could still be involved in glucose regulation although its DNA binding does not change with nutritional conditions. This seems to be the case with USF, which is found in both the fasted and fed states, but is a necessary component of the protein complex bound to DNA in the glucose regulation of FAS (Latasa, et al, 2003) and probably G6PDH. The situation may be the same for SPI.

We not only examined the possible involvement of SPI through mutation of a putative binding site, we also sought to discover if SREBP induced G6PDH transcription. Supershift EMSA assays and cotransfection studies were unable to determine if G6PDH is a target of SREBP. Due to the low abundance, SREBP is difficult to detect in nuclear extracts, as reported by other research groups (Briggs, et al., 1993; Fortez, et al., 1999; Koo, et al., 2001; Towle, 2003; Latasa, et al., 2003). Briggs, et al. stated that within the numerous proteins that bind to the SRE, they had difficulty in identifying a DNA binding protein that exhibited binding specifically to the SRE and correlated with SRE-dependent transcription (Briggs, et al., 1993). A study
by Koo, et al. acknowledged that their attempts to detect SREBP binding using nuclear extracts from liver were unsuccessful due to low abundance of the protein, therefore they prepared nuclear extracts from COS cells transfected with a vector overexpressing SREBP (Koo, et al., 2001). Lastly, Latasa, et al. declared that the binding of endogenous SREBP to any SREBP-regulated gene has never been shown by EMSA or chromatin immunoprecipitation (ChIP) studies and that binding of the protein to specific sites has been detected mainly by using \textit{in vitro}-transcribed and -translated SREBP (Latasa, et al., 2003).

Our attempt to overexpress SREBP was unsuccessful as well. A different delivery system, such as adenoviral infection, may have proven more productive than lipid-mediated transfection. This has been used successfully to overexpress SREBP in primary rat hepatocytes. Nevertheless, there are still inherent problems with overexpression studies for such experiments do not necessarily reflect the conditions normally found in the cell. The abnormally high levels of a protein, such as SREBP, can have other unforeseen effects. In addition, these studies are performed \textit{in vitro}, which may not mirror the environment \textit{in vivo}.

We have shown that glucose induces G6PDH expression, but is it glucose itself or a metabolite that provides the necessary stimulus? It is believed that glucose, \textit{per se}, is not the signaling molecule, however there is conflicting evidence as to which glucose metabolite actually regulates the glucose-induced transcription. It has been suggested that either glucose-6-phosphate (Prip-Buus, et al., 1995), a metabolite of pyruvate, such as phosphoenolpyruvate (PEP) (Kang, et al., 1996) or xylulose-5-
phosphate (Doiron, et al., 1996) could be the glucose-signaling molecule. Therefore, we tested whether the G6PDH promoter carbohydrate responsiveness was specific for glucose by using pyruvate. Pyruvate alone did not induce the G6PDH promoter, although there was a significant response with pyruvate plus insulin. The combined response is due to the insulin because the response is the same, irrespective of the pyruvate concentration. Our results are similar to those of Matsuda, et al. who showed that pyruvate, in approximately the same concentration range as used in our studies (5-20 mM), could not induce PK in primary rat hepatocytes (Matsuda, et al., 1990). The fact that pyruvate did not stimulate G6PDH expression is in agreement with the hypothesis that glucose itself, or a metabolite of glucose, such as glucose-6-phosphate (G-6-P) may be the carbohydrate-signaling molecule. Xylitol (5 mM) has been shown to induce the accumulation of PK mRNA in primary hepatocytes to the same level as 20 mM glucose in the absence of insulin by Doiron, et al. This concentration of xylitol did not significantly affect the intracellular concentration of glucose-6-phosphate. The response to xylitol, as well as glucose, requires a functional GRE. These researchers proposed that X-5-P mediates the glucose effects in these cells (Doiron, et al., 1996). Unfortunately, the intracellular concentration of X-5-P was not measured in this study. Experiments in adult rat hepatocyte cultures have questioned the view that X-5-P is the metabolite regulating glucose-induced transcription. The accumulation of FAS mRNA in response to increasing levels of xylitol (1-5 mM) was associated with levels of G-6-P, but not levels of X-5-P, phosphoenolpyruvate, 3-phosphoglycerate or fructose-6-phosphate. Thus, there is evidence that favors the idea that G-6-P is the
glucose intermediate that regulates gene transcription in response to glucose (Girard, et al., 1997). To my knowledge, xylitol has not been studied as to its effects on G6PDH induction, although I suspect that it would have little effect. Xylitol is not only metabolized to X-5-P, but also to lactate by way of glycolysis (Girard, et al., 1997). Lactate is converted to pyruvate, which we have already shown has no effect on G6PDH transcription. Consequently, our data supports the view that G-6-P is the glucose-signaling molecule. Glucose-6-phosphate is the substrate for G6PDH, which converts G-6-P to 6-phosphogluco-lactone and produces NADPH. NADPH is necessary for fatty acid synthesis and the enzymes involved in this synthesis, ACC and FAS, are also regulated by glucose (glucose metabolism). In addition, high G-6-P levels activate glycogen synthase and inactivate phosphorylase a (Alston, et al., 2004), thus promoting glycogen production and inhibiting glycogenolysis. It seems appropriate that these metabolic pathways should all be coordinately regulated and therefore, G-6-P is more likely to be the signaling molecule.

Collier and Scott speculate that one could possibly test if X-5-P is the signaling molecule by overexpression of G6PDH, which may be able to drive the glucose flux through the pentose phosphate pathway and generate X-5-P and perhaps other signaling metabolites. Since X-5-P alleviates ChREBP phosphorylation and inhibition, one should observe increased activity of this transcription factor. One might predict that increased ChREBP would be able to prevent streptozotocin-induced diabetes, diet-induced diabetes, or both (Collier and Scott, 2004). The fact that we show that G6PDH is itself regulated by glucose argues against the view that X-5-P is the
signaling molecule and ChREBP is the universal glucose-responsive transcription factor.

Taken together, our results parallel those from a recent study by Latasa, et al., who showed that in the liver, USF is bound to the -65 E-box in the FAS promoter in both fasted and refeed mice, while SREBP is bound to the -150 sterol response element (SRE) only in the refeed mice (Latasa, et al., 2003). We showed that in liver nuclear extracts from refeed rats, there was increased protein bound to an E-box oligonucleotide corresponding to the E-box region of the G6PDH promoter. Using supershift EMSA, we identified USF as one of the proteins bound to this E-box. Latasa, et al. demonstrated that mutation of either the SRE or the E-box abolishes the feeding-induced activation of the FAS promoter (Latasa, et al., 2003). Feeding increases both glucose and insulin levels. Similarly, we showed that mutation of the E-box in a reporter gene containing the G6PDH promoter completely abolished both the insulin and glucose responsiveness. They conclude that the E-box alone is not sufficient for the feeding and insulin activation of FAS, however it is required for the SREBP binding to the SRE and activating FAS (Latasa, et al., 2003). We also concluded that the G6PDH E-box alone is not sufficient for the insulin and glucose responses, because the insertion of four E-boxes into a non-responsive reporter construct, 4EBpGL2, did not confer insulin or glucose responsiveness. However, mutation of the E-box abolishes the G6PDH glucose and insulin responses, so the E-box is necessary, but not sufficient for these responses. Latasa, et al. mutated the SRE within the FAS promoter. We did not examine this region in the G6PDH promoter in
these studies, but it is very likely that we would find that the SRE is also necessary for the insulin responsiveness of G6PDH. The scenario with G6PDH could be very similar to that found with FAS. If one speculates that the SRE, as well as the E-box, is necessary for G6PDH insulin responsiveness, then it can be surmised that USF probably binds to the E-box, which is necessary for the binding of SREBP to the SRE to elicit the insulin response. Examination of the G6PDH SRE is a critical step toward unraveling the functions of both the SRE and the E-box.

The E-box, as well as an additional unknown *cis*-element(s), are necessary for G6PDH glucose responsiveness. Possible candidates for the additional glucose *cis*-element(s) or transcription factor(s) include nuclear factor Y (NFY), which is necessary for the carbohydrate activation of FAS in primary hepatocytes through its interaction with SREBP and SP1 (Magaña, et al., 2000). There are three putative NFY binding sites within the G6PDH promoter at -660, -591 and -221. The proximity to the E-box may be important, which would advocate the -591 NFY site.

Hepatic nuclear factor 4 (HNF4), previously called LF-A1, is another accessory factor that could be involved in the glucose regulation of G6PDH. It has been demonstrated to be necessary for the glucose response of PK in primary hepatocytes (Bergot, et al., 1992; Liu, et al, 1993) and is required for repression of PK transcription by cAMP (Vaulont and Kahn, 1994) and PUFA (Jump and Clarke, 1999). HNF4 has been shown to cooperatively bind with USF to activate the apolipoprotein A-II gene (Ribeiro, et al, 1999). The G6PDH promoter contains one
potential HNF4 binding site at -417 which is within the glucose-responsive region of the promoter.

SREBP is necessary for the glucose response of the PK and FAS genes (Foretz, et al., 1999) although no effects of glucose on SREBP expression in hepatocytes have been found (Koo, et al., 2001). It is possible that SREBP could be involved in the glucose responsiveness of G6PDH. As previously suggested, one could test its involvement in the glucose and insulin responsiveness of the gene by mutation of the SRE within the promoter.

Lastly, ChREBP is regulated by X-5-P and thus is an unlikely candidate for glucose-regulation of G6PDH. However, further studies are necessary to determine if it is involved in the regulation of G6PDH.

Our work is only the beginning of an intricate and interesting story. Although a great deal of progress has been made in recent years in understanding the regulation of metabolic genes, knowledge of the regulation of G6PDH is just beginning to approach the level of understanding of other metabolic genes, such as PK, S14, FAS and ACC. Further studies are crucial in order to decipher what other players are involved in the glucose and insulin regulation of this unique and important enzyme.
Appendix A

Putative *Cis*-elements in the Glucose-6-phosphate Dehydrogenase Promoter
<table>
<thead>
<tr>
<th>Cis-element</th>
<th>Sequence</th>
<th>Position</th>
<th>String</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax4 (MPAX4)</td>
<td>AGAAGTGAAGAGGGGCAGGAGC</td>
<td>-876 to -856</td>
<td>+</td>
</tr>
<tr>
<td>Pax4 (MPAX4)</td>
<td>ATCCTAGTCAGGGCGGTCACCC</td>
<td>-829 to -819</td>
<td>+</td>
</tr>
<tr>
<td>API</td>
<td>CAGGCGGTCACC</td>
<td>-821 to -810</td>
<td>-</td>
</tr>
<tr>
<td>SPI</td>
<td>AGGCGG</td>
<td>-820 to -815</td>
<td>-</td>
</tr>
<tr>
<td>NFkB</td>
<td>AGTGGAAAAC</td>
<td>-794 to -785</td>
<td>-</td>
</tr>
<tr>
<td>ELK1</td>
<td>CTTCCGGCTTCCCGGT</td>
<td>-780 to -767</td>
<td>-</td>
</tr>
<tr>
<td>ELK1</td>
<td>GTGGGATCCCGGAAG</td>
<td>-757 to -744</td>
<td>+</td>
</tr>
<tr>
<td>NFY (nuclear factor Y)</td>
<td>CTGCCGTTGGCCAG</td>
<td>-660 to -648</td>
<td>NA</td>
</tr>
<tr>
<td>ELK1</td>
<td>GTTGCCAGCGGA</td>
<td>-656 to -643</td>
<td>-</td>
</tr>
<tr>
<td>FOXD3 (forkhead box D3)</td>
<td>CACACAAATTTTT</td>
<td>-601 to -590</td>
<td>-</td>
</tr>
<tr>
<td>NFY</td>
<td>TTTTTTTGGACAG</td>
<td>-591 to -579</td>
<td>NA</td>
</tr>
<tr>
<td>NF1 (nuclear factor 1)</td>
<td>GGTC</td>
<td>-578 to -575</td>
<td>+</td>
</tr>
<tr>
<td>H4TF2 (histone H4 transcription factor 2)</td>
<td>TACGT</td>
<td>-573 to -568</td>
<td>+</td>
</tr>
<tr>
<td>SPI</td>
<td>CTAGCCTCG</td>
<td>-560 to -552</td>
<td>-</td>
</tr>
<tr>
<td>USF</td>
<td>CTCAGAGATCCACCTGCC</td>
<td>-549 to -532</td>
<td>+</td>
</tr>
<tr>
<td>Ebox</td>
<td>CACCTG</td>
<td>-539 to -534</td>
<td>+/-</td>
</tr>
<tr>
<td>ELK1</td>
<td>GCCTTTTTTCCCG</td>
<td>-534 to -523</td>
<td>-</td>
</tr>
<tr>
<td>Bicoid</td>
<td>CTACTGGG</td>
<td>-521 to -514</td>
<td>-</td>
</tr>
<tr>
<td>GCN4</td>
<td>AAAGCAGTGGA</td>
<td>-510 to -500</td>
<td>-</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Cis-element</th>
<th>Sequence</th>
<th>Position</th>
<th>String</th>
</tr>
</thead>
<tbody>
<tr>
<td>API</td>
<td>AGTGAGTCACC</td>
<td>-505 to -495</td>
<td>+/-</td>
</tr>
<tr>
<td>ELF1</td>
<td>CCTCACTAAAAACACCC</td>
<td>-453 to -438</td>
<td>-</td>
</tr>
<tr>
<td>HNF4 (hepatic nuclear factor 4)</td>
<td>CCCTACTAAGTTAAAAGTC</td>
<td>-417 to -399</td>
<td>+</td>
</tr>
<tr>
<td>SREBP</td>
<td>GGGGTCATCC</td>
<td>-324 to -314</td>
<td>-</td>
</tr>
<tr>
<td>NFY</td>
<td>CACCAATGCAAGC</td>
<td>-221 to -209</td>
<td>-</td>
</tr>
<tr>
<td>GATA-binding factor 1</td>
<td>CATTTATCTCTA</td>
<td>-142 to -131</td>
<td>+</td>
</tr>
<tr>
<td>SP1</td>
<td>CTCCCCCCCCCTC</td>
<td>-126 to -114</td>
<td>-</td>
</tr>
<tr>
<td>SP1</td>
<td>CCCTCCTCCCCCG</td>
<td>-118 to -106</td>
<td>-</td>
</tr>
<tr>
<td>SP1</td>
<td>AGCTCCTCCCCGCT</td>
<td>-86 to -74</td>
<td>-</td>
</tr>
<tr>
<td>API</td>
<td>GGTCAGCTCAGTCAA</td>
<td>-43 to -57</td>
<td>-</td>
</tr>
</tbody>
</table>
Appendix B

Protocol Clearance From the Institutional Animal Care and Use Committee
WESTERN MICHIGAN UNIVERSITY
Institutional Animal Care and Use Committee

ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

PROJECT OR COURSE TITLE: Actions Of Insulin And Metals On Gspdh Expression in Rat Hepatocytes
IACUC Protocol Number: 00-07-01
Date of Review Request: Date of Last Approval: 9/18/01
Purpose of project (select one): ☑ Teaching ☑ Research ☑ Other (specify)

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Susan Stapleton
Department: CHEM
Electronic Mail Address: susan.stapleton@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Title: Select one
Department: Electronic Mail Address:

1. The research, as approved by the IACUC, is completed:
   ☑ Yes (Continue with items 4-5 below.) ☑ No (Continue with items 2-5 below.)

   If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☑ Yes ☑ No
3. Have there been any new findings or publications relative to this research? ☑ Yes ☑ No

   Describe the sources used to determine the availability of new findings or publications:
   ☑ Animal Welfare Information Center (AWIC)
   ☑ Search of literature databases (select all applicable)
     AGRICOLA ☑ Biological Abstracts ☑ Current Research Information Service (CRIS)
     Medline ☑ Other (please specify):
     Date of search: 1/1/2001-12/31/2001 Years covered by the search: 1995-
     Key words: ☑ Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research? ☑ Yes ☑ No

   Cumulative number of mortalities:

5. Animal usage: Number of animals used during this quarter (3 months): 8
   Cumulative number of animals used to date: 40

   [Signature] [Signature] [Date]

IACUC REVIEW AND APPROVAL
Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

[Signature] [Date]

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Biobase (2004). Transplorer software [demo version].


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Towle, H.C. (2003). Personal communication.


