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REGULATION OF GLUT4 EXPRESSION *IN VIVO* AND *IN VITRO*

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1. ABSTRACT

The GLUT4 gene is subject to complex tissue-specific and metabolic regulation that has a profound impact on insulin-mediated glucose disposal. The regulation of this gene is of special clinical interest because insulin-mediated glucose homeostasis is highly sensitive to the levels of GLUT4 protein in muscle and adipose tissue. For this reason, the mechanisms of regulated expression of the GLUT4 gene have been intensively studied over the past decade. Understanding the transcriptional mechanisms that underlie the regulated expression of this highly differentiated gene have been slow to emerge, due to the paucity of suitable model systems available for detailed investigation. The development of transgenic mouse models to understand the mechanisms of transcriptional regulation has greatly enhanced our understanding of this gene. Information gained about the regulation of the GLUT4 gene has provided insight into mechanisms by which complex gene regulation occurs through a small number of cis-acting regulatory elements.

2. INTRODUCTION

The weight of evidence indicates that GLUT4 is the major insulin responsive glucose transporter mediating insulin-dependent glucose disposal. This proposal was originally based on the observation that GLUT4 is the predominant glucose transporter isoform in insulin-responsive tissues. Proof that GLUT4 is the primary effector molecule for insulin-mediated glucose disposal was obtained using transgenic animal models. Mice genetically engineered to overexpress an exogenous GLUT4 gene in the major GLUT4-expressing tissues, or in skeletal muscle or adipose tissue alone, displayed enhanced insulin responsiveness and enhanced peripheral glucose utilization (1-4). The high levels of transporters enhanced insulin responsiveness in genetic and experimental models of diabetes, reversing the diabetic phenotype. (5-7). Thus the expression of the GLUT4 gene is a clinically relevant molecule to target for treatment of insulin resistant disease states.

3. REGULATION OF GLUT4 EXPRESSION DURING INSULIN-DEFICIENCY

Expression of GLUT4 mRNA is subject to tissue-specific, hormonal and/or metabolic regulation. GLUT4 mRNA expression is largely restricted to brown and white adipose tissue, and skeletal and cardiac muscle tissues, although GLUT4 mRNA and protein have been detected in specialized cell types of other tissues. Changes in GLUT4 gene expression are observed in physiologic states of altered glucose homeostasis. In general, GLUT4 mRNA expression is reduced in states of relative insulin-deficiency such as STZ-induced diabetes and chronic fasting (for review see ref (8, 9)), and regulated steady state GLUT4 mRNA levels differ in GLUT4 expressing tissues. For example, changes in GLUT4 mRNA expression occur much more rapidly in adipose tissue than skeletal muscle (10). Chronic fasting markedly reduces GLUT4 mRNA levels in adipose tissue, but has little or no effect on GLUT4 mRNA in skeletal muscle (11). Changes in steady state levels of GLUT4 mRNA could potentially be the result of changes in either the rate of synthesis of GLUT4 mRNA (gene transcription) or changes in stability of messenger RNA. Nuclear run-on assays measuring the rate of GLUT4 mRNA transcription demonstrate that transcription is decreased in both adipose tissue and skeletal muscle in STZ-induced diabetic animals (12, 13), while the rate of the GLUT4 gene transcription in skeletal muscle of fasted animals is increased (13). Thus, changes in GLUT4 mRNA steady state levels reflect changes in the rate of mRNA synthesis.

The molecular basis for regulation of GLUT4 gene expression in states of relative insulin deficiency *in vivo* has been very difficult to resolve. *In vivo*, the insulin-

deficient state is complicated by the elevation of counter-regulatory hormones. In addition, insulin-deficiency is tightly coupled to plasma glucose levels and intracellular glucose utilization. To illustrate, STZ-diabetic animals are hyperglycemic and insulinopenic whereas fasted animals are hypoglycemic and insulinopenic. This might suggest that insulin rather than circulating glucose levels are responsible for the regulation of GLUT4 expression in adipose tissue. This hypothesis was supported in studies using phlorizin to increase urinary output of glucose in diabetic rats. In contrast to insulin, phlorizin-induced normalization of glycemia in these insulin deficient animals was not able to restore GLUT4 mRNA expression in adipose tissue to normal levels (14-16). In contrast, insulinopenia resulting from fasting is not associated with down regulation of GLUT4 mRNA in skeletal muscle, suggesting that insulin levels per se do not have a role in regulation of GLUT4 gene expression.

Confirmation that the GLUT4 gene is not directly regulated by insulin signaling has come from tissue-specific knockouts of the insulin receptor in transgenic mouse. Using these models, the expression of GLUT4 in the presence or absence of a functional insulin receptor can be measured directly. The insulin receptor has been knocked out in each the major GLUT4-expressing tissues individually, and in each case ablation of the insulin receptor has had either no effect (17, 18) or has increased GLUT4 expression (19). It is clear that down-regulation of the GLUT4 gene during insulin-deficiency is controlled by factors other than a lack of insulin receptor activation, and this may be related to factors present during a state insulin deficiency in every tissue in the body.

Chronically fasted and STZ-diabetic animals each represent states of insulin deficiency showing inhibition of peripheral glucose metabolism. The production of transgenic mice overexpressing the GLUT4 gene has provided a model of insulinopenia in which peripheral glucose utilization is enhanced. Overexpression of the human GLUT4 protein in these animals markedly enhanced glucose uptake and utilization in the fed state, resulting in hypoglycemia and hypoinsulinemia (2). The hypoinsulinemia in these animals is physiologically relevant in that it is sufficient to interfere with lipid metabolism, similar to that observed during insulin deficiency (2, 5). In spite of the state of relative hypoinsulinemia, expression of endogenous mouse GLUT4 mRNA was unaffected by the presence of the human GLUT4 protein (20). This transgenic mouse model represents a rare physiologic state in which hypoinsulinemia is accompanied by increased glucose utilization. In this case, GLUT4 gene expression is unaltered, suggesting that the predominant metabolic control of GLUT4 gene expression is linked to intracellular glucose metabolism. The divergent affect of the hypoinsulinemia due to fasting compared to STZ-diabetes in skeletal muscle may be linked to differences in energy metabolism occurring in muscle in these different states.

Although not as extensively studied, dietary manipulation has also been shown to affect GLUT4 mRNA expression. Rats fed a high fat diet (80% of total calories) display a marked down regulation of GLUT4 mRNA expression in both adipose tissue and skeletal muscle (21-23). Interpretation of these feeding studies is complicated by the fact that fed plasma insulin levels were reduced in these animals relative to rats fed either standard chow diets or high carbohydrate diets. Further confounding the interpretation is the fact that the carbohydrate content of the diet is reduced and intracellular glucose metabolism is likely occurring at a reduced level. Thus the high fat feeding study may mimic the overall metabolic state found in insulin-deficiency of Type II diabetes.

4. REGULATION OF GLUT4 EXPRESSION BY EXERCISE

In contrast to the down-regulation of GLUT4 observed in insulin-deficiency, GLUT4 expression is increased at the transcriptional level by endurance exercise (13). The increase in GLUT4 gene expression in response to exercise is rapid, occurring after only one session of aerobic exercise (24). The increase in GLUT4 protein induced by endurance exercise training is on the order of 1.5 to 2-fold, but this is enough to modify carbohydrate metabolism (24). After cessation of endurance training, GLUT4 expression reverts to normal levels in two to four days (25, 26). Activation of the AMP-activated protein kinase (AMPK) is known to occur in contracting muscle as a result of increases in AMP and decreases in phosphocreatine and ATP. For this reason, activation of AMPK has been hypothesized to mediate the exercise-induced changes in GLUT4 expression. In support of this, several laboratories have demonstrated that chronic administration of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an activator of AMPK, increases GLUT4 synthesis in rodents and L6 myotubes (27-30). While these experiments demonstrate that AICAR treatment can increase expression of GLUT4, they do not conclusively prove that AMPK activity is the mechanism by which exercise increases GLUT4 levels. Proof of this hypothesis will require a model system in which AMPK activity can be antagonized concomitantly with exercise training.

5. REGULATION OF GLUT4 EXPRESSION *IN VITRO*

In vitro models for GLUT4 gene regulation are limited by the small number of cultured cell types expressing the GLUT4 gene. Differentiated murine 3T3-L1 and F442A adipocytes express GLUT4 protein and mRNA at levels similar to that observed in primary isolated adipocytes or adipose tissue (31-33). GLUT4 mRNA is detected approximately 4 days after the onset of differentiation in these cell types (31). Unlike primary adipocytes or adipose tissue, the 3T3-L1 adipocytes also express high levels of the GLUT1 glucose transporter isoform (32). These cell lines have been used as *in vitro* models to study several aspects of glucose transporter regulation, including regulation of gene expression. Interestingly, chronic exposure of 3T3-L1 cells to insulin has differential effects on GLUT4 gene expression *in vivo*

and *in vitro*. Animals chronically treated with insulin show increased GLUT4 mRNA in adipose (34, 35) whereas chronic insulin treatment of 3T3-L1 adipocytes results in either no change or in a marked reduction in GLUT4 mRNA levels (36, 37). The differential response of GLUT4 mRNA to chronic insulin treatment *in vivo* and *in vitro* suggests that GLUT4 mRNA does not respond to insulin levels directly, al least in adipose tissue. In contrast, incubation of 3T3-L1 adipocytes in glucose-free medium reduces GLUT4 mRNA levels about 10-fold, accompanied by increased steady state levels of GLUT1 mRNA (38). Reintroduction of glucose to the starved adipocytes restored GLUT4 mRNA to pre-starvation levels. Supplementation of glucose-free media with either fructose or pyruvate as an alternative energy source maintained the steady-state level of GLUT4 mRNA. These data support a metabolic regulation of GLUT4 transcription as opposed to models based strictly on hormonal regulation.

Changes in insulin levels are accompanied by compensatory alterations in several counterregulatory hormones *in vivo*, particularly those that elevate cAMP levels in adipocytes (39). Thus, cAMP levels become elevated when circulating insulin levels are low. Elevated cAMP may be responsible for decreased GLUT4 transcription during insulin-deficiency. This was demonstrated *in vivo* by showing that an adenosine receptor agonist that prevented elevation of cAMP levels in adipose tissue of diabetic rats also prevent the decrease in GLUT4 transcription that accompanies insulin deficiency (12). This is consistent with the observation that cAMP inhibited GLUT4 transcription in 3T3-L1 adipocytes (40). Thus, these data suggest that modulation of GLUT4 gene expression in states of insulin deficiency may be due in part to changes in intracellular cAMP levels and not due to direct effects of insulin on GLUT4 gene transcription.

Thyroid hormone is another example of a hormone that regulates of GLUT4 gene expression in adipose tissue, heart and skeletal muscle. Fetal rodent muscle primarily expresses the GLUT1 gene while expressing GLUT4 at relatively low levels (41). During postnatal life, GLUT1 expression decreases with a concomitant increase in GLUT4 mRNA. Hypothyroidism in the perinatal period reduces GLUT4 mRNA steady state levels in heart and brown adipose tissue, while GLUT1 mRNA levels remain elevated (42). This condition is reversed by treatment of hypothyroid neonatal rats with injections of thyroid hormone. Similarly, adult hypothyroid rats show reduced GLUT4 mRNA levels in both skeletal muscle and heart (43, 44). The profound effects of thyroid hormone on GLUT4 mRNA levels suggest that this hormone plays an important role in the developmental pattern of tissue-specific GLUT4 gene expression. The mechanism by which thyroid hormone modulates GLUT4 mRNA expression is unknown. The transcription rate of the GLUT4 gene was significantly higher in hypothyroid rats treated with thyroid hormone compared to untreated hypothyroid control animals (45). Although an initial report suggested the rat GLUT4 promoter could be transactivated by co-expression of thyroid hormone receptor (46), these data were not reproducible (47). It is possible that thyroid hormone may indirectly regulate GLUT4 transcription through changes in intracellular metabolism, since many metabolic processes are profoundly influenced by this hormone.

6. TRANSCRIPTIONAL REGULATION OF THE GLUT4 GENE PROMOTER

6.1. Identification of promoter regulatory elements

To understand how the conditions described influence GLUT4 gene transcription, it is necessary to identify the molecular elements (cis-DNA sequences and trans-acting factors) responsible for regulation of the gene. Traditionally, promoter studies are carried out in model cell culture systems owing to their ease of transfection and experimental manipulation. The general strategy of these studies is to clone specific portions of a given promoter upstream of an easily measured reporter gene, followed by transfection into a suitable cell line. Levels of reporter mRNA or protein are then used as indirect measurements of the transcriptional activity of the promoter fragment under study. The value of tissue culture systems in transcription promoter analyses is undisputed, and a great deal of understanding has been achieved by employing these systems. However, additional information can be obtained by analyzing promoter activity in transgenic animals. In the case of GLUT4, we observe a complex pattern of gene expression in various physiologic states that are not possible to represent using tissue culture models. In addition, transcription analysis in transgenic mice allows us to measure effects of specific promoter elements in a variety of tissues under the influences of various hormonal, nutritional and metabolic factors.

To date, we have analyzed 12 different transgenic constructs for tissue-specific and hormonal-dependent GLUT4 gene regulation (Figure 1). The first established transgenic line was engineered to express a human GLUT4 mini-gene consisting of the entire coding region of the gene and 5.3 kb of 5' flanking DNA (20). Assay of the expression of this construct indicated that the human gene, expressed in a mouse background, was transcribed at levels identical to those of the mouse GLUT4 gene under a variety of conditions. Furthermore, the complex pattern of human GLUT4 transcription initiation site selection was observed in these transgenic mice. The feasibility of studying a promoter-reporter fusion construct was demonstrated by generating a second line of transgenic mice carrying a DNA construct in which the chloramphenicol acetyltransferase (CAT) gene was expressed from 2.4 kb of human GLUT4 DNA located immediately 5' of the transcription start site (48). The ability of the 2.4 kb fragment to promote transcription was measured by directly comparing CAT mRNA expression to mouse GLUT4 mRNA. These data demonstrated that 2.4 kb of the 5' region was sufficient to confer not only tissue specific expression, but also regulated expression of the GLUT4 gene in chronic fasted and STZ-induced diabetic mice (20, 48).

Since all of the apparent regulatory cis-DNA elements were accounted for within 2.4 kb of the

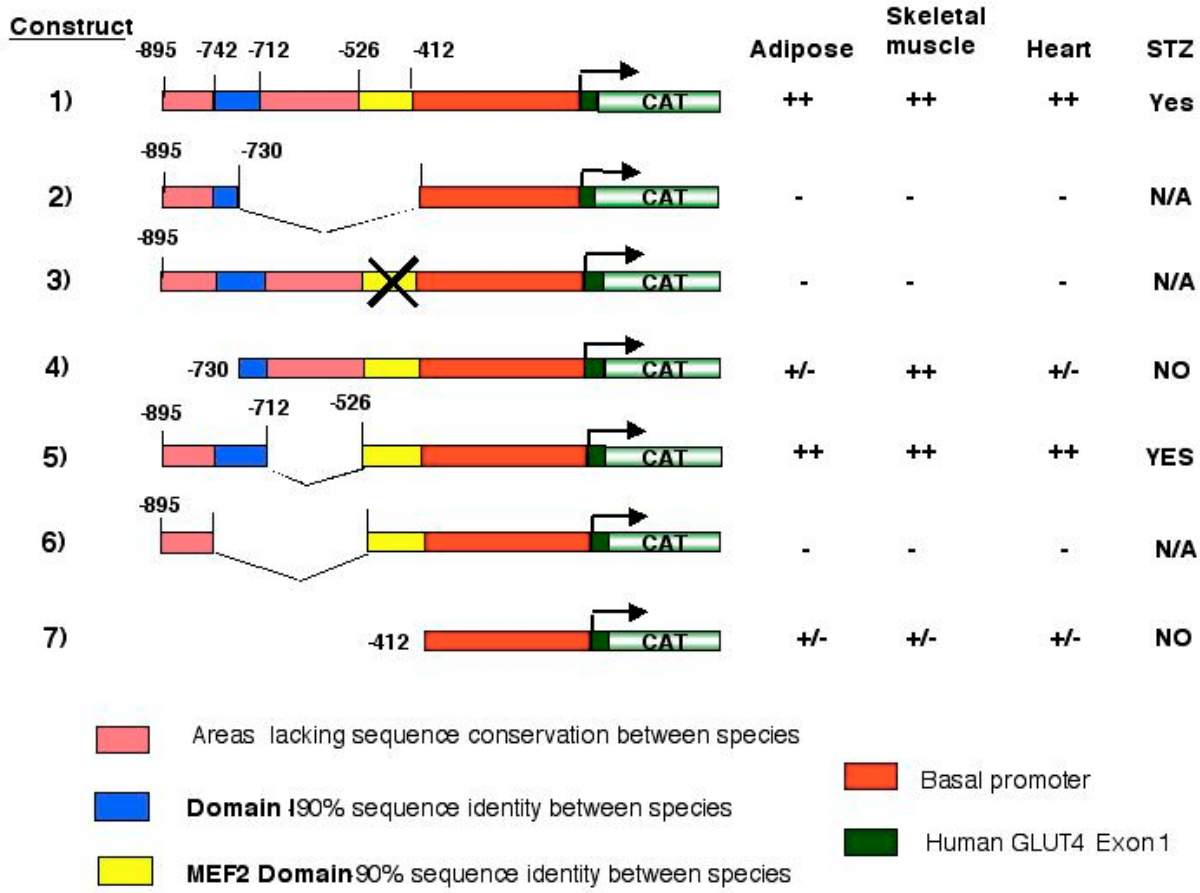

Figure 1. Summary of functional analysis of the human GLUT4 gene promoter in transgenic mice. A schematic depiction of transgenic constructs is depicted on the left. The colored bars indicate various structural domains of the GLUT4 gene. The bent arrow refers to the transcription initiation site. Expression of transgenic mRNA in adipose tissue (brown and white), hindquarter skeletal muscle, and heart are indicated to the right. A designation of $^{++}$ indicates full expression, - indicates no expression, and $^{+}$/- indicates a low level of expression relative to the endogenous GLUT4. STZ indicates whether the construct is regulated in STZ-induced diabetes.

transcription initiation site, we concentrated exclusively on the 5' flanking DNA of the human GLUT4 promoter in an effort to define the transcription regulatory elements. The initial approach was to precisely define the cis-acting sequences required to support a full program of gene expression. This was accomplished by generating transgenic constructs fusing CAT with portions of the 5' GLUT4 regulatory region. Using this approach, we have begun to define the structure of the GLUT4 gene promoter and its regulatory regions (Figure 1) with respect to both tissue-specific expression and regulation under conditions of experimental insulin-deficiency (i.e. streptozotocin-induced diabetes). This model system has proved to be immensely valuable in addressing the mechanisms responsible for the transcriptional regulation of the GLUT4 gene.

A series of deletions in the 5' end of the human GLUT4 promoter allowed us to map important promoter regulatory elements to the first 895 bp upstream of the major transcription initiation site (49). A comparison of sequences in this region of the human gene with the analogous regions of the mouse and rat genes revealed highly conserved regions containing greater than 90% sequence identity (50). The conserved region farthest from the transcription initiation site did not possess a binding site for any known transcription factors. We refer to this region as Domain I. The conserved region nearest the transcription initiation site contained a perfectly matched binding site for MEF2 transcription factors and we refer to this site as the MEF2 domain. The high sequence conservation in these regions suggested that they were important functional sites for regulation of Glut4 gene transcription. Removal of the unconserved sequences (less than 85%) between these domains had no effect on transcriptional activity (Figure 1, compare constructs 1 and 5). A 5' deletion removing both of these domains left a basal promoter that expressed at a very low level in all tissues, including those that do not normally express Glut4 (Figure 1, construct 7 and (51)). A construct deleting most of Domain I but leaving the MEF2 domain intact supported a high level of CAT expression in the skeletal muscle.

However, expression by this construct was aberrant with respect to the intact promoter (Figure 1, construct 4 and (51)). Transgenic expression was not restricted to the tissues normally expressing GLUT4, and expression in heart and adipose tissues was very low. Importantly, this construct (containing an intact MEF2 domain but not Domain I) was not able to support regulated expression of transgenic mRNA under conditions of STZ-deficiency in skeletal muscle or any other tissues. Thus, by our criteria, the MEF2 domain by itself does not constitute a fully functional promoter. Although the MEF2 domain was not sufficient to support full transgenic expression in heart and adipose tissue, deletion of this region completely ablated transgenic mRNA expression (Figure 1, construct 2). Thus the MEF2 domain is necessary, but not sufficient for full function of the Glut4 promoter (49). A point mutation in this MEF2 domain preventing MEF2 transcription factor binding had the same effect as deletion of the entire conserved region, demonstrating that the MEF2 binding site was the functional element within this region (Figure 1, compare construct 2 and 3) (49). The MEF2 domain functions cooperatively with Domain I to support regulated transcription of the human Glut4 promoter (Figure 1, construct 6 and (52)). Thus, an internal deletion of Domain I that leaves the MEF2 binding domain intact is not expressed in mice. Taken together, our data demonstrate that both Domain I and the MEF2 domain are necessary to provide full Glut4 promoter function.

6.2. MEF2 domain binding proteins

The MEF2 binding site has also been shown to be important for expression of the rat GLUT4 promoter in C2C12 myotubes (46, 47) and L6E9 myotubes (53). In these studies, the MEF2 domain of the rat promoter appears to be sufficient to support gene expression. Given the low level of GLUT4 expression in these tissues, the MEF2 binding domain may have been functioning at the low level observed in transgenic mice carrying the MEF2 binding site alone (Figure 1, construct 4). In mice, this construct was sufficient for detection of mRNA in the skeletal muscle, but was not subject to normal regulation in the experimental diabetic state. Thus the cultured cell model appears to be a minimal expression system that has limited utility for understanding the full complexity of GLUT4 regulation *in vivo.*

The MEF2 DNA binding site is known to bind isoforms of the MEF2 family of DNA binding proteins (54). Four MEF2 isoforms (MEF2A, MEF2B, MEF2C and MEF2D) are members of a multi-gene family of DNA binding proteins belonging to a larger family of MADS-box domain transcription factors. These transcription factors have been studied primarily within the context of myogenesis, however the expression of these proteins extends beyond muscle tissues. Using isoform specific antibodies in electrophoretic mobility shift assays, we showed that MEF2A and MEF2D isoforms in skeletal muscle, heart and adipose tissue bind the GLUT4 MEF2 binding domain (49). These studies established a requirement for MEF2 binding activity in gene expression in adipose tissue. This is not unexpected, since adipose tissue and skeletal muscle both arise from the embryonic mesoderm.

Regulated transcription of the GLUT4 gene may occur in part through binding of a MEF2 protein isoform to the MEF binding site. Two reports demonstrate independently that MEF2A binding activity is downregulated in heart and skeletal muscle nuclear extracts from streptozotocin-induced diabetic mice and rats (49, 55). In each of these reports, MEF2D binding was not changed in diabetes; therefore, it was hypothesized that either MEF2A alone or the MEF2A/MEF2D heterodimer are important for GLUT4 transcriptional regulation. In contrast to the down-regulation observed for MEF2A protein levels during insulin deficiency, MEF2A/MEF2D binding activity is increased in nuclear extracts from normal and genetically diabetic (ob/ob) mice chronically treated with AICAR (30, 56). Thus the MEF2 protein isoforms appear to play a central role in tissue-specific and metabolic regulation of the GLUT4 gene. While MEF2 proteins and their cognate DNA binding site clearly play a role, it must be reiterated that these proteins alone are not sufficient to impart regulated expression of the GLUT4 gene. Overexpression of MEF2A in transgenic mice has a limited effect on expression of GLUT4 mRNA and protein in these animals (57). Thus, either additional protein(s) are required to work in concert with MEF2A at the MEF2 binding domain (such as MEF2D), or additional transcriptional activators may work through other DNA regulatory elements. The second hypothesis is consistent with our observations of the human GLUT4 promoter showing that the MEF2 domain is necessary, but not sufficient to support a full program of GLUT4 transcriptional regulation (52). Supporting a role for factors other than MEF2 in regulated GLUT4 transcription, Santalucia *et al*, have shown that MEF2 functions cooperatively with MyoD and thyroid receptor-alpha to support GLUT4 expression in L6E9 cells (53). It is clear that there may be multiple mechanisms by which several types of transcription factors to work in conjunction with MEF2A to regulate GLUT4 transcription.

6.3. Domain I binding proteins

The DNA sequence of Domain I does not contain any known transcription factor binding sites (52). Thus, identification and characterization of Domain I binding proteins has been ongoing. Interestingly, analysis of the mouse GLUT4 promoter in 3T3-L1 adipocytes revealed that Domain I of the mouse gene is responsible for down-regulation of GLUT4 gene expression occurring following chronic insulin treatment (58). Although the effects of insulin *in vivo* and *in vitro* are dissimilar, it remains possible that the insulin responsive element defined in 3T3-L1 cells may also mediate effects of insulin-deficiency *in vivo.* In 3T3-L1 adipocytes, several proteins including NF1 and Olf-1/Early B Cell Factor have been shown to support down-regulation of the mouse GLUT4 promoter under chronic insulin treatment (59, 60). However, these factors have not been shown to be required for either tissue-specific or differentiation-specific regulation of the GLUT4 promoter *in vivo* or in 3T3-L1 cells. We have identified a novel zinc-finger transcription factor termed GEF (for GLUT4 enhancer factor) that may play a pivotal role in the physiologic function of Domain I (52). This protein was shown to bind Domain I in a sequence specific manner, and

an antibody raised against the GEF binding domain specifically reacts with nuclear proteins bound to Domain I DNA in electrophoretic mobility shift assays. Experiments are currently underway to evaluate the physiologic role of this novel transcription factor. Establishing functional Domain I binding proteins will pave the way for understanding the complex regulation of the GLUT4 gene. A thorough knowledge of the molecular basis for expression of the GLUT4 gene will be important for targeting the expression of this gene in a manner appropriate for treatment of insulin resistant glucose transport.

7. PERSPECTIVES

Through the use of transgenic mice, GLUT4 has been shown to play a key role in insulin-mediated glucose uptake. As a result of these studies, it has become evident that the GLUT4 protein is a potential therapeutic target for treating insulin resistance. The realization that GLUT4 expression is subject to both down-regulation and up-regulation at the level of gene transcription suggests that this process can be modulated by pharmaceutical interventions.

The use of transgenic animals to study the transcriptional mechanisms of this highly differentiated gene represents a major advance in our approach to understanding aspects of promoter function. The GLUT4 promoter does not appear to function through a series of non-cooperative linear regulatory elements, but rather through communication between two regulatory elements operating in conjunction with their cognate binding proteins. This interaction between two regulatory elements provides the framework for a model to explain both the tissue specific and metabolic regulation of the GLUT4 gene. We speculate that the restricted pattern of expression of the GLUT4 gene may be, in part, explained by the overlapping patterns of expression of MEF2 and GEF. This speculation is supported by our observations that GLUT4 is expressed at high levels only in tissues expressing both GEF and MEF2, specifically heart, skeletal muscle, and adipose tissue. Metabolic regulation of the GLUT4 gene may arise through regulated binding of MEF2 proteins and/or the Domain I binding proteins to the GLUT4 promoter. Evidence has been presented showing that MEF2A binding to the GLUT4 promoter changes in a manner consistent with the regulated expression of the GLUT4 gene in both insulin-deficiency and chronic AICAR treatment. Future work in understanding the Domain I binding proteins and how they work with MEF2A may be the key to understanding the regulation of GLUT4 expression.

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