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# Identification of a 30-Base Pair Regulatory Element and Novel DNA Binding Protein That Regulates the Human GLUT4 Promoter in Transgenic Mice*

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**We have previously demonstrated that the important *cis*-acting elements regulating transcription of the human *GLUT4* gene reside within 895 base pairs (bp) upstream of the transcription initiation site (Thai, M. V., Guruswamy, S., Cao, K. T., Pessin, J. E., and Olson, A. L. (1998) *J. Biol. Chem.* 273, 14285–14292). Our studies demonstrated that an MEF2 binding site within this region was necessary, but not sufficient, for GLUT4 promoter function in transgenic mice. We have identified a second regulatory element (Domain I) that functions cooperatively with the MEF2 domain in regulating GLUT4 transcription. Using a yeast-one hybrid screen, we obtained a partial cDNA and generated an antibody directed against a protein binding specifically to Domain I. Sequence analysis of the partial cDNA indicates that the protein binding to Domain I is a novel protein. The antibody specifically labels two proteins of approximately 70 and 50 kDa in Western blot analysis. These molecular masses correspond to Domain I binding proteins identified by UV-cross-linking nuclear extracts to a Domain I probe. The antibody raised against the Domain I binding protein inhibited formation of a Domain I-protein complex in electrophoretic mobility shift assays. We conclude that we have identified an authentic, novel, Domain I binding protein required for transcriptional regulation of the human GLUT4 promoter.**

One of the many physiologic actions of insulin is to promote glucose uptake in skeletal muscle, heart, and adipose tissue. Several lines of evidence have shown that the effect of insulin on glucose uptake in these tissues results directly from the recruitment of the GLUT4[1] facilitative glucose transporter from an intracellular vesicle pool to the plasma membrane (for recent reviews see Refs. 1 and 2). GLUT4 was first implicated as the major insulin responsive glucose transporter when it was shown to be the predominant isoform expressed in tissues exhibiting insulin-stimulated glucose uptake (3–6). In addition, GLUT4 protein was shown to move to the plasma membrane in response to insulin receptor activation in insulin responsive tissues (7, 8). Proof that GLUT4 is the primary effector molecule for insulin-mediated glucose disposal came from transgenic animals. Mice genetically engineered to overexpress an exogenous *GLUT4* gene in all of the major *GLUT4*-expressing tissues, or in skeletal muscle or adipose tissue alone, display enhanced insulin responsiveness and enhanced peripheral glucose utilization (9–12).

Insulin-mediated glucose homeostasis is extremely sensitive to GLUT4 levels in the major insulin responsive tissues, including heart, skeletal muscle, and adipose tissue. In rodent models of insulin deficiency (fasting or streptozotocin (STZ)-induced diabetes), GLUT4 protein and mRNA expression are markedly reduced and together account for the reduced insulin-dependent glucose transport in response to insulin under these conditions (13–19). Genetic studies strengthen the relationship between insulin-mediated glucose homeostasis and the levels of GLUT4 protein. Transgenic mice expressing only one allele of the *GLUT4* gene were shown to express GLUT4 protein at reduced levels, resulting in a progressive diabetic phenotype characterized by impaired glucose homeostasis (20). In STZ-diabetic mice, overexpression of GLUT4 in either fat or skeletal muscle, under the control of heterologous tissue-specific promoters, improves glucose homeostasis (21, 22). Finally, expression of the human *GLUT4* gene within the context of the genetically diabetic *db/db* strain of mice improved glycemic control and insulin sensitivity (23). The close association between the GLUT4 protein pool size and physiologic changes in glucose homeostasis make manipulation of *GLUT4* gene expression a potentially important target for therapeutic interventions designed to treat insulin resistance associated with diabetes.

To manipulate *GLUT4* gene expression as a therapeutic strategy, a detailed understanding of the regulation of GLUT4 transcription at the molecular level is required. *GLUT4* mRNA expression is subject to tissue-specific, hormonal, and metabolic regulation. Changes in *GLUT4* gene expression occur *in vivo* during states of altered glucose homeostasis. In general, *GLUT4* mRNA expression is down-regulated in states of relative insulin-deficiency such as STZ-induced diabetes and chronic fasting (for review see Refs. 24 and 25). The observed differences in steady-state levels of *GLUT4* mRNA are attributed to changes in mRNA synthesis, with rates determined by nuclear run-on transcription assays demonstrating that

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF249267.*

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[1] The abbreviations used are: GLUT4, adipose/muscle-specific glucose transporter; STZ, streptozotocin; bp, base pair(s); TBE, Tris/borate EDTA; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis, MEF2, myocyte enhancer factor 2; GST, glutathione *S*-transferase; $GEF_{db}$, GLUT4 enhancer factor DNA binding domain; kb, kilobase(s); PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; NF-1, nuclear factor 1.

*GLUT4* gene transcription in STZ-induced diabetic animals is decreased in both adipose tissue and skeletal muscle (26, 27). These results underscore the importance of understanding the molecular mechanisms underlying function of the *GLUT4* promoter, if pharmacological methods for increasing GLUT4 expression are to be developed.

Transgenic mice have provided a valuable model system for studying the *GLUT4* gene promoter within a natural physiologic context. Using transgenic mice, we have shown previously that regions regulating expression of the human *GLUT4* gene are located within 895 base pairs (bp) of DNA positioned upstream of the major transcription initiation site (28–31). The 895-bp regulatory region supported a pattern of gene expression identical to that of the mouse *GLUT4* gene, both in terms of tissue specificity and regulation, in fasting and STZ-induced models of insulin deficiency (31). We determined in those studies that an MEF2 DNA binding domain performs a critical role in regulation of the human *GLUT4* gene promoter; however, our data indicated that the MEF2 binding site alone is not sufficient to support a regulated and tissue-specific program of gene expression (30, 31). In the current study, we have identified a second regulatory region within the functional portion of the human GLUT4 promoter that acts synergistically with the MEF2 binding domain to support a pattern of transcription identical to the endogenous *GLUT4* gene. In addition, we have identified a novel DNA binding protein that interacts with this region *in vitro*. Our experimental evidence indicates that the two regulatory regions of the human GLUT4 promoter function cooperatively to support transcription in transgenic mice.

## MATERIALS AND METHODS

*Preparation of Transgenic Mice*—cDNA constructs used to generate transgenic mice were derived from the plasmid hGLUT4[2.4]CAT (28). Two internal deletions in hGLUT4[2.4]CAT were generated by polymerase chain reaction. Internal deletions were made between nucleotides −711 and −526 (Δ711/526) and between nucleotides −742 to −526 (Δ742/526). The promoter-reporter constructs were obtained by digesting plasmid DNA with AVRII and *Hin*dIII so that the 5′-end of each reporter construct corresponded to nucleotide position −895 relative to the major transcription initiation site of the human *GLUT4* gene. These constructs are referred to as 895Δ711/526-hG4-CAT and 895Δ742/526-hG4-CAT, respectively. The DNA fragments containing the promoter-reporter construct were isolated by agarose gel electrophoresis and injected into the pronucleus of fertilized mouse embryos at the Microinjection Core Facility at the Oklahoma Medical Research Foundation, in Oklahoma City (Dr. Brian Sauer, director). Transgenic animals carrying the appropriate constructs were identified by slot blot analysis of isolated tail DNA using the 4.6-kilobase (kb) *Sac*I/*Hin*DIII fragment of hGLUT4[2.4]CAT as a probe.

*STZ-induced Diabetes*—Insulin-deficient diabetes was induced by a single intraperitoneal injection of STZ (200 mg/kg of body weight) following an overnight fast as described previously (32). 72 h after injection, tail vein blood samples were assayed for glucose concentration using chemstrips (Roche Molecular Biochemicals). Animals with blood glucose levels greater than 400 mg/dl were considered diabetic. The diabetic animals were either left untreated or were treated with 1 unit of regular insulin per day for 2 days. The mice were killed 5 days after STZ injection, and the tissues were frozen in liquid nitrogen and stored at −70 °C until analyzed.

*RNA Isolation and RNase Protection Assay*—Total cellular RNA was isolated from frozen tissues using guanidinium isothiocyanate extraction followed by purification on a CsCl gradient (33) as described previously (34). RNA was quantified spectrophotometrically by absorbance at 260 nM and stored as an ethanol precipitate at −70 °C. RNase protection analysis was carried out as described previously (31).

*Preparation of Nuclear Extracts*—Tissues were harvested and frozen in liquid nitrogen. The frozen tissues were pulverized in liquid nitrogen and homogenized in 10 volumes of homogenization buffer A (250 mM sucrose; 10 mM Hepes, pH 7.6; 25 mM KCl; 1 mM EDTA; 10% glycerol; 0.1 mM phenylmethylsulfonyl fluoride (PMSF); 0.5 mM benzamidine; 2 μg/ml each of aprotinin, leupeptin, and pepstatin A; 2 mM levamisole; 10 mM β-glycerophosphate; and 1 mM sodium vanadate) with 10 strokes of a Teflon pestle. The homogenate was centrifuged 10 min at 2400 × *g* at 4 °C. The pellet was resuspended in 10 ml of homogenization buffer A and homogenized with 10 strokes of a Dounce homogenizer with a tight fitting pestle (pestle B). The homogenate was layered over one-half volume of buffer A-1.0 (buffer A with addition of 1.0 M sucrose) and centrifuged at 2400 × *g* for 10 min at 4 °C. The pellet was resuspended in 2 volumes of buffer A-1.0, and repelleted by microcentrifugation at 16,000 × *g* for 20 s. The crude nuclear pellet was then resuspended in 1 volume of nuclear extraction buffer (10 mM Hepes, pH 7.6; 325 mM NaCl; 3 mM $MgCl_2$; 0.1 mM EDTA; 10% glycerol; 1 mM dithiothreitol (DTT); 0.1 mM PMSF; 0.5 mM benzamidine; and 2 μg/ml each of aprotinin, leupeptin, and pepstatin A) and incubated on ice for 20 min, and the particulate material was removed by centrifugation at 16,000 × *g* in a microcentrifuge for 10 min at 4 °C. The supernatant was dialyzed against buffer C (25 mM Hepes, pH 7.6; 100 mM KCl; 0.1 mM EDTA; 10% glycerol; 1 mM DTT; 0.1 mM PMSF; 0.5 mM benzamidine; 2 μg/ml each of aprotinin, leupeptin, and pepstatin A; 2 mM levamisole; 10 mM β-glycerophosphate; and 1 mM sodium vanadate) for 4 h. The dialysate was assayed for total protein (Bradford) and stored at −70 °C.

*DNase I Footprinting and Electrophoretic Mobility Shift Assays*—DNase I footprinting was performed using a DNA fragment corresponding to nucleotides −800 to −412 of the human GLUT4 promoter with an internal deletion of nucleotides −711 to −526. The probe was prepared by end labeling the *Avr*II/*Bss*HI fragment cleaved from the parental 2.4-hGR-CAT plasmid carrying the internal deletion. The fragment was end-labeled with T4 polynucleotide kinase, cut with *Bam*HI, and gel-isolated so that the probe was labeled at only the 3′-end. DNase I footprinting was performed without or with nuclear extracts from heart and liver, using a Core Footprinting system (Promega, Madison, WI). Electrophoresis of the DNase I footprint was performed on a 7.5 M urea/6% acrylamide sequencing gel adjacent to a Maxam and Gilbert guanine sequencing ladder of the fragment. The gel was fixed, dried, and exposed to film at −70 °C with intensifying screens.

Oligonucleotides containing the human GLUT4 Domain I DNA binding site (CTTGTCCCTCGGACCGGCTCCAGGAACCAA and its complement) were custom-synthesized (Life Technologies, Inc.). The oligonucleotides containing the OCT1 DNA binding site were commercially prepared (Santa Cruz). Oligonucleotides were end-labeled with T4 polynucleotide kinase. Labeled probes (0.5 ng) were incubated with 10 μg of total protein isolated from nuclei in a 10-μl reaction containing 2 μg of poly(dI-dC), 40 mM KCl, 5 mM $MgCl_2$, 15 mM Hepes, pH 7.9, 1 mM EDTA, 0.5 mM DTT, and 5% glycerol for 20 min at room temperature. For competition studies, extracts were preincubated with various concentrations of unlabeled oligonucleotide as indicated for 5 min before addition of the radiolabeled probe. For competition studies using antibodies, extracts were preincubated with 2.5 μg of either preimmune IgG, anti-GST-$GEF_{db}$ IgG, or IgG from an irrelevant antiserum raised against a glutathione *S*-transferase (GST) fusion protein. Preincubation was carried out for 1 h on ice before addition of the radiolabeled probe. Samples were electrophoresed on a non-denaturing 6% polyacrylamide gel (29:1 acrylamide:bis acrylamide) buffered with Tris borate/EDTA (TBE; 22 mM Tris, 22 mM boric acid, and 0.5 mM EDTA) at 300 V for 25 min at room temperature. Gels were then dried and an exposure of them was made on film at room temperature.

*Yeast One-hybrid Screen*—The MATCHMAKER one-hybrid system (CLONTECH) was used in screening a human skeletal muscle cDNA library to identify a protein that binds to the Domain I regulatory element of the human GLUT4 promoter. A Domain I/pHISi-1 reporter construct was generated by inserting three tandem repeats of the Domain I sequence (AATTCCCTCGGACCGGCTCCACCAATCCCTCGG-ACCGGCTCCAGGAATCCCTCGGACCGGCTCCAGGAA) into the *Sma*I/*Eco*RI sites of the pHISi-1 integration vector. PHISi-1 was also used to make a reporter construct containing a mutant Domain I sequence (Mut1) target element (AATTCCCTCGGGTTAACTCCAGGA-ATCCCTCGGGTTAACTCCAGGAATCCCTCGGGTTAACTCCAGG-AA) that was unable to bind nuclear proteins during electrophoretic mobility shift assay (EMSA). pHISi-1 expresses the *HIS3* gene from a minimal promoter that allows a low level of *HIS3* expression. A large scale transformation of the yeast strain YM4271 ($his^-$, $ura^-$, $leu^-$) with the target vector constructs was performed on medium lacking histidine. A stable transformant growing on $His^-$ medium that did not grow on $His^-$ medium supplemented with 15 mM 3-amino-1,2,4-triazole (added to suppress growth resulting from low levels of *HIS3* expression from the minimal promoter) was used as the Domain I/pHISi-1 reporter strain for library screening. The Mut1/pHISi-1 stable transformant was constructed using the same procedure. An adult human skeletal muscle cDNA library (CLONTECH) was probed by transformation of the Domain I/pHISi-1 reporter strain. Transformants expressing library proteins that activated transcription of the Domain I/pHISi-1 construct

were selected on Leu$^-$/His$^-$/3-amino-1,2,4-triazole dropout medium. cDNA from positive transformants was isolated and retransformed into the Mut1/pHISi-1 reporter strain. Transformants that failed to grow in the Mut1/pHISi-1 reporter strain were selected for further characterization.

*Production of GST Fusion Proteins and Antibodies*—A cDNA clone (GenBank™ accession number AF249267) isolated from the yeast one-hybrid screen was subcloned in-frame into the GST-3X fusion protein vector (Amersham Pharmacia Biotech). The fusion protein (GST-$GEF_{db}$) was isolated from *Escherichia coli* (DH5-$\alpha$ strain) by lysing cells in radioimmune precipitation buffer (10 mM Tris, pH 7.4, 0.15 M NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 2 mM PMSF) followed by affinity purification over a GST-Sepharose column according to the manufacturer's specifications. The fusion protein was eluted from the column with 20 mM glutathione (pH 8.0) and was concentrated using Centricon-30 filters (Amicon Inc.). The concentrated samples were dialyzed overnight against 1000 sample volumes of Tris-buffered saline (TBS, 20 mM Tris, pH 7.5, 150 mM NaCl). Antibodies against the isolated fusion protein were raised in rabbits (Cocalico Biologicals). IgG proteins were purified from preimmune rabbit serum and GST-$GEF_{db}$ antiserum by protein-G affinity chromatography.

*Western Blot Analysis*—30 $\mu$g of total nuclear extract protein were solubilized in an equal volume of 2× Laemmli sample buffer (120 mM Tris, pH 6.8, 4% SDS, 20% glycerol, and 200 mM DTT) and separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 7.5 acrylamide gels. Separated proteins were transferred to polyvinylidene difluoride membranes (Millipore) in buffer (25 mM Tris and 190 mM glycine, pH 8.5) overnight at 0.2 A at 4 °C. Polyvinylidene difluoride membranes were blocked 1 h at room temperature with the TBS solution containing 0.2% Tween 20 and 7% dried milk (Carnation). Blocked membranes were probed with the rabbit polyclonal antibody raised against GST-$GEF_{db}$ (0.2 $\mu$g/ml) in TBS containing 0.1% Tween 20 and 2% bovine serum albumin (Sigma) for 1 h at room temperature. To determine specificity, the polyclonal antibody solution was also preincubated with 15 $\mu$g of GST-$GEF_{db}$ fusion protein immobilized on agarose beads. The solution (depleted of GST-$GEF_{db}$-specific IgG) was used for Western blot analysis. Immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce) following incubation of the blot with a goat-anti-rabbit secondary antibody conjugated to horseradish peroxidase (Pierce).

## RESULTS

*Tissue-specific Expression of the Human GLUT4 Gene in Transgenic Mice*—In our previous studies, we demonstrated that the smallest promoter-enhancer construct capable of supporting regulated, tissue-specific expression of the *GLUT4* gene (895-hG4-CAT) consisted of 895 bp of DNA located immediately upstream of the transcription initiation site (30, 31). Two lines of evidence suggested that functional activity of 895-hG4-CAT was regulated by two regulatory domains consisting of a proximal MEF2 binding domain and an uncharacterized upstream element located in the vicinity of nucleotide −730 (relative to the major transcription initiation site). First, transgenic mRNA expression was ablated upon introduction of a loss-of-function mutation in the MEF2 binding domain in the 895-hG4-CAT reporter construct (31). Second, a 5′-deletion of all sequences upstream of −730 resulted in a pattern of expression that was not regulated under STZ diabetes and was not tissue-specific (30).

The focus of the current study was to confirm that an additional regulatory element upstream of the MEF2 regulatory domain was required for GLUT4 promoter function in transgenic mice, and to define that element. Sequence analysis of the region upstream of the MEF2 binding domain did not reveal any known transcription factor binding domains. We assumed, however, that an important functional domain would coincide with a region of significant sequence conservation between species. A 60-bp region with greater than 90% sequence homology exists between nucleotides −712 and −772 (186 bp upstream of the MEF2 binding domain) (30). We refer to this region as Domain I. The 186 bp of DNA intervening between Domain I and the MEF2 binding domain showed little sequence conservation, suggesting that it may not be functionally

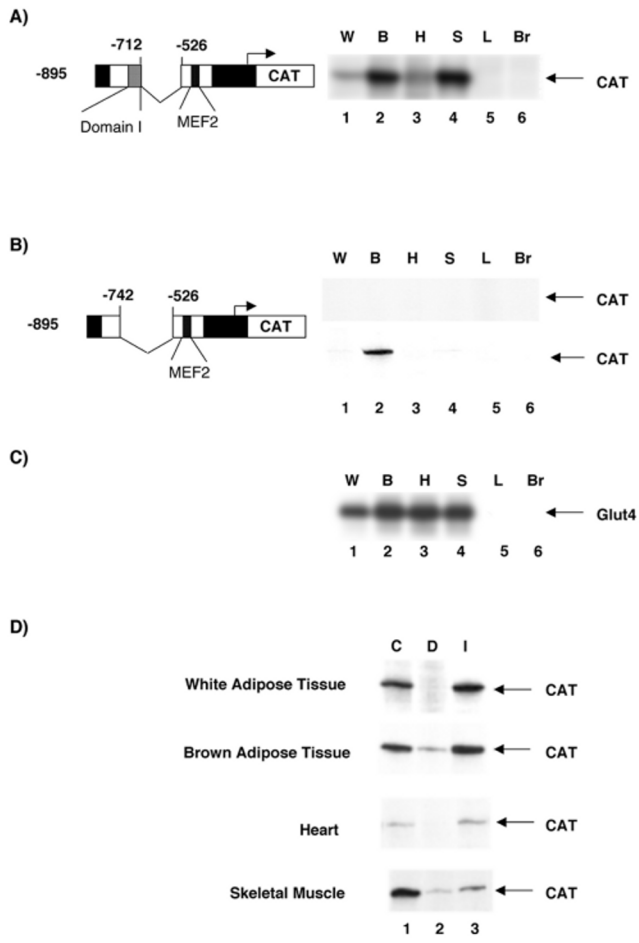


FIG. 1. **Tissue-specific expression of *CAT* mRNA in transgenic mice carrying different human *GLUT4* 5′-flanking DNA.** 10 $\mu$g of total RNA isolated from white adipose tissue (*W*), brown adipose tissue (*B*), heart (*H*), hindquarter skeletal muscle (*S*), liver (*L*), and brain (*Br*) was analyzed for *CAT* mRNA (*panels A* and *B*) and endogenous mouse *GLUT4* mRNA (*panel C*) using RNase protection as described under "Materials and Methods." The schematic representation and names of constructs used to generate transgenic mice are shown in *A* and *B*. The major transcription start site of the human *GLUT4* gene is indicated by the *arrow*. Nucleotide positions for each construct are indicated relative to the transcription start site, assigned +1. Expression of 895Δ711/526-hG4-CAT in streptozotocin-induced diabetes is shown in *D*. Total cellular RNA was isolated from control (*C*), untreated diabetic (*D*), or insulin-treated diabetic (*I*) female mice. Diabetic animals had blood glucose levels greater than 400 mg/dl following treatment with a single dose of 200 mg/ml streptozotocin and killed 5 days later. Insulin-treated diabetic animals received 0.5 unit of regular insulin twice a day for the last 2 days of the diabetic period.

important. To test our hypothesis that the unconserved sequences did not contain a regulatory element, we mutated the 895-hG4-CAT construct, deleting the unconserved sequences between −526 and −711 (895Δ711/526-hG4-CAT). RNA expression from this construct was studied in eight independent founder lines using an RNase protection assay. This assay was designed to measure transgenic mRNA in conjunction with endogenous *GLUT4* mRNA. The pattern of tissue-specific expression from 895Δ711/526-hG4-CAT is shown in Fig. 1*A*. RNA expression from the modified *GLUT4* promoter-enhancer in this construct was restricted to GLUT4-expressing tissues (Fig. 1*A*, *lanes 1–4*). Importantly, transgenic CAT mRNA was not expressed in liver and brain, tissues that express little or no GLUT4 mRNA. The pattern of transgene expression from 895Δ711/526-hG4-CAT is identical to that observed for previous constructs carrying the full-length human GLUT4 regulatory region (30, 31), indicating that the unconserved region is

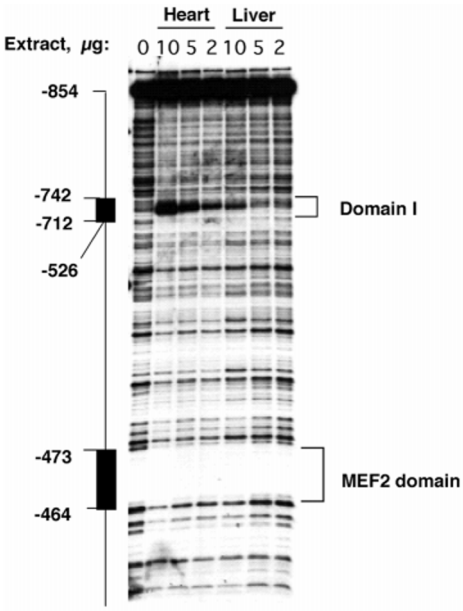


FIG. 2. **DNase I footprint analysis of the human GLUT4 promoter.** A DNA fragment containing bases −800 to −412 with an internal deletion of bases −711 to −526 was labeled at the 3′-end. The labeled probe was incubated with varying amounts of heart or liver nuclear extracts as indicated. The samples were then digested with DNase I and separated on a denaturing acrylamide gel. The *boxes* highlight a DNase I hypersensitivity site corresponding to Domain I and the MEF2 binding site. The *numbers* indicate the nucleotide number in the promoter-enhancer region of the human *GLUT4* gene as determined by concurrent running of sequencing reactions.

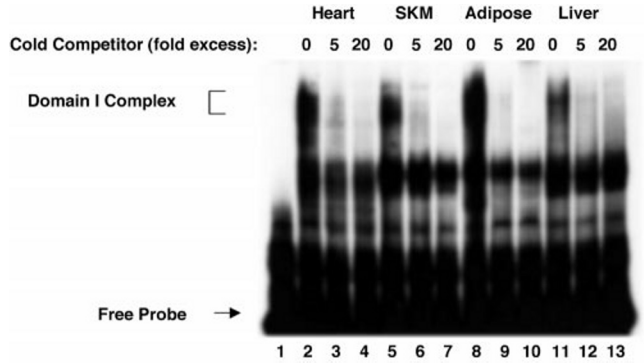


FIG. 3. **The Domain I binding complex is present in heart, skeletal muscle, adipose tissue, and liver nuclear extracts.** The 30-bp Domain I oligonucleotide was labeled with [$\gamma$-$^{32}$P]ATP and incubated in the absence (*lane 1*) or presence of 10 $\mu$g of heart (*H*, *lanes 2–4*), hindquarter skeletal muscle (*S*, *lanes 5–7*), subscapular brown adipose tissue (*B*, *lanes 8–10*), or liver (*L*, *lanes 11–13*) nuclear extracts. Competition for DNA-protein complex binding was performed by preincubating varying amounts of unlabeled oligonucleotide with extracts for 5 min prior to addition of labeled probe. Samples were resolved by nondenaturing gel electrophoresis (EMSA) and autoradiographed as described under "Materials and Methods."

not required for regulated expression of *GLUT4*.

*Expression of 895Δ711/526-hG4-CAT in Insulin-deficient Diabetic Mice*—It is well established that in both rats and mice, *GLUT4* gene expression is down-regulated in insulin-deficient states. This has been shown to be due to a decrease in the rate of transcription (26, 29). Decreased transcription is an insulin-dependent response, because the transcription rate and *GLUT4* mRNA levels were fully restored following insulin therapy (26, 29). To determine if 895Δ711/526-hG4-CAT was regulated by insulin deficiency in a manner analogous to the endogenous *GLUT4* gene, we examined transgenic *CAT* mRNA expression in STZ-induced diabetes. Expression of *CAT* mRNA in white and brown adipose tissue, heart, and hindquarter skeletal muscle was reduced in diabetic mice identically to that observed for the endogenous mouse *GLUT4* gene (Fig. 1*D*) (30). Treatment of these diabetic mice with insulin restored *CAT* mRNA to normal levels (Fig. 1*D*). These data demonstrate that the unconserved sequences between positions −711 to −526 do not contribute to the function of the *GLUT4* promoter with respect to either tissue-specific expression or regulated expression during insulin deficiency.

*DNase I Footprint Analysis*—Having substantially narrowed the control region of the human *GLUT4* gene responsible for regulation of transcription, we employed DNase I footprint analysis to determine the specific site(s) that bind nuclear proteins and hence confer function. Footprint analysis was performed on a 202-bp DNA fragment corresponding to nucleotides −412 to −800 with an internal deletion of nucleotides −711 to −526 (corresponding to the unconserved 186-bp region). Footprints were carried out using nuclear extracts prepared from heart and liver (Fig. 2). Analysis by this method revealed a strong DNase I hypersensitivity site upstream of a protected region spanning the DNA between nucleotides −712 and −742. This hypersensitivity site was apparent in footprints with extracts from both heart and liver, however, hypersensitivity was much less prominent using liver extracts.

*Nuclear Proteins Bind to Domain I*—The presence of a DNase I hypersensitivity site in Domain I prompted us to carry out an electrophoretic mobility shift assay (EMSA) to determine if a sequence-specific protein complex formed in this region. Several oligonucleotides spanning the region between −800 and −712 were screened for ability to bind nuclear proteins (data not shown). Only an oligonucleotide corresponding to the hypersensitivity site (nucleotides −742 to −712) bound nuclear proteins in EMSA and was used for further studies. EMSA analysis of nuclear extracts from heart, skeletal muscle, adipose tissue, and liver (Fig. 3) revealed that a sequence-specific protein complex formed with each of the extracts. Competition analysis using a 5- and 20-fold molar excess of unlabeled oligonucleotide confirmed that the observed complex bound the oligonucleotide in a sequence-specific manner. The presence of a small amount of the binding complex forming in liver nuclear extracts suggests that the Domain I binding site may be a ubiquitous promoter element, implying that the MEF2 binding domain plays a role in conferring tissue-specific gene activation.

*Functional Analysis of the Human GLUT4 Promoter-Enhancer Lacking Domain I*—To test whether Domain I was a functional promoter element, we commissioned a second line of transgenic mice carrying an internal deletion of the −895-hG4-CAT construct from nucleotides −742 to −526. This construct deleted the unconserved region between Domain I and the MEF2 domain, with an additional deletion of the Domain I elements defined in EMSA (−895Δ742/526-hG4-CAT) (Fig. 1*B*). Message levels expressed from this construct were compared with levels from the construct −895Δ712/526-hG4-CAT, which deletes only the unconserved sequences between the regulatory domains (Fig. 1, *A* and *B*). In contrast to the latter two reporter constructs, the construct deleting Domain I (−895Δ742/526-hG4-CAT) was not able to support expression of the transgene. Results from two independent founder lines expressing this construct are shown in Fig. 1*B*. In one founder line, transgenic mRNA was expressed at low levels in brown adipose tissue. This is probably due to an artifact of transgene integration, because transgenic mRNA does not show up in any other tissue, and is not expressed in any tissues from the other founder line. Thus, the Domain I promoter element is required for full promoter function, even in the presence of an intact

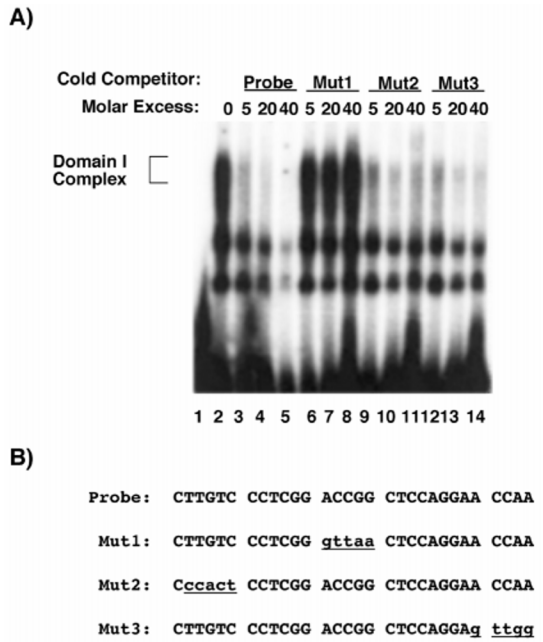


FIG. 4. **Identification of nucleotide sequences required for Domain I-protein complex formation.** *A*, the 30-bp Domain I oligonucleotide probe was labeled with [$\gamma$-$^{32}$P]ATP and incubated without (*lane 1*) or with 10 $\mu$g of heart nuclear extract (*lanes 2–14*). Nuclear extracts were also preincubated for 5 min with varying amounts of unlabeled wild type oligonucleotide (*lanes 3–5*) or mutant oligonucleotide before adding the labeled Domain I oligonucleotide probe. Specific mutations are shown in *B*. Mutated sequences are shown in *lowercased letters* and are *underlined*. Samples were resolved by non-denaturing gel electrophoresis (EMSA) and autoradiographed as described under "Materials and Methods."

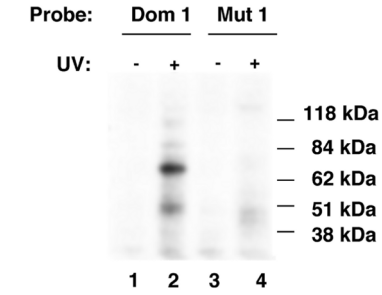


FIG. 5. **UV cross-linking of nuclear proteins to Domain I oligonucleotide.** Domain I (*lanes 1* and *2*) and Mut1 (*lanes 3* and *4*) oligonucleotides (modified as described under "Materials and Methods") were incubated with heart nuclear extracts. The binding reactions were either left untreated (*lanes 1* and *3*) or exposed to broad range UV light (*lanes 2* and *4*). The samples were resolved by 7.5% SDS-PAGE and autoradiographed as described under "Materials and Methods."

MEF2 binding site. These data suggest that the Domain I regulatory site and the MEF2 binding site have a functionally cooperative relationship in transcriptional activation of the *GLUT4* gene. We show here that, within the context of −895-hG4-CAT, the MEF2 binding site can not function without a Domain I site (Fig. 1*B*), and we showed previously that Domain I could not function in the presence of a mutated MEF2 binding domain (31). Taken together, each domain is necessary, but not sufficient for transcriptional activity of the human GLUT4 promoter in transgenic mice.

*Characterization of the Domain I Protein Binding Site*—To begin identifying individual protein components of the Domain I nuclear protein complex, we needed to define the sequence required for formation of the DNA-protein complex. To this end, we carried out competition binding in EMSA analysis using a series of mutated oligonucleotides spanning Domain I (Fig. 4). A mutation between −725 and −729 (Mut1) within the Domain I oligonucleotide rendered the oligonucleotide incapable of forming a complex with nuclear proteins, as determined by its inability to compete with the wild-type Domain I probe for complex formation (Fig. 4). In contrast, oligonucleotide mutations at either the 5′-end (Mut2) or 3′-end (Mut3) competed for binding with the wild-type probe, indicating that these sequences do not participate in formation of the DNA-protein complex (Fig. 4).

*Identification of Domain I Binding Protein(s)*—EMSA is limited in its ability to resolve the size(s) of the specific nuclear protein(s) that directly bind to Domain I. To address this, we performed experiments in which the DNA-protein complex was UV cross-linked using a radiolabeled double-stranded oligonucleotide corresponding to Domain I. A modified oligonucleotide was prepared substituting bromodeoxyuridine for thymidine in the non-coding strand of the wild-type Domain I 30-mer, or the Mut1 30-mer that was unable to form a shifted complex in EMSA. The DNA-protein complex was allowed to form under the same conditions used in EMSA, except that after incubation, the binding reaction was either left untreated or cross-linked by exposure to UV light at 260–500 nm for 25 min. The binding reactions were then subjected to 7.5% SDS-PAGE to resolve the cross-linked DNA-protein complex. A major UV-dependent band, binding specifically to the wild type oligonucleotide, migrated at a position corresponding to approximately 70 kDa (Fig. 5, compare *lanes 1* and *2*). A less abundant band was observed to migrate just below the 51-kDa marker.

The Domain I sequence was examined for known transcription factor binding motifs, and none were identified. This analysis indicates that the DNA-protein complex contains a novel DNA binding protein(s) or that Domain I is a novel site binding a known protein or complex of proteins. To identify proteins binding to Domain I, we used a yeast one-hybrid screen of a human skeletal muscle library that employed three tandem repeats of the Domain I binding site as the target promoter. To eliminate false positives, cDNA clones from the first screen were tested for their inability to promote transcription from three tandem repeats of the mutant Domain I binding site (Fig. 4). We characterized one positive clone that we call $GEF_{db}$ (GLUT4 Enhancer Factor DNA binding domain) to determine whether this clone encoded a protein present in the Domain I DNA binding complex.

$GEF_{db}$ consisted of approximately 1100 bp encoding a small (28 kDa) peptide, which encompasses the DNA binding domain of the full-length protein. To determine whether this cDNA encoded an authentic Domain I DNA binding domain, we subcloned the $GEF_{db}$ cDNA in-frame with glutathione *S*-transferase to form a fusion protein, GST-$GEF_{db}$. GST-$GEF_{db}$ and GST alone were analyzed by EMSA in the presence or absence of a 20-fold molar excess of either unlabeled Domain I oligonucleotide or unlabeled Mut1 oligonucleotide (Fig. 6*A*). GST alone did not bind to the Domain I DNA probe (Fig. 6, *lane 5*). In contrast, GST-$GEF_{db}$ (Fig. 6*A*, *lane 8*) was able to bind DNA, and binding was specific for Domain I, because it could be competed by unlabeled wild type oligonucleotide, but not by unlabeled Mut1 oligonucleotide (Fig. 6A, compare *lanes 9* and *10*). The DNA-protein complex formed with GST-$GEF_{db}$ migrated differently than the complex formed from tissue nuclear extracts (Fig. 6*A*, *lane 2*). This was expected, because GST—$GEF_{db}$ was generated from a partial cDNA and therefore would not be expected to have the same mobility as the endogenous protein complex.

The sequence for the partial cDNA that $GEF_{db}$ is given in Fig. 6*B*. A search of data bases showed that the $GEF_{db}$ cDNA does not match any known gene, but does match with several human EST clones. Analysis of the protein by BLAST search

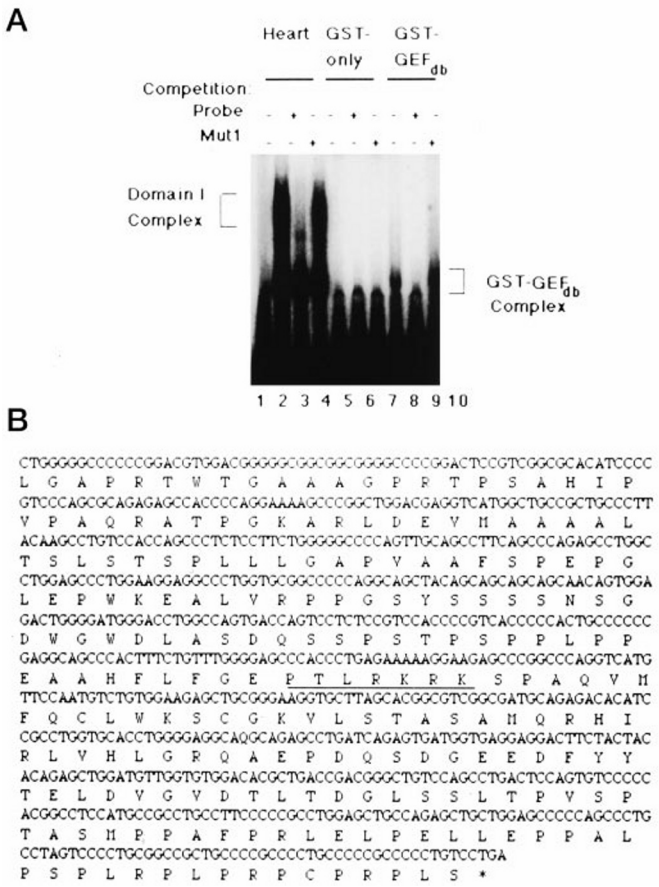


FIG. 6. *A*, GST-GEF$_{db}$ specifically binds Domain I. The 30-bp Domain I oligonucleotide was labeled with [$\gamma$-$^{32}$P]ATP and incubated without (*lane 1*) or with 10 $\mu$g of heart nuclear extracts (*lanes 2–4*), 1 $\mu$g of GST alone (*lanes 5–7*), or 1 $\mu$g of GST-GEF$_{db}$ (*lanes 8–10*). Binding reactions were competed with a 20-fold molar excess of unlabeled wild-type oligonucleotide (*lanes 3*, *6*, and *9*) or Mut1 oligonucleotide (*lanes 4*, *7*, and *10*) for 5 min prior to addition of radiolabeled probe. Samples were resolved by non-denaturing gel electrophoresis (EMSA) and autoradiographed as described under "Materials and Methods." *B*, the cDNA sequence and deduced amino acid sequence of the partial cDNA encoding the GEF$_{db}$. The amino acids are given as a *single-letter code*. The nuclear localization signal sequence is shown *underlined*.

does not show homology to any known protein. Our data show that the GST-GEF$_{db}$ fusion protein binds the Domain I in the same sequence-specific manner as nuclear proteins bind the oligo. Therefore, the GEF$_{db}$ contains a Domain I-specific DNA binding motif that represents a novel DNA binding protein. The partial cDNA does contain a conventional nuclear localization sequence indicating that this protein is localized to the nucleus (Fig. 6*B*).

A polyclonal antibody was raised against GST-GEF$_{db}$ and was used for Western blot analysis of heart nuclear extracts (Fig. 7, *lane 1*). Two specific immunolabeled bands were observed. The larger band migrated at approximately 70 kDa and a smaller band migrated just below 50 kDa, coinciding with the molecular masses observed for the UV cross-linked DNA-protein complexes (Fig. 5). To demonstrate that the bands were labeled specifically by IgG directed against the GEF$_{db}$ peptide, a control blot was performed with IgG that had been preabsorbed with GST-GEF$_{db}$ fusion protein (Fig. 7, *lane 2*).

To confirm that the GEF$_{db}$ antibody was directed against a constituent of the *in vivo* DNA binding complex, we preincubated nuclear extracts with preimmune IgG, GST-GEF$_{db}$ IgG, or IgG from an irrelevant GST fusion protein before carrying out EMSA (Fig. 8*A*). Because the GST-GEF$_{db}$ IgG is directed against the DNA binding site, we predicted that the antibody

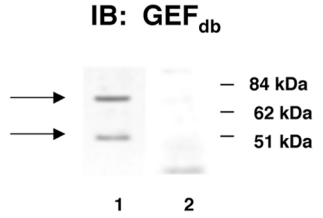


FIG. 7. **Immunoblot analysis of nuclear extracts.** Duplicate samples of heart nuclear extract were immunoblotted with anti-GEF$_{db}$ antibody that was left untreated (*lane 1*) or preabsorbed with GST-GEF$_{db}$ fusion protein (*lane 2*). Immunolabeled bands were detected using goat anti-rabbit IgG conjugated with HRP and detected by chemiluminescence.

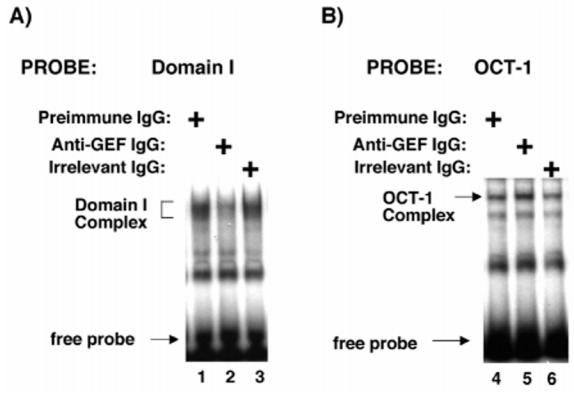


FIG. 8. **Anti-GEF$_{db}$ IgG specifically competes for Domain I in EMSA.** Heart nuclear extracts (10 $\mu$g) were pretreated in binding buffer for 1 h with 3 $\mu$g of preimmune IgG, anti-GEF$_{db}$ IgG, or IgG raised against an irrelevant GST fusion protein. Following incubation with antibody, radiolabeled Domain I probe or OCT-1 probe was added. Samples were resolved by non-denaturing gel electrophoresis (EMSA) and autoradiographed as described under "Materials and Methods."

would compete with the Domain I oligonucleotide for binding to the Domain I binding protein. We found that complex binding was specifically competed by the anti-GST-GEF$_{db}$ IgG, but not the preimmune IgG or IgG raised against an irrelevant fusion protein (Fig. 8*A*). The anti-GST-GEF$_{db}$ IgG competed specifically with the Domain I oligonucleotide for protein binding, because this antibody had no effect when antibody-treated nuclear extracts were incubated with an OCT-1 oligonucleotide probe (Fig. 8*B*).

## DISCUSSION

To date, we have analyzed 13 different transgenic constructs for bonafide tissue-specific and hormonal/metabolic *GLUT4* gene regulation. Through mutational analysis of the region controlling transcription of the human *GLUT4* gene, we found that all of the apparent *cis*-acting regulatory elements are located within 895 bp of the transcription initiation site. Over the course of these studies, we observed that mRNA expression from a given construct is not in itself a sufficient criterion to determine whether a promoter-enhancer fragment functions in gene expression. We have, therefore, adopted the following criteria to characterize a region as regulatory. First, at least two independent founder lines must be studied to account for possible aberrations in gene expression due to copy number or point of insertion of the transgene into the mouse genome. Second, we look for a pattern of transgene expression that mirrors the restricted pattern of expression observed for endogenous *GLUT4* mRNA. Finally, we study regulation of transgenic mRNA under conditions of STZ diabetes to determine whether transgene expression is regulated similarly to the endogenous gene. Thus, a construct supporting expression of transgenic mRNA that is also regulated under STZ diabetes is

considered functional, whereas a construct that is not regulated is not considered to be fully functional.

Our analysis indicates that transcriptional control of the human GLUT4 promoter requires two DNA binding domains: Domain I located between nucleotides −724 and −712, and the MEF2 binding domain located between nucleotides −473 and −464 (both regions are numbered relative to the transcription start site). We showed previously that a mutation in the MEF2 binding domain ablated transgene expression in all tissues (31). In the current study we show that deletion of Domain I also prevents transgene expression, regardless of the presence of the MEF2 domain (Fig. 1). In a previous study, we showed that a *GLUT4* control region deleted to position −730 supported a very low level of transgene expression that was not regulated in STZ diabetes (30). This deletion bisected Domain I but left the MEF2 binding domain intact. Taken together, results obtained from the deletion mutants we have analyzed strongly support the hypothesis that Domain I and the MEF2 binding domain function together to support regulated expression of the *GLUT4* gene.

Our observation that a deletion of either the 30-bp Domain I or mutation of the MEF2 binding domain, within the context of the 895-hG4-CAT parental promoter construct, completely abolishes transgenic expression. This is consistent with previous data in which we deleted both elements together (31) in the context of 895-hG4-CAT. In contrast, deletion of these regulatory elements from the 5′end (−895) to either position −730 or −412, causes an unrestricted, unregulated basal expression of transgenic mRNA (30). It is likely that sequences upstream of Domain I (present in all constructs analyzed in this paper) may function to restrict basal promoter activity in the absence of transcriptional enhancers. Support for this hypothesis comes from our previous observation that the addition of sequences upstream of Domain I to the −412 deletion (895Δ730/412-hG4-CAT) inhibited expression of transgene sequences presumably by inhibiting the basal promoter (31). This may be part of the mechanism for tissue-specific regulation of the *GLUT4* gene promoter.

The sequence of the Domain I regulatory element does not match any known transcription factor binding motifs. To identify the DNA binding proteins interacting with Domain I, we used a yeast one-hybrid screen in which the Domain I binding site served as the target DNA-binding site. To eliminate false positives from this screen, positive cDNAs were tested for their inability to support growth of yeast that contained a mutant Domain I binding site as the target DNA-binding site. In the current study we report the characterization of one cDNA clone identified by this method. The sequence of this partial cDNA (Fig. 6*B*) does not coincide with any known cDNA; however, it is represented in multiple human expressed sequence tag clones of similar size. The amino acid sequence deduced from the open reading frame encoded by this partial cDNA does not match any known transcription factor or DNA binding protein. To confirm that this cDNA encoded an authentic Domain I binding site, we cloned the cDNA in-frame to generate a GST fusion protein (GST-$GEF_{db}$). The ability of GST-$GEF_{db}$ to bind to Domain I was demonstrated in EMSA. Specificity of this binding was demonstrated by the fact that formation of a DNA-protein complex could be competed by unlabeled wild-type oligonucleotide, but not by a mutant (Mut 1) Domain I oligonucleotide.

We generated an antibody against GST-$GEF_{db}$ to determine if this antibody would react with any proteins in the Domain I DNA binding complex. Several lines of evidence indicate that this antibody specifically reacts with a component of the Domain I-protein complex. The anti-GST-$GEF_{db}$ antibody detected proteins from heart nuclear extracts migrating at the same molecular mass as proteins that cross-linked to the Domain I binding site under ultraviolet radiation; two bands of approximate 70 and 50 kDa were observed by both immunoblotting and UV cross-linking analyses (Figs. 5 and 7). It is not known whether these bands arise from a single polypeptide, or if they represent conserved isoforms.

Pretreatment of nuclear extracts with anti-GST-$GEF_{db}$ IgG specifically interfered with Domain I binding in EMSA analysis but did not interfere with formation of other DNA-protein complexes (for example, OCT-1). Because our anti-GST-$GEF_{db}$ was raised against a fragment of the full-length protein containing the DNA binding domain, we expected that the antibody would compete for Domain I binding, reducing the formation of the shifted complex in the EMSA assay rather than producing a supershifted band. As expected, the antibody inhibited formation of the Domain I-protein complex, however, it was unable to completely inhibit binding. This result could be anticipated under conditions in which the affinity of the antibody for the Domain I binding protein is not high enough to quantitatively bind the antigen.

Interestingly, analysis of the mouse *GLUT4* promoter in 3T3-L1 adipocytes revealed an element responsible for insulin-mediated down-regulation of a *GLUT4* promoter-reporter gene in those cells (35). The element described in that study coincides exactly with the Domain I element described in the current study. We have shown that Domain I of the human *GLUT4* promoter is required for accurate promoter function in GLUT4-expressing tissues of transgenic mice. This same element appears to be required for down-regulation of promoter function during chronic insulin treatment in 3T3-L1 adipocytes. The relationship between the different functions of Domain I in these two very different systems is not immediately obvious. It is possible and perhaps likely that transcriptional regulation of the *GLUT4* gene occurs by somewhat different mechanisms in whole animals *versus* cultured cells. Consistent with this, mRNA expression from the *GLUT4* gene in response to chronic insulin treatment is different in the mouse *versus* 3T3-L1 adipocytes (reviewed in Ref. 36). Also consistent with the notion of differences in the two systems with respect to transcription factor binding, the mobility of the DNA-protein complex obtained from white adipose tissue nuclear extracts was different from that observed using extracts prepared from 3T3-L1 adipocytes (35).

It has been reported that in 3T3-L1 adipocytes, a member of the NF-1 family of transcription factor binds to a region of the mouse GLUT4 corresponding to Domain I (37). Although the NF-1 consensus binding site is not conserved in the human GLUT4 Domain I element, we have not ruled out that one or more NF-1 isoforms could bind the human Domain I element. More importantly, Cooke and Lane (37) clearly demonstrate that the NF-1 transcription factor represents only a fraction of the mouse Domain I binding complex formed in 3T3-L1 adipoctyes. In the same report they show that mutation of the NF-1 binding site did not reduce normal expression of the reporter construct, but instead prevented down-regulation of reporter expression in the presence of chronic insulin treatment, an effect that is observed in 3T3-L1 adipocytes and other cell lines. The DNA sequence and deduced amino acid sequence of our clone indicate that we have not identified a member of the NF-1 family of transcription factors. Thus, we have identified a novel DNA binding protein that is a direct Domain I binding protein in nuclear extracts obtained from tissues. Our data do not preclude that other proteins, including NF-1, are also involved in the Domain I binding *in vivo*.

Domain I binding activity is not strictly restricted to the

GLUT4-expressing tissues, because we observed binding activity in liver, a tissue that does not express the *GLUT4* gene. We have shown previously that high levels of MEF2 binding activity are restricted to GLUT4-expressing tissues, suggesting a potential role for MEF2 in tissue-specific expression (31). The data presented in this paper demonstrating that Domain I and MEF2 binding domains must function together indicates that tissue specificity is not likely to result from MEF2 levels alone. We predict that regulated and tissue-specific expression of the *GLUT4* gene occurs only in tissues where sufficient Domain I binding activity and MEF2 binding activity coincide. Regulation of the *GLUT4* gene under insulin-deficient conditions may reflect the levels of either Domain I binding activity or MEF2 binding activity. Support for this model comes from our previous observation that MEF2 binding activity is decreased during STZ diabetes, thus correlating with the down-regulation of *GLUT4* gene expression (31).

In summary, we have determined that the human GLUT4 promoter-enhancer is regulated by the functional cooperativity of two regulatory domains separated by 186 bp of intervening sequence. The regulatory element proximal to the site of transcription initiation is an MEF2 binding domain that complexes with isoforms of the MEF2 family of transcription factors (31). The distal regulatory element (Domain I) is a novel DNA binding element that does not share sequence similarity with any known transcription factor binding motifs. We have generated an antibody that reacts against at least one protein binding to Domain I. Isolation of the full-length cDNA clone encoding the Domain I binding protein is underway. Further studies are also underway to determine the mechanisms by which the two *cis*-acting regulatory elements function together to promote GLUT4 transcriptional activity.

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