Myocyte Enhancer Factor 2 (MEF2)-Binding Site Is Required for GLUT4 Gene Expression in Transgenic Mice

REGULATION OF MEF2 DNA BINDING ACTIVITY IN INSULIN-DEFICIENT DIABETES

(Received for publication, February 10, 1998, and in revised form, March 16, 1998)

Martin V. Thai‡, Suresh Guruswamy‡, Kim T. Cao‡, Jeffrey E. Pessin§, and Ann Louise Olson‡‖

From the ‡Department of Biochemistry and Molecular Biology, the University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190 and the §Department of Physiology and Biophysics, the University of Iowa, Iowa City, Iowa 52242

We have previously demonstrated that important regulatory elements responsible for regulated expression of the human GLUT4 promoter are located between −1154 and −412 relative to transcription initiation (Olson, A. L., and Pessin, J. E. (1995) J. Biol. Chem. 270, 23491–23495). Through further analysis of this promoter regulatory region, we have identified a perfectly conserved myocyte enhancer factor 2 (MEF2)-binding domain (−CTAAAATTAG−), that is necessary, but not sufficient, to support tissue-specific expression of a chloramphenicol acetyltransferase reporter gene in transgenic mice. Biochemical analysis of this DNA element demonstrated the formation of a specific DNA-protein complex using nuclear extracts isolated from heart, hindquarter skeletal muscle, and adipose tissue but not from liver. DNA binding studies indicated that this element functionally interacted with the MEF2A and/or MEF2C MADS family of DNA binding transcription factors. MEF2 DNA binding activity was substantially reduced in nuclear extracts isolated from both heart and skeletal muscle of diabetic mice, which correlated with decreased transcription rate of the GLUT4 gene. MEF2 binding activity completely recovered to control levels following insulin treatment. Together these data demonstrated that MEF2 binding activity is necessary for regulation of the GLUT4 gene promoter in muscle and adipose tissue.

To promote the storage of metabolic energy in the form of glycogen and triglycerides, insulin increases glucose uptake in skeletal muscle, heart, and adipose tissue. The GLUT41 facilitative glucose transporter protein is the main glucose transporter in fat and muscle tissue (1–6). GLUT4 protein is localized to the interior of the cell and moves to the plasma membrane in response to insulin receptor activation (7, 8). Several lines of evidence have shown that the effect of insulin on glucose uptake in these tissues results directly from the recruitment of GLUT4 from an intracellular vesicle pool to the plasma membrane (for recent reviews see Refs. 9 and 10).

Insulin-mediated glucose homeostasis is extremely sensitive to the overall GLUT4 protein pool size in the major insulin responsive tissues that include heart, skeletal muscle, and adipose tissue. In rodent models of insulin deficiency (fasting- or streptozotocin (STZ)-induced diabetes), the expression of GLUT4 protein and mRNA is markedly reduced and accounts for the insulin resistance of glucose transport under these conditions (11–17). Genetic studies strengthen the relationship between insulin-mediated glucose homeostasis and the cellular levels of GLUT4 protein. Mice engineered to express only one allele of the GLUT4 gene were shown to have diminished GLUT4 protein levels resulting in a progressive diabetic phenotype characterized by impaired glucose homeostasis (18). On the other hand, an increase in GLUT4 protein pool size enhances insulin-sensitive glucose uptake. Transgenic mice expressing the human GLUT4 gene specifically in adipose tissue or in both adipose and muscle tissues displayed a marked increase in basal glucose disposal and insulin-sensitive glucose uptake (19–25). In STZ diabetic mice, overexpression of GLUT4 in either fat or skeletal muscle under the control of heterologous tissue-specific promoters improves glucose homeostasis (26, 27). Finally, expression of the human GLUT4 gene in the genetically diabetic db/db strain of mice improved glycemic control and insulin sensitivity (28). The close association of GLUT4 protein pool size with physiologic changes in glucose homeostasis makes physiologic manipulation of GLUT4 gene expression an attractive target for therapeutic interventions designed to treat the insulin resistance associated with diabetes.

To achieve this goal, a detailed molecular understanding of the regulation of GLUT4 gene expression in a natural physiologic context is required. By using transgenic mice, we have previously reported that regions regulating expression of the human GLUT4 gene are found within 1154 bp of DNA located 5′ to the major transcription initiation site (29–31). The 1154-bp fragment regulatory DNA supported a pattern of gene expression that mimics the mouse GLUT4 gene both in terms of tissue-specific and regulated expression in both fasting and STZ-induced models of insulin deficiency (31). In the current study, we have determined an essential role for an MEF2 DNA-binding domain in regulation of the human GLUT4 gene promoter. In addition, we have begun to characterize the nuclear proteins that interact with this binding domain in the major GLUT4 expressing tissues.

MATERIALS AND METHODS
Preparation of Transgenic Mice—The CDNA constructs used to generate transgenic mice were derived from the plasmid hGLUT4(2.4)CAT containing 2400 bp of GLUT4 5′-flanking DNA (29). This plasmid was

* This work was supported by Research Grants DK47894, DK44612, and DK42452 from the National Institutes of Health and Award 196085 from the Juvenile Diabetes Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, The University of Oklahoma Health Sciences Center, P. O. Box 26891, Oklahoma City, Oklahoma 73190. Tel.: 405-271-2227; Fax: 405-271-3092.
‡ The abbreviations used are: GLUT4, adipose/muscle-specific glucose transporter; STZ, streptozotocin, bp base pair; SRF, serum response factor; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis, MCK, muscle creatinine kinase; MEF2, myocyte enhancer factor 2; Pipes, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.
used to generate 895-h4-G4-CAT by digestion of the parental plasmid with AvrII and SacI. An internal deletion in hGLUT4(2.4)CAT was made by digestion with RsaII and BssHIII, followed by treatment with T4 DNA polymerase in the presence of nucleotides to fill in the remaining nucleotide overhangs. The plasmid was then religated to form hGLUT4(2.4)CAT. This plasmid was used to generate 412-h4-G4-CAT by digestion with BclI and HindIII and 895S730/412-h4-G4-CAT by digestion with AvrII and HindIII. A third plasmid was generated in which the MEF2-binding site of hGLUT4(2.4)CAT (CTAAATAAG) was mutated to form an Apal site (CTGGGGCCCAG). This plasmid hGLUT4(2.4)apaCAT was used to generate 895APA-h4-G4-CAT by digestion with AvrII and HindIII. The DNA fragments were isolated by agarose gel electrophoresis and injected into the pronuclei of fertilized mouse embryos at the University of Iowa Transgenic Animal Facility (Iowa City, IA). Transgenic animals carrying the appropriate constructs were identified by slot blot analysis of isolated tail DNA using the 4.6-kilobase pair SacHindIII 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 body weight) following an overnight fast as described previously (32). Seventy-two hours after injection, tail vein blood samples were assayed for glucose concentration using chemstrips (Boehringer Mannheim). Animals with blood glucose levels greater than 400 mg/dl were considered diabetic. The diabetic animals were either left untreated or treated with 1 unit of insulin for a week to term. All experiments were performed on these diabetic mice.

Preparation of Nuclear Extracts—Tissues were harvested and snap-frozen in liquid nitrogen. The frozen tissues were pulverized in liquid nitrogen and homogenized in 10 volumes of homogenization buffer A (10 mM Hepes, pH 7.6, 100 mM KCl, 1 mM EDTA, 10% glycerol, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM PMSF, 2 μg/ml each aprotinin, leupeptin, and pepstatin A, and 6 μg/ml each leupeptin and aprotinin). The homogenate was centrifuged with a microcentrifuge at 13,000 × g for 10 min at 4 °C. The supernatant was dialyzed against buffer C (25 mM Hepes, pH 7.6, 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM PMSF, 2 μg/ml each aprotinin, leupeptin, and pepstatin A, and 6 μg/ml each leupeptin and aprotinin). N. Olson, University of Texas, Southwestern Medical Center, Dallas).

RNA Isolation—Total cellular RNA was isolated from snap-frozen tissue using the guanidium 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 until prepared for analysis.

RNase Protection Assay—An 890-bp SalI/EcoRI fragment of the mouse GLUT4-CAT plasmid, p69GLUT4-CAT (obtained from Dr. M. D. Lane, Johns Hopkins Medical School, Baltimore, MD) (35), was subcloned into the SalI/EcoRI site of pBluescript II (BS). This plasmid was linearized with BssH1 and used as a template to generate a 616-nucleotide antisense RNA probe that is able to anneal to either the 5′ end of mouse GLUT4 sequences or the 5′ end of CAT mRNA. The antisense RNA was labeled with [α-32P]UTP using T3 polymerase in an in vitro transcription assay (Promega). Ten μg of total RNA was hybridized with 5 × 10^5 cpm of labeled probe in 30 μl of hybridization buffer (80% deionized formamide, 0.4× NaCl, 40× M2.5, pH 6.4, and 1 mM EDTA) at 65 °C. A mixture of either left untreated or treated with 100 μl of a stock of regular insulin per day for 2 days. The mice were killed 5 days after STZ injection, and the tissues were snap-frozen in liquid nitrogen and stored at −70 °C until prepared for analysis.

In Vitro Translation—To generate in vitro translation products, cDNAs corresponding to human MEF2A and mouse MEF2C isoforms were subcloned into expression vectors, linearized, in vitro transcribed, and quantitated on a 1% agarose-formaldehyde gel. Equivalent amounts of RNA were used to program rabbit reticulocyte lysates in the presence and absence of radiolabeled [35S]methionine. The radiolabeled in vitro translation products were analyzed and quantitated by SDS-PAGE followed by autoradiography and by immunoblotting with the αMEF2A antibody (data not shown).

Electrophoretic Mobility Shift Assays—The human GLUT4 MEF2 DNA-binding site double-stranded oligonucleotide (GGGAGCTAA-GGATCCATGAC-NH2) was purchased from LifeTechnologies, Inc.). The MEF2F2 and OCT1 DNA-binding site oligonucleotides were commercially prepared (Santa Cruz). The oligonucleotides were end-labeled with T4 polynucleotide kinase. The probes (0.5 μg) were incubated with up to 14 μg of nuclear extracts in a 10-μl reaction containing 2 μg poly(dI-dC), 40 μM KCl, 5 μM MgCl2, 0.5 μM Hepes, pH 7.9, 1 M DTT, 10 μM PMSF, 2 μg/ml each aprotinin, leupeptin, and pepstatin A, and 6 μg/ml each aprotinin and leupeptin. The dialysate was assayed for total protein (Bradford) and stored at −70 °C.

RESULTS

Tissue-specific Expression of the Human GLUT4 Gene in Transgenic Mice—To identify the DNA sequences responsible for tissue-specific expression of the human GLUT4 gene, we have generated multiple lines of transgenic mice carrying CAT reporter gene constructs containing various deletions in the 5′-flanking DNA. In a previous report, we demonstrated that the smallest construct capable of supporting full promoter function required 1154 bp of the human GLUT4 5′-flanking DNA (31). To localize further functional elements within this region, we created transgenic mice carrying 895 bp of DNA fused to the CAT reporter gene (895-h4-G4-CAT). RNase protection analysis of these mice displayed a pattern of CAT expression that par-
Fig. 1. Tissue-specific expression of CAT mRNA in transgenic mice carrying different human GLUT4 5′-flanking DNA. Ten μg of total RNA isolated from white adipose tissue (W), brown adipose tissue (B), hindquarter skeletal muscle (S), liver (L), and brain (Br) was analyzed for CAT mRNA (A) and endogenous mouse GLUT4 mRNA (B) using RNAse protection assay as described under “Materials and Methods.” The schematic representation and names of constructs used to generate transgenic mice are shown in A. The solid line represents the human GLUT4 5′-flank, and major transcription start site of the human GLUT4 gene is indicated by the bent arrow. Nucleotide sequence for each construct is indicated relative to the transcription start site as +1.

alleled the expression of the endogenous murine GLUT4 mRNA (Fig. 1, A and B). The transgenic CAT and endogenous murine GLUT4 mRNAs were expressed at highest levels in brown adipose tissue and skeletal muscle with slightly lower levels in cardiac muscle followed by white adipose tissue. This protein is identical to transgene expression observed for larger segments of regulatory DNA (30). Importantly, there was no measurable expression of the 895-hG4-CAT transgenic mRNA in liver or brain, tissues which normally do not express GLUT4. Since expression of reporter genes in transgenic animals may be affected by the location of genomic integration and copy number, we analyzed five independent founder lines with essentially identical results (Table I). From these data we conclude that at least 895 bp of 5′-flanking DNA are required for expression of the GLUT4 gene.

In a previous study we reported that a deletion in the human GLUT4 promoter to position −730 relative to the transcription initiation site was only partially functional (31). This construct supported an unrestricted pattern of transgene expression that was quite high in skeletal muscle but low in heart and adipose tissue. This unregulated expression and regulated expression during insulin deficiency. The data implied that a regulatory element important for skeletal muscle-specific expression of GLUT4 resides within bp −730 and −412. To examine the contribution of this region for human GLUT4 promoter function, we prepared two constructs in which the DNA sequences between positions −730 and −412 were deleted (Fig. 1A). This internal deletion was created in two different plasmids with 5′ boundaries at either position −1154 (1154Δ730/412-hG4-CAT) or −895 (895Δ730/412-hG4-CAT). Deletion of these internal sequences ablated transgene irrespective of their precise 5′ boundaries in all of five independent transgenic lines (Fig. 1A, Table I).

Inspection of the −730 to −412 sequences revealed the presence of a consensus (CTAAAATAG) myocyte enhancer factor 2 (MEF2)-binding site located from −473 to −464. To determine if this potential MEF2-binding site contributed to GLUT4 promoter function, we mutated this site in 895-hG4-CAT to produce an ApaI site in its place (895[APA]-hG4-CAT) by replacement of the core AAAAT sequence with GGGCCC. Analysis of six independent founder lines demonstrated that 895[APA]-hG4-CAT was not significantly expressed in any tissues (Fig. 1A, Table I). These data indicate that the putative MEF2-binding site located within the region −730 to −412 is required for GLUT4 gene expression in heart, skeletal muscle, and adipose tissue. However, since 730 bp of the GLUT4 5′-flanking DNA, which encompasses this MEF2-binding site, do not support the full pattern of gene expression, this indicates that the MEF2 site is necessary but not sufficient for the normal tissue-specific GLUT4 promoter activity.

Regulated Expression of 895-hG4-CAT in Insulin-deficient Diabetic Mice—It was well established that in both rats and mice GLUT4 gene expression is down-regulated in insulin-deficient states due to a decrease in transcription rate of the gene (30, 36). This is an insulin-specific response since the decrease in transcription and loss of GLUT4 mRNA are fully restored following insulin therapy. To determine if 895-hG4-CAT was regulated by insulin deficiency in an analogous manner to the endogenous GLUT4 gene, we compared the expression of the mouse GLUT4 and transgenic CAT mRNAs in STZ-induced diabetes. Expression of CAT mRNA in white and brown adipose tissue, heart, and hindquarter skeletal muscle was reduced in diabetic mice in a manner that paralleled the down-regulation of the mouse GLUT4 gene (Fig. 2). Treatment of these diabetic mice with insulin restored both CAT mRNA and GLUT4 mRNA levels to normal or above normal levels (Fig. 2). These data, in conjunction with the 5′ and internal deletion analysis, suggest that the regulatory and tissue-specific elements are located downstream of nucleotide −895 and that sequences upstream of this position do not contribute to the function of the GLUT4 promoter with respect to tissue-specific expression and regulated expression during insulin deficiency.

Tissue-specific Expression of the MEF2A and MEF2C Isoforms—The MEF2-binding site in the human GLUT4 promoter is perfectly conserved in both the mouse and rat genes (37). This type of domain has been reported to specifically bind MADS box proteins of the MEF2 family of transcription factors (38). Although first described in nuclear extracts from cultured myoblasts, skeletal muscle, heart, and brain, some MEF2 isoforms such as MEF2A are more broadly expressed (39). The presence of MEF2 isoform mRNAs and immunoreactive proteins in adipose tissue has not been reported. Therefore, Northern blot analysis was performed using probes specific for either MEF2A or MEF2C to determine the tissue distribution of these genes with respect to the major GLUT4-expressing tissues.

| Construct | Number of founders tested | Number of founders that express transgenic mRNA |
|-----------|--------------------------|-----------------------------------------------|
| hGLUT4 [2.4] CAT<sup>a</sup> | 2 | 2 |
| 1975-hG4-CAT<sup>b</sup> | 4 | 4 |
| 1639-hG4-CAT<sup>b</sup> | 6 | 4 |
| 1154-hG4-CAT<sup>b</sup> | 3 | 3 |
| 730-hG4-CAT<sup>b</sup> | 6 | 6 |
| 412-hG4-CAT<sup>b</sup> | 5 | 5 |
| 895-hG4-CAT<sup>b</sup> | 5 | 5 |
| 1154Δ730/412-hG4-CAT<sup>b</sup> | 3 | 0 |
| 895Δ730/412-hG4-CAT<sup>b</sup> | 2 | 0 |
| 895[APA]hG4-CAT<sup>b</sup> | 6 | 0 |

<sup>a</sup> Transgenic mRNA expression was measured by RNase protection assay.

<sup>b</sup> Expression of this transgenic construct was originally reported in Ref. 28.

<sup>c</sup> Expression of this transgenic construct was originally reported in Ref. 30.
MEF2C mRNA was detected at highest levels in skeletal muscle and brain, with much lower levels in white and brown adipose tissue and heart (Fig. 3A). On the other hand, MEF2A mRNA was highly expressed in brown and white adipose tissue, heart, and skeletal muscle as well as in brain, whereas a much lower level of mRNA was detected in liver (Fig. 3B). To determine the levels of MEF2A protein, nuclear extracts prepared from heart, skeletal muscle, and brown adipose tissue were also subjected to Western blot analysis using an antibody against MEF2A. Western blot analysis confirmed the presence of MEF2A immunoreactive material in brown adipose tissue, heart, and skeletal muscle but not in liver (Fig. 3C). Although the MEF2A antibody can cross-react with MEF2C (see below), the presence of both MEF2A mRNA and protein in the major GLUT4 expressing tissues coupled with the functional requirement of the MEF2-binding site strongly suggests that this isoform is likely to play a role in GLUT4 gene expression.

**Specificity of Nuclear Proteins That Bind the GLUT4 MEF2 Site**—To characterize proteins that bind to the human GLUT4 promoter MEF2-binding site, heart nuclear extracts were incubated with a radiolabeled 18-bp oligonucleotide corresponding to the GLUT4 MEF2 site. Electrophoretic mobility shift assay (EMSA) revealed the presence of a diffuse band that was specific to the MEF2-binding site. As a control, an oligonucleotide corresponding to the MEF2-binding site of the muscle creatinine kinase (MCK) MEF2 site was used. The MCK MEF2 site contains a GC-rich sequence that binds the ubiquitously expressed family of OCT1 transcription factors, was unable to compete for the heart nuclear extract binding to the radiolabeled GLUT4 MEF2 oligonucleotide (Fig. 4A, lanes 8–10). These data confirm that this nuclear binding activity was specific for the MEF2 DNA-binding domain.

Since mutation of the MEF2-binding consensus site resulted in a loss of function in the 895[APAl]hG4-CAT transgenic construct, we also examined the ability of an 18-bp oligonucleotide carrying the mutated MEF2-binding site to compete for heart nuclear extract binding (Fig. 4B). Incubation with either a 2-, 5-, 10-, or 20-fold molar excess of the unlabeled GLUT4 MEF2 oligonucleotide effectively prevented the formation of this DNA-protein complex in a dose-dependent manner (Fig. 4B, lanes 5–10). These data are consistent with the hypothesis that a DNA-protein complex must form at this site to permit function of the GLUT4 promoter.

**Biochemical Analysis of Nuclear Proteins That Bind the GLUT4 MEF2 Site**—To identify the nature of this protein-DNA complex, we next preincubated the heart nuclear extracts with the cMEF2A antibody or a control IgG (Fig. 5). As observed in Fig. 4, incubation of heart nuclear extracts with the 18-bp MEF2 oligonucleotide resulted in the formation of a DNA-protein complex that was specific for MEF2A. The presence of both MEF2A mRNA and protein in the major GLUT4 expressing tissues coupled with the functional requirement of the MEF2-binding site strongly suggests that this isoform is likely to play a role in GLUT4 gene expression.
MEF2 probe (molar excess of unlabeled oligonucleotides corresponding to the GLUT4 binding domain complexed with heart nuclear extracts. The samples were then incubated with 1 mM-32P]ATP and incubated without (lane 1) or with 10 μg of heart nuclear extract (lanes 2–10). The heart nuclear extracts were also preincubated for 5 min in the absence (lanes 2, 5 and 8) or presence of a 5-fold (lanes 3, 6 and 9) or a 30-fold (lanes 4, 7 and 10) molar excess of unlabeled oligonucleotides corresponding to the GLUT4 MEF2 probe (lanes 3 and 4), the MCK MEF2-binding site (lanes 6 and 7), or an OCT1-binding site (lanes 9 and 10). The samples were then resolved by non-denaturing gel electrophoresis (EMSA) and autoradiography as described under “Materials and Methods.”

FIG. 4. Heart nuclear extracts contain proteins that specifically bind the consensus GLUT4 MEF2-binding domain. A, an 18-bp oligonucleotide probe corresponding to the GLUT4 MEF2 domain was labeled with [-32P]ATP and incubated without (lane 1) or with 10 μg of heart nuclear extract (lanes 2–10). The heart nuclear extracts were also preincubated for 5 min in the absence (lanes 2, 5 and 8) or presence of a 5-fold (lanes 3, 6 and 9) or a 30-fold (lanes 4, 7 and 10) molar excess of unlabeled oligonucleotides. The formation of this complex was increased in direct proportion to the concentration of heart nuclear extract incubated with the GLUT4 MEF2 oligonucleotide (Fig. 5, lanes 3, 5, 7, and 9). Although the αMEF2A antibody was capable of inducing a supershift at all these concentrations of extract, only a fraction of this complex was affected (Fig. 5, lanes 2, 4, 6, and 8). Thus, it appears that although the formation of this complex was, in part, due to the binding of an MEF2 transcription factor, an unidentified complex can form independently of MEF2A immunoreactive protein.

In addition to the heart, nuclear extracts from skeletal muscle and brown adipose tissue also contained proteins that interacted with the GLUT4 MEF2-binding sites (Fig. 6A, lanes 3, 5, and 7). GLUT4 MEF2 binding activity was not observed in extracts isolated from liver nuclei (Fig. 6A, lane 9). The DNA-protein complexes generated by the heart, skeletal muscle, and brown adipose tissue nuclear extracts were all similar in abundance and mobility. Furthermore, pretreatment of nuclear extracts with the αMEF2A antibody supershifted a similar portion of these complexes demonstrating that MEF2A immunoreactive material was partially responsible for the total binding activity in all of these tissues (Fig. 6A, lanes 2, 4 and 6). Since no binding activity was observed in the liver nuclear extracts, we next probed these extracts with an oligonucleotide corresponding to the OCT1-binding site (Fig. 6). Electrophoretic mobility assay of the labeled OCT1-binding site demonstrated that the liver extract was fully competent for DNA binding activity. Thus the GLUT4 MEF2 site binding activity was specific for GLUT4-expressing tissues.

To confirm further the involvement of the MEF2 transcription factor in GLUT4 DNA binding, we compared the binding activity of heart nuclear extracts with that of in vitro translated MEF2C and MEF2A protein (Fig. 7). As previously observed, incubation of heart nuclear extracts with the labeled GLUT4 MEF2 oligonucleotide resulted in the appearance of a DNA-protein complex that was partially supershifted in the presence of the αMEF2A antibody (Fig. 7A, lanes 1–3). Incubation of the oligonucleotide probe with in vitro translated MEF2A resulted a diffuse DNA-protein complex that migrated similarly to the heart nuclear extract complex (Fig. 7A, lane 5). However, in contrast to the heart nuclear extract, preincubation with the αMEF2 antibody resulted in a complete mobility shift of the entire complex (Fig. 7A, lane 4). The MEF2C in vitro translation products also formed a diffuse complex that migrated more rapidly than the MEF2A complex (Fig. 7A, lane 7). Similar to MEF2A, preincubation with the αMEF2A antibody resulted in the complete retardation in electrophoretic mobility of the MEF2C-DNA complex (Fig. 7A, lane 6). The ability of the αMEF2A antibody to cross-react with MEF2C was not surprising in that the epitope used to generate this antibody is largely conserved in these two MEF2 isoforms. As controls, unprogrammed lysates from the in vitro translation reactions confirmed the requirement of MEF2A and MEF2C protein for these DNA binding interactions (Fig. 7A, lanes 8 and 9). Additionally, the sequence specificity of these binding events was demonstrated by the ability of a 5- and 30-fold molar excess of the unlabelled GLUT4 MEF2 oligonucleotide to compete for the DNA binding of the heart nuclear extract (Fig. 7B, lanes 2–4), in vitro translated MEF2A (Fig. 7B, lanes 5–7), and in vitro translated MEF2C (Fig. 7B, lanes 8–10).

STZ Diabetes Decreases MEF2 Binding Activity—Since the MEF2-binding site appeared to be critical for GLUT4 promoter function, we postulated that this binding site may be necessary for regulated expression during states of insulin deficiency. Nuclear extracts were prepared from heart and skeletal muscle obtained from control, STZ diabetic mice, and diabetic mice that were subsequently treated with insulin (Fig. 8). Electro-
and subscapular brown adipose tissue (B, lanes 2, 4, 6, and 8) or with 1 μg of rabbit IgG (IgG, lanes 3, 5, 7, and 9) for 1 h at 4 °C. The samples were then resolved by non-denaturing gel electrophoresis (EMSA) and autoradiography as described under “Materials and Methods.” Lane 1 is probe incubated without nuclear extract. The consensus OCT1-binding domain was 32P-labeled and incubated without (lane 1) or with 10 μg of total nuclear extracts from heart (lane 2), hindquarter skeletal muscle (lane 3), subscapular brown adipose tissue (lane 4), and liver (lane 5). The samples were then resolved by non-denaturing gel electrophoresis (EMSA) and autoradiography as described under “Materials and Methods.”

Previously, we examined the tissue-specific and hormonal/metabolic regulation of GLUT4 gene expression by generating transgenic mice carrying various 5’ GLUT4 promoter CAT reporter gene constructs (31). In that study, we determined that a fragment consisting of 1154 bp of the GLUT4 5’-flanking DNA was sufficient to support the full pattern of gene expression observed for the endogenous gene. Although a construct containing 730 bp of 5'-flanking data was able to support expression of the reporter gene in hindquarter skeletal muscle, the levels of expression were low in heart and adipose tissue, and expression was observed in tissues that do not normally express high levels of GLUT4 mRNA. Furthermore, we also noted that a deletion of the DNA upstream of position −412 relative to the site of transcription initiation functioned as a basal promoter that was not tissue-specific.

To define more precisely the functional GLUT4 regulatory sequences, we have now demonstrated that 895 bp of 5'-flanking DNA of the human GLUT4 gene is sufficient to support
expression of a CAT reporter gene that mirrors the expression of the mouse GLUT4 gene. Having established a more proximal 5’ boundary, internal deletions between positions −730 and −412 resulted in a complete loss of detectable GLUT4 promoter activity. Within this region, the GLUT4 gene contains a highly conserved 10-bp MEF2-binding sequence (CTA(A/T)TA(G/A)) that is completely conserved in the GLUT4 gene (CTA-AAAATAG) from humans, rats, and mice (37). Furthermore, in the context of the functional 885-bp GLUT4 promoter, a 6-bp substitution of the core MEF2-binding sequence (CTGGGC-CCAG) resulted in a complete abrogation of promoter function. Together, these data provide compelling evidence for the functional significance of the MEF2-binding site in GLUT4 gene expression, which is critical for the tissue-specific and hormonal/metabolic regulation of GLUT4 expression.

The functional significance of the MEF2-binding site in GLUT4 gene expression prompted us to determine the nature of DNA-protein binding complexes in the major GLUT4-expressing tissues, adipose and muscle. Electrophoretic mobility gel shift assays demonstrated the formation of a specific DNA-protein complex between the GLUT4 MEF2-specific oligonucleotide and nuclear extracts from heart, skeletal muscle, and brown adipose tissue. Although there was no apparent qualitative or quantitative difference in the shifted complexes obtained with these nuclear extracts, this complex was completely absent from liver, a tissue that does not express the GLUT4 gene.

Although the precise composition of this binding complex has not been fully elucidated, our data support a role for the MEF2A and/or MEF2C isoforms in both the binding and expression of the GLUT4 gene. Northern blot analysis demonstrated that MEF2A was highly expressed in muscle and adipose tissue, whereas the MEF2C isoform was only abundant in muscle and is therefore unlikely to contribute to GLUT4 expression in adipose tissue. However, since the MEF2A antibody was found to cross-react with the MEF2C isoform, the relative levels of protein expression in these tissues could not be determined. Nevertheless, the ability of MEF2A and MEF2C to specifically interact with the GLUT4 MEF2-binding sequence was also substantiated by in vitro translation of these transcription factors. In vitro translated MEF2A and MEF2C were both fully capable of inducing specific binding to the GLUT4 MEF2-binding sequence and underwent a complete mobility shift upon incubation with the MEF antibody (αMEF2).

Consistent with these observations, heart, skeletal muscle, and adipose tissue nuclear extracts also demonstrated an αMEF2 antibody-induced supershift. Surprisingly, however, only a fraction of the endogenous nuclear extract complex displayed reduced mobility, suggesting the presence of other unknown factors and/or additional complexes. At present, we have not ruled out the two other MEF2 isoforms (MEF2B and MEF2D), which have significantly different mobilities than either MEF2A and MEF2C in gel shift assays (40, 41). It is also unlikely that these complexes contain the serum response factor (SRF) since the upstream and downstream nucleotides flanking the core (AT)6 sequence of the GLUT4 MEF2-binding domain are incompatible for SRF binding (42). In addition, supershift binding with an SRF antibody was completely unable to induce a supershift of these complexes (data not shown). Further study will be required to determine the precise nature of the DNA-protein complex identity of this DNA-binding protein.

In any case, further support for the involvement of MEF2A and/or MEF2C was obtained by examining the regulation of GLUT4 gene expression in insulin-deficient diabetic mice. It has been well established that in states of insulin deficiency, there is marked reduction in GLUT4 gene expression due to a decrease in GLUT4 gene transcription, which can be restored upon insulin treatment (31, 36). In parallel to the inhibition of GLUT4 transcription, we have also observed that the formation of the GLUT4 MEF2 binding activity was decreased and also recovered following insulin therapy. Concomitant with the changes in binding activity, the protein levels of MEF2 were decreased in the diabetic state and recovered following insulin treatment. Since adipose tissue and heart do not express the MEF2C isoform, these changes appear to be specific for MEF2A. Based on these data, we hypothesize that the level of GLUT4 gene expression may be modulated, at least in part, by the level of MEF2A binding activity in adipose tissue and probably in skeletal muscle as well.

In addition to the complexity of regulation at the GLUT4 MEF2 binding consequence, it is clear that other elements of the GLUT4 promoter have significant impact on expression. For example, expression of the CAT reporter gene containing 730 bp of GLUT4 5’-flanking DNA resulted in normal expression in skeletal muscle but not in heart or adipose tissue (31). This construct also failed to display hormonal/metabolic regu-
Transcriptional Regulation of the Human GLUT4 Gene

In summary, the data presented in this study demonstrate through functional analysis of the GLUT4 promoter that a conserved MEF2 binding is necessary but not sufficient to support expression of the GLUT4 gene. Nuclear extracts from the major GLUT4-expressing tissues have significant MEF2 binding activity which appears to be qualitatively similar. This binding activity is absent in liver, a tissue which does not express the GLUT4 gene. MEF2 binding activity is specifically decreased in extracts obtained from STZ diabetic mice but is fully restored following insulin treatment. This decrease and subsequent recovery in binding activity is attributable to changes in MEF2A and/or MEF2C nuclear protein levels. Taken together, the functional and biochemical analyses of the GLUT4 MEF2-binding site support a role for this domain in the GLUT4 gene expression in skeletal muscle, heart, and adipose tissue.

REFERENCES

1. James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) Nature 333, 183–185
2. Birnbaum, M. J. (1989) Cell 57, 305–315
3. Charrot, M. J., Brosius, P. C., Ill, Alger, S. L., and Lodish, H. F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5253–5259
4. Fukumo, H., Kayano, T., Buse, J. B., Edwards, Y., Pilch, P. F., Bell, G. I., and Seino, S. (1989) J. Biol. Chem. 264, 17771–17774
5. Kaestner, K. H., Christy, B. J., McLennan, J. C., Braiterman, L. T., Corneliou, P., Pekala, P. J., and Lane, M. D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3150–3154
6. James, D. E., Strube, M., and Mueckler, M. (1989) Nature 338, 83–87
7. Cushman, S. W., and Wardzala, L. J. (1989) J. Biol. Chem. 265, 4758–4762
8. Suzuki, K., and Kono, T. (1986) Proc. Natl. Acad. Sci. U. S. A. 77, 2542–2545
9. Olson, A. L., and Pessin, J. E. (1996) Annu. Rev. Nutr. 16, 235–256
10. Stephens, J. M., and Pilch, P. F. (1995) Endocr. Rev. 16, 529–546
11. Berger, J., Biawas, C., Vicario, P. P., Strout, H. V., Saperstein, R., and Pilch, P. F. (1989) Nature 340, 70–72
12. Garvey, W. T., Huecksteadt, T. P., and Birnbaum, M. J. (1989) Science 245, 60–62
13. Garvey, W. T., Maianu, L., Huecksteadt, T. P., Birnbaum, M. J., Molina, J. M., and Ciaraldi, T. P. (1991) J. Clin. Invest. 87, 1072–1081
14. Sivitz, W. I., DeSautel, S. L., Kayano, T., Bell, G. I., and Pessin, J. E. (1989) Nature 340, 72–74
15. Sivitz, W. I., DeSautel, S. L., Kayano, T., Bell, G. I., and Pessin, J. E. (1990) Mol. Endocrinol. 4, 583–588
16. Charro, M. J., and Kahn, B. B. (1990) J. Biol. Chem. 265, 7994–8000
17. Bourre, R. E., Koranyi, L., James, D. E., Mueckler, M., and Permutt, M. A. (1990) J. Clin. Invest. 86, 542–547
18. Stenbit, A. E., Tso, T.-S., Li, J., Burellin, R., Grenen, D. L., Factor, S. M., Houseknicht, K., Katz, E. B., and Charro, M. J. (1997) Nat. Med. 3, 1096–1101
19. Liu, M.-L., Gibbs, E. M., McDaid, S. C., Milici, A. J., Stuenkenbrok, H. A., McPherson, R. K., Treadway, J. L., and Pessin, J. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 11346–11350
20. Hansen, P. A., Guel, E. A., Marshall, B. A., Gao, J., Pessin, J. E., Holloszy, J. O., and Mueckler, M. (1995) J. Biol. Chem. 270, 1679–1684
21. Shepherd, P. R., Gudri, L., Tuzo, E. Yang, H., Leach, F., and Kahn, B. B. (1993) J. Biol. Chem. 268, 22243–22246
22. Ren, J. M., Marshall, B. A., Guel, E. A., Gao, J., Johnson, D. W., Holloszy, J. O., and Mueckler, M. (1993) J. Biol. Chem. 268, 16113–16115
23. Ren, J. M., Marshall, B. A., Mueckler, M. M., McCalcule, M., Amatruca, J. M., and Shulman, G. I. (1995) J. Clin. Invest. 95, 429–432
24. Marshall, B. A., Ren, J. M., Johnson, D. W., Gibbs, E. M., Lillicio, J. S., Soeller, W. C., Holloszy, J. O., and Mueckler, M. (1993) J. Biol. Chem. 268, 18442–18445
25. Gue, E. A., Ren, J. M., Marshall, B. A., Gao, J., Hansen, P. A., Holloas, J. O., and Mueckler, M. (1994) J. Biol. Chem. 269, 18366–18370
26. Leturque, A., Loizau, M., Vautont, S., Salmimken, M., and Girard, J. (1996) Diabetes 45, 23–27
27. Tuzo, E., Gudri, L., and Kahn, B. B. (1997) Endocrinology 138, 1604–1611
28. Gibbs, E. M., Stote, J. L., McCoid, S. C., Stuenkenbrok, H. A., Pessin, J. E., Stevenson, R. W., Milici, A. J., and McNeish, J. D. (1995) J. Clin. Invest 95, 1512–1518
29. Liu, M.-L., Olson, A. L., Moe-Maye-Roww, W. S., Bus, J. B., Bell, G. I., and Pessin, J. E. (1992) J. Biol. Chem. 267, 11673–11676
30. Olson, A. L., Liu, M.-L., Moe-Maye-Roww, W. S., Bus, J. B., Bell, G. I., and Pessin, J. E. (1993) J. Biol. Chem. 268, 9839–9846
31. Olson, A. L., and Pessin, J. E. (1995) J. Biol. Chem. 270, 23491–23495
32. LeMarchand-Brustel, Y., Olichon-Berthe, C., Gremeaux, T., Tant, J. F., Rochet, N., and Van Obberghen, E. (1990) Endocrinology 127, 2687–2685
33. Chirgwin, J. M., Przybyla, A. E., Macdonald, R. J., and Butter, W. J. (1979) Biochemistry 18, 5294–5299
34. Olson, A. L., Perlam, S., and Robillard, J. E. (1990) Pediatr. Res. 28, 183–185
35. Kaestner, K. H., Christy, B. J., and Lane, M. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 87, 251–255
36. Gerrits, P. M., Olson, A. L., and Pessin, J. E. (1993) J. Biol. Chem. 268, 640–644
37. Liu, M.-L., Olson, A. L., Edgington, N. P., Moe-Maye-Roww, W. S., and Pessin, J. E. (1994) J. Biol. Chem. 269, 28514–28521
38. Shore, P., and Sharrack, A. D. (1995) Eur. J. Biochem. 229, 1–13
39. Doudou, E., Sparrrow, D. B., Mohun, T., and Treisman, R. (1995) Nucleic Acids Res. 23, 4277–4274
40. Han, T.-H., and Pyrwy, R. (1995) Mol. Cell. Biol. 15, 2907–2915
41. Molkenstain, J. D., Firulli, A. B., Black, B. L., Martin, J. F., Husted, C. M., Copeland, N., Jenkins, N., Lyons, G., and Olson, E. (1996) Mol. Cell. Biol. 16, 3814–3824
42. Pellegrini, L., Tan, S., and Richards, T. J. (1995) Nature 376, 409–408
43. Molkenstain, J. D., Black, B. L., Martin, J. F., and Olson, E. (1998) Cell 83, 1125–1136
44. Mao, Z., and Nadal-Giraud, B. (1996) J. Biol. Chem. 271, 14371–14375