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# A Sequence Element in the GLUT4 Gene That Mediates Repression by Insulin*

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**Prolonged treatment of 3T3-L1 adipocytes decreases expression of GLUT4, the insulin-responsive glucose transporter. Expression of promoter-reporter gene constructs that contained 2900 or 785 base pairs of 5′-flanking region of the murine GLUT4 gene was down-regulated by insulin ($p < 0.0005$), whereas expression of constructs that contained 641, 469, or 78 base pairs of 5′-flanking region was not. Nuclear extract from 3T3-L1 adipocytes protected the region from −707 to −681 in the GLUT4 5′-flanking region from DNase I digestion. Using an oligonucleotide probe that corresponded to this footprinted region, two major protein-DNA complexes were identified by a gel mobility shift assay. Southwestern analysis identified four protein bands with molecular masses from 38 to 46 kDa that bound to the insulin-responsive region probe. A reporter gene construct in which bases −706 to −676 were deleted was not repressed by insulin treatment, confirming that this sequence is necessary for the repression of the GLUT4 promoter by insulin in 3T3-L1 adipocytes. This sequence does not show homology to previously described insulin response elements and thus represents a distinct mechanism of gene regulation by insulin.**

GLUT4 is a member of the protein family of glucose transporters that mediates facilitated diffusion of glucose across cell membranes. GLUT4, the insulin-responsive glucose transporter, is present in adipose and muscle cells, where it catalyzes the rate-limiting step for glucose uptake and metabolism (1). Insulin increases glucose uptake into these cell types by stimulating the translocation of GLUT4 from an intracellular microsomal compartment to the plasma membrane. Perturbation of GLUT4 expression through overexpression in transgenic mice and underexpression through targeted gene disruption has been shown to increase and decrease insulin-stimulated glucose uptake, respectively, in these animals. In fact, even modest increases in GLUT4 expression have been shown to ameliorate insulin resistance in the db/db mouse (2) and to completely alleviate the insulin resistance that develops in mice fed a high fat diet (3).

Alterations in GLUT4 expression have been found in many models of insulin resistance (4). GLUT4 expression is decreased in adipose tissue in human obesity and type 2 diabetes mellitus (5, 6). The decreased expression of GLUT4 correlates with the decrease in insulin-stimulated glucose uptake in these adipocytes. However, GLUT4 expression is not decreased in muscle tissue in these states, and muscle tissue, not adipose tissue, accounts for the majority of whole body insulin-stimulated glucose uptake. Therefore, decreased expression of GLUT4 in adipocytes is not the primary site of decreased insulin-stimulated glucose uptake in insulin resistant states. Nonetheless, changes in glucose uptake into adipocytes can affect whole body glucose disposal as shown with transgenic mice in which a targeted increase in GLUT4 expression in adipose tissue increased whole body glucose disposal in both the basal and insulin-stimulated state (7). In addition, altered lipid metabolism in the adipocyte could affect glucose uptake into muscle (8, 9), and changes in GLUT4 expression in the adipocyte can alter lipid metabolism in the animal (10). Finally, the level of GLUT4 expression in adipocytes may play a role in the development of obesity by regulating adipose tissue development, because GLUT4-null mice have markedly decreased adipose tissue mass (11), and mice that overexpress GLUT4 in adipocytes have two to three times more adipose tissue (7). In addition, young fa/fa rats show increased expression of GLUT4 in adipocytes at a time when they are rapidly accumulating adipose tissue (12). Because changes in GLUT4 expression are able to alter so many aspects of energy utilization, better understanding of the mechanisms that mediate regulation of GLUT4 expression will be needed as attempts are made to modulate insulin sensitivity and to affect changes in the development of obesity.

A number of hormonal and metabolic stimuli have been found to regulate GLUT4 expression in adipose tissue (13). Expression of GLUT4 in adipocytes is decreased in obese, high fat fed and fasted animals. Furthermore, insulin deficiency in streptozotocin-induced diabetes leads to decreased GLUT4 expression in adipose tissue as well as in muscle tissue. Although insulin acutely stimulates an increase in GLUT4 activity at the plasma membrane, it has been shown to decrease GLUT4 gene expression in the 3T3-L1 adipocyte (14), an *in vitro* model of tissue adipocytes. This effect suggests that the decreased expression of GLUT4 found in adipose tissue in obesity and type 2 diabetes mellitus might be due to suppression of GLUT4 expression by the hyperinsulinemia that occurs in both of these conditions.

The effect of insulin on GLUT4 expression in 3T3-L1 adipocytes is rapid, with a decrease in GLUT4 mRNA levels occurring within 4 h of insulin treatment and a decrease in GLUT4 gene transcription occurring within 1 h. This effect is most likely mediated through the insulin receptor, because the down-regulation is seen with a relatively low level of insulin and requires a much higher level of IGF-I[1] (14). We have investigated the molecular mechanism underlying this action

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[1] The abbreviations used are: IGF-1, insulin-like growth factor-1; CAT, chloramphenicol acetyltransferase; RT, reverse transcriptase; PCR, polymerase chain reaction; SCD2, stearoyl CoA desaturase 2; C/EBP, CCAAT/enhancer-binding protein; kb, kilobase(s); bp, base pair(s); PEPCK, phospho*enol*pyruvate carboxykinase; IGFBP, insulin-like growth factor-binding protein.

of insulin on GLUT4 expression to identify the cis-acting elements in the GLUT4 gene that mediate this response and the trans-acting factors that interact with these elements.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum. The cells were induced to differentiate into the adipocyte phenotype by treating confluent cells with 0.5 mM isobutylmethylxanthine, 1 $\mu$M dexamethasone, and 167 nM insulin in 10% fetal bovine serum for 2 days, followed by treatment with 167 nM insulin in 10% fetal bovine serum for 2 days. The cells were then maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and fed every other day. Eight days after induction of differentiation a medium change was made, and on the ninth day, adipocytes were treated with 1 $\mu$M insulin. Cells were harvested 4 h after insulin treatment for preparation of nuclear extract or after 8 h for preparation of RNA.

*Plasmid Constructs*—Murine GLUT4/chloramphenicol acetyltransferase (CAT) reporter plasmids contained the 5′-flanking region of the GLUT4 gene, the GLUT4 transcription initiation site, 171 bp of GLUT4 5′-untranslated sequence, and the coding sequence for the bacterial CAT gene. The −785, −469, and −78 GLUT4/CAT plasmids were prepared as described previously (15). The −2,900 GLUT4/CAT plasmid was constructed by digesting −7000 GLUT4/CAT (15) with *Apa*I, filling in the ends using the Klenow fragment of DNA polymerase I, and re-ligating the plasmid. The −641 GLUT4/CAT plasmid was constructed by digesting −785 GLUT4/CAT with *Bss*H II and *Hin*dIII, filling in the ends using the Klenow fragment of DNA polymerase I, and re-ligating the plasmid. The −785ΔA GLUT4/CAT plasmid was constructed by digesting the −785 GLUT4/CAT plasmid with *Hin*dIII and *Sma*I to remove bases −785 to −469 of GLUT4 promoter. This sequence was then replaced with an insert generated by the polymerase chain reaction (PCR) using *Taq* and Pwo DNA polymerase (Expand, Boehringer Mannheim) to amplify two fragments encompassing bases −785 to −707 and bases −675 to −469 of the GLUT4 promoter. These fragments were connected at an *Eco*RI site within one of the primers for each fragment (the 3′-primer for the −785 to −707 fragment and the 5′-primer for the −675 to −469 fragment). The resulting plasmid was identical to the −785 GLUT4/CAT plasmid except that bases −706 to −676 had been replaced with the sequence 5′-GAATTC-3′. This plasmid was sequenced to confirm the ligated ends, the deleted sequence, and the PCR-amplified sequence.

*Stable Transfections*—Transfection was performed using the calcium/phosphate co-precipitation method (16). Subconfluent 3T3-L1 preadipocytes were transfected with 25 $\mu$g of the reporter construct and 2.5 $\mu$g of the pSV2Neo plasmid. The cells were incubated with the precipitate for 4 h and then shocked with 10% glycerol in phosphate buffered saline for 2 min. Cells were selected and maintained in 300 $\mu$g/ml G418 beginning 24 h after transfection. After 7–10 days, resistance foci of clones were pooled and maintained for further study. Two independent pools of 20–50 foci were studied for each construct.

*Quantitation of mRNA*—Cells were lysed in 5 M guanidinium isothiocyanate, 0.5% sarcosyl, and 5% $\beta$-mercaptoethanol, and total RNA was isolated by centrifugation over a 5.7 M CsCl cushion. For quantitation of the endogenous GLUT4 message, 10 $\mu$g of total RNA was separated by electrophoresis through a 1.2% agarose gel containing 6.5% formaldehyde. The RNA was transferred to a nylon membrane (Hybond-N; Amersham Corp.), fixed by UV irradiation, and hybridized with a 1.7-kb murine GLUT4 cDNA ($2 \times 10^6$ cpm/ml) that was labeled by random hexamer priming (Decaprime; Ambion, Inc.). Hybridization was performed in a solution containing 50% formamide, 4× SSC, 5× Denhardt's solution, 50 mM phosphate buffer (pH 7.0), 100 $\mu$g/ml yeast tRNA, 0.5 mg/ml sodium pyrophosphate, and 1% SDS at 42 °C for 16 h. The filter was washed at high stringency (0.1× SSC, 0.1% SDS at 65 °C) for 1 h. Band intensity of the 2.7-kb GLUT4 mRNA was quantitated on a Fujifilm BAS2000 bio-imaging autoanalyzer.

To quantitate the reporter mRNA levels, a quantitative reverse transcriptase (RT)-PCR assay was developed. Total RNA was treated with RNase-free DNase to remove contaminating DNA and then quantitated by UV spectroscopy. The quality and quantitation of the DNase-treated RNA was confirmed by examination of the 28 and 18 S RNA on an ethidium bromide-stained agarose gel. The competitor RNA was prepared by inserting a 14-bp linker sequence into the *Puv*II site in the coding sequence of the CAT gene. The *Msc*I to *Eco*RI fragment containing portions of the GLUT4 5′-flanking region, GLUT4 5′-untranslated region, and CAT coding region with the 14-bp linker sequence was inserted into the polylinker site of the pGEM2 plasmid. RNA was prepared from the *Nhe*I linearized plasmid from the T7 polymerase site using the Maxiscript kit per the manufacturer's directions (Ambion Inc.). This cRNA was quantitated by UV spectroscopy, aliquoted, and stored at −80 °C. 100 ng of total RNA from control or treated cells plus an aliquot of cRNA ($3 \times 10^{-3}$ to 0.8 pg) were reverse transcribed together using a CAT-specific primer (5′-CATCAGGCGGGCAAGAATGTGAATAAAGGC-3′) in a 20-$\mu$l reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 5 units RNase inhibitor, and 12.5 units MuLV reverse transcriptase (Perkin-Elmer). The RT product was then amplified using the same CAT-specific primer as the downstream primer and using a GLUT4-specific primer that hybridized to the GLUT4 5′-untranslated sequence as the upstream primer (5′-CAGGCCCGGACCCTATACCCTATTCATTTT-3′). The PCR reaction was performed in a 100-$\mu$l reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5.5 mM $MgCl_2$, 0.0008% gelatin, 200 nM of each dNTP (dATP, dCTP, dGTP, and dTTP), 2 $\mu$Ci of [$\alpha$-$^{32}$P]dATP and 1.25 units AmpliTaq DNA polymerase (Perkin-Elmer). The PCR was carried out for 40 cycles of 94 °C × 45, 65 °C × 30, and 72 °C × 20 s. Samples not treated with reverse transcriptase were included as negative controls. The PCR products were separated on a 6% acrylamide/8 M urea gel. The intensities of the product bands were quantitated on a Fujifilm BAS2000 bio-imaging analyzer. The quantity of the reporter gene RNA product was calculated by extrapolating between the sample where the 319-bp PCR product predominated (the cRNA product) and the sample where the 305-bp PCR product predominated (the GLUT4/CAT reporter gene product). Representative results are illustrated in Fig. 1.

*DNase I Footprinting Analysis*—DNase I footprint analysis was performed using the technique described by Brenowitz *et al.* (17). The *Hin*dIII − *Sma*I fragment of the GLUT4 5′-flanking region (bases −785 to −469) was ligated into the polycloning site of the Bluescript KS plasmid. To label the coding strand, the plasmid was linearized with *Hin*dIII, labeled with a fill-in reaction using the Klenow fragment of DNA polymerase (Megaprime; Ambion Inc.) in the presence of [$\alpha$-$^{32}$P]dATP, and digested from the plasmid with *Bam*HI. To label the non-coding strand, the plasmid was linearized with *Bam*HI, labeled with a fill-in reaction, and digested from the plasmid with *Hin*dIII. The probes were purified on a 6% acrylamide gel, followed by purification using a NACS-52 column (Life Technologies, Inc.). Nuclear extract was prepared from ninth day 3T3-L1 adipocytes following the method described by Lavery and Schibler (18). The following were added to each of the solutions during purification as inhibitors of protein phosphatase activity: 30 mM $\beta$-glycerophosphate (Sigma), 1 mg/ml *p*-nitrophenyl phosphate (Sigma), and 5 mM sodium vanadate (Fisher). The protein concentration of the nuclear extract was quantitated using the BCA Protein Assay Reagent (Pierce). 20 $\mu$l of 1× NUN solution (0.3 M NaCl, 1 M urea, 1% Nonidet P-40, 25 mM Hepes (pH 7.9), and 1 mM dithiothreitol) containing the indicated quantity of nuclear protein was added to 180 $\mu$l of binding buffer containing radiolabeled probe (100,000 cpm), 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM dithiothreitol, 50 $\mu$g/ml bovine serum albumin, 2 $\mu$g/ml calf thymus DNA, 100 mM KCl, and 2 $\mu$g of poly[d(I-C)]. The binding reaction was incubated on ice for 1 h. Probe/nuclear extract samples were brought to room temperature and 0.25–0.33 units of DNase I (Pharmacia) was added to the samples. The digestion reaction was stopped after 3 min by the addition of 200 $\mu$l of 1.3% SDS, 27 mM EDTA, 270 mM NaCl, and 50 $\mu$g yeast tRNA. The samples were extracted with phenol/chlorophorm/isoamyl alcohol (25:24:1) and precipitated in ethanol. Samples were resuspended in formamide loading dye and electrophoresed on a 6 or 10% acrylamide/8 M urea gel. A sequencing reaction was run concurrently to correlate protected regions with the corresponding GLUT4 sequence.

*Gel Mobility Shift Assay*—A 41-bp double-stranded oligonucleotide probe (probe a; see Table I), corresponding to bases −710 to −674 of the 5′-flanking region of the GLUT4 gene plus GATC on the 3′ end for labeling, was synthesized and labeled with [$\alpha$-$^{32}$P]dATP by a fill-in reaction (Megaprime; Ambion, Inc.) The probe for the corresponding region of the human gene was also synthesized (probe h) and included GATC on the 3′ end for labeling; the sequence encompassed bases 1264–1300 from Buse *et al.* (19) (see Table I; bold capital letters indicate bases that differ from the murine sequence.) Mutant probes (probes m1, m2, and m3), with base changes from the murine sequence introduced as indicated by bold capital letters in Table I, were also synthesized. $50–100 \times 10^3$ cpm of labeled probe was incubated with 2 $\mu$g of nuclear extract in a 30 $\mu$l solution containing 0.33× NUN buffer, 8.3% glycerol, 20 mM Hepes (pH 7.6), 2 mM dithiothreitol, 10 $\mu$g of bovine serum albumin, and 2 $\mu$g of poly[d(I-C)]. The binding reaction was incubated on ice for 1 h and then separated by electrophoresis on a polyacrylamide gel in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA). For competition reactions, 100 ng of unlabeled oligonucleotide (approximately 100-fold excess over the labeled probe) was added to the binding

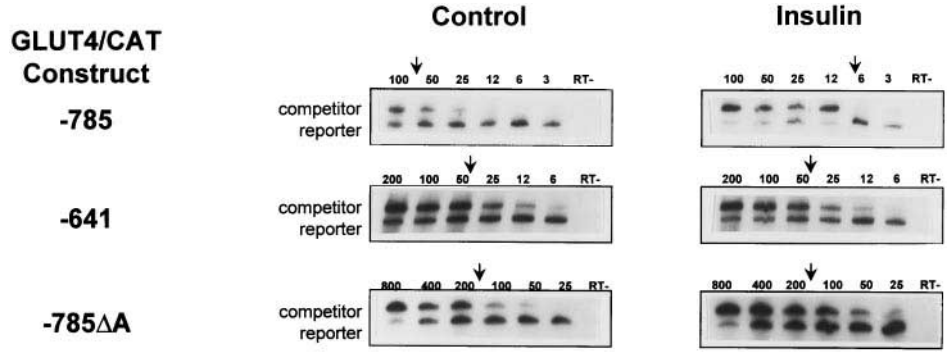


FIG. 1. **Insulin regulation of GLUT4/CAT reporter mRNA in stably transfected 3T3-L1 adipocytes.** Representative results of the competitive RT-PCR assay for the −785 GLUT4/CAT, −641 GLUT4/CAT, and −785ΔA GLUT4/CAT constructs are shown. Competitive RT-PCR assay was performed as described. RNA was prepared from 3T3-L1 adipocyte stable cell lines expressing GLUT4/CAT reporter genes. Treated cells were exposed to 1 μM insulin for 8 h. Quantity of competitor RNA is shown in $10^{-3}$ pg. Reporter quantitation (*arrows*) was calculated by interpolating between the adjacent samples where the first has a predominance of the competitor band and the next has a predominance of the reporter gene product band. *RT−* indicates negative control where no reverse transcriptase enzyme was added.

reaction. The −710 to −674 GLUT4 double-stranded oligonucleotide (probe a) was used as a specific competitor, and a 24-bp double-stranded oligonucleotide including the unrelated DNA binding sequence of the stearoyl-CoA desaturase 2 (SCD2) gene corresponding to bases −437 to −421 of the SCD2 5′-flanking region was used as a nonspecific competitor. Nuclear extract was prepared from adult mouse epididymal adipose tissue using the same technique described above for 3T3-L1 adipocyte nuclear extract except that the protein phosphatase inhibitors were not included in the preparation.

*Southwestern Analysis*—50 μg of nuclear extract was boiled for 10 min in 6% SDS and 60 mM dithiothreitol and then separated on a polyacrylamide gel. The stacking gel contained 5% acrylamide, 125 mM Tris (pH 6.8), and 0.1% SDS; the resolving gel contained 12% acrylamide, 375 mM Tris (pH 8.8), and 0.1% SDS. After separation, the proteins were transferred to a nylon membrane (Imobilon; Millipore) in 20% methanol, 25 mM Tris, 0.2 M glycine by electrotransfer at 15 V overnight. The filters were washed in denaturing buffer (25 mM Hepes (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol) with 6 M guanidine HCl and then renatured by five successive washings in 2-fold dilutions of the guanidine HCl in denaturing buffer, with a final incubation in denaturing buffer with no guanidine HCl. All washes were for 10 min at 4 °C. The filters were blocked in 5% nonfat dry milk in binding buffer (50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) for 1 h at room temperature and then hybridized overnight at 4 °C in binding buffer with 0.25% nonfat dry milk and $1 \times 10^6$ cpm/ml of labeled probe a. Competition was performed by adding 1 μg/ml of unlabeled double-stranded oligonucleotide (approximately 200-fold excess). Filters were washed with 0.25% nonfat dry milk in binding buffer at 4 °C for 30 min. Autoradiography was performed at −80 °C with Kodak X-Omat AR film (Eastman Kodak Co.) and an intensifying screen for the indicated times.

## RESULTS

*Identification of an Insulin-responsive Region in the 5′-Flanking Region of the GLUT4 Gene*—Previous studies showed that transcription of the GLUT4 gene is down-regulated by insulin in 3T3-L1 adipocytes (14). To locate the cis-acting elements of the GLUT4 gene responsible for the insulin-induced decrease in GLUT4 expression, a series of GLUT4 5′-flanking region-reporter gene constructs was prepared. These constructs contained various lengths of the murine GLUT4 5′-flanking region, the GLUT4 transcription start site, 171 bp of GLUT4 5′-untranslated region, and the coding sequence for bacterial CAT. The constructs were stably transfected into 3T3-L1 preadipose cells, after which two independent pools of foci for each construct were collected and used for study.

The stable cell lines expressing the GLUT4/CAT reporter constructs were induced to differentiate into cells exhibiting the adipocyte phenotype. On day 9 of the differentiation protocol, cells were treated with 1 μM insulin for 8 h and compared with untreated control cells. Expression of the endogenous GLUT4 gene was quantitated by Northern analysis of total

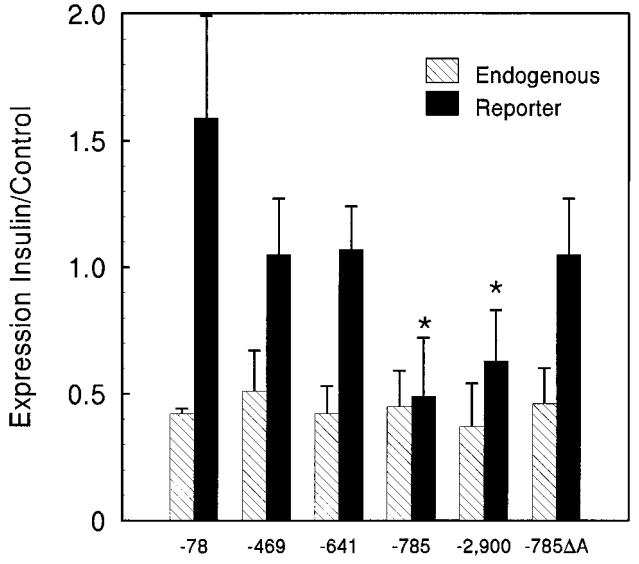


FIG. 2. **Effect of insulin on endogenous GLUT4 and GLUT4/CAT reporter gene mRNA levels.** Stable 3T3-L1 cell lines expressing GLUT4 promoter/CAT reporter gene constructs with 5′ ends at nucleotides −78, −469, −641, −785, and −2900 and a common 3′ end at +172 were prepared. −785ΔA GLUT4/CAT is identical to the −785 GLUT4/CAT plasmid except that bases −706 to −676 were replaced with the sequence 5′-GAATTC-3′. The cells were induced to differentiate into cells expressing the adipocyte phenotype, and on day 9 of the differentiation protocol, insulin was added to treated cells to a concentration of 1 μM. Cells were harvested after 8 h, and RNA was isolated. Endogenous GLUT4 mRNA was analyzed by Northern analysis and quantitated using a Fujifilm BAS2000 bio-imaging autoanalyzer. GLUT4 reporter/CAT mRNA was quantitated by a competitive RT-PCR technique (see "Experimental Procedures"). Results are expressed as a ratio of the expression level in treated cells to the expression level in control cells (mean ± S.D. of five or more independent experiments). *, $p < 0.0005$ different from 1.0. The absolute expression level in control cells for each of the constructs ($10^{-6}$ pg reporter/pg total RNA) was: −78 GLUT4/CAT = 31 ± 12; −469 GLUT4/CAT = 0.47 ± 0.27; −641 GLUT4/CAT = 0.58 ± 0.34; −785 GLUT4/CAT = 0.23 ± 0.14; −2,900 GLUT4/CAT = 0.88 ± 0.48; −785ΔA GLUT4/CAT = 1.5 ± 0.44.

RNA. In all transfected cell lines, insulin treatment reduced GLUT4 mRNA levels by 50–60%. Expression of the GLUT4/CAT reporter genes was quantitated by a competitive RT-PCR assay. Fig. 1 shows representative results of the RT-PCR assay for the reporter constructs that define the insulin-responsive region in the gene. As shown in Fig. 2, reporter genes containing 785 bp or 2.9 kb of GLUT4 5′-flanking region were repressed by insulin to a level similar to that of the endogenous GLUT4 gene. In contrast, reporter genes containing 641 bp or

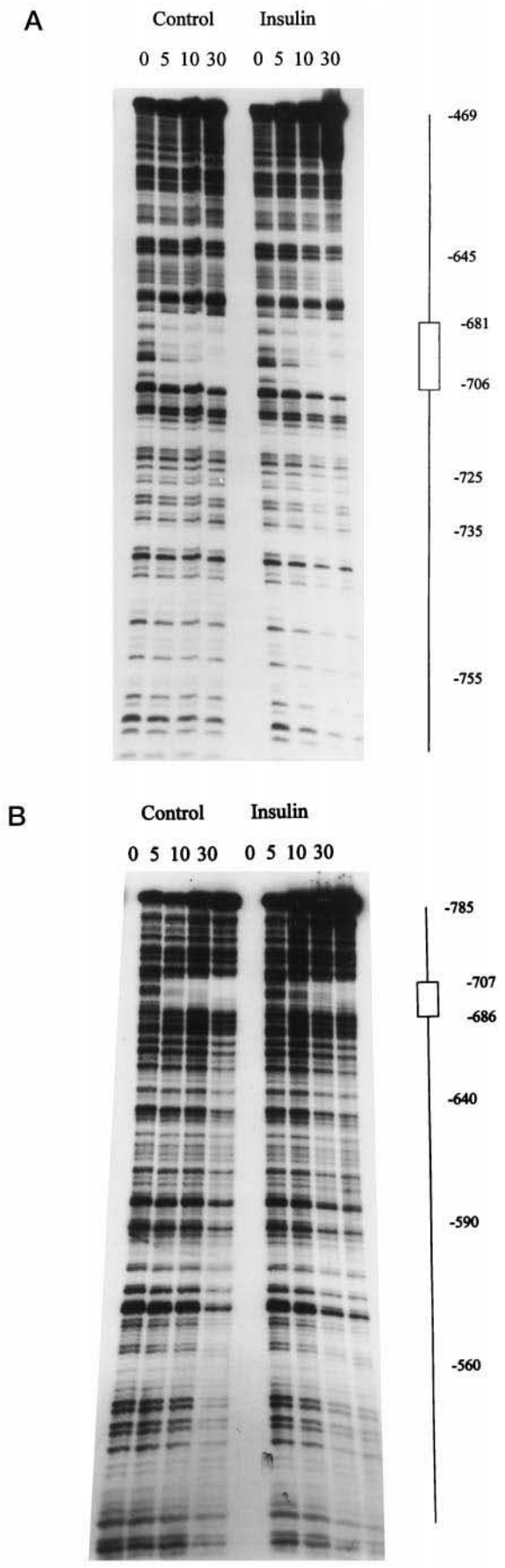


FIG. 3. **DNase I footprint analysis of the yGLUT4 promoter.** Nuclear extracts were prepared from control day 9 3T3-L1 adipocytes or from cells that were treated with 1 $\mu$M insulin for 4 h. The coding (*A*) or non-coding strand (*B*) of a fragment containing bases −785 to −469 of the 5′-flanking region of the murine GLUT4 gene was labeled on one end with [$\alpha$-$^{32}$P]dATP and incubated with the indicated quantity of nuclear extract ($\mu$g). The samples were then digested with DNase I and separated on a denaturing acrylamide gel. The *box* highlights the footprinted region protected from DNase I digestion. The *numbers* indicate the nucleotide number in the 5′-flanking region of the murine GLUT4 gene, as determined by concurrently run sequencing reactions.

less of 5′-flanking region were not repressed by insulin treatment. Thus, the region between −785 and −641 bp in the 5′-flanking region of the GLUT4 gene contains sequence necessary for insulin-induced down-regulation of GLUT4 expression.

*Identification of a Sequence Element in the Insulin-responsive Region Protected from DNase I Digestion*—To identify sites of nuclear protein-DNA interaction within the −785 to −641 bp region of the GLUT4 gene, DNase I footprinting analysis was conducted. The *Hin*dIII-*Sma*I fragment of the GLUT4 5′-flanking region, containing bases −785 to −469, was labeled on one end with [$\alpha$-$^{32}$P]dATP. The labeled probe was incubated with 3T3-L1 adipocyte nuclear extract and then subjected to digestion with DNase I. A single protected region was identified when either the coding or non-coding strand was labeled. Bases −681 to −706 were protected on the coding strand, and bases −686 to −707 were protected on the non-coding strand (Fig. 3). To determine if insulin treatment of 3T3-L1 cells altered the binding of nuclear proteins detected by the footprinting assay, the reaction was also carried out using nuclear extract from 3T3-L1 adipocytes that had been treated with insulin. As seen in Fig. 3, the footprint pattern was the same when the probe was incubated with nuclear extract prepared from control 3T3-L1 adipocytes or from cells treated with 1 $\mu$M insulin for 4 h.

*Identification of Nuclear Protein-DNA Complexes Binding to the Insulin-responsive Element*—To further characterize the interaction of specific nuclear proteins with the DNA sequence identified by DNase I footprinting, gel mobility shift assays were performed. A 41-bp double-stranded oligonucleotide was prepared that corresponded to the sequence from −710 to −674 bp in the 5′-flanking region of the murine GLUT4 gene. This oligonucleotide was labeled with [$\alpha$-$^{32}$P]dATP and then incubated with 2 $\mu$g of 3T3-L1 adipocyte nuclear extract after which the protein-DNA complexes were resolved by polyacrylamide gel electrophoresis. As shown in Fig. 4*A*, two major protein-DNA complexes were identified. Each of these complexes resulted from specific interactions of nuclear proteins with the labeled oligonucleotide, as they were competed away with an excess of unlabeled oligonucleotide but not by an excess of an unlabeled, unrelated oligonucleotide. As with the DNase I footprint pattern, the gel shift pattern did not differ when the oligonucleotide was incubated with nuclear extract from control 3T3-L1 adipocytes compared with that from cells treated with 1 $\mu$M insulin for 4 h.

To further define the sequence necessary for binding to the nuclear proteins, three mutant oligonucleotides (Table I) were tested for their ability to bind to 3T3-L1 adipocyte nuclear proteins and to compete for binding by the wild-type insulin-response region probe. As shown in Fig. 4*B*, mutant 1 (m1), with three base changes in the 5′ end of the oligonucleotide, was completely unable to bind the nuclear proteins in direct binding studies and was also unable to compete for binding with the wild-type probe. Mutant 2 (m2), with six base changes in the middle of the oligonucleotide, was able to bind, and to compete with the wild-type probe for binding, to the nuclear protein(s) that form the slower migrating major protein-DNA complex. However, m2 was unable to bind, or to compete for

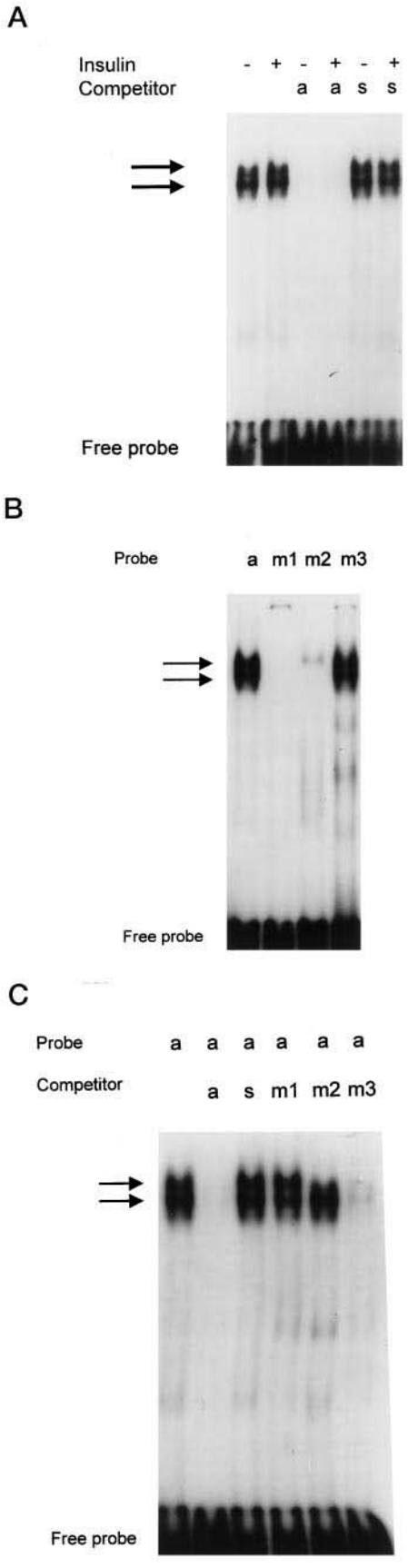


FIG. 4. **Binding of nuclear proteins from 3T3-L1 adipocytes to bases −710 to −684 of the 5′-flanking region of the GLUT4 gene.** *A*, 2 μg of nuclear extract from control day 9 3T3-L1 adipocytes (−) or from cells treated with 1 μM insulin for 4 h (+) was incubated with [α-$^{32}$P]dATP-labeled double-stranded oligonucleotide for 1 h on ice. 100-fold excess unlabeled oligonucleotide (*a*) was added to the incubation as a specific competitor as indicated. 100-fold excess of an unlabeled, unrelated oligonucleotide (SCD2, *s*) was added as a nonspecific competitor as indicated. *B*, 2 μg of nuclear extract from control day 9 3T3-L1 adipocytes was incubated with [α-$^{32}$P]dATP-labeled double-stranded oligonucleotide for 1 h on ice. Probes used were the native insulin-response region probe (*a*) and mutant probes m1, m2, and m3. *C*, for competition studies, 2 μg of nuclear extract was incubated with [α-$^{32}$P]dATP-labeled probe *a*, and 100-fold excess unlabeled probe was added as indicated. The protein-DNA mixtures were resolved on an 8% polyacrylamide gel. Gels were exposed to x-ray film at −80 °C for 1–4 h. These results were confirmed with four different preparations of nuclear extracts. *Arrows* indicate specific protein-DNA complexes.

TABLE I

*Oligonucleotide probes and footprinted sequence in the insulin-responsive region of the murine GLUT4 5′-flanking region*

Residues that differ from the wild-type murine sequence are shown in bold capital letters. The footprinted region (bases −707 to −681) is indicated for reference. a, wild-type murine GLUT4 sequence; h, human GLUT4 sequence; m1, m2, and m3, mutations of wild-type murine GLUT4 sequence.

| Probe | Sequence |
|---|---|
| a | 5′-cacctgtcccttgggtcccctccaagaaccagtgtaggatc-3′ |
| h | 5′-cac**T**tgtccct**C**gg**AC**c**GG**ctccaGgaacca**ACC**t**G**ggatc-3′ |
| m1 | 5′-cacctgtc**T**ctt**A**g**A**tcccctccaagaaccagtgtaggatc-3′ |
| m2 | 5′-cacctgtcccttgggtcc**TTCTTG**agaaccagtgtaggatc-3′ |
| m3 | 5′-cacctgtcccttgggtcccctccaaga**GTTGAC**gtaggatc-3′ |
| Footprint | 5′-ctgtcccttgggtcccctccaagaacc-3′ |
| | ↑ −707 ↑ −681 |

binding, to the protein(s) that form the faster migrating major complex. Mutant 3 (m3), with six base changes in the 3′ end of the oligonucleotide, was able to form the same protein-DNA complexes as the native probe and was able to completely compete for the complexes formed by the wild-type probe. Thus, the ability of the 3T3-L1 adipocyte nuclear proteins to bind to the insulin-responsive region is highly sequence-specific, as shown by the inability of proteins to bind to mutant 1. In addition, the results from the studies using the m2 probe show that the two major protein-DNA complexes include proteins that have different sequence specificities for their binding to the insulin-responsive region.

The sequence of the murine and human GLUT4 genes is highly conserved in the region we have identified as being insulin-responsive (Fig. 5). To determine whether the corresponding sequence of the human gene can bind 3T3-L1 adipocyte nuclear proteins and therefore can be a potential site for regulation, we prepared an oligonucleotide containing the sequence of the human GLUT4 5′-flanking region corresponding to the footprinted region from the murine gene. This sequence is 74% identical between the two species, with 20 of the 27 bases in the footprint region being identical. When the oligonucleotide with the human sequence was used as the probe in the gel shift assay, a similar pattern of protein-DNA complexes was identified using 3T3-L1 nuclear extract (Fig. 6*A*). However, whereas the murine probe produced two major protein-DNA complexes, the human oligonucleotide bound only to the faster migrating complex in both direct and competition binding experiments.

Nuclear extract from mouse adipose tissue was also tested to determine whether protein-DNA complexes similar to those produced by nuclear extracts from 3T3-L1 adipocytes would be generated. Nuclear extract from mouse adipose tissue incubated with the murine insulin-responsive region probe gave rise to a number of specific protein-DNA complexes, including one complex that corresponded to one of the major protein-DNA complexes formed with 3T3-L1 adipocyte nuclear extract (Fig. 6*B*). This appears to be identical to that produced by the human sequence probe and 3T3-L1 nuclear extract.

```
Murine  5′-ctgtcccttgggtcccctccaagaacc-3′
            ::::::: ::  :  ::::: :::::
Human   5′-ttgtccctcggaccggctccaggaacc-3′
```

FIG. 5. **Comparison of the sequence of the footprinted element in the insulin-responsive region of the murine GLUT4 5′-flanking region with the corresponding bases from the human GLUT4 gene.** Bases −707 to −681 of the 5′-flanking region of the murine GLUT4 gene are compared with the homologous bases from the human gene (bases 1267–1293 from Buse *et al.* (19)). The *dots* between the two sequences indicate identical bases.

To estimate the molecular mass of the nuclear proteins that interact with the insulin-responsive element, Southwestern analysis was performed. When nuclear proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nylon membrane, four specific protein bands were identified by hybridization with the labeled insulin-responsive region oligonucleotide (Fig. 7). The estimated sizes of these proteins were 38, 40, 45, and 47 kDa. The specificity of this binding was verified by the competition with unlabeled oligonucleotide and the absence of inhibition by an unrelated oligonucleotide. In some experiments, nuclear extract prepared from cells treated with insulin showed an increase in the amount of oligonucleotide hybridizing to the nuclear proteins; the signal was 2.1 ± 0.5 (mean ± S.D., $n = 4$) higher for all four bands in the insulin-treated lane compared with that of the control lane.

*Verification of the Identification of an Insulin-responsive Element*—The importance of the sequence identified by DNase I footprinting in mediating the response to insulin was assessed by reporter gene analysis with a mutated GLUT4 promoter-reporter construct. When the sequence from −676 to −706 bp was deleted from the −785 GLUT4/CAT reporter, down-regulation by insulin of the −785 bp of GLUT4 5′-flanking region was lost (Figs. 1 and 2). This confirms that bases −676 to −706 are essential for the repression of the GLUT4 promoter by insulin in 3T3-L1 adipocytes and thus constitute an insulin-responsive element.

## DISCUSSION

We have used promoter-reporter gene constructs to identify an insulin-responsive region in the GLUT4 gene. Our results show that, like the endogenous GLUT4 gene (14), expression of promoter-reporter constructs containing at least 785 bp of 5′-flanking region of the GLUT4 gene is decreased by insulin treatment in 3T3-L1 adipocytes, whereas expression of constructs containing 641 bp or less of 5′-flanking region is not altered by treatment with insulin. Previously, this laboratory had been unable to demonstrate repression by insulin of a transfected GLUT4 minigene that included 7-kb of 5′-flanking region (20). In contrast with the present work, those studies were performed with a single clonal cell line that had been specifically selected for a high level of GLUT4 expression and therefore may have selected against regulated expression. The present study was performed on duplicate independently prepared pools of transfected cells to minimize the effect of integration site-specific events.

The sequence from bases −707 to −681 is protected from DNase I digestion by nuclear proteins from 3T3-L1 adipocytes. Deletion of this sequence results in loss of reporter gene regulation by insulin. Gel mobility shift assays using a probe with a block mutation of bases −683 to −678 showed the same pattern as that for the wild-type sequence, further delineating the DNA-binding sequence to bases −707 to −684. Thus, it appears that bases −707 to −684 in the GLUT4 5′-flanking region contain a sequence element necessary for the repression of GLUT4 expression by insulin in 3T3-L1 adipocytes. Gel mobility shift studies showed that the oligonucleotide corresponding to the insulin-responsive region bound to nuclear proteins from murine adipose tissue and produced complexes similar to those produced from 3T3-L1 adipocytes, suggesting that this element also functions *in vivo*. In addition, an oligonucleotide corresponding to the homologous human gene sequence was also

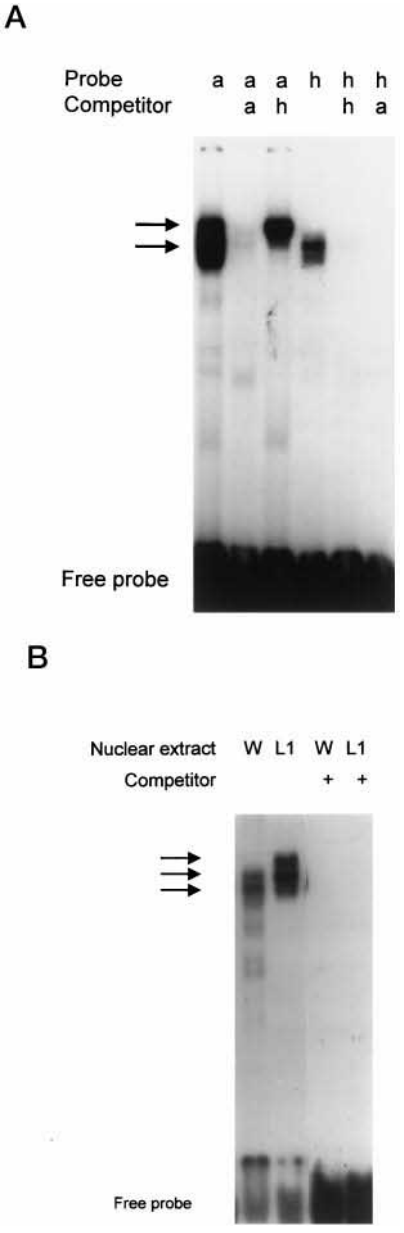


FIG. 6. **Binding of nuclear proteins from 3T3-L1 adipocytes and murine white adipose tissue to the insulin-responsive region of the murine GLUT4 gene and to the corresponding sequence of the human GLUT4 gene.** *A*, gel mobility shift assay comparing the insulin-responsive region of the murine GLUT4 gene to the homologous human sequence. 2 μg of nuclear extract from day 9 3T3-L1 was incubated with [α-$^{32}$P]dATP-labeled double-stranded oligonucleotide for 1 h on ice. The nuclear extract was incubated with a probe containing bases −710 to −684 of the 5′-flanking region of the murine GLUT4 gene (*a*) or with a probe containing the homologous sequence from the human GLUT4 gene (*h*). Competition studies were performed by adding 100-fold excess unlabeled oligonucleotide to the incubation as indicated. *B*, gel mobility shift assay comparing nuclear extract from 3T3-L1 adipocytes with that of mouse adipose tissue. 2 μg of nuclear extract from day 9 3T3-L1 (*L1*) or 10 μg nuclear extract from mouse epididymal white adipose tissue (*W*) was incubated with [α-$^{32}$P]dATP-labeled double-stranded oligonucleotide (probe a) for 1 h on ice. 100-fold excess unlabeled oligonucleotide was added as a specific inhibitor, as indicated. The protein-DNA mixtures were resolved on an 8% polyacrylamide gel. Gels were exposed to x-ray film at −80 °C for 2–4 h. *Arrows* indicate specific protein-DNA complexes.

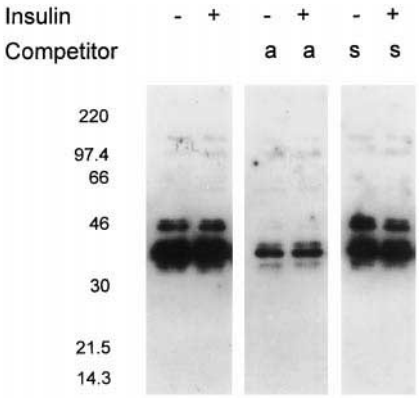


FIG. 7. **Southwestern analysis to detect proteins that bind to bases −710 to −684 of the 5′-flanking region of the GLUT4 gene.** 50 μg of nuclear extract from control day 9 3T3-L1 adipocytes (−) or from cells treated with 1 μM insulin for 4 h (+) was separated on a denaturing SDS-polyacrylamide gel. After transfer to nylon membrane, the immobilized proteins were denatured, renatured, and then hybridized with [α-$^{32}$P]dATP-labeled double-stranded oligonucleotide probe. The hybridization was carried out in the absence or the presence of excess unlabeled oligonucleotide (*a*) or excess of an unlabeled, unrelated oligonucleotide (SCD2, *s*). After washes, the filters were exposed to x-ray film at −80 °C for 16 h. Apparent molecular masses (kDa) of protein standards are shown. These results were confirmed with four different preparations of nuclear extracts.

able to bind to 3T3-L1 adipocyte nuclear proteins, indicating that this element may regulate GLUT4 expression in human adipose tissue.

As an initial attempt at elucidating the mechanism for the change in expression induced by insulin, we looked for changes in the DNA sequence footprinted by nuclear proteins from insulin-treated cells compared with that from control cells. No difference was observed. Similarly, no difference in the protein-DNA complexes identified by the gel mobility shift assay was seen between nuclear extract prepared from insulin-treated cells compared with control cells. Finally, Southwestern analysis identified the same set of nuclear proteins from control and insulin-treated cells. The lack of an increase in binding detected by the gel mobility shift assay suggests that the small and variable increase in the quantity of the probe bound to nuclear proteins from insulin-treated cells in the Southwestern analysis is not a significant finding. The lack of insulin-induced changes in protein-DNA interactions detected by DNase I footprinting or gel mobility shift assays has been observed with insulin-responsive sequences from other genes. Perhaps the most extensively examined insulin-responsive gene is that for phospho*enol*pyruvate carboxykinase (PEPCK). Repression of PEPCK expression is not correlated with changes in gel mobility shift pattern of the insulin-responsive sequence (21, 22). Similarly no change is observed in the gel mobility shift pattern for the insulin-responsive sequences of the IGFBP-1 (22, 23) or glucagon (24) genes, genes that are repressed by insulin, or for the pattern for the insulin-responsive sequence of the amylase gene (25, 26), a gene that is transcriptionally activated by insulin treatment. In contrast, insulin was shown to alter the protein-DNA complexes identified by gel mobility shift in a number of other genes whose expression is stimulated by insulin, including the IGFBP-3 (27), prolactin (28), fatty acid synthase (29), c-*fos* (30), and glyceraldehyde-3-phosphate dehydrogenase (31) genes.

We previously showed that the repression of GLUT4 expression by insulin in 3T3-L1 adipocytes was not dependent on new protein synthesis, because the effect was not blocked by cycloheximide (15). It is likely, therefore, that the effect is mediated by post-translational modification of an existing transcription factor, with phosphorylation or dephosphorylation being a possible mechanism. The present results that show no change in the patterns on the DNase I footprint analysis or gel mobility shift assay induced by insulin treatment suggest that this modification does not alter the DNA binding of the transcription factor *per se* but probably affects the interaction of the transcription factor with other proteins involved in transcription. Alternatively, it is possible that DNA binding activity is altered by a change in phosphorylation state *in vivo* but that the phosphorylation state was not maintained during the nuclear extract preparation, despite the use of protein phosphatase inhibitors.

We have identified four nuclear proteins that show specific interactions with the insulin-responsive sequence of the GLUT4 gene. These proteins have molecular masses ranging from 38 to 47 kDa (Fig. 7). A number of proteins have now been identified that are involved in mediating the regulation of gene expression induced by insulin. HNF-3 and possibly members of the C/EBP family interact with the insulin-responsive sequence of the PEPCK (22, 32) and IGFBP-1 (22) genes. Ets-related proteins have been implicated in mediating the regulation of the prolactin and somatostatin genes by insulin (33, 34), sterol regulatory element binding proteins mediate low density lipoprotein receptor expression regulation by insulin (35), and upstream stimulatory factors binding to E-box sequence (29, 36) and possibly Sp1 (37) mediate the stimulation of fatty acid synthase by insulin. In addition, a number of uncharacterized proteins have also been identified as mediating insulin-induced changes in gene expression including 90- and 70-kDa (IGFBP-3 (27)), 57-kDa (glyceraldehyde-3-phosphate dehydrogenase (38)), and 70−80-kDa (growth hormone (39)) proteins. The insulin-responsive sequence of the GLUT4 gene does not have significant homology to the PEPCK/IGFBP-1 core insulin response sequence (T(G/A)TTTTG (22)), Ets-related transcription factor response element (CGGA (33)), Sp1 binding site (GGGCGG), sterol regulatory element sequence (CACCCCAC (40)), or E-box sequence (CANNTG (36)). The only similarity between the insulin-responsive sequence of the GLUT4 gene to insulin response sequences from other genes (41) is homology to the insulin/glucose response element of the L-type pyruvate kinase gene and to the insulin response element of the thyrotropin receptor. However, the response of the thyrotropin receptor gene appears to be mediated by the IGF-I receptor, not the insulin receptor, and the regulation of both the thyrotropin receptor and L-type pyruvate kinase genes is dependent on new protein synthesis, which is not required for the regulation of GLUT4 by insulin. Finally, the proteins we have identified by Southwestern analysis do not correspond in size to the other identified proteins. Therefore, the regulation of GLUT4 by insulin appears to involve a unique DNA-binding protein.

Several pieces of evidence suggest that C/EBP-α could be a regulator of GLUT4 expression. C/EBP-α has been shown to have the ability to transactivate the GLUT4 promoter (42). In addition, C/EBP-α may be involved in the regulation of PEPCK gene expression by insulin (22), and Hemati *et al.* (45) have recently found that repression of GLUT4 expression by insulin in 3T3-L1 adipocytes correlates with the dephosphorylation of C/EBP-α. However, as noted above, there is no sequence with homology to a C/EBP binding site in the insulin-responsive sequence we have identified. The studies that demonstrated transactivation of GLUT4 by C/EBP-α found the effect to be mediated by the proximal 469 bp of 5′-flanking region of the GLUT4 gene and identified a C/EBP binding site at bases −254 to −262. Nonetheless, we were interested in determining whether C/EBP-α was present in the protein-DNA complexes

identified by the gel mobility shift assay. C/EBP-$\alpha$ antibody altered the mobility of protein-DNA complexes from 3T3-L1 adipocyte nuclear extract incubated with a consensus C/EBP binding site oligonucleotide but did not alter the mobility of the protein-DNA complexes identified using the −676 to −706 bp oligonucleotide of the insulin-responsive region.[2] Therefore, C/EBP-$\alpha$ does not appear to be present in the protein-DNA complexes. Although these findings show that C/EBP-$\alpha$ does not interact directly with the insulin-responsive region of the GLUT4 gene, it is still possible that C/EBP-$\alpha$ is involved in the regulation of GLUT4 by insulin, but this would have to occur through the interaction of the protein-DNA complexes at the insulin-responsive region with C/EBP-$\alpha$ that is bound at a separate site.

We have found that bases −707 to −684 in the GLUT4 5′-flanking region mediate the regulation of GLUT4 by insulin in 3T3-L1 cells. These bases are within a region that has a high degree of sequence homology between the human and murine genes and has been identified as a region that mediates regulation of GLUT4 by a number of other stimuli. The regulation of GLUT4 in skeletal muscle by exercise and in skeletal muscle and white adipose tissue by a high fat diet has been localized to bases −441 to −1000 of the murine GLUT4 gene using transgenic mice (43). Olson and Pessin studied the regulation of the human GLUT4 promoter using reporter genes in transgenic mice (44). They found that 1154 bp of the 5′-flanking region was sufficient to confer regulation in muscle and adipose tissue by uncontrolled diabetes. A reporter with only 730 bp of 5′-flanking region, which by sequence homology would interrupt the insulin-responsive region of the murine gene at base −698, was not regulated. We have identified only a single DNase I protected site in the sequence from −785 to −480 using nuclear extract from 3T3-L1 nuclear extract, raising the possibility that these different effectors converge to regulate GLUT4 expression through a common region of the promoter.

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