Hormonal/Metabolic Regulation of the Human GLUT4/Muscle-fat Facilitative Glucose Transporter Gene in Transgenic Mice*

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To examine the hormonal/metabolic as well as tissue-specific expression of the GLUT4/muscle-fat facilitative glucose transporter gene, we have generated several transgenic mouse lines expressing a human GLUT4 mini-gene which extends 5.3 kilobases (kb) upstream of transcription start and terminates within exon 10. This construct (hGLUT4-11.5) was expressed in a tissue-specific pattern identical to the endogenous mouse GLUT4 gene. The transcription initiation sites of the transgenic construct were similar to the GLUT4 gene expressed in human tissues. To investigate the hormonal/metabolic-dependent regulation of GLUT4, the transgenic animals were made insulin-deficient by streptozotocin (STZ) treatment. In these animals, STZ-induced diabetes resulted in a parallel decrease in endogenous mouse GLUT4 mRNA and the transgenic human GLUT4 mRNA in white adipose tissue, brown adipose tissue, and cardiac muscle. Similarly, insulin treatment of the STZ-diabetic animals restored both the endogenous mouse and transgenic human GLUT4 mRNA levels. To further define cis-regulatory regions responsible for this hormonal/metabolic regulation, the same analysis was performed on transgenic animals which carry 2.4 kb of the human GLUT4 5'-flanking region fused to a CAT reporter gene (hGLUT4[2.4]-CAT). This reporter construct responded similarly to the human GLUT4 mini-gene demonstrating that the element(s) controlling hormonal/metabolic regulation and tissue specificity all reside exclusively within 2.4 kb of the transcriptional initiation site.

The GLUT4/muscle-fat facilitative glucose transporter is specifically expressed in muscle and adipose tissue and is primarily responsible for mediating insulin-stimulated glucose uptake (1). In these tissues, acute insulin stimulation of glucose transport results from a rapid translocation of pre-formed GLUT4 proteins from an intracellular pool to the plasma membrane (2–6). In addition to the insulin-regulated GLUT4 protein translocation, insulin-deficient states such as fasting and streptozotocin (STZ)[1]-induced diabetes mellitus are also associated with altered levels of GLUT4 mRNA and protein in adipose tissue and skeletal and cardiac muscle (7–11). Recently, we have observed that the decrease in white adipose tissue GLUT4 mRNA can be primarily accounted for by a decrease in the transcription rate of the GLUT4 gene (12).

To investigate the molecular basis for tissue-specific and hormonal/metabolic regulation of GLUT4 gene expression in insulin-responsive tissues such as adipose and muscle, we have isolated and characterized the human GLUT4 gene (13). However, studies of the expression of a 2.4-kb fragment of the hGLUT4 promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene showed extremely weak promoter activity when transfected into cultured mouse 3T3-L1 adipocytes, a result inconsistent with the high levels of expression of this gene *in vivo* (13). Thus, in order to assess the tissue-specific as well as hormonal/metabolic regulation of GLUT4 gene expression in an appropriate physiological context, we have generated lines of transgenic mice carrying different amounts of human GLUT4 sequences. In this study, we have demonstrated that the mRNA and protein expression of a human GLUT4 mini-gene construct parallels that for the endogenous mouse gene and that sequences responsible for both tissue specificity as well as hormonal/metabolic regulation reside within 2.4 kb of the start of transcription.

EXPERIMENTAL PROCEDURES

Preparation of Transgenic Mice—An 11.5-kb *KpnI/EcoRI* fragment of the human GLUT4 gene was inserted into the vector pHSS6 (14) which was modified to include a *KpnI* site in the polylinker and to include the SV40 polyadenylation site (15) inserted at the *EcoRI* and *HindIII* sites, respectively. The human GLUT4 construct (hGLUT4-11.5) includes 5.3 kb of the 5'-flanking region and extends through exon 10; the *EcoRI* site is in the 3'-untranslated region. This construct codes for the full-length GLUT4 protein. The human GLUT4 gene, the 5'-flanking region and SV40 polyadenylation sequences were removed from the vector by *NotI* digestion. The fragment was gel-purified using Tris-acetate-EDTA agarose gel electrophoresis and injected into the pronucleus of fertilized mouse embryos (Jackson Laboratories Transgenic Mouse Facility, Bar Harbor, ME). Transgenic animals were identified by PCR amplification of tail DNA using primers 5'-GAG TATTTAGGGCCAGATGAGAAC-3' and 5'-GG TTACAAATAAAGCAATAGCATCAC-3' which amplify a 590-base pair region unique to the hGLUT4-11.5 construct that spans the junction of the human GLUT4 and SV40 sequences. The construction and isolation of mice carrying the hGLUT4[2.4]-CAT reporter gene

[1] The abbreviations used are: STZ, streptozotocin; CAT, chloramphenicol acetyltransferase; hGLUT4, human GLUT4.

was described previously (16). Heterozygous F1 and F2 transgenic progeny were obtained by mating the founder animals with C57/BL6 mice. Animals were studied between 6 and 12 weeks of age.

STZ-induced Diabetes—Animals were treated with 200 mg/kg STZ via intraperitoneal injection following a 6-h fasting period as described by LeMarchand-Brustel *et al.* (17). Sixty to 65 h post-injection, tail vein blood samples were tested for glucose concentration. Animals with blood glucose concentrations greater than 400 mg/dl were considered diabetic. Some diabetic animals were treated with 2 units of regular insulin and 3 units of long-acting insulin per day for 48 h. Diabetic animals were studied 84–89 h following STZ treatment.

Human RNA—Human fat tissue was obtained from patients undergoing gastroplasty. Informed consent was obtained from patients prior to surgery. A sample of omental fat (12–15 g) was obtained following completion of the surgical procedure. The sample was snap-frozen in liquid nitrogen until analysis.

RNA Isolation—Total cellular RNA was isolated from snap-frozen tissues using the guanidinium isothiocyanate-CsCl method (18) as described previously (19). RNA was quantified spectrophotometrically by absorbance at 260 nM and stored as an ethanol precipitate at $-70\ ^\circ C$.

Northern Blotting and Ribonuclease (RNase) Protection Assay—A probe (pJW3) was constructed which contained a 624-base pair *PstI/PvuII* fragment of the human GLUT4 gene. This probe contains all of exon 1 and includes sequences that are not conserved between human and rodent GLUT4 transcripts. The *PstI/PvuII* fragment was cloned into the *PstI/SmaI* site of Bluescript SK– (Stratagene). Plasmid DNA was linearized by digestion with *EcoRI* and T3 polymerase was used to generate a 682-nucleotide probe. A mouse GLUT4-specific probe was kindly provided by Dr. M. D. Lane (20). The ribonuclease (RNase) protection assays and Northern blot analysis were performed as described previously (16).

Western Blot Analysis—Crude membranes were prepared from tissues snap-frozen in liquid nitrogen. Briefly, the various tissues were homogenized in 10 volumes of ice-cold TES buffer (250 mM sucrose, 10 mM Tris, 2 mM EDTA, pH 7.4) containing a mixture of protease inhibitors (21). The homogenates were cleared by centrifugation at $3,000 \times g$ for 10 min followed by centrifugation of the supernatant at $200,000 \times g$ for 70 min 4 °C. The pellet containing the crude membranes were then resuspended in homogenization buffer. The samples (25–100 μg of protein) were fractionated on 10% reducing SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. The nitrocellulose membranes were then incubated with a rabbit GLUT4 polyclonal antibody (insulin-regulated glucose transporter, East Acres), followed by a 1-h incubation with 5×10^6 cpm/ml of ^{125}I-labeled protein A (Amersham Corp.) and autoradiography.

RESULTS

Tissue-specific Expression of a Human GLUT4 Transgene— To assess the tissue-specific and hormonal/metabolic-dependent regulation of the human GLUT4 gene, two different constructs were used to derive several lines of transgenic mice (Fig. 1). The hGLUT4-11.5 construct spans 11.5 kb of the human GLUT4 gene which contains 5.3 kb of 5'-flanking DNA and all of the protein coding region (13). The other

transgenic animals carried the hGLUT4[2.4]-CAT construct which consists of 2.4 kb of 5'-flanking DNA fused to the bacterial chloramphenicol acetyltransferase gene. To provide for appropriate mRNA processing, the CAT construct was engineered with an SV40 polyadenylation signal including the small t antigen intron. The hGLUT4-11.5 construct was constructed with the SV40 polyadenylation signal without the SV40 splice site. As described previously (16), two independent founder lines were established for hGLUT4[2.4]-CAT with copy numbers of 2 and 18. Similarly, four independent founder lines were obtained for the hGLUT4-11.5 construct with transgene copy numbers ranging from 25 to 64 (data not shown).

It has been well established that GLUT4 expression is restricted to tissues that display insulin-stimulated glucose transport activity such as adipose tissue (white and brown) and skeletal and cardiac muscle (22–26). To determine the pattern of expression of the hGLUT4-11.5 construct, we designed a ribonuclease protection assay using the unique 5'-end of the human gene which will not protect the endogenous mouse GLUT4 mRNA (13). The hGLUT4-11.5 mRNA was specifically detected in white adipose tissue (Fig. 2A, lane 5), brown adipose tissue (Fig. 2A, lane 6), heart (Fig. 2A, lane 7), and hindquarter skeletal muscle (Fig. 2A, lane 8), but not in brain (Fig. 2A, lane 3) or liver (Fig. 2A, lane 4). The three specific bands identified (251, 227, and 218 nucleotides) correspond to the major transcription start sites used in the human GLUT4 gene *in vivo* (see Fig. 3). Identical tissue-specific expression and transcriptional start site usage was observed in all four hGLUT4-11.5 transgenic lines. The amino acid sequences of the human and mouse GLUT4 proteins are 95% identical (27) and as a consequence both cross-react with GLUT4 antibodies. Thus, to assess the expression of human GLUT4 protein in our transgenic animals, we compared the levels of GLUT4 protein in tissues from transgenic and non-transgenic mice (Fig. 2B). The transgenic mice displayed higher levels of GLUT4 protein in white adipose tissue (Fig 2B, lanes 5 and 6), brown adipose tissue (Fig 2B, lanes 7 and 8), heart (Fig 2B, lanes 9 and 10), and to a smaller extent, hindquarter skeletal muscle (Fig 2B, lanes 11 and 12) compared with nontransgenic animals. Similar to the endogenous GLUT4 protein, there was no detectable expression of GLUT4 protein in brain (Fig 2B, lanes 1 and 2) and liver (Fig 2B, lanes 3 and 4) in the transgenic animals. Taken together, these data demonstrate the tissue-specific expression of the human transgenic GLUT4 gene which encodes a protein having the appropriate immunoreactivity and mobility by SDS-polyacrylamide gel electrophoresis.
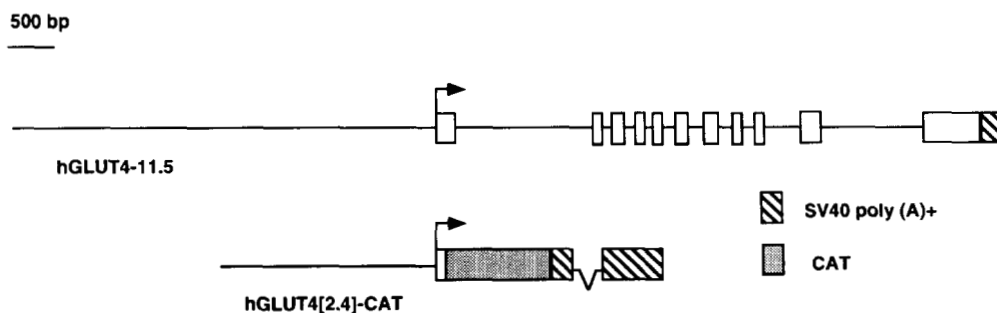

FIG. 1. **Schematic representation of the human muscle-fat/GLUT4 gene constructs used to generate lines of transgenic animals.** The top drawing depicts the hGLUT4-11.5 construct. This construct contains 5.3 kb of the human 5'-flanking DNA, all the exons and introns through the protein coding region of exon 10, plus an SV40 polyadenylation signal. The *open boxes* represent exons. The bottom drawing depicts the hGLUT4[2.4]-CAT reporter gene. This construct contains 2.4 kb of human GLUT4 5'-flanking DNA fused to the CAT reporter gene. The SV40 polyadenylation signal and small t antigen splice site were used in the CAT reporter construct.
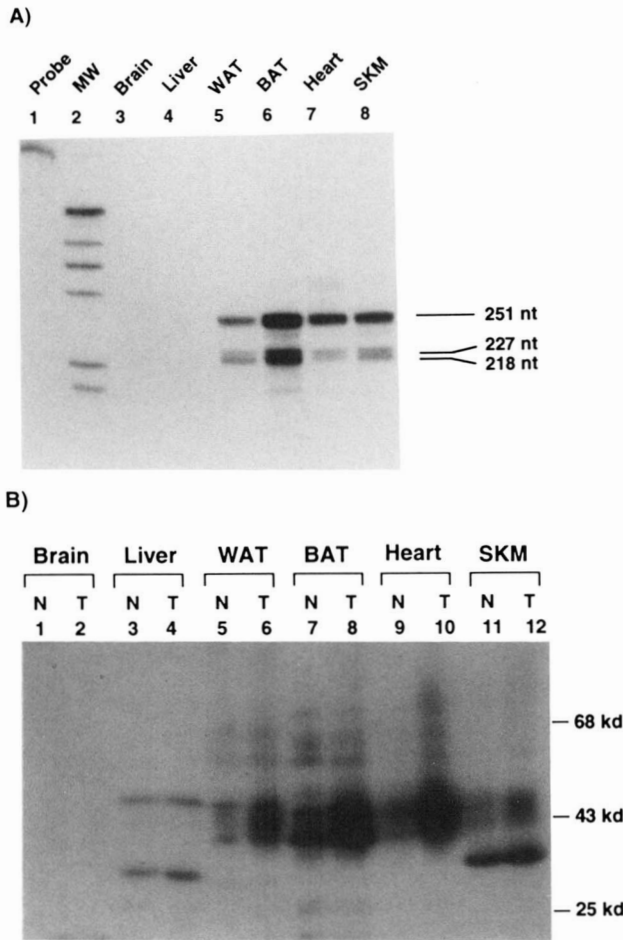
A)

B)

FIG. 2. **Tissue distribution of hGLUT4-11.5 RNA and protein in transgenic animals.** *A*, hGLUT4-11.5 RNA was measured by an RNase protection assay using a human GLUT4-specific probe directed to the 5'-end of the transgenic mRNA. Total RNA was isolated from brain (*lane 3*), liver (*lane 4*), white adipose tissue (*WAT, lane 5*), brown adipose tissue (*BAT, lane 6*), heart (*lane 7*), and hindquarter skeletal muscle (*SKM, lane 8*). Five µg of total RNA was used for each assay. Undigested probe (*Probe*) and molecular weight (*MW*) markers (1-kb ladder) are shown in *lanes 1* and *2*, respectively. The transgenic mRNA probe protects fragments of 251, 227, and 218 nucleotides (*nt*) as indicated. Identical results were obtained in two other independent founder lines examined. *B*, GLUT4 protein was detected by Western blot analysis using a GLUT4 polyclonal antibody (insulin-regulated glucose transporter) which detects both the mouse and human proteins as described under "Experimental Procedures." GLUT4 was measured in preparations of total membranes using 100 µg of total protein from brain (*lanes 1* and *2*), liver (*lanes 3* and *4*), white adipose tissue (*WAT, lanes 5* and *6*), brown adipose tissue (BAT, *lanes 7* and *8*), heart (*lanes 9* and *10*), and hindquarter skeletal muscle (*SKM, lanes 11* and *12*). The nontransgenic mice samples (*N*) were run in *lanes 1, 3, 5, 7, 9,* and *11*, and the transgenic mice samples (*T*) were run in *lanes 2, 4, 6, 8, 10,* and *12*. Two independent founder lines were examined and identical results were obtained.

Mapping Transcriptional Start Sites in the Transgenic Mice—Previous studies have demonstrated that the mouse GLUT4 gene has a unique transcription initiation site (28), whereas the human GLUT4 gene utilizes multiple start sites (13). To determine whether the three major bands identified by RNase protection correspond to these start sites, we compared the protected fragments generated using human adipose tissue mRNA with those from transgenic mouse mRNA (Fig. 3*A*). Three protected bands, corresponding to 251, 227, and 218 nucleotides were observed in both human adipose tissue mRNA (Fig. 3*A*, lane 1) and in adipose tissue mRNA from transgenic mice (Fig. 3*A, lane 2*). However, the 218-nt pro-
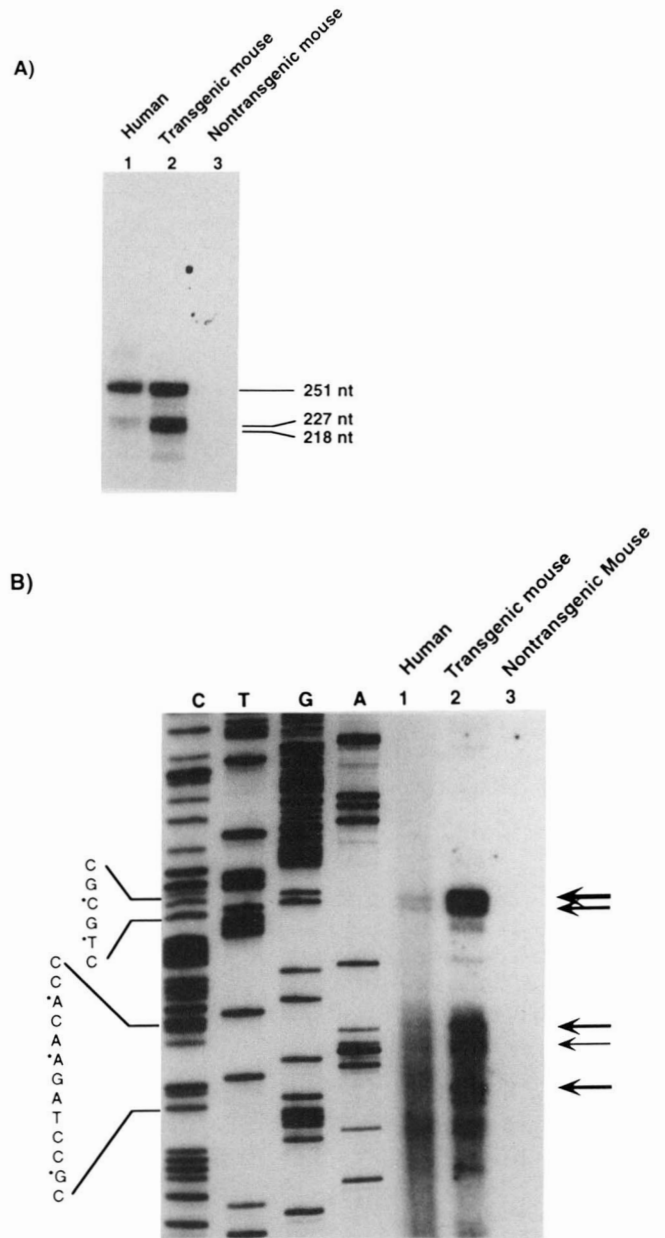
A)

B)

FIG. 3. **Comparison of transcription start site usage for human and hGLUT4-11.5 transgenic GLUT4 mRNA.** A, RNase protection assay was used to characterize the 5'-ends of human GLUT4 mRNA in total RNA from female human omental (*lane 1*) and female transgenic mouse perimetrial white adipose tissue (*lane 2*). Fifty µg of total RNA from human fat and 5 µg of total RNA from mouse fat were assayed. To demonstrate the specificity of the probe for human GLUT4 mRNA, 5 µg of total RNA isolated from nontransgenic mouse adipose tissue was also assayed (*lane 3*). B, primer extension analysis was carried out using 50 µg of human (*lane 1*) and 5 µg each of transgenic (*lane 2*) and nontransgenic (*lane 3*) total RNA from the tissues described above. The mRNA was hybridized to a synthetic oligonucleotide complementary to nucleotides (*nt*) 3–40 of the human GLUT4 cDNA (25). The sequencing ladder was obtained using the same oligonucleotide primer in a dideoxy sequencing reaction of the *PstI/PvuII* fragment of the human GLUT4 gene as described under "Experimental Procedures." The primer extension products were analyzed on a 6% acrylamide, 7.5 M urea gel. The major start points are indicated by the *asterisk* on the deduced nucleotide sequence given at the *left*.

tected fragment was present in lower abundance in the human white adipose tissue RNA than in RNA from the transgenic mice (compare Fig. 3*A, lanes 1* and *2*). The specificity of the RNase protection assay was confirmed by the absence of these

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bands in adipose tissue mRNA isolated from nontransgenic mice (Fig. 3A, lane 3).

The results of the RNase protection studies were confirmed by primer extension analysis (Fig. 3B). Five transcription initiation sites were observed for the human GLUT4 mRNA in adipose tissue from the transgenic mice (Fig. 3B, lane 2). These start sites were clustered into three regions corresponding to the three bands identified by RNase protection (Fig. 3A). Similar extension products were also observed in human adipose tissue mRNA (Fig. 3B, lane 1), demonstrating the fidelity of transcription initiation of the hGLUT4-11.5 construct in these transgenic animals. Since the oligonucleotide used was specific for the human GLUT4 5'-untranslated region, no extension products were detected in mRNA prepared from nontransgenic mouse adipose tissue (Fig. 3B, lane 3). The relative stronger intensity of the largest sized protected fragment identified by RNase protection assay was also reflected in the primer extension analysis, indicating that the major transcriptional start site was located at base pair +1 (Fig. 3B).

Sex-related Differences in Expression of the GLUT4 Transgene—During these studies we consistently had difficulty in detecting the presence of the hGLUT4-11.5 transcript in white adipose tissue from male mice, suggesting a possible sex difference in expression of the transgene. Thus, we compared expression of hGLUT4-11.5 mRNA between male and female littermates (Fig. 4A). RNase protection analysis showed no significant difference in steady-state hGLUT4-11.5 mRNA levels between male and female brown adipose tissue (Fig. 4A, lanes 5 and 6), heart (Fig. 4A, lanes 7 and 8) and hindquarter skeletal muscle (Fig. 4A, lanes 9 and 10). In contrast, the transgene was poorly expressed in male epididymal adipose tissue (Fig. 4A, lane 3), whereas high levels of expression were detected in female perimetrial adipose tissue (Fig. 4A, lane 4). This marked difference was also reflected in GLUT4 protein levels (Fig. 4B, lanes 1 and 2). Similar to the hGLUT4-11.5 mRNA, GLUT4 protein levels between male and female littermates were not significantly different in brown adipose tissue (Fig. 4B, lanes 3 and 4) and heart and hindquarter skeletal muscle (data not shown). This apparent sex-related difference in expression of the transgene in white adipose tissue was also observed in the three transgenic lines examined (data not shown).

In contrast, Northern blot analysis of mouse GLUT4 mRNA from male and female nontransgenic mice did not indicate any sex-related difference in GLUT4 mRNA (Fig. 5A) or protein levels (Fig. 5B) in any of the tissues examined. Since it was possible that the high levels of hGLUT4-11.5 expression could have down-regulated the expression of the endogenous mouse GLUT4 gene in male epididymal adipose tissue, RNase protection was used to determine the levels of the endogenous mouse GLUT4 mRNA in the hGLUT4-11.5 transgenic background (Fig. 5C). In these animals, the endogenous mouse GLUT4 mRNA was not significantly different in either the male epididymal or female perimetrial adipose tissue (Fig. 5C, lanes 7 and 8), whereas expression of the transgenic construct was almost undetectable in the male epididymal adipose tissue (Fig. 5C, lane 3), but highly expressed in female perimetrial adipose tissue (Fig. 5C, lane 4).

Expression of the GLUT4 Transgene in Diabetic Animals—Previous studies have documented that short term insulin-deficient diabetes in rats, induced by STZ-treatment (2–3 days), results in a marked decrease in steady-state levels of GLUT4 mRNA in adipose tissue (7–11). To examine the hormonal/metabolic regulation of the hGLUT4-11.5 transgene in insulin-deficient diabetes, RNA was isolated from
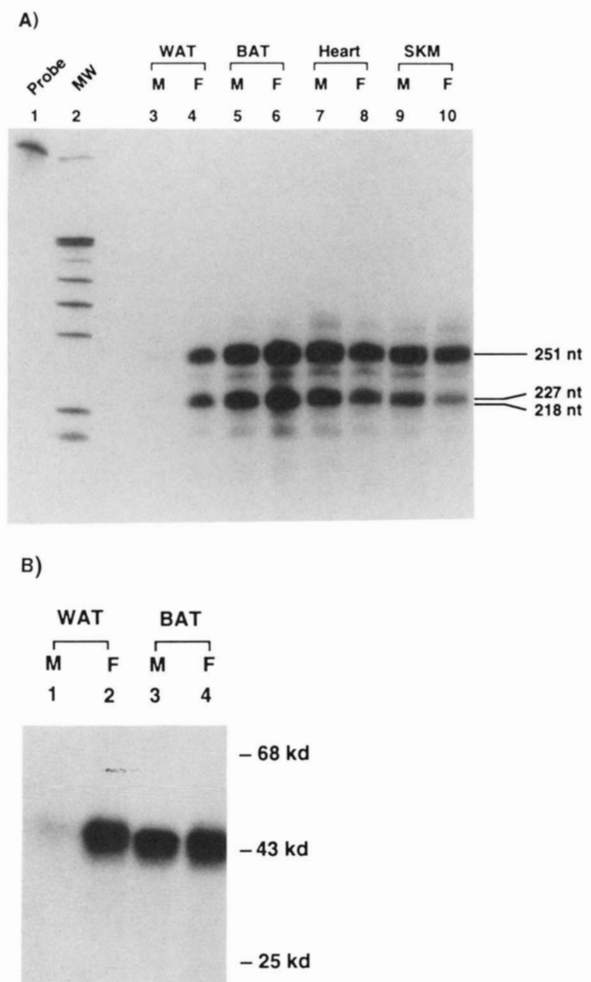

FIG. 4. **Comparison of human GLUT4 mRNA and protein abundance in male and female hGLUT4-11.5 transgenic mice.** *A*, Transgenic mRNA was measured using an RNase protection assay as described in Fig. 2. Total cellular RNA was isolated from white adipose tissue (*WAT, lanes 3 and 4*), brown adipose tissue (*BAT, lanes 5 and 6*), heart (*lanes 7 and 8*), and hindquarter skeletal muscle (*SKM, lanes 9 and 10*) from male (*M, lanes 3, 5, 7 and 9*) and female (*F, lanes 4, 6, 8, and 10*) transgenic mice. Five µg of total RNA was analyzed in each lane. Undigested probe (*Probe*) and molecular weight (*MW*) markers (1-kb ladder) are shown in *lanes 1* and *2*, respectively. This is a representative experiment and identical results obtained in three independent founder lines. *B*, GLUT4 protein was measured in 100 µg of total membrane protein isolated from white adipose tissue (*WAT, lanes 1 and 2*) and 25 µg of total membrane protein isolated from brown adipose tissue (*BAT, lanes 3 and 4*) as described in Fig. 2. Total membrane protein isolated from male (*lanes 1 and 2*) and female (*lanes 2 and 4*) transgenic mice were subjected to Western blot analysis using a polyclonal GLUT4 antibody (insulin-regulated glucose transporter). *nt*, nucleotides.

control, STZ-diabetic, and insulin-treated STZ-diabetic female transgenic animals (Fig. 6A). RNase protection analysis of the human GLUT4 transcript demonstrated the presence of the expected three protected fragments in white adipose tissue (Fig. 6A, lanes 3–5), brown adipose tissue (Fig. 6A, lanes 6–8), and heart (Fig. 6A, lanes 9–11). STZ-induced diabetes resulted in a marked decrease in the hGLUT4-11.5-derived transcript which recovered upon insulin treatment in white (Fig. 6A, lanes 4 and 5) and brown adipose tissues (Fig. 6A, lanes 7 and 8). Insulin-deficient diabetes also caused a small decrease in heart tissue (Fig. 6A, lane 10), which also returned to control levels following insulin therapy (Fig. 6A, lane 11). The relative changes in the transgenic human GLUT4 mRNA from these tissues occurred in parallel to the
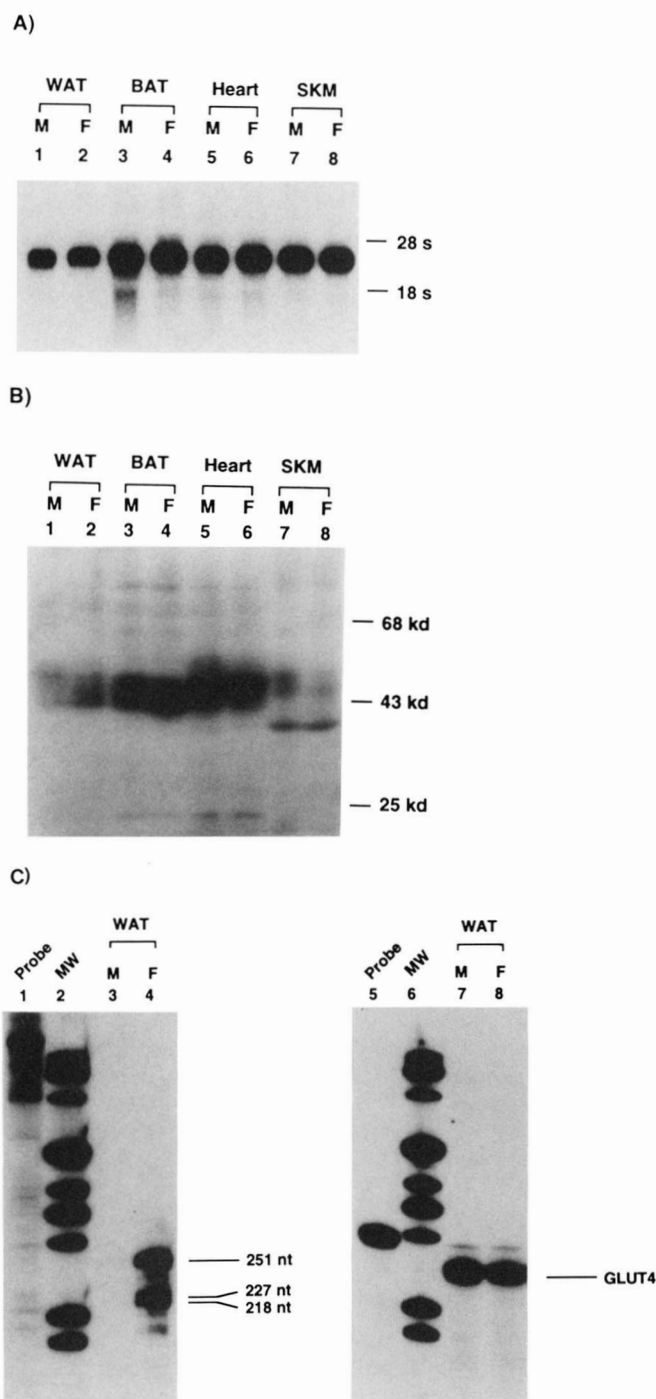
A)

WAT   BAT   Heart   SKM
M   F M   F M   F   M   F
1   2 3   4 5   6   7   8

— 28 s

— 18 s

B)

WAT   BAT   Heart   SKM
M   F M   F M   F   M   F
1   2 3   4 5   6   7   8

— 68 kd

— 43 kd

— 25 kd

C)

WAT   WAT
Probe MW M   F   Probe MW M   F
1   2   3   4   5   6   7   8

— 251 nt

— 227 nt
— 218 nt

—— GLUT4

FIG. 5. **Comparison of endogenous mouse GLUT4 mRNA and protein in nontransgenic and hGLUT4-11.5 transgenic mice.** *A*, endogenous mouse GLUT4 mRNA was analyzed by Northern blot analysis. Total cellular RNA was isolated from white adipose tissue (*WAT, lanes 1* and *2*), brown adipose tissue (*BAT, lanes 3* and *4*), heart (*lanes 5* and *6*), and hindquarter skeletal muscle (*SKM, lanes 7* and *8*) from male (*M, lanes 1, 3, 5,* and *7*) and female (*F, lanes 2, 4, 6,* and *8*) nontransgenic mice. Ten µg of total RNA was fractionated on a 1% agarose-formaldehyde gel electrophoresis, transferred to a nylon filter, and probed with a ^{32}P-labeled antisense GLUT4 RNA probe. Following high stringency washes, the hybridized filter was exposed to film at −70 °C. *B*, the endogenous mouse GLUT4 protein was analyzed by Western blotting using a polyclonal GLUT4 antibody as described in the legend to Fig. 2. GLUT4 was measured in 75 µg of total membrane protein isolated from white adipose tissue (*WAT, lanes 1* and *2*), brown adipose tissue (*BAT, lanes 3* and *4*), heart (*lanes 5* and *6*), and hindquarter skeletal muscle (*SKM, lanes 7* and *8*) isolated from male (*M, lanes 1, 3, 5,* and *7*) and female (*F, lanes 2, 4, 6,* and *8*) nontransgenic mice. *C*, transgenic and endogenous

changes in the endogenous mouse GLUT4 transcript (Fig. 6*B*). Consistent with previous studies (21, 29), neither short term STZ-induced diabetes nor insulin treatment had significant effect on the endogenous mouse GLUT4 or hGLUT4-11.5 transcript in skeletal muscle (data not shown).

To begin narrowing down the region responsible for this hormonal/metabolic regulation of the human GLUT4 gene, we examined the effect of STZ-induced diabetes in transgenic mice carrying only 2.4 kb of the human GLUT4 5′-flanking DNA fused to the CAT reporter gene. We have reported previously (16) that this transgenic construct was also expressed in an appropriate tissue-specific pattern. RNase protection was used to identify transcripts from the transgene and the mouse GLUT4 gene (Fig. 7); the two bands above the 176-nt mouse GLUT4 mRNA-specific fragment represent products of incomplete RNase digestion as they were only present in GLUT4-expressing tissues (Fig. 7, *lanes 12* and *13*), and changes in their levels paralleled the changes in abundance of the 176-nt fragment. As observed in Fig. 4*A* for the hGLUT4-11.5 transcript, CAT mRNA was not detectable in male white adipose tissue from control animals, whereas high levels of endogenous mouse GLUT4 mRNA were readily apparent (Fig. 7, *lane 3*). Although CAT mRNA transcripts were not evident in the male white adipose tissue of control or diabetic animals (Fig. 7, *lanes 3* and *4*), expression of the transgene was superinduced following insulin treatment in a fashion similar to that seen with the hGLUT4-11.5 transgene (Fig. 7, *lane 5*). STZ-induced diabetes also resulted in decreased expression of CAT mRNA in brown adipose tissue (Fig. 7, *lane 7*) and heart (Fig. 7, *lane 10*), which recovered following insulin treatment (Fig. 7, *lanes 8* and *11*). These alterations in CAT mRNA levels paralleled the changes in the endogenous mouse GLUT4 mRNA, albeit to a greater extent than either the endogenous gene or the hGLUT4-11.5 transgenic construct.

DISCUSSION

The GLUT4/muscle-fat glucose transporter is unique among the family of mammalian facilitative glucose transporters with respect to its unusal tissue-specific expression and hormonal/metabolic regulation (1). The GLUT4 mRNA and protein are expressed predominantly in white and brown adipose tissue as well as in skeletal and cardiac muscle (22–26). These are the major tissues that display insulin-stimulated glucose transport activity and GLUT4 has been documented to be the protein isoform primarily accounting for this response (4, 6, 30). Furthermore, expression of the GLUT4 mRNA and protein in these tissues is modulated by various complex hormonal and metabolic states. For example, several studies have demonstrated that insulin-deficient diabetes in rats resulted in a marked decrease in white adipose tissue GLUT4 mRNA and protein which may account for the insulin resistance that occurs under this condition (7–11). Recently, we have demonstrated that the decrease in white adipose tissue steady-state GLUT4 mRNA levels primarily results from a decrease in transcription rate (12).

To investigate the molecular basis for GLUT4 gene expres-

GLUT4 mRNA was measured in RNA isolated from white adipose tissue of male (*M, lanes 3* and *7*) and female (*F, lanes 4* and *8*) transgenic mice. Transgenic GLUT4 mRNA (*lanes 3* and *4*) and endogenous mouse GLUT4 (*lanes 7* and *8*) were measured in 5 µg of total RNA by an RNase protection assay using a human GLUT4-specific probe and a mouse GLUT4-specific probe as described under "Experimental Procedures." Undigested probe (*Probe*) is shown in *lanes 1* and *5* for the human-specific and mouse-specific probes, respectively. Molecular weight markers (*MW, lanes 2* and *6*) are the end-labeled 1-kb ladders. *nt*, nucleotides.

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A)

FIG. 6. **Endogenous and transgenic GLUT4 mRNA in STZ-diabetic hGLUT4-11.5 transgenic mice.** Transgenic female mice were either left untreated (*Control, lanes 3, 6, and 9*) treated with STZ to induce diabetes for 3 days (*Diabetic, lanes 4, 7, and 10*) or following 3 days of diabetes treated with insulin for 48 h (*Insulin, lanes 5, 8,* and *11*) as described under "Experimental Procedures." Total cellular RNA was isolated from white adipose tissue (*WAT, lanes 3–5*), brown adipose tissue (*BAT, lanes 6–8*), and heart (*lanes 9–11*). This is a representative experimented performed four times in two independent founder lines. *A,* Five μg of total RNA from each tissue was analyzed for transgenic GLUT4 mRNA by RNase protection assay using a probe specific for the 5′-end of the human GLUT4 mRNA. *Lane 1* is undigested probe. The molecular weight marker (*MW, lane 2*) is the end-labeled 1-kb ladder. *B,* Five μg of total RNA was analyzed for endogenous mouse GLUT4 by RNase protection assay using a probe specific for mouse GLUT4 mRNA (20). *Lane 1* is the undigested probe. The molecular weight marker (*MW, lane 2*) is the end-labeled 1-kb ladder. *nt,* nucleotides.

B)
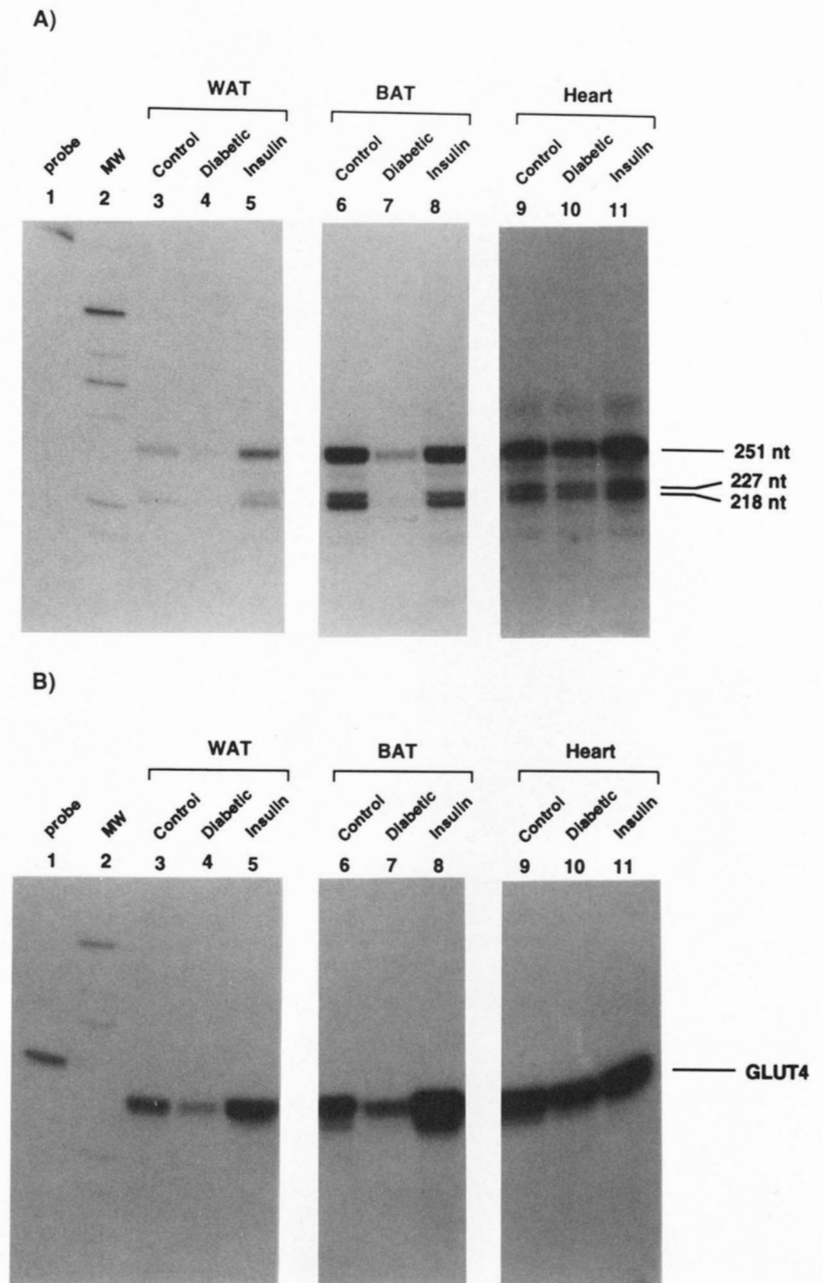
sion, several laboratories have begun to analyze the GLUT4 promoter. However, these studies are hampered by both the paucity of cells that express GLUT4 mRNA and the limited ability to observe reporter expression in some tissue culture cell lines, particularly 3T3-L1 adipocytes (13). Nevertheless, co-transfection studies in 3T3-L1 fibroblasts have suggested that a specific C/EBP binding site is required for adipose tissue expression of the mouse GLUT4 gene (28). In contrast, although we have also observed that C/EBP will transactivate the mouse GLUT4 promoter, we have been unable to detect any significant effect of C/EBP on expression of the human GLUT4 gene.[2] Thus, to overcome the difficulties associated with the analysis of this gene in cultured cells and to provide a more appropriate physiological context to examine the regulation of GLUT4 expression, we have prepared several lines of transgenic mice carrying two different human GLUT4 constructs.

In this study, we examined the tissue-specific and hormonal/metabolic regulation in transgenic mice carrying 5.3 kb of the promoter and all the protein coding portions of the GLUT4 gene, termed hGLUT4-11.5. This transgenic construct was expressed in an appropriate tissue-specific manner, with high levels of hGLUT4-11.5 mRNA in white adipose tissue, brown adipose tissue, skeletal muscle, and heart. Surprisingly, although this human GLUT4 transgene was efficiently expressed in female perimetrial adipose tissue there was only low level expression in male epididymal adipose tissue. This phenomenon was not a result of the site of chromosomal integration as three independent founder lines examined were all found to express the hGLUT4-11.5 transcript in this manner. In addition, the smaller hGLUT4[2.4]-CAT transgenic construct was also expressed at a substantially reduced levels in male *versus* female white adipose tissue.[2] In contrast, the endogenous mouse GLUT4 gene was not differentially expressed in male and female adipose tissue in either nontransgenic or transgenic mice. Taken together,

[2] A. L. Olson, M.-L. Liu, W. S. Moye-Rowley, J. B. Buse, G. I. Bell, and J. E. Pessin, unpublished results.
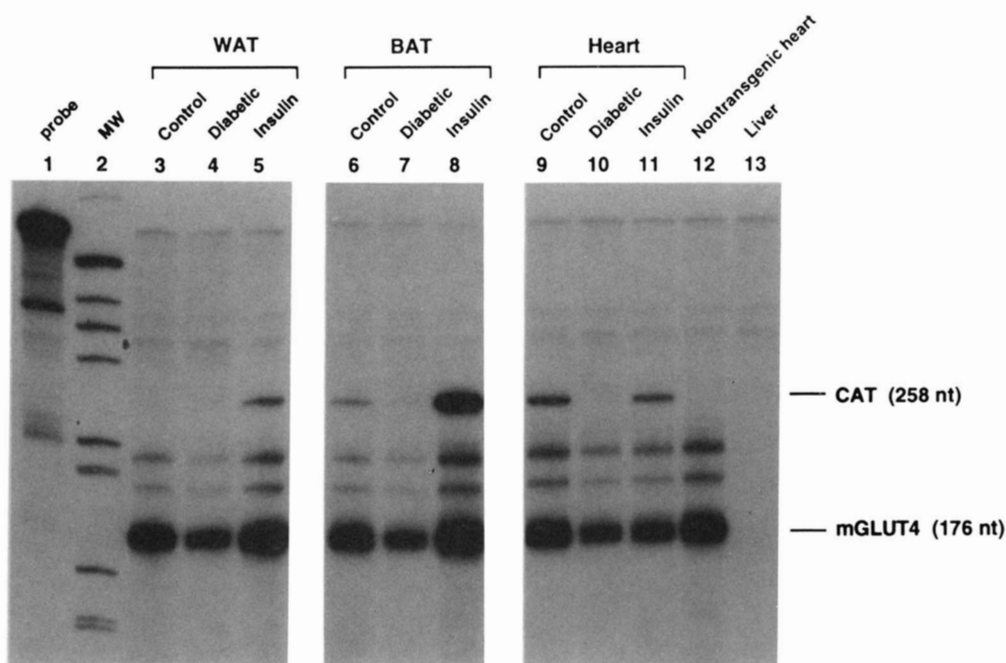
FIG. 7. **Endogenous and transgenic GLUT4 mRNA in STZ-diabetic hGLUT4[2.4]-CAT transgenic mice.** Transgenic male mice were either left untreated (*Control, lanes 3, 6,* and *9*), treated with STZ to induce diabetes for 3 days (*Diabetic, lanes 4, 7,* and *10*), or following 3 days of diabetes treated with insulin for 48 h (*Insulin, lanes 5, 8,* and *11*) as described under "Experimental Procedures." Total cellular RNA was isolated from white adipose tissue (*WAT, lanes 3–5*), brown adipose tissue (*BAT, lanes 6–8*), and heart (*lanes 9–11*). Ten μg of total RNA from each tissue was analyzed for CAT mRNA and endogenous mouse GLUT4 mRNA by RNase protection assay using a probe specific for both mRNA species (16). The CAT and mouse GLUT4 mRNA specific fragments are 258 and 176 nucleotides (*nt*), respectively. Heart tissue RNA (*lane 12*) from a nontransgenic littermate was included as a control to confirm the specificity of the RNase protection assay for CAT mRNA. Liver RNA (*lane 13*) was included to confirm specificity of the assay for mouse GLUT4 mRNA. *Lane 1* is undigested probe. The molecular weight marker (*MW, lane 2*) is the end-labeled 1-kb ladder. This is a representative experiment which was carried out three times.

these data suggest that either the hGLUT4-11.5 construct is lacking an adipose tissue-specific element required for expression in male epididymal adipose tissue or that this sex-related difference is specific for humans and does not occur in mice.

The human GLUT4 gene also differs from the mouse and rat genes in having multiple closely spaced transcriptional start sites, whereas there is a single unique start site in both the mouse (28) and rat GLUT4 genes (31). The molecular basis for the different transcription start site usage in the human *versus* rodent GLUT4 genes is not understood at the present time but may reflect specific differences in either gene structure or regulation between these species.

STZ-induced insulin deficiency in rats has been documented to decrease GLUT4 mRNA and protein levels in white adipose tissue, brown adipose tissue, and cardiac muscle (7–11). Although skeletal muscle GLUT4 levels were also found to decline, this required a substantially longer duration of STZ-induced diabetes than used in this study (21, 29). Following acute STZ-induced diabetes, we have observed that expression of the hGLUT4-11.5 transgenic construct was regulated in a similar manner as the endogenous mouse GLUT4 gene. Surprisingly, in the STZ-diabetic mouse hearts, the decrease of both the endogenous and hGLUT4-11.5 transgenic GLUT4 mRNA levels were smaller than that reported for STZ-diabetic rat hearts (11). Consistent with this study, we have also observed a significantly greater decrease in GLUT4 mRNA levels in STZ-diabetic rat hearts compared with STZ-diabetic mouse hearts.[2] Even though it remains to be determined whether heart muscle regulation of GLUT4 expression in humans more closely reflects the changes observed in mouse or rat hearts, these data further underscore the apparent species specific differences in GLUT4 expression

between humans, rats, and mice.

Similar to the hGLUT4-11.5 construct, expression of the hGLUT4[2.4]-CAT reporter gene also decreased in the STZ-diabetic transgenic mice and recovered following insulin treatment. In addition, 2.4 kb of human GLUT4 5'-flanking DNA was sufficient to convey appropriate regulation of reporter expression by fasting and refeeding (16). Interestingly, the change in magnitude of the CAT mRNA was consistently greater than that of the endogenous mouse GLUT4 or hGLUT4-11.5 mRNA. These larger effects on the hGLUT4[2.4]-CAT reporter mRNA most likely reflect differences between CAT and GLUT4 mRNA turnover rates. In any case, these data directly demonstrate that all of the sequence information required for tissue-specific and insulin regulation *in vivo* reside within 2.4 kb of the GLUT4-flanking DNA.

In summary, the human GLUT4 gene can be appropriately expressed and regulated in transgenic mice. Unlike the rodent GLUT4 genes, the human gene displays 5'-mRNA heterogeneity due to the use of multiple transcription initiation sites which are recapitulated in the transgenic mice. Furthermore, the low level of expression of the transgene in male compared with female adipose tissue suggests a fundamental difference in expression between humans and rodents. Whether the molecular basis for this phenomenon results from differences in gene structure or the cellular context between human and rodent adipocytes remains to be determined. Finally, these studies have localized the cis-acting DNA sequences responsible for both tissue-specific and hormonal/metabolic regulation of human GLUT4 expression to within a 2.4-kb region of the 5'-flanking DNA.

REFERENCES

1. Pessin, J. E., and Bell, G. I. (1992) *Annu. Rev. Physiol.* **54**, 911–930
2. James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) *Nature* **333**, 183–185
3. Marette, A., Richardson, J. M., Ramlal, T., Balon, T. W., Vranic, M., Pessin, J. E., and Klip, A. (1992) *Am. J. Physiol.* **263**, C443–C452
4. Zorzano, A., Wilkinson, W., Kotliar, N., Thoidis, G., Wadzinkski, B. E., Ruoho, A. E., and Pilch, P. F. (1989) *J. Biol. Chem.* **264**, 12358–12363
5. Hirshman, M. F., Goodyear, L. J., Wardzala, L. J., Horton, E. D., and Horton, E. S. (1990) *J. Biol. Chem.* **265**, 987–991
6. Holman, G. D., Kozka, I. J., Clark, A. E., Flower, C. J., Saltis, J., Habberfield, A. D., Simpson, I. A., and Cushman, S. W. (1990) *J. Biol. Chem.* **265**, 18172–18179
7. Sivitz, W. I., DeSautel, S. L., Kayano, T., Bell, G. I., and Pessin, J. E. (1989) *Nature* **340**, 72–74
8. Berger, J., Biswas, C., Vicario, P. P., Strout, H. V., Saperstein, R., and Pilch, P. F. (1989) *Nature* **340**, 70–72
9. Garvey, W. T., Huecksteadt, T. P., and Birnbaum, M. J. (1989) *Science* **245**, 60–63
10. Kahn, B. B., Charron, M. J., Lodish, H. F., Cushman, S. W., and Flier, J. S. (1989) *J. Clin. Invest.* **84**, 404–411
11. Camps, M., Castello, A., Munoz, P., Monfar, M., Testar, X., Palacin, M., and Zorzano, A. (1992) *Biochem. J.* **282**, 765–772
12. Gerrits, P. M., Olson, A. L., and Pessin, J. E. (1993) *J. Biol. Chem.* **268**, 640–644
13. Buse, J. B., Yasuda, K., Lay, T. P., Leo, T. S., Olson, A. L., Pessin, J. E., Karam, J. H., Seino, S., and Bell, G. I. (1992) *Diabetes* **41**, 1436–1445
14. Seifert, H. S., Chen, E. Y., So, M., and Heffron, F. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 735–739
15. Harland, R., and Weintraub, H. (1985) *J. Cell Biol.* **101**, 1094–1099
16. Liu, M.-L., Olson, A. L., Moye-Rowley, W. S., Buse, J. B., Bell, G. I., and Pessin, J. E. (1992) *J. Biol. Chem.* **267**, 11673–11676
17. LeMarchand-Brustel, Y., Olichon-Berthe, C., Gremeaux, T., Tant, J. F., Rochet, N., and Van Obberghen, E. (1990) *Endocrinology* **127**, 2687–2695
18. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
19. Olson, A. L., Perlman, S., and Robillard, J. E. (1990) *Pediatr. Res.* **28**, 183–185
20. Kaestner, K. H., Flores-Riveros, J. R., McLenithan, J. C., Janicot, M., and Lane, M. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 1933–1937
21. Richardson, J. M., Balon, T. W., Treadway, J. L., and Pessin, J. E. (1991) *J. Biol. Chem.* **266**, 12690–12694
22. James, D. E., Strube, M., and Mueckler, M. (1989) *Nature* **338**, 83–87
23. Birnbaum, M. J. (1989) *Cell* **57**, 305–315
24. Charron, M. J., Brosius, F. C., III, Alper, S. L., and Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 2535–2539
25. Fukumoto, H., Kayano, T., Buse, J. B., Edwards, Y., Pilch, P. F., Bell, G. I., and Seino, S. (1989) *J. Biol. Chem.* **264**, 7776–7779
26. Kaestner, K. H., Christy, R. J., McLenithan, J. C., Braiterman, L. T., Cornelius, P., Pekala, P. J., and Lane, M. D. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 3150–3154
27. Burant, C. F., Sivitz, W. I., Fukumoto, H., Kayano, T., Nagamatsu, S., Seino, S., Pessin, J. E., and Bell, G. I. (1991) *Recent Prog. Horm. Res.* **47**, 349–387
28. Kaestner, K. H., Christy, R. J., and Lane, M. D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 251–255
29. Kahn, B. B., Rosetti, L., Lodish, H. F., and Charron, M. J. (1991) *J. Clin. Invest.* **87**, 2197–2206
30. Calderhead, D. M., and Lienhard, G. E. (1988) *J. Biol. Chem.* **263**, 12171–12174
31. Olson, A. L., Edgington, N., Moye-Rowley, W. S. (1992) *Diabetes* **41**, Suppl. 1, 88A