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Expression and Regulation of the Human GLUT4/Muscle-Fat Facilitative Glucose Transporter Gene in Transgenic Mice*

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Min-Ling Liu‡§, Ann Louise Olson‡¶, W. Scott Moye-Rowley‡, John B. Buse‖, Graeme I. Bell‖, and Jeffrey E. Pessin‡**

From the ‡Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242 and ‖Howard Hughes Medical Institute, the Department of Biochemistry and Molecular Biology, and the Department of Medicine, The University of Chicago, Chicago, Illinois 60637

To study the molecular basis of tissue-specific expression of the GLUT4/muscle-fat facilitative glucose transporter gene, we generated lines of transgenic mice carrying 2.4 kilobases of the 5'-flanking region of the human GLUT4 gene fused to a chloramphenicol acetyltransferase (CAT) reporter gene (hGLUT4[2.4]-CAT). This reporter gene construct was specifically expressed in tissues that normally express GLUT4 mRNA, which include both brown and white adipose tissues as well as cardiac, skeletal, and smooth muscle. In contrast, CAT reporter activity was not detected in brain or liver, two tissues that do not express the GLUT4 gene. In addition, the relative levels of CAT mRNA driven by the human GLUT4 promoter in various tissues of these transgenic animals mirrored those of the endogenous mouse GLUT4 mRNA.

Since previous studies have observed alterations in GLUT4 mRNA levels induced by fasting and refeeding (Sivitz, W. I., DeSautel, S. L., Kayano, T., Bell, G. I., and Pessin, J. E. (1989) *Nature* 340, 72–74), the regulated expression the hGLUT4[2.4]-CAT transgene was also assessed in these animals. Fasting was observed to decrease CAT activity in white adipose tissue which was super-induced upon refeeding. These alterations in CAT expression occurred in parallel to the changes in endogenous mouse GLUT4 mRNA levels. Although CAT expression in skeletal muscle and brown adipose tissue was unaffected, the endogenous mouse GLUT4 mRNA was also refractory to the effects of fasting/refeeding in these tissues. These data demonstrate that 2.4 kilobases of the 5'-flanking region of the human GLUT4 gene contain all the necessary sequence elements to confer tissue-specific expression and at least some of the sequence elements controlling the hormonal/metabolic regulation of this gene.

Facilitative glucose uptake in mammalian cells is mediated by a family of structurally related proteins with distinct physical and kinetic properties as well as tissue-specific patterns of expression (Burant *et al.*, 1991). The isoform designated as GLUT4/muscle-fat glucose transporter is specifically expressed in muscle and adipose tissue and is the protein primarily responsible for mediating insulin-stimulated glucose uptake by these tissues (Birnbaum, 1989; Charron *et al.*, 1989; Kaestner *et al.*, 1989; James *et al.*, 1989; Fukumoto *et al.*, 1989). The levels of GLUT4 mRNA and protein are decreased in insulin-deficient states such as diabetes and fasting (Berger *et al.*, 1989; Garvey *et al.*, 1989; Kahn *et al.*, 1989; Sivitz *et al.*, 1989; Richardson *et al.*, 1991). This decrease in GLUT4 expression contributes, at least in part, to the impaired insulin-stimulated glucose uptake by peripheral tissues that is associated with these altered metabolic states (Holman *et al.*, 1990). Moreover, GLUT4 expression is similarly regulated in adipose tissue of both humans and rats, suggesting a common underlying mechanism controlling GLUT4 expression under these conditions (Garvey *et al.*, 1991; Sinha *et al.*, 1991).

The mouse (Kaestner *et al.*, 1990) and human[1] GLUT4 genes and their putative 5'-flanking transcriptional regulatory regions have been isolated and characterized. The promoters of these genes lack canonical TATA sequence elements, and studies of the promoter of the mouse GLUT4 gene have suggested that the transcription factor C/EBP may contribute to the differentiation-specific expression of GLUT4 in adipose tissue (Kaestner *et al.*, 1990). In addition, elevation of cAMP levels in differentiated 3T3-L1 adipocytes has been observed to decrease transcription of the endogenous mouse GLUT4 gene (Kaestner *et al.*, 1991).

In these as well as in most other studies examining gene expression, model tissue culture cell lines are typically transfected with promoter-reporter gene constructs. However, DNA regions that are crucial for promoter function *in vivo* may not always be evident in transient transfection assays performed in cultured cell lines (Pinkert *et al.*, 1987; Ross *et al.*, 1990). Furthermore, transfection with a 2.4-kb[2] fragment of the hGLUT4 promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene in cultured mouse 3T3-L1 adipocytes displays only weak promoter activity.[1] This result suggests that the trans-acting factors necessary for high level expression of the hGLUT4 gene may not be present or are limiting in 3T3-L1 adipocytes. In addition, model cell lines can not be used to mimic the altered hormonal and/or meta-

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** Recipient of National Institute of Health Research and Career Development Award DK01822. To whom correspondence should be addressed: Dept. of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242. Tel.: 319-335-7823; Fax: 319-335-7330.

[1] J. B. Buse, K. Yasuda, T. P. Lay, T. S., Seo, A. L. Olson, J. E. Pessin, J. H. Karam, S. Seino, and G. I. Bell, manuscript in preparation.

[2] The abbreviations used are: kb, kilobase pair(s); bp, base pair(s); CAT, chloramphenicol acetyltransferase; hGLUT4, human GLUT4; PCR, polymerase chain reaction; Pipes, 1,4-piperazinediethanesulfonic acid.

bolic environment associated with various physiological states of insulin deficiency.

Thus, in order to assess the tissue-specific as well as regulated expression of the GLUT4 gene in an appropriate physiological context, we have generated lines of transgenic mice carrying 2.4 kb of the 5′-flanking promoter region of the hGLUT4 gene fused to a CAT reporter gene. In this study, we demonstrate that 2.4 kb of upstream sequence is sufficient to direct tissue-specific CAT expression that mirrors the expression of the endogenous mouse GLUT4 gene. In addition, the regulation of this fusion gene by fasting/refeeding paralleled the changes observed for the endogenous mouse GLUT4 mRNA.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of the Human GLUT4 Gene—The human GLUT4 gene was isolated as a series of overlapping clones from the genomic library of Lawn *et al.* (1978) by hybridization with human GLUT4 cDNA probes. The genomic clones span 32 kb, including 15 kb of 5′-flanking region. The sequence of 2.0 kb of the 5′-flanking region and 6.3 kb representing the 11 exons and 10 introns of the gene was determined.

Preparation of Transgenic Mice—A 2.4-kb *Apa*I fragment containing the promoter region of the human GLUT4 gene was blunt-ended and inserted into the *Hin*cII site of pGEM-4Z™ to generate ph-GLUT4-ApaI[2.4]. This fragment includes nucleotides −2.4 kb to +84 bp relative to the major transcription start site (+1). The orientation of this fragment in pGEM-4Z placed a unique *Xba*I site located in the polylinker site immediately downstream of nucleotide +84. The hGLUT4[2.4]-CAT construct was prepared by transferring a 2.4-kb *Hin*dIII-*Xba*I fragment from phGLUT4-ApaI[2.4] into the CAT expression vector pUC19-CAT (kindly provided by Dr. Richard Maurer, University of Iowa, Iowa City, IA). A 4.6-kb *Sac*I and *Hin*dIII fragment containing the hGLUT4[2.4]-CAT reporter gene construct was purified by 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′-AGGGGGCGTGGCCTTCTGGGGT-3′ and 5′-TGAGCATTCAT-CAGGCGGGCAAGA-3′ which amplify a 439-bp region spanning the junction between the hGLUT4 promoter and the CAT reporter gene. Heterozygous F1 and F2 transgenic progeny were obtained by mating the founder animals with C57BL6 mice and were studied at 6–12 weeks of age. Transgenic animals in these matings were identified by PCR amplification of tail DNA as described above.

CAT Activity Assay—Transgenic animals were killed by cervical dislocation following Metofane inhalation. Tissues were isolated by rapid dissection and then snap-frozen in liquid nitrogen. In initial studies, the samples for measurements of CAT activity were prepared by pulverizing the frozen tissue with a ceramic mortar. Two hundred and fifty μl of lysis buffer (20 mM Tris, pH 7.4, 2 mM $MgCl_2$, and 0.1% Triton X-100) was added to the powdered tissue, and this suspension was freeze-thawed 3 times at −70 °C. In subsequent studies, lysis buffer without Triton X-100 was used as its presence resulted in a small decrease in CAT activity. The suspensions were cleared by microcentrifugation at 12,000 × *g* for 10 min and the protein concentration determined by the method of Lowry *et al.* (1951). CAT activity from 30–500 μg of protein extract was determined as described by Gorman *et al.* (1982) for 2 h. The reaction products were extracted with ethyl acetate and separated on grooved silica gel 150A thin layer chromatography plates (Whatman).

RNA Isolation—Total cellular RNA was isolated from snap-frozen tissues using the guanidinium isothiocyanate-CsCl method (Chirgwin *et al.*, 1979) as previously described (Olson *et al.*, 1990). RNA was quantified spectrophotometrically by absorbance at 260 nM and stored as an ethanol precipitate at −70 °C.

RNase Protection Assay—The mouse GLUT4-CAT plasmid p-469GLUT4.CAT (a kind gift of Dr. M. Daniel Lane, Johns Hopkins Medical School, Baltimore, MD) was linearized with *Bsu*36I, and T3 RNA polymerase was used to generate a 616-nucleotide antisense RNA probe. The antisense RNA probe was labeled with [α-^{32}P]UTP (Melton *et al.*, 1984) and 2 × 10^6 cpm of the labeled probe was hybridized with 10–20 μg of total RNA in 30 μl of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 40 mM Pipes, pH 6.4, and 1 mM EDTA). Hybridization was carried out overnight at 57 °C. Non-hybridized RNA was digested for 1 h at 30 °C in buffer (300 mM NaCl, 10 mM Tris-Cl, pH 7.4, 5 mM EDTA) containing 5 μg/ml RNase T1 and 40 μg/ml RNase A. The protected RNA fragments were separated using 6% acrylamide, 7.5 M urea gel electrophoresis and identified by exposure to Kodak XAR film at −70 °C. The size of the protected fragments was estimated using a DNA 1-kb ladder (GIBCO-BRL) that was end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase (Sambrook *et al.*, 1989).

Northern Blotting—Aliquots of 10 μg of total RNA were fractionated by 1% agarose-formaldehyde gel electrophoresis (Lehrach *et al.*, 1977). After electrophoresis, RNA was transferred to Nytran filters (0.45 μm) (Schleicher & Schuell). The filters were prehybridized for 1 h at 60 °C in a solution of 50% deionized formamide, 5 × Denhardt's reagent, 1.0% sodium dodecyl sulfate, and 200 μg/ml denatured salmon sperm DNA. Hybridization of filters was carried out with fresh prehybridization buffer solution containing 2 × 10^6 cpm/ml radiolabeled probe. The probe was an antisense RNA derived from rat GLUT4 cDNA (pSM1–1) that was linearized with *Eco*RV and transcribed using T7 polymerase (Birnbaum, 1989). Hybridization was carried out overnight at 60 °C. Filters were washed according to manufacturer's specifications, which included a high stringency wash with RNase A (10 μg/ml in 2 × SSPE) at 37 °C for 15 min. The washed filters were exposed to Kodak XAR film at −70 °C.

RESULTS AND DISCUSSION

The sequences responsible for tissue-specific expression of the GLUT4 gene in muscle and adipose tissue are unknown. In order to identify the cis-acting regulatory elements that control GLUT4 expression *in vivo*, we prepared a reporter gene construct containing 2.4 kb of the promoter region of the hGLUT4 gene fused to the CAT gene, designated hGLUT4[2.4]-CAT. This construct was injected into the pronucleus of mouse embryos, and, of the 25 animals that were born, PCR amplification of mouse tail DNA demonstrated that two (8%) female founders contained the transgene. Southern blot analysis indicated that one animal (2.4hF3) had integrated two copies of the transgene and the other (2.4hF16) carried approximately 18 copies (data not shown).

To assess the expression of the reporter gene, CAT activity of tissue extracts from heterozygous transgenic and control mice was measured (Fig. 1). CAT activity was readily detected in extracts of brown adipose tissue from both transgenic mouse lines but the levels of CAT activity were markedly greater in tissues obtained from the 2.4hF16 line than from the 2.4hF3 animals (compare *lanes 2* and *3*). As expected, there was no CAT activity present in extracts of brown adipose and liver tissue from non-transgenic control animals (*lanes 1* and *4*) or in liver extracts from transgenic mice (*lanes 5* and *6*). Since the levels of expression of the hGLUT4-CAT construct were higher in the 2.4hF16 transgenic mice, we focused our studies on this line of animals.

RNA and protein blotting studies have indicated that

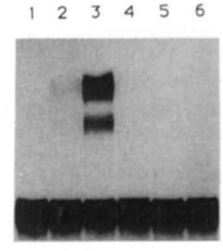

FIG. 1. **Expression of hGLUT4[2.4]-CAT reporter activity in transgenic mice.** The CAT activity in 0.5 mg of protein extract prepared from brown adipose tissue (*lanes 1–3*) and liver (*lanes 4–6*) isolated from control (*lanes 1* and *4*), transgenic mouse 2.4hF3 (*lanes 2* and *5*), and transgenic mouse 2.4hF16 (*lanes 3* and *6*) was determined as described under "Experimental Procedures" with buffer containing Triton X-100.

GLUT4 is expressed at highest levels in brown and white adipose tissue as well as in cardiac and skeletal muscle (Birnbaum, 1989; Fukumoto *et al.*, 1989; James *et al.*, 1988, 1989; Charron *et al.*, 1989). Lower levels of mRNA have been detected in kidney and small intestine; however, the cellular sites of expression of GLUT4 in these latter tissues are unknown. In the 2.4hF16 transgenic mice, CAT activity was detected in tissues which typically express GLUT4 mRNA such as diaphragm, heart, quadriceps skeletal muscle, white and brown adipose tissue, and uterus (Fig. 2*A*). There was no CAT activity in tissues which do not express GLUT4 mRNA, such as liver or brain. This pattern of tissue-specific expression of the hGLUT4[2.4]-CAT construct was similar in young (5 weeks) and old (15 months) mice as well as in male and female animals.

These results indicate that 2.4 kb of the 5′-flanking region of the hGLUT4 gene are sufficient to confer tissue-specific expression. However, the relative levels of CAT activity did not appear to quantitatively reflect the endogenous steady-state levels of GLUT4 mRNA previously reported for these tissues (Birnbaum, 1989; Charron *et al.*, 1989; Fukumoto *et al.*, 1989; James *et al.*, 1989). For example, the relative abundance of GLUT4 mRNA is similar in white and brown adipose tissue as well as heart and skeletal muscle; however, CAT activity in heart extracts was significantly lower than skeletal muscle or brown and white adipose tissue (Fig. 2*A*). The reasons for this discrepancy are unknown but may reflect differences in stability of CAT mRNA or CAT protein compared with GLUT4 mRNA.

Therefore, the levels of CAT and GLUT4 mRNA in tissues of the 2.4hF16 transgenic animals were assessed using an RNase protection strategy (Fig. 2*B*). The endogenous mouse GLUT4 mRNA was detected as a 176-nucleotide fragment which was present at similar levels in diaphragm, heart, quadriceps skeletal muscle, and white and brown adipose tissue and at much lower levels in kidney. There was no detectable GLUT4 mRNA specific signal in liver or brain. It should be noted that the two bands above the 176-nucleotide mouse GLUT4 mRNA specific fragment represent incomplete RNase digestion as these bands were not observed in other experiments. The tissue distribution of the 258-nucleotide fragment specific for CAT mRNA was identical to that seen for CAT activity. CAT mRNA was present in diaphragm, heart, skeletal muscle, and brown and white adipose tissue. However, the CAT mRNA was disproportionately higher in the heart (Fig. 2*B*, *lane 5*) compared with the relative level of measured CAT activity (Fig. 2*A*, *lane 5*). There was no detectable CAT mRNA in liver, brain, or kidney, consistent with the absence of CAT activity in these tissues. Thus, the pattern of tissue-specific expression and levels of CAT mRNA paralleled the expression levels of the endogenous mouse GLUT4 mRNA. These data indicate that the CAT reporter activity qualitatively reflects the appropriate tissue-specific activity of GLUT4 promoter constructs. However, in some tissues such as heart, the levels of CAT activity may be influenced by post-transcriptional mechanisms and thus may not quantitatively reflect the degree of transcriptional activity present.

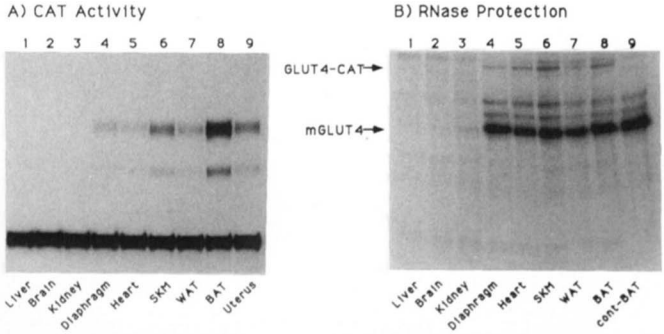

FIG. 2. **CAT reporter activity, steady-state CAT, and GLUT4 mRNA levels in transgenic mice.** *A*, CAT activity was determined in Triton X-100 tissue extracts (0.5 mg) from a heterozygous female 2.4hF16 transgenic mouse. The percent conversion of chloramphenicol was: liver (0.1%), brain (0.4%), kidney (0.1%), diaphragm (4.5%), heart (2.9%), hindquarter skeletal muscle (*SKM*, 8.6%), white adipose tissue (*WAT*, 4.8%), brown adipose tissue (*BAT*, 28.7%) and uterus (7.3%). *B*, steady-state CAT and endogenous mouse GLUT4 mRNA levels were determined by RNase protection from total RNA pooled from three 2.4hF16 animals. Ten μg of total RNA was used for the RNA protection assay, except for white adipose tissue, in which 20 μg was used because of the relative low levels of CAT and GLUT4 mRNA present. The CAT and mouse GLUT4 mRNA specific fragments are 258 and 176 nucleotides, respectively. Brown adipose tissue RNA from a non-transgenic littermate (cont-BAT, *lane 9*) was included as a control to confirm the specificity of the RNase protection assay for CAT mRNA; the 258-nucleotide fragment specific for CAT mRNA was not detected.

Previous studies have demonstrated that in white adipose tissue GLUT4 mRNA levels are decreased in insulin-deficient states such as streptozotocin-induced diabetes and fasting (Berger *et al.*, 1989; Garvey *et al.*, 1989; Kahn *et al.*, 1989; Sivitz *et al.*, 1989). To assess the hormonal/metabolic regulation of the hGLUT4[2.4]-CAT reporter gene, mice were either untreated, fasted for 24 h, or fasted for 24 h followed by 24 h of refeeding (Fig. 3). In this experiment, fasting resulted in an approximately 2-fold decrease in CAT activity in white adipose tissue. Following 24 h of refeeding, the CAT activity was super-induced approximately 4-fold greater than control values (Fig. 3, *lanes 1–3*). In five independent experiments the percent chloramphenicol conversions (CAT activity) were control, 13 ± 2.1; fasting, 9.2 ± 1.7; and fasting/refeeding, 50 ± 6.0. This decrease and subsequent recovery of CAT activity qualitatively paralleled the changes observed for endogenous mouse GLUT4 mRNA levels (Fig. 4, *lanes 1–3*). The smaller decrease of CAT activity compared with GLUT4 mRNA following 24 h of fasting may reflect the relative greater stability of CAT protein. In contrast to white adipose tissue, there was no significant effect of fasting/refeeding on CAT activity in brown adipose tissue (Fig. 3, *lanes 4–6*) and hindquarter skeletal muscle (Fig. 3, *lanes 7–9*). Fasting/refeeding also did not alter the levels of endogenous mouse GLUT4 mRNA in these tissues (Fig. 4, compare *lanes 4–6* and *lanes 7–9*). Although fasting has been reported to increase GLUT4 mRNA levels in skeletal muscle of fasted rats (Charron and Kahn, 1990), we have not observed this phenomenon in fasted mice. Nevertheless, these data demonstrate that 2.4

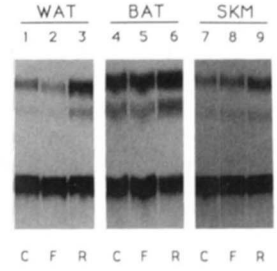

FIG. 3. **CAT reporter activity in untreated, fasted, and refed transgenic mice.** Heterozygous female 2.4hF16 transgenic mice were either untreated (*C*, *lanes 1*, *4*, and *7*), fasted for 24 h (*F*, *lanes 2*, *5*, and *8*), or fasted for 24 h then refed for 24 h (*R*, *lanes 3*, *6*, and *9*). CAT activity was measured in tissue extracts from 300 μg of white adipose tissue (*WAT*, *lanes 1–3*), 30 μg of brown adipose tissue (*BAT*, *lanes 4–6*), and 300 μg of hindquarter skeletal muscle (*SKM*, *lanes 7–9*).

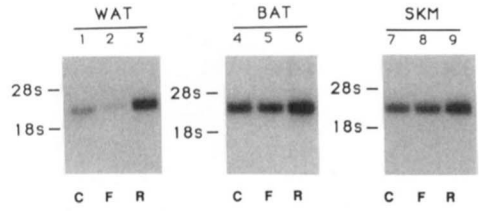

FIG. 4. **Endogenous GLUT4 mRNA in untreated, fasted and refed transgenic mice.** Heterozygous female 2.4hF16 transgenic mice were either untreated (*C, lanes 1, 4,* and *7*), fasted for 24 h (*F, lanes 2, 5,* and *8*), or fasted for 24 h then refed for 24 h (*R, lanes 3, 6,* and *9*). Total cellular RNA was isolated from white adipose tissue (*WAT, lanes 1–3*), brown adipose tissue (*BAT, lanes 4–6*), and hindquarter skeletal muscle (*SKM, lanes 7–9*). Ten μg of total RNA was fractionated on a 1% agarose-formaldehyde gel, 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.

kb of the 5′-flanking region of the hGLUT4 gene provide, at least in part, some of the cis-regulatory elements responsible for hormonal/metabolic-dependent regulation that occurs in white adipose tissue *in vivo*.

In summary, a 2.4-kb region flanking the 5′-end of the human GLUT4 gene appears sufficient to direct tissue-specific expression of a reporter gene in transgenic mice. Although the tissue levels of CAT activity in these transgenic animals are consistent with the relative levels of endogenous GLUT4 mRNA, the CAT activity is markedly reduced in the heart. Thus, studies using CAT activity as a measure of promoter activity in heart compared to other tissues need to be interpreted with caution and suggest that CAT mRNA levels may be a more appropriate measure of these transcriptional events. In addition to be being sufficient for conferring tissue-specific expression in transgenic animals, the 2.4-kb hGLUT4 promoter fragment also displays appropriate hormonal/metabolic regulation compared with the endogenous mouse GLUT4 mRNA. Although these studies were performed on a single transgenic founder line, the high degree of tissue specificity and appropriate hormonal/metabolic regulation of the reporter construct strongly suggests that the site of chromosomal integration did not influence expression. Currently, additional GLUT4 promoter constructs are being expressed in transgenic mice to precisely identify the DNA elements responsible for both tissue-specific and regulated expression of this gene.

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