Identification of a Skeletal Muscle-specific Regulatory Domain in the Rat GLUT4/Muscle-Fat Gene*

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To identify sequences responsible for the muscle-specific expression of the rat GLUT4/muscle-fat gene, we examined the transcriptional regulation of this gene in the differentiating murine C2C12 skeletal muscle cell line. Differentiated myofibers displayed a 4–5-fold increase in GLUT4 mRNA compared with undifferentiated myoblasts which paralleled the conversion from non-muscle β-actin mRNA to muscle-specific α-actin mRNA expression. Transient transfection of progressive 5' and 3' deletions of the GLUT4 5'-flanking DNA identified a 281-base pair region located between -517 and -237 relative to the transcription start site which conferred myotube-specific expression. This region increased reporter activity in the context of the GLUT4 minimal promoter in an orientation-independent manner and, in addition, onto the heterologous thymidine kinase promoter. Myotube-specific expression of both GLUT4 reporter constructs and the endogenous mouse GLUT4 mRNA was also observed to be thyroid hormone-dependent. Further, cotransfection of reporter constructs containing the 281-base pair GLUT4 differentiation-specific enhancer with the thyroid hormone receptor specifically increased luciferase activity in myotubes approximately 12-fold. Thus, these data demonstrate the presence of a proximal skeletal muscle-specific activation domain that is necessary for both myotube-specific GLUT4 expression and thyroid hormone responsiveness.

Facilitative glucose transport plays a central role in energy metabolism by mediating the concentration-dependent uptake of circulating glucose into mammalian cells. Currently, there are four members of the facilitative glucose transporter family, each characterized by having distinct kinetic properties as well as tissue-specific patterns of expression (1). The insulin-responsive glucose transporter, GLUT4, is expressed primarily in adipose tissue and skeletal and cardiac muscle, tissues that display insulin-stimulated glucose transport (2–6). Recently, several studies have demonstrated that insulin-deficient diabetes results in decreased expression of both muscle and adipose tissue GLUT4 protein and mRNA (7–11). Since skeletal muscle is quantitatively the major tissue responsible for insulin-mediated glucose disposal in vivo (12), the decreased skeletal muscle expression of GLUT4 may account for some forms of peripheral insulin resistance which are associated with diabetes.

In addition to hormonal/metabolic control, the expression of GLUT4 in cardiac and skeletal muscle is developmentally regulated (13). The predominant glucose transporter in fetal muscle is the GLUT1 isoform. However, during late neonatal development and within the first weeks of postnatal life there is a marked decrease in GLUT1 levels with a concomitant increase in GLUT4 expression. This pattern of developmental regulation is observed with many muscle-specific genes including those encoding enzymes involved in energy metabolism as well as contractile proteins (14). Although the mechanism responsible for this isoform exchange is unknown, a number of myogenic transcription factors have been described which appear to play a major functional role in the expression of at least some muscle-specific genes. The helix-loop-helix myogenic factors, MyoD (15), myogenin (16, 17), MRF4 (18), and myf-5 (19), have been shown to bind to specific regulatory elements in several muscle-specific genes including muscle creatine kinase (20, 21), myosin light chain (22), and the acetylcholine receptor α and δ subunits (23, 24). Although the GLUT4 gene contains several E box motifs with the potential to interact with these factors, cotransfection studies have not demonstrated any specific trans-activation by these DNA binding proteins.

To begin to understand how tissue-specific transcription of the GLUT4 gene is regulated during skeletal muscle development, we have cloned portions of the 5'-flanking sequence of the rat GLUT4 gene upstream of the luciferase reporter gene. Since the murine skeletal muscle differentiating cell line C2C12 has been used extensively to analyze muscle-specific expression of a number of genes, we utilized this tissue culture model system for transient transfection assays of both 5' and 3' GLUT4 deletion reporter constructs. In this manner we have identified a muscle differentiation-specific activating domain located from -517 to -237 relative to the transcription start site of the rat GLUT4 gene. This region, which contains a thyroid hormone response element (TRE) homology, displayed myotube-specific activation by the thyroid hormone receptor α1 (TRα1) which is consistent with the observed T3 induction of endogenous GLUT4 mRNA.

EXPERIMENTAL PROCEDURES

Cell Culture—the C2C12 mouse muscle cell line (American Type Culture Collection) was maintained at subconfluent densities in growth media consisting of Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 25 mM glucose and supplemented with 20% fetal calf serum. The cells were induced to differentiate by exchanging the growth medium for differentiation medium consisting of 1% horse serum, 1% fetal calf serum, 0.05% insulin, 100 nM T3, 10 ng/ml dexamethasone, 100 nM triiodothyronine, 50 μg/ml ascorbic acid, 50 μg/ml gentamicin, and 10 μg/ml transferrin.
of Dulbecco's modified Eagle's medium with 5.5 mM glucose supplemented with 2% horse serum following cell confluence. HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 25 mM glucose plus 10% fetal calf serum. Where indicated thyroid hormone was added to the medium to be harvested as myoblasts or myotubes, respectively. The cells were transiently transfected by the calcium phosphate method (26) at 24 h (myoblasts) and 72-96 h (>50% fusion into multinucleated myotubes) after transfection. In some experiments, fully differentiated fused myotube cultures were directly transfected with GLUT4 luciferase reporter constructs with similar results.

RESULTS

GLUT4 Gene Expression in C2C12 Cells—The GLUT4 glucose transporter is expressed in adult skeletal muscle, heart, and adipose tissue (4). However, in fetal rat skeletal muscle the GLUT4 protein is only detectable following 21 days of gestation at which time levels account for approximately 6% of adult values (13). Subsequently, an increase in GLUT4 expression occurs with a concomitant decrease in GLUT1 levels as development proceeds into the adult state. To determine whether the differentiating murine C2C12 skeletal muscle cell line is an appropriate model for the developmental regulation of GLUT4 expression, steady-state levels of GLUT4 mRNA were determined by Northern blot analysis (Fig. 1A). The GLUT4 transcript, which was detectable at relatively low levels in poly(A)* RNA from myoblasts (Fig.

![Northern blot analysis of endogenous GLUT4 and actin mRNA levels.](image-url)
1A, lane 1) was increased approximately 4-fold in differenti- 
tiated myotubes (Fig. 1A, lane 2). The amount of GLUT4 
RNA in the myofibers was estimated to be approximately 
2–5% of that present in adult mouse skeletal muscle based 
upon the signal intensity obtained with 5 μg of total mouse 
hindquarter skeletal muscle RNA (Fig. 1A, lane 3). HeLa cells 
were observed to express substantially lower levels of GLUT4 
mRNA (Fig. 1A, lane 4) which were unchanged by treatment 
with the same differentiation medium that induced C2C12 
myotube formation (Fig. 1A, lane 5).

As an independent measure of muscle cell differentiation, 
an identical blot was hybridized with a probe for actin mRNA 
(Fig. 1B). This probe recognizes both the muscle-specific α- 
actin (1.5 kb) as well as the non-muscle β-actin species (2.1 
kb). Under these conditions, C2C12 myoblasts predominantly 
expressed β-actin mRNA (Fig. 1B, lane 1) and following 
myotube differentiation primarily expressed α-actin mRNA 
(Fig. 1B, lane 2). The switch from β-actin to α-actin expres-
sion documents the phenotypic conversion of the C2C12 cells 
from a non-muscle (myoblast) to muscle (myofiber) state as 
has been described in developing muscle tissue (36, 37) and 
in myogenic cells in culture (38, 39). As expected, only the α- 
actin mRNA species was detected in adult skeletal muscle 
(Fig. 1B, lane 3), whereas β-actin was expressed in both 
untreated and differentiation medium-treated HeLa cells (Fig. 
1B, lanes 4 and 5).

The 5’ Flanking Region of the GLUT4 Gene Confers Myofi-
ter-specific Expression.—To determine whether myotube-spe-
cific gene expression is mediated by distinct cis-acting ele-
ments within the 5’ regulatory region of the rat GLUT4 gene, 
three luciferase reporter constructs were prepared containing 
68, 149, or 2,212 bp of upstream GLUT4 sequences relative 
to the transcription start site (Fig. 2A). These constructs, 
including the promoterless pLucLink vector, were transfect-
ed into HeLa cells and C2C12 cells that were maintained in 
either the myoblast state or differentiated into multinucleated 
myotubes. In myotubes the −2212Luc reporter displayed a 
3.6-fold increase of luciferase reporter activity compared with 
the −149Luc construct. In contrast, expression of the 
−149Luc reporter was essentially identical to the expression 
of −2212Luc in HeLa cells and myoblasts. In all three cell 
types examined, deletion from −149 to −68 resulted in an 
additional 95% decrease in transcription activity. These data 
suggest that a positive regulatory region is present between 
−2212 and −149 which confers a differentiation-specific in-
crease in GLUT4 transcription in C2C12 cells. An additional 
activating domain is present between −149 and −68 which 
is essential for minimal GLUT4 expression in all three cell 
types. Thus, the minimal GLUT4 promoter can be function-
ally defined by the −149 5’ boundary.

To determine whether the correct site of transcription 
initiation was utilized in the reporter constructs, primer ex-
tension analysis of the −2212Luc mRNA was performed (Fig. 
3). RNA isolated from C2C12 myotubes transfected with 
−2212Luc yielded several extension products (Fig. 3, lane 1) 
that are not present in RNA from untransfected cells (Fig. 3, 
lane 2). The largest and most abundant band (arrow) corre-
sponds to an A/T base pair which represents the transcription 
initiation site of the mouse (40) and rat α GLUT4 genes.

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manuscript in preparation.
Identification of a Myofiber-specific GLUT4 Activating Region—To define further the putative muscle-specific activating region within the GLUT4 upstream sequence, a series of 5′ deletion constructs were prepared using convenient restriction sites (Fig. 2B), including a construct containing an additional 2100 bp of 5′ sequence which extends 4300 bp upstream from the transcription start site. Transient transfection of C2C12 cells with GLUT4 promoter constructs extending from −149 bp to −4300 bp resulted in similar luciferase activities in the undifferentiated myoblasts (Fig. 4). However, myotubes displayed a 3–4-fold enhancement of luciferase activity with the −4300Luc, −2212Luc, −813Luc, and −517Luc reporter constructs compared with the −500Luc and −149Luc reporter constructs. These results demonstrate that the pattern of expression in C2C12 myoblasts, which express very low levels of GLUT4 mRNA, was similar for constructs spanning −149 to −4300 bp of GLUT4 upstream sequence. However, a significant increase in transcription was observed in C2C12 myotubes transfected with constructs containing a minimum of 517 bp of flanking sequence, suggesting that an activating domain resides in a region that has −517 as its 5′ boundary.

To define the 3′ boundary of the myotube-specific activating region, a 3′ deletion set was generated which had a shared 5′ end at −2212 and included −149 bp of minimal GLUT4 promoter sequence (Fig. 5A). In myoblasts, internal deletion of the sequence between −149 and −237 had no significant effect on myotube-specific expression, which was similar to the intact −2212Luc reporter. However, internal deletion of −149 to −501 or −149 to −813 resulted in luciferase activities that were indistinguishable from the −149Luc reporter. Similarly, a 3′ deletion set using −812 as the 5′ boundary also demonstrated a loss of myotube-specific expression in the −149 to −501 internal deletion (Fig. 5B). In myoblasts, there were no significant differences in the expression of any of the 3′ deletion constructs. These data demonstrate the presence of a myotube activating region that has −237 as its 3′ boundary. Taken together, the results of the 5′ and 3′ deletion sets define the activating region as a 281-bp EcoNI–BstXI fragment (−517 to −237).

An enhancer is characterized by its ability to increase transcriptional activity of heterologous promoters in an orientation- and distance-independent manner. To determine if the 281-bp fragment has the characteristics of an enhancer, the EcoNI–BstXI promoter domain was cloned upstream of the −149Luc GLUT4 minimal promoter construct both in the 5′ to 3′ and the 3′ to 5′ orientation as well as upstream of the TK105Luc promoter construct in the 5′ to 3′ orientation. Luciferase activities of the transfected constructs in myoblasts and myotubes are normalized to the −149Luc construct. The data represent the mean ± S.E. from two transfection experiments, performed in triplicate, using two preparations of each DNA construct.
negative orientations compared with the parental −149Luc vector alone. As expected, transcriptional activation by this region only occurred in C2C12 myotubes and was not observed in myoblasts.

GLUT4 Reporter Gene Expression Is Thyroid Hormone-responsive—Inspection of the proximal GLUT4 promoter revealed a sequence motif having strong identity with TREs described previously (41, 42). Further, digestion with Smal (−500) bisected this putative element and resulted in reporter constructs that did not display myotube-specific expression (Figs. 4 and 5). We therefore next examined the T3 dependence of GLUT4 reporter expression in the myotube cultures depleted of T3 (Fig. 7). Under these conditions, the C2C12 were morphologically observed to fuse into myotubes as well as to display the appropriate conversion of β- to α-actin (see Fig. 9B). Treatment of these myotube cultures for 24 h with 50 nM T3 resulted in a 4- and 5-fold greater expression of the −517Luc and (−517/−237)149Luc reporter genes, respectively, compared with the −500Luc and −149Luc constructs (Fig. 7). This pattern of expression was similar to that observed for these reporter constructs in C2C12 myotubes under standard differentiation conditions (Fig. 6). In contrast, in the absence of T3, the expression of the −517Luc and (−517/−237)149Luc reporter genes was decreased approximately 2.5-fold with no effect on the −500Luc and −149Luc reporters.

Since a putative TREV was contained within the myotubesspecific 281-bp enhancer domain, C2C12 cultures were cotransfected with an expression vector encoding the rat TRa1 cDNA and the (−517/−237)149Luc reporter genes, respectively, with or without (−T3) set to a value of 1. These results were obtained from two transfection experiments, performed in duplicate.

Fig. 7. Thyroid hormone regulation of GLUT4 reporter constructs. C2C12 cells were transfected with the (−517/−237)149Luc reporter or 5' deletion constructs containing −517, −500, or −149 bp of GLUT4 promoter. Cells were allowed to differentiate in T3-depleted medium and then treated with (+T3) or without (−T3) 50 nM T3 24 h prior to harvest. The data are expressed as relative luciferase activity/β-galactosidase activity ± S.E. with the −149Luc construct (−T3) set to a value of 1. These results were obtained from two transfection experiments, performed in duplicate.

mRNA by T3 (Fig. 9). As observed in Fig. 1, standard differentiation conditions resulted in an approximate 5-fold increase in GLUT4 mRNA in myotubes compared with myotubes (Fig. 9A, lanes 1 and 2). In contrast, differentiation of the myotubes in the absence of T3 (Fig. 9A, lane 3) resulted in a reduced expression of GLUT4 mRNA to levels similar to those observed in the myoblast cultures (Fig. 9A, lane 1).

Fig. 9. Thyroid hormone regulation of endogenous GLUT4 mRNA. Steady-state levels of GLUT4 mRNA (A), and actin mRNA (B) were assessed in C2C12 myoblasts (MB, lane 1), myotubes (MF lane 2), myotubes differentiated in T3-depleted medium (lane 3), or parallel plates treated with T3 (50 nM) for 12 h (lane 4) or 24 h (lane 5) as indicated. C, densitometric quantitation of the above GLUT4 blot relative to an identical blot that was hybridized with a cDNA probe for glyceraldehyde phosphate dehydrogenase. Five μg of poly(A)+ RNA was fractionated on a 1% agarose gel, transferred to nitrocellulose membrane, and hybridized with the indicated probes as described under "Experimental Procedures." This is a representative blot that independently performed three times.
However, T₃ treatment for 12 or 24 h resulted in a 5-fold induction of GLUT4 mRNA levels (Fig. 9A, lanes 4 and 5), which was equivalent to that observed in the standard myotube cultures (Fig. 9A, lane 2). The increase in myofiber GLUT4 mRNA levels was a direct effect of T₃ treatment as the T₃-depleted cultures both morphologically fused into myofibers and demonstrated the appropriate conversion of β- to α-actin (Fig. 9B). Because of the conversion from β- to α-actin mRNA, densitometric quantitation of GLUT4 expression was normalized to the invariant gene glyceraldehyde-3-phosphate dehydrogenase as indicated in Fig. 9C.

**DISCUSSION**

To define cis-DNA regions that regulate tissue-specific expression of the GLUT4 gene it is necessary to identify a suitable host cell system. The murine 3T3-L1 cell line has been well documented as expressing high levels of GLUT4 mRNA and protein when differentiated into the adipocyte phenotype (6, 43). To obtain a GLUT4-expressing muscle cell line, we have screened various cells including BC3H1, L6, L8, and 23A2. Although studies have observed GLUT4 mRNA and protein in the rat L6 and L8 muscle cell lines (44, 45), we were unsuccessful in detecting sufficient levels of GLUT4 mRNA for transcriptional analysis in these cells. In contrast, we have observed that multinucleated C2C12 myotubes were found to express GLUT4 mRNA levels approximately 4-5-fold greater than in proliferating C2C12 myoblasts. It should also be noted, however, that even following the myotube-specific induction of GLUT4 mRNA, the levels of this transcript were still substantially lower than that observed for in vivo skeletal muscle tissue. Nevertheless, since GLUT4 mRNA levels were induced in a myoﬁbr-speciﬁc manner, we reasoned that this cell line would provide a useful system in which to study the muscle differentiation-induced expression of this gene.

Based upon the availability of a suitable model system, we undertook a systematic study to identify sequence regions that control muscle-specific GLUT4 expression. Luciferase reporter constructs containing progressive amounts of the rat GLUT4 5’-flanking region were transfected into C2C12 cells and HeLa cells. Proliferating C2C12 myoblasts and HeLa cells, which do not express appreciable amounts of GLUT4 mRNA, displayed essentially identical luciferase activity whether the reporter constructs contained −149 or −2212 bp of GLUT4 upstream sequence. However, deletion to within 68 bp of the transcription start site resulted in a 95% decrease in luciferase activity in all cell types studied, suggesting that this proximal region from −149 to −68 provides minimal promoter function. In this regard, there are several consensus sequences for general control factors within this region which include a CCAAT homology element at −94 and two GC-rich sequences located at −129 and −65.2

In contrast, a 4-5-fold myotube-specific increase in luciferase activity was observed for the −4300Luc, −2212Luc, −813Luc, and −517Luc reporter constructs compared with the −5000Luc and −149Luc constructs. This increase in reporter activity directly correlated with the myotube induction of endogenous GLUT4 mRNA. Importantly, primer extension analysis demonstrated that transcription from the transfected GLUT4-luciferase construct was correctly initiated and therefore represented appropriate transcriptional activation. These data establish that the 5’ boundary of the myotube-specific activation region lies within −517 bp of transcription start. Further, 3’ deletion analysis has defined the 3’ boundary of this myotube activating region to within −237 bp of transcription start. The functional importance of this region was confirmed by its ability to activate myotube-specific expression in an orientation- and promoter-independent manner, and therefore the EcoNI-BstXI promoter region can be classified as an enhancer domain.

Thyroid hormone has been well established as modulating metabolic rate and whole body glucose utilization (46, 47) as well as increasing markedly glucose uptake in primary isolated rat skeletal muscle cell cultures (48). In addition, thyroid hormone has been shown to have a positive regulatory effect on GLUT4 mRNA and protein content in the skeletal muscle of hypothyroid rats (49). Consistent with these data, we have observed T₃-dependent regulation of both GLUT4 mRNA levels as well as the (−517−237)149Luc and −517Luc reporters in C2C12 myotubes. It is of interest to note that the effects of T₃ on the endogenous myoﬁbr GLUT4 mRNA levels (Fig. 9) were greater than those observed for the transfected GLUT4-Luc reporter genes (Fig. 7). Although we can not provide a mechanism accounting for this observation at the present time, this phenomenon has been characterized to report for several other thyroid hormone-responsive genes (50).

Within the rat GLUT4 281-bp myotube-specific enhancer region is the sequence GGAGACggggGAGACgccGGCCA which is highly conserved among the three, mouse (40), and human GLUT4 genes (52). These three hexamer repeat sequences in the GLUT4 gene have close identity with vitamin D, retinoic acid, and thyroid hormone response core element consensus sequences. The thyroid hormone response element consensus sequence proposed by Leidig (G/AGGTCA-N₉G/AGGA/CCN) is composed of two direct hexamer repeats separated by any three to five nucleotides (41). Additional evidence has suggested that the thyroid hormone receptor shows a selective preference for a separation between the AGGTCA-related sequences of four nucleotides (42). The three hexamer repeat motif in the GLUT4 promoter appears to be a reasonable candidate for a functional TRE. In support of this conclusion, both the (−517−237)149Luc and −517Luc reporters, but not the −500Luc and −149Luc constructs, displayed T₃ sensitivity. In addition, cotransfection of these reporters with TRLA1 demonstrated specific trans-activation in C2C12 myoblasts.

In summary, we have identified a 281-bp region within the GLUT4 promoter which plays a role both in the myotube-specific activation and the thyroid hormone responsiveness of GLUT4 transcription in C2C12 cells. The specific transcription factor interactions that mediate this activation remain to be characterized and will contribute toward an understanding of the mechanisms that control GLUT4 gene expression during skeletal muscle development.

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