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Differential Regulation of the Muscle-specific GLUT4 Enhancer in Regenerating and Adult Skeletal Muscle*

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We have reported a novel functional co-operation among MyoD, myocyte enhancer factor-2 (MEF2), and the thyroid hormone receptor in a muscle-specific enhancer of the rat GLUT4 gene in muscle cells. Here, we demonstrate that the muscle-specific enhancer of the GLUT4 gene operates in skeletal muscle and is muscle fiber-dependent and innervation-independent. Under normal conditions, both in soleus and in extensor digitorum longus muscles, the activity of the enhancer required the integrity of the MEF2-binding site. Cancellation of the binding site of thyroid hormone receptor enhanced its activity, suggesting an inhibitory role. Muscle regeneration of the soleus and extensor digitorum longus muscles caused a marked induction of GLUT4 and stimulation of the enhancer activity, which was independent of innervation. During muscle regeneration, the enhancer activity was markedly inhibited by cancellation of the binding sites of MEF2, MyoD, or thyroid hormone receptors. Different MEF2 isoforms expressed in skeletal muscle (MEF2A, MEF2C, and MEF2D) and all members of the MyoD family had the capacity to participate in the activity of the GLUT4 enhancer as assessed by transient transfection in cultured cells. Our data indicate that the GLUT4 enhancer operates in muscle fibers and its activity contributes to the differences in GLUT4 gene expression between oxidative and glycolytic muscle fibers and to the GLUT4 up-regulation that occurs during muscle regeneration. The activity of the enhancer is maintained in adult muscle by MEF2, whereas during regeneration the operation of the enhancer depends on MEF2, myogenic transcription factors of the MyoD family, and thyroid hormone receptors.

The GLUT4 glucose transporter gene is expressed mainly in muscle and adipose cells. GLUT4 expression is exquisitely regulated in skeletal muscle so that its level determines the whole-body glucose disposal in response to insulin. GLUT4 is differentially expressed in oxidative and glycolytic muscle fibers in the rat (1–3), undergoes up-regulation in muscle by thyroid hormones (4–6), and is repressed by muscle denervation (7–9), in experimental diabetes (2, 10, 11) or in response to cyclic AMP treatment (12). In addition, agonists of AMP-activated protein kinase enhance GLUT4 transcription in a muscle fiber-dependent manner (13, 14).

As to the regulation of GLUT4 gene transcription, different studies performed in transgenic mice have shown that a 5′-flanking region of 1154 bp in the GLUT4 gene is sufficient to drive muscle-, heart-, and adipose tissue-specific GLUT4 expression (15, 16). In addition, several regulatory elements have been identified within this region. Initial studies performed in cultured muscle cells identified the region −522/−402 as necessary for muscle-specific expression and a myocyte enhancer factor-2 (MEF2)[1]-binding site that was critical for its transcriptional activity (17). Disruption of this MEF2-binding site ablated tissue-specific GLUT4 expression in transgenic mice (18). Recently, it has been suggested that the transcriptional co-activator peroxisome proliferator activator protein-γ co-activator-1 participates in GLUT4 gene transcription by interacting with MEF2 transcription factors (19). The Krüppel-like factor, KLF15, binds to a site near the MEF2-binding element and induces GLUT4 gene expression in 3T3-L1 cells (20).

Another relevant region is located at −742/−712 relative to the transcription initiation site. Different factors such as NF1 (nuclear factor I) (21) and a partially characterized protein (22) bind to this region. NF1 seems to participate in the effects of insulin on GLUT4 gene expression in adipose cells (21) and the uncharacterized protein binding activity seems to interact with the MEF2-binding site mentioned previously (22). In addition, a region located between −125 and −112 of the mouse promoter has been reported to bind a 96-kDa protein, which seems to act as a repressor selectively in pre-adipocytes but not in adipocytes (23).

We have reported tripartite co-operation between MyoD, MEF2, and the thyroid hormone receptor (TRα1) that takes place in the context of an 82-bp muscle-specific enhancer in the rat GLUT4 gene (at −502/−420), which is active in both cardiac and skeletal muscle (24). In the L6E9 skeletal muscle cell

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[1] The abbreviations used are: MEF2, myocyte enhancer factor-2; EDL, extensor digitorum longus; TRα1, thyroid hormone receptor; TRE, thyroid hormone receptor element; MRF, myogenic regulatory factor; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; T_3, triiodothyronine.

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line and in 10T1/2 fibroblasts, a powerful synergistic activation of the GLUT4 enhancer relied on the over-expression of MyoD, MEF2, and TRα1 and the integrity of their respective binding sites. This is in keeping with the capacity of the E-box and the thyroid response element (TRE) present in the enhancer to bind MyoD and thyroid hormone receptors (24, 25). Furthermore, we have shown that in 10T1/2 fibroblasts, the forced over-expression of MyoD, MEF2, and TRα1 induces the expression of the endogenous, otherwise silent, GLUT4 gene (24).

In this study, we provide evidence that the −502/−420 enhancer acts in skeletal muscle under *in vivo* conditions and that its activity depends on the muscle fiber context, is up-regulated during muscle regeneration, and is independent of innervation.

MATERIALS AND METHODS

Reporter and Expression Vectors—The −502/−420 TKCAT and the −502/−420-pGL3-luciferase reporter constructs were obtained by the annealing of three overlapping pairs of synthetic oligonucleotides that encompassed the DNA sequence, comprising positions −502 to −420 in the rat GLUT4 5′-flanking region. The oligonucleotides flanking the enhancer were designed so that upon annealing, cohesive *Bam*HI ends would be incorporated into both ends of the reconstituted enhancer to allow the cloning into the *Bam*HI-digested TKCAT or luciferase reporter vectors. All oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). TKCAT, a gift from Dr. Nadal-Ginard (Cardiovascular Research Institute, New York Medical College, Valhalla, NY), is a reporter vector that allows the analysis of putative transcriptionally regulatory regions because of their effect on the basal transcription of the CAT gene driven by the −109/+51 region of the herpesvirus thymidine kinase gene (26). Mutant versions of the −502/−420 enhancer, which contained nucleotide substitutions in the sequence of putative binding sites for known transcription factors, were made by substituting, in the annealing reaction, a new pair of oligonucleotides containing the desired mutation for the wild type pair. The mutant forms of the enhancer were subsequently cloned into the same *Bam*HI site of the TKCAT vector by the same method used for the wild type enhancer. The sequence and orientation of the insert in all constructs was confirmed by sequencing.

The expression vectors for mouse MyoD, mouse myogenin, and rat muscle regulatory factor-4 (MRF4) were obtained from Dr. H. Weintraub (Fred Hutchinson Cancer Research Center, Seattle,WA), Dr. E. N. Olson (M. D. Anderson Cancer Center, Houston, TX), and Dr. S. F. Konieczny (Purdue University, West Lafayette, IN), respectively. The cDNA of human Myf5 obtained from ATCC was cloned into an eukaryotic expression vector. The expression vectors for TRα1 (pMT2-TRα1), MEF2A, MEF2C, and MEF2D were obtained from Dr. B. Nadal-Ginard (Harvard Medical School, Boston, MA), Dr. P. Ruiz-Lozano (University of California at San Diego, La Jolla, CA), Dr. J. McDermott (York University, Toronto, Canada), and Dr. E.N. Olson (M. D. Anderson Cancer Center), respectively.

Muscle Regeneration and Denervation—Muscle regeneration was induced in 200–250-g male Wistar rats by intramuscular injection of bupivacaine as described (27). Denervation was produced by cutting the sciatic nerve high in the thigh.

Transfection of Regenerating and Adult Muscles—Regenerating innervated or denervated soleus or extensor digitorum longus muscles were injected with plasmid DNA (50 μg) at day 3 after bupivacaine treatment as described (27). We have shown previously that gene transfer efficiency is high after DNA injection at day 3, when the regenerating muscle is composed mostly of small myotubes, but is very poor after DNA injection at day 1, when only mononucleated myoblasts are present (27). Muscles were removed at day 10 after injury (day 7 after transfection) and frozen in isopentane cooled in liquid nitrogen. Adult muscles were transfected by intramuscular injection of plasmid DNA (20 μg) followed by electroporation to increase gene transfer efficiency. The electroporation procedure was similar to that described by Mir *et al.* (28). Co-transfection of RSV-CAT or RSV-luciferase expression plasmids was used to normalize for transfection efficiency. Muscles were removed at day 7 after transfection and frozen in isopentane cooled in liquid nitrogen. Muscle extracts were obtained, and reporter gene activity (luciferase and CAT) was measured by standard procedures. All data were normalized for protein concentration on muscle extracts.

Cell Culture and Transfections—The C3H10T1/2 cell line (10T1/2) was purchased from ATCC (Manassas, VA) and cultured as described (29). Cells were transfected with the Fugene™ transfection enhancer reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. CAT activity was measured in cytoplasmic extracts as described (29). Transfections with the CAT reporter vector included an *Escherichia coli* β-galactosidase expression vector (pON249) under the control of the cytomegalovirus promoter (24). β-Galactosidase activity was measured in cytoplasmic extracts to determine the efficiency of transfection. Protein concentration was measured with the BCA protein assay reagent (Pierce). When T_3 was administered to cells, medium containing T_3-depleted serum was used. T_3-depleted serum was prepared by anion-exchange chromatography as described (30).

Western Blotting—Western blotting was performed essentially as described (9). Twenty-five μg of membrane protein obtained from regenerating or control muscles was loaded onto 10% SDS-polyacrylamide gels to detect GLUT4 and the α_1 subunit of the Na^+-K^+-ATPase. Proteins were detected by using specific antibodies.

RNA Isolation and Northern Blot Analysis—Total RNA was extracted using the acid guanidinium thiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi (31). All samples had an A_{260}/A_{280} ratio above 1.8. After quantification, total RNA (30 μg) was denatured at 65 °C in the presence of formamide, formaldehyde, and ethidium bromide to allow the visualization of RNA. RNA was separated on a 1.2% agarose-formaldehyde gel and blotted on Hybond N filters. The RNA in gels and in filters was visualized with ethidium bromide by UV transillumination to ensure the integrity of RNA, to check the loading of equivalent amounts of total RNA, and to confirm proper transfer. Northern blot was performed as reported (4). The rat cDNA probe for GLUT4, a 2470-bp *Eco*RI fragment obtained from Dr. M. Birnbaum (University of Pennsylvania), was labeled with [^{32}P]dCTP by random oligonucleotide priming.

RESULTS

The Muscle-specific Enhancer of the GLUT4 Gene Operates in Skeletal Muscle under in Vivo Conditions and Is Muscle Fiber-dependent—We have previously reported that the −502/−420 enhancer regulates the transcriptional activity of the GLUT4 gene in muscle cells in culture (24). Here, we studied the relevance of this enhancer under *in vivo* conditions in rat skeletal muscle. In initial studies, the −502/−420-TKCAT plasmid or the parental promoter vector (as a control) were transfected by electroporation into adult rat soleus (mainly composed of slow-twitch oxidative muscle fibers) and extensor digitorum longus (EDL) (mainly composed of fast-twitch glycolytic muscle fibers) muscles. Our data indicate that the −502/−420 enhancer is operative under *in vivo* conditions both in soleus and in EDL muscles and the activity of the enhancer was about 7–11-fold that of the control vector (Fig. 1). The data also indicate that the enhancer activity was significantly higher in soleus muscle than in EDL (Fig. 1). Similar observations were obtained after transfection with a plasmid −502/−420-luciferase, *i.e.* the enhancer is operative in skeletal muscles, and soleus showed a greater activity than EDL muscles (data not shown). This is consistent with greater GLUT4 gene expression in soleus compared with EDL muscles (1–3).

Muscle Regeneration Stimulates the Activity of the GLUT4 Enhancer—Damaged skeletal muscle is able to regenerate by activation of satellite cells. These cells are quiescent under basal conditions; however, their activation induced by trauma causes proliferation and further differentiation into myotubes and muscle fibers. The myogenic factors MyoD and Myf5 are induced early during muscle regeneration, whereas MRF4 and myogenin show a later induction (32). It has also been suggested that during muscle regeneration, the activity of MEF2 is enhanced (33). On this basis, we explored the impact of muscle regeneration on GLUT4 enhancer activity. To this end, we tested the enhancer activity in intact or regenerating soleus or EDL muscles at day 7 after bupivacaine injection. Transfection of the enhancer linked to the CAT reporter indicated a marked stimulation (5-fold stimulation) of transcriptional activity during regeneration (Fig. 2). Similar data were obtained when the GLUT4 enhancer was linked to a luciferase reporter, *i.e.* activ-

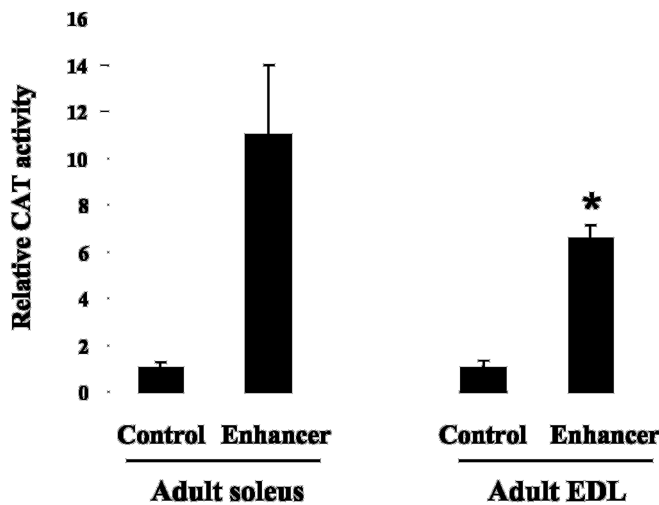


FIG. 1. **The muscle-specific GLUT4 enhancer (−502/−420) is active in rat skeletal muscle in a fiber-dependent manner.** Soleus and EDL muscles from adult male rats were transfected *in vivo* by electroporation with 20 μg of empty vector (*Control*) and 20 μg of the −502/−420-TKCAT construct (*Enhancer*) along with a luciferase reporter plasmid as a transfection control. We collected muscles 7 days after transfection to obtain extracts and further assay luciferase and CAT activity. CAT activity was corrected by luciferase activity and protein and expressed as values relative to control (empty vector) activity. Results are the mean ± S.E. of at least 10 observations/group. *, statistically significant difference between soleus and EDL muscles, $p < 0.05$.

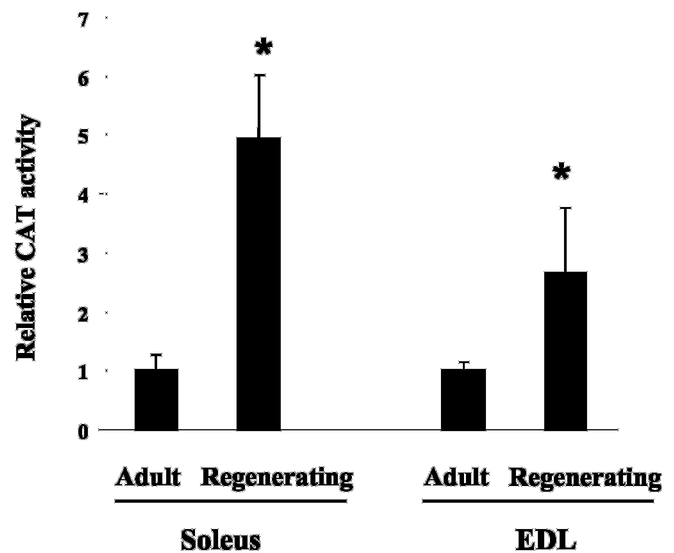


FIG. 2. **The muscle-specific GLUT4 enhancer (−502/−420) is activated during muscle regeneration.** Soleus and EDL muscles from adult male rats or from regenerating muscles were transfected *in vivo* with the −502/−420-TKCAT construct together with a luciferase reporter plasmid as a transfection control. After 7 days of transfection, muscles were collected, extracts were obtained, and enzymatic activities were assayed. Results are the mean ± S.E. from five observations/group. *, statistically significant difference between adult muscle and regenerating muscle, $p < 0.05$.

ity was enhanced 5-fold after muscle regeneration (data not shown). Muscle regeneration also promoted marked stimulation of the GLUT4 enhancer activity in EDL muscles (Fig. 2).

The GLUT4 Enhancer Activity Is Independent of Muscle Innervation—Muscle denervation causes a marked repression of GLUT4 in skeletal muscles (7–9). Thus, we explored the effect of denervation in the activity of the GLUT4 enhancer. To this end, innervated or denervated soleus and EDL muscles were transfected by electroporation with the −502/−420-TKCAT plasmid and reporter activity was assayed at day 7 (Fig. 3). No significant differences were detected between innervated and denervated groups in either soleus or EDL muscles (Fig. 3). Under these conditions, GLUT4 expression was largely reduced by denervation in both muscle types (data not shown).

We also explored the effect of denervation on enhancer activity in regenerating muscles. To this end, soleus or EDL muscles were induced to regenerate in the absence or presence of innervation. Reporter gene analyses in transfected muscles indicated that the activity of the enhancer was again independent of innervation in both soleus and EDL muscles (Fig. 3).

Expression of GLUT4 in Regenerating Muscle—Based on the regulatory pattern of the GLUT4 enhancer, we explored the impact of muscle regeneration on GLUT4 expression. To this end, soleus and EDL muscles were induced to regenerate by bupivacaine injection in the absence or presence of innervation. At different times of initiation of regeneration, muscles were collected and GLUT4 protein and mRNA quantitated by Western and Northern blot. We also processed in parallel muscles from adult rats that were subjected to denervation for 7 days. Expression of GLUT4 protein was very low after 3 days of regeneration compared with adult levels (Fig. 4). GLUT4 protein markedly increased from day 3 to day 6 of regeneration and remained relatively stable from day 6 to day 10 (Fig. 4). Denervation did not prevent a robust induction of GLUT4 protein at day 6. However, denervation was associated to lower levels of GLUT4 expression during the regeneration period (Fig. 4). These effects were specific, and no substantial alteration of the α_1 subunit of the Na^+-K^+-ATPase was detected under these conditions (Fig. 4). An induction of GLUT4 mRNA was also detected with regeneration, which was progressive during time, so that maximal levels were attained only at day 10 (data not shown). In addition, maximal levels of GLUT4 mRNA at day 10 of regeneration were markedly lower in the denervated group (data not shown). Thus, during muscle regeneration GLUT4 is markedly induced and whereas an initial GLUT4 induction wave is largely independent of innervation, at later times it becomes dependent on muscle innervation.

Different Requirements of the Muscle-specific GLUT4 Enhancer in Adult and in Regenerating Muscles—We studied the active elements that were responsible for the activity of the enhancer in adult and in regenerating muscles. In some studies, we transfected soleus and EDL muscles with different mutant versions of the enhancer (*i.e.* mutations in the E-box, the MEF2-binding site, or the TRE). Analysis in cultured cells has demonstrated that the mutations used completely cancel the three binding sites (24). Both in soleus and in EDL muscles from adult rats, we detected a similar profile of changes to the transcriptional output of the reporter (Fig. 5). Cancellation of the E-box did not alter the transcriptional activity of the enhancer in soleus or EDL muscles. In contrast, mutation of the MEF2-binding site caused a marked reduction of the activity of the enhancer both in soleus and in EDL muscles (Fig. 5). Mutation of the TRE doubled the transcriptional activity of the enhancer in soleus and EDL muscles (Fig. 5), suggesting that the thyroid hormone receptors may play a negative role in adult muscles.

We also studied the requirements of the GLUT4 enhancer during muscle regeneration. To this end, we transfected innervated or denervated regenerating soleus or EDL muscles with the different mutant versions of the enhancer. Cancellation of the E-box or the TRE caused a marked reduction in the activity of the enhancer in innervated or denervated regenerating soleus or EDL muscles (Figs. 6 and 7), which is in contrast to what we found in adult muscle (Fig. 5). In keeping with the observations in adult muscle, mutation of the MEF2-binding site caused a marked reduction of the activity of the enhancer

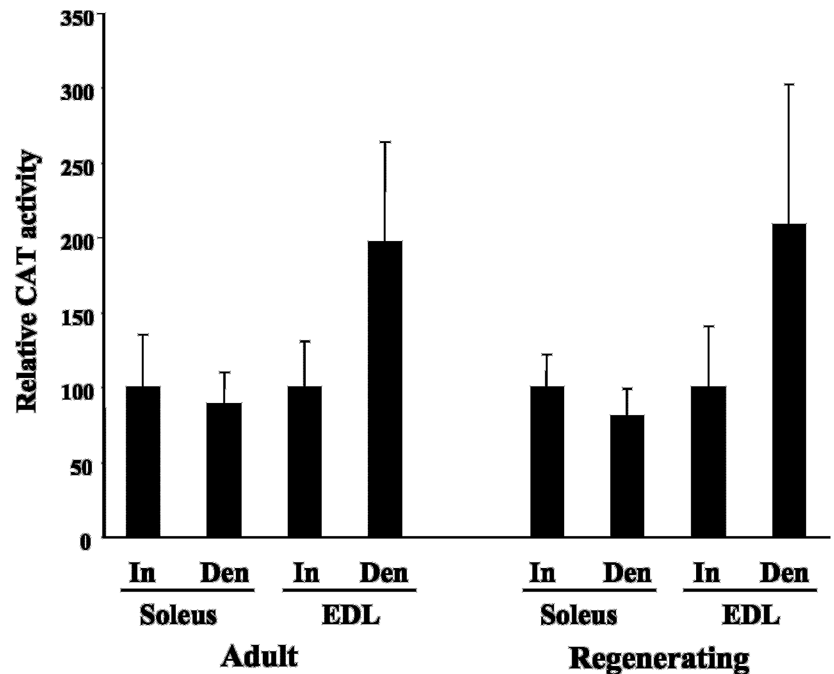
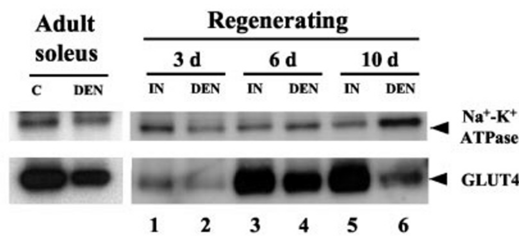


FIG. 3. **The activity of the muscle-specific GLUT4 enhancer (−502/−420) is independent of innervation.** Adult and regenerating soleus and EDL muscles were transfected with the −502/−420-CAT construct together with a luciferase reporter plasmid as a transfection control. Simultaneous with transfection, muscles were denervated (*Den*) by sciatic nerve sectioning, or they remained innervated (*In*). Seven days after transfection, muscles were collected, extracts were obtained, and luciferase and CAT activity were assayed. Specific CAT activity was expressed as a percentage of activity in the innervated groups. Results are mean ± S.E. from five observations/group. Differences between innervated and denervated muscles were not statistically significant.

A. Soleus



B. EDL

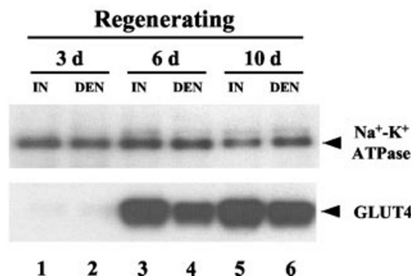


FIG. 4. **GLUT4 expression is induced during muscle regeneration.** Total membrane fractions were obtained from control (*C*), denervated, regenerating (*IN*) or regenerating-denervated (*DEN*) soleus (*panel A*) or extensor digitorum longus muscles (*panel B*). Denervation of adult nonregenerating muscle was done 7 days prior to muscle collection. Muscle regeneration was studied on days 3, 6, and 10. Total membranes were subjected to SDS-PAGE and further immunoblotting using specific antibodies directed against GLUT4 or the α_1 subunit of Na^+-K^+-ATPase. Panels show representative autoradiograms.

in innervated or denervated regenerating soleus or EDL muscles (Figs. 6 and 7).

Different MEF2 Isoforms or MRF Myogenic Proteins Operate on the GLUT4 Enhancer—We have previously shown that MEF2A and MyoD transactivate the muscle-specific GLUT4 enhancer and that MEF2A synergizes with MyoD and TRα1 on the activity of the enhancer in muscle and non-muscle cells (24). Based on the role of the E-box and the MEF2 element in the control of the activity of the muscle-specific GLUT4 enhancer in the muscle fiber under different conditions, we studied whether other MEF2 isoforms expressed in muscle, *i.e.* MEF2C and MEF2D (34), or other MRF myogenic factors, *i.e.* myogenin, Myf5, or MRF4, also transactivate the enhancer.

In some studies 10T1/2 cells were transiently transfected with MEF2A, MEF2C, or MEF2D either alone or in combination with TRα1 and/or MyoD (Fig. 8*A*). MEF2A, MEF2C, and MEF2D behaved similarly when transfected on their own (3–4-fold induction) (Fig. 8*A*). In addition, all MEF2 isoforms synergized with MyoD and TRα1 (Fig. 8*A*). Under these conditions, MEF2C was most efficient in synergizing with TRα1 or with MyoD plus TRα1 (Fig. 8*A*). This was followed by MEF2A and MEF2D (Fig. 8*A*). We also tested whether co-transfection with different MEF2 isoforms could modify, through the generation of heterodimers, the functional tripartite cooperativity with MyoD or TRα1. Transfection of cells with expression vectors for MEF2A and MEF2D in the presence of MyoD and TRα1 activated the enhancer up to levels comparable with MEF2A, which were greater than the values obtained in the presence of MEF2D (Fig. 8*B*). Similarly, transfection with MEF2C and MEF2D in the presence of MyoD and TRα1 raised the enhancer activity up to values similar to those of the MEF2C group and again greater than the values of the MEF2D group (Fig. 8*C*).

In other experimental series, we transiently co-transfected 10T1/2 cells with different combinations of cDNA expression vectors for members of the MyoD family of MRF, MEF2A, and TRα1. In some experiments we used MyoD or myogenin as MRF proteins and in other experiments MyoD or MRF4 (Fig. 9). Myogenin and MRF4 transfected on their own showed a moderate activation of the enhancer, which was comparable with the effect of MyoD (Fig. 9). Transfection of TRα1 caused a repression of the enhancer when cells were maintained in the absence of T_3 (data not shown), whereas a substantial activation was detected in the presence of T_3 (Fig. 9). In addition, and most importantly, both myogenin and MRF4 activated the enhancer synergistically with TRα1 and with TRα1 plus MEF2A (Fig. 9). The synergistic effect displayed by myogenin or MRFR4 was similar to that of MyoD (Fig. 9). Myf5 behaved similarly (data not shown). Furthermore, we examined whether the effects of myogenin synergizing with TRα1 in

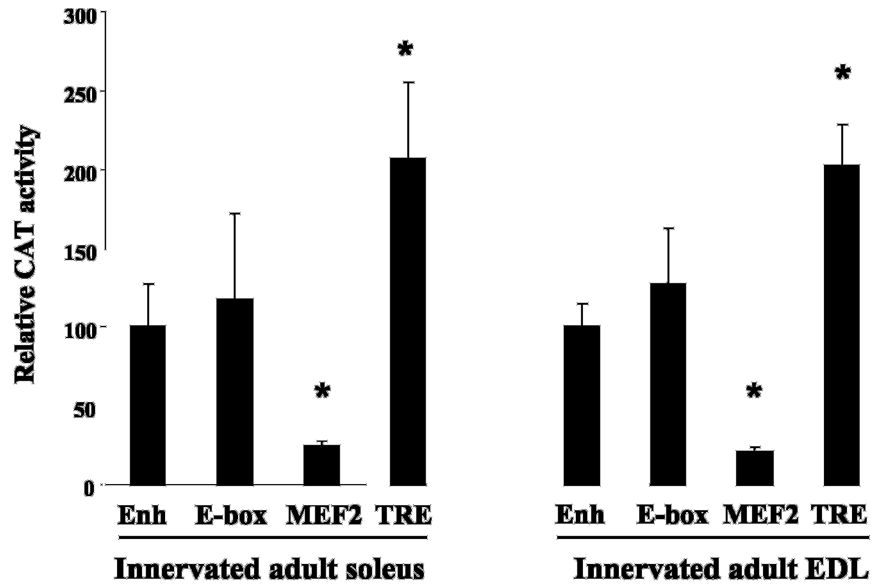


FIG. 5. **Effect of mutations in the E-box, MEF2, and TRE on the activity of the muscle enhancer in adult rat skeletal muscles.** Soleus and EDL muscles from adult male rats were *in vivo* transfected by electroporation with −502/−420-TKCAT constructs containing either the wild type enhancer (*Enh*) or mutated versions at the E-box, MEF2 box, or TRE together with a luciferase reporter plasmid as a transfection control. After 7 days of transfection, muscles were collected, extracts were obtained, and enzymatic activities were assayed. CAT activity was expressed as a percentage of wild type enhancer activity. Results are mean ± S.E. from five observations/group. *, statistically significant difference compared with the wild type enhancer group, $p < 0.05$.

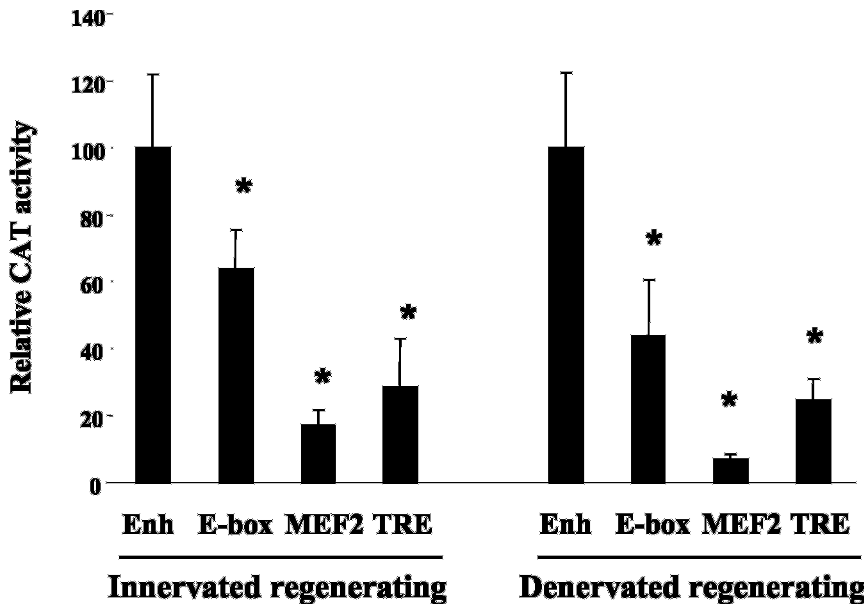


FIG. 6. **Effect of mutations in the E-box, MEF2, and TRE on the activity of the muscle enhancer in regenerating soleus muscle.** Soleus muscles from regenerating muscles were *in vivo* transfected with −502/−420TK-CAT construct, containing either the wild type enhancer (*Enh*) or mutated versions at the E-box, MEF2 box, or TRE together with a luciferase reporter plasmid as a transfection control. After 7 days of transfection, muscles were collected, extracts were obtained, and enzymatic activities were assayed. CAT activity was expressed as a percentage of wild type enhancer activity. Results are the mean ± S.E. from five observations/group. *, statistically significant difference compared with the wild type enhancer group, at $p < 0.05$.

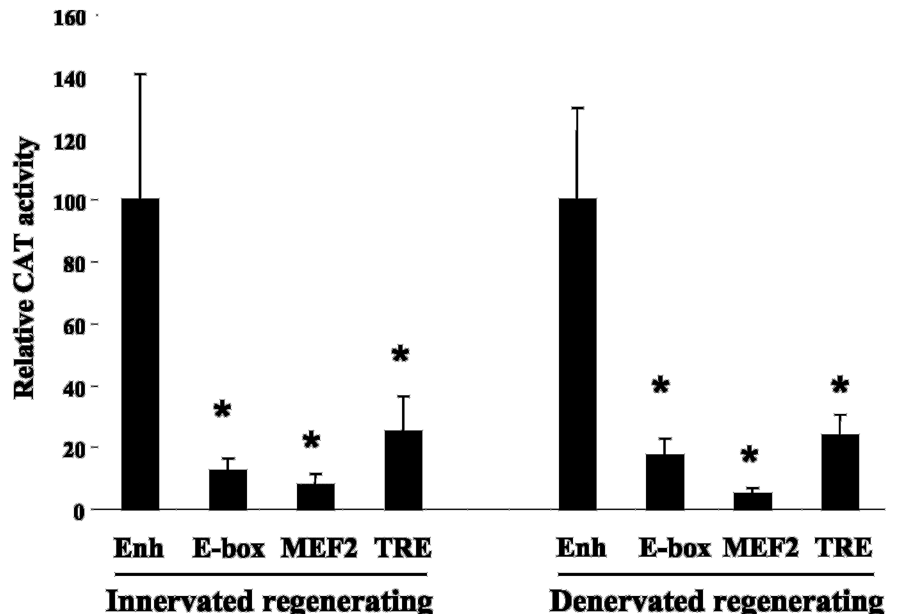


FIG. 7. **Effect of mutations in the E-box, MEF2, and TRE on the activity of the muscle enhancer in regenerating extensor digitorum longus muscle.** EDL muscles from regenerating muscles were transfected *in vivo* with the −502/−420TK-CAT construct containing either the wild type enhancer (*Enh*) or mutated versions at the E-box, MEF2 box, or TRE together with a luciferase reporter plasmid as a transfection control. After 7 days of transfection, muscles were collected, extracts were obtained, and enzymatic activities were assayed. CAT activity was expressed as a percentage of wild type enhancer activity. Results are the mean ± S.E. from five observations/group. *, statistically significant difference compared with the wild type enhancer group, $p < 0.05$.

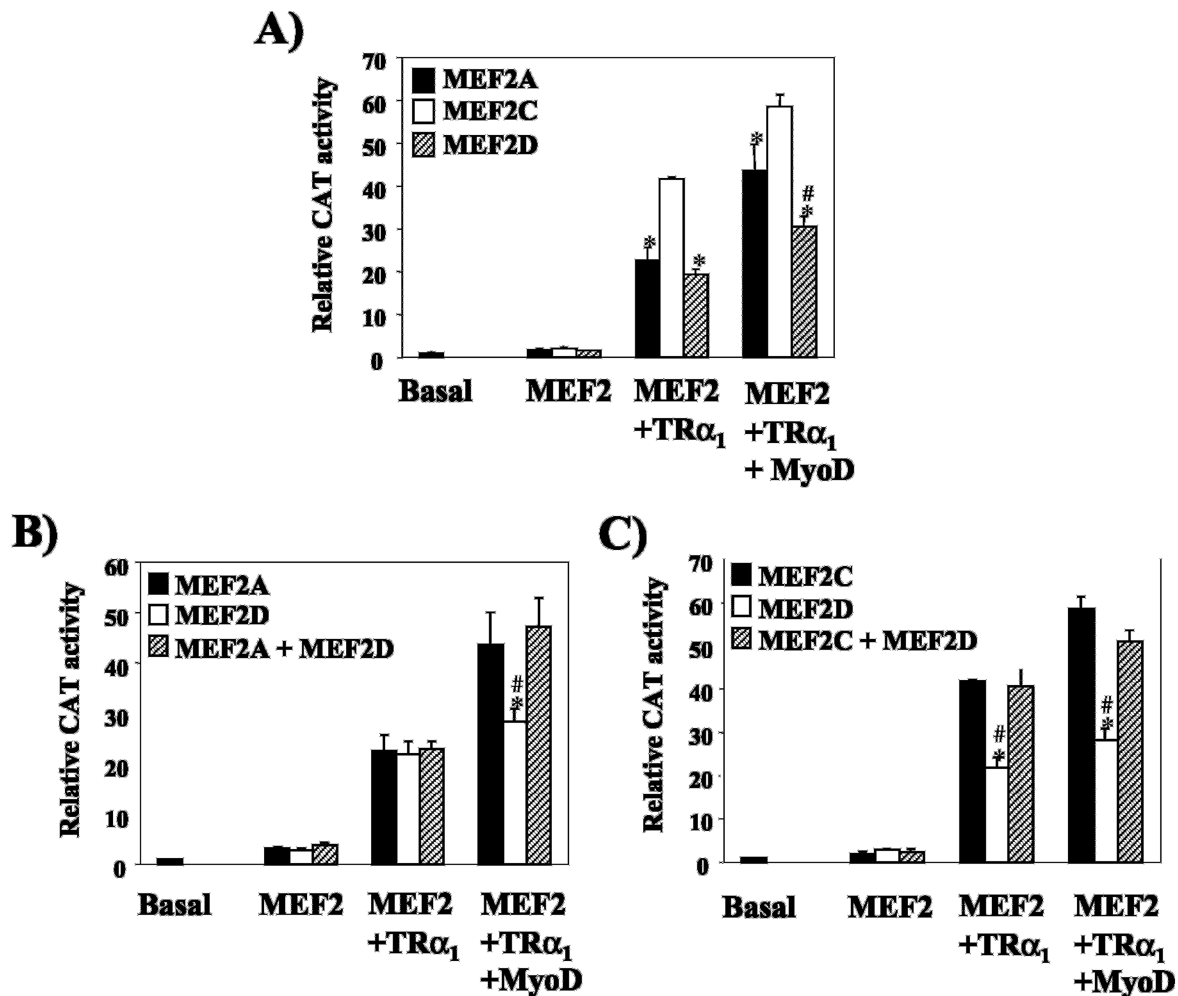


FIG. 8. **MEF2A, MEF2C, and MEF2D synergize with MyoD and TRα1 in the *trans*-activation of the GLUT4 −502/−420 TKCAT reporter system.** 10T1/2 fibroblasts were transfected with 100 ng of the −502/−420 TKCAT construct and 50 ng of expression vectors for MyoD, MEF2A, MEF2C, MEF2D, or TRα1 with Fugene™ reagent. T_3 at a final concentration of 100 nM was added to cells to which TRα1 had been transfected. Cells were harvested 36 h after transfection. *A*, co-transfections were done by combining MyoD and TRα1 with MEF2A (*filled bars*), MEF2C (*open bars*), or MEF2D (*hatched bars*). Data are the means ± S.E. of relative CAT activity over basal −502/−420 TKCAT levels from seven independent experiments performed in triplicate. Basal levels showed an enzymatic activity of 3.9 ± 0.5 nmol of acetylated chloramphenicol/μg of protein/h and was 3.8 times above background (CAT activity in non-transfected cells). *, significantly different from the MEF2C group, $p < 0.05$. #, a significant difference between the MEF2A and MEF2D groups, $p < 0.05$. *B* shows the results obtained by transfecting with MEF2A (*filled bars*), MEF2D (*open bars*), or MEF2A and MEF2D (*hatched bars*). Data are the means ± S.E. of relative CAT activity over basal −502/−420 TKCAT levels from seven independent experiments performed in triplicate. *, significantly different from the MEF2A group, $p < 0.05$. #, significantly different from the MEF2A/MEF2D group, $p < 0.05$. *C* shows the results obtained by transfecting with MEF2C (*filled bars*), MEF2D (*open bars*), or MEF2C and MEF2D (*hatched bars*). *, significantly different from the MEF2C group, $p < 0.05$. #, significantly different from the MEF2C/MEF2D group, $p < 0.05$.

activating the enhancer were dependent on the integrity of the E-box, as we have previously reported for MyoD (24). Thus, transient co-transfection studies were done using either a wild type or a mutated version of the enhancer plus a combination of expression vectors for the transcription factors. Data indicated that both the effects of myogenin or MyoD on the enhancer activity as well as the synergy between myogenin and TRα1 or MyoD and TRα1 were abolished by mutating the E-box (data not shown). Altogether, these data demonstrate that the integrity of the E-box is required for the effects of the MRF protein family on the activity of the muscle-specific GLUT4 enhancer and reveal the existence of the functional redundancy of the different members of the MRF family synergizing with MEF2 and TRα1 on the muscle-specific GLUT4 enhancer.

DISCUSSION

The results of this study indicate that the muscle-specific GLUT4 enhancer located at −502/−420 operates under *in vivo* conditions in maintaining the expression of GLUT4 in skeletal muscle, contributes to the differences in GLUT4 expression detected in glycolytic and oxidative muscle fibers, and is activated in regenerating muscles under conditions in which GLUT4 is induced. In adult muscle, the activity of the enhancer requires an intact MEF2-binding site, and the TREs play an inhibitory role. However, during muscle regeneration, the MEF2 site, the E-box, and the TREs are crucial in maintaining a high activity. On the other hand, the activity of the enhancer is independent of muscle innervation both in adult muscle and during muscle regeneration.

We have previously demonstrated that the muscle-specific GLUT4 enhancer is activated synergistically by MyoD, MEF2, and TRα1 in muscle and nonmuscle cells in culture and that this requires the integrity of the respective DNA-binding elements (24). In addition, mutation of the MEF2-binding element or the TRE caused a reduction of transcriptional output in cultured cardiomyocytes (24). In this study, we show that in adult skeletal muscle, under normal conditions, the activity of the muscle-specific GLUT4 enhancer is essentially maintained by the MEF2-binding element, and so cancellation of the ele-

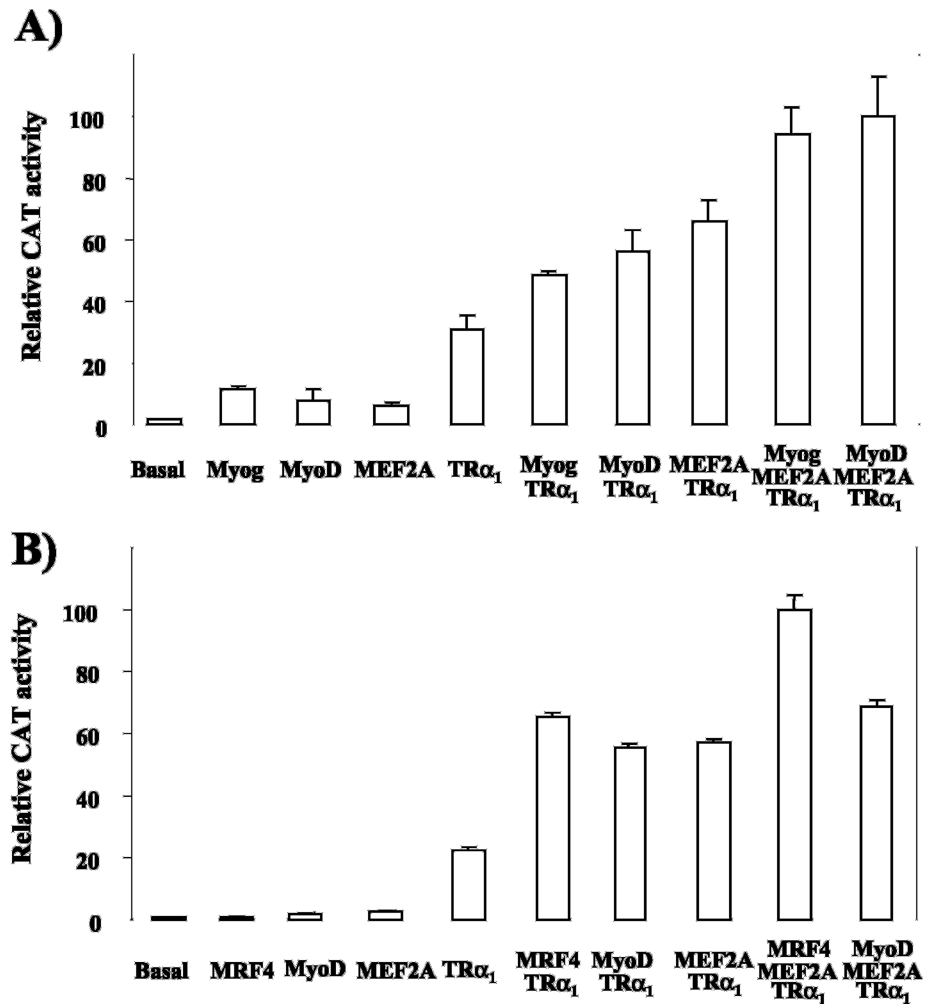


FIG. 9. **MyoD, myogenin, and MRF4 synergize with MEF2A and TRα1 in the GLUT4 −502/−420 TKCAT reporter system.** 10T1/2 fibroblasts were transfected with 100 ng of the −502/−420 TKCAT construct and 50 ng of expression vectors for MyoD, myogenin, MRF4, MEF2A, or TRα1 with Fugene™ reagent. T_3 at a final concentration of 100 nM was added to cells when TRα1 was transfected. Cells were harvested 36 h after transfection. Data are the means ± S.E. of relative CAT activity over basal −502/−420 TKCAT levels from six independent experiments performed in triplicate. *A* shows results obtained by transfection with myogenin (*Myog*) or MyoD. *B* shows results obtained by transfection with MRF4 or MyoD.

ment causes a major repression of activity. In addition, we have found that the enhancer is subjected to repression via the TRE, so that its cancellation doubles the activity of the enhancer. The relevance of the MEF2-binding site was also reported in a study using transgenic mice, in which a marked repression of the transcriptional activity driven by 895 bp of DNA encompassing the 5′-flanking region of GLUT4 gene was detected in adipose tissues or in muscles after mutation of the MEF2-binding site (18). It is surprising to find that the TRE inhibits the activity of the enhancer under normal conditions in adult muscle because: on the one hand, the administration of T_3 to rats is known to cause the induction of GLUT4 gene expression in certain muscle types (5, 6); in addition, the concentration of T_3 in rat skeletal muscle lies within the nanomolar range (35), *i.e.* high enough to activate to some degree thyroid hormone receptors. In any case, the reason that the muscle-specific GLUT4 enhancer remains inhibited through the TRE in skeletal muscle remains unexplained.

In contrast with adult muscle, muscle regeneration caused a dramatic change in the mode of operation of the GLUT4 enhancer. Thus, during muscle regeneration, cancellation of the three elements detected previously, *i.e.* the MEF2-binding site, the E-box, and the TRE, blocked the activity of the enhancer, consistent with the idea that they play an stimulatory role under those conditions.

The different pattern shown by the GLUT4 enhancer indicates that the E-box of the enhancer remains inactive in adult skeletal muscle, whereas it is operative in regenerating muscle. This is consistent with the low expression of MRFs in skeletal muscle during adult life (36, 37) and with their induction during regeneration (32). In addition, our data suggest a powerful inhibitory effect of the TRE in adult muscle and a stimulatory role during regeneration.

A corollary of the activity profile of the muscle-specific GLUT4 enhancer is that its activity is low in adult skeletal muscle, under normal conditions, compared with the maximal potential activity that it can attain at high levels of the MRF transcription factors, MEF2, and TRα1. This suggests the possibility of major up-regulation of GLUT4 gene transcription via activation of this enhancer by an increase in the levels of expression or activity of such transcription factors.

We have observed that the activity of the enhancer is greater in soleus than in EDL muscles, which helps to explain the differences in GLUT4 gene expression and gene transcription that exist between oxidative and glycolytic muscles (1–3). We found that the profile of changes in the activity of the enhancer in response to mutations in the E-box, MEF2 site, or TRE was similar in both muscle types. These data suggest that oxidative muscles show a greater activity of the muscle-specific GLUT4 enhancer than glycolytic muscles as a consequence of a greater activity of the MEF2-binding site. This is in keeping with observations indicating that the soleus muscle displays a greater expression of MEF2A and MEF2D and a lower phosphorylation level and greater activity than proteins obtained from gastrocnemius muscle (38).

The damage of skeletal muscle triggers the activation of otherwise quiescent satellite cells. These are mononucleated stem cells that are situated between the basal lamina of the extracellular matrix and the plasma membrane of muscle fibers. After proliferation, satellite cells exit the cell cycle and

fuse, forming multinucleated myotubes, which replace the damaged muscle fibers. We provide evidence that muscle regeneration induced after bupivacain treatment causes a dramatic induction of GLUT4 expression, which is consistent with the fact that GLUT4 is induced along with myogenesis in cultured cells (29, 39). This finding coincides with a very strong stimulation of the muscle-specific GLUT4 enhancer relative to the intact muscle and with the functional operation of all three elements (MEF2, E-box, and TRE). We propose that under these conditions, the enhancer is activated via induction of myogenic MRFs (32, 40) and by activation of the MEF2 factors (33). In this regard, we have demonstrated that all members of the MRF protein family are equally able to cooperate functionally with MEF2 and thyroid hormone receptors in the context of the muscle-specific GLUT4 enhancer. Based on the fact that muscle regeneration recapitulates muscle development, it is possible that the muscle-specific GLUT4 enhancer participates in the progressive induction of GLUT4 that occurs during perinatal development in skeletal muscle (4, 41, 42).

It is well known that muscle denervation causes a dramatic repression of muscle GLUT4 gene expression (7–9) because of repressed transcription (43). In this study, we have clearly shown that muscle denervation is not detrimental to the activity of the muscle-specific GLUT4 enhancer either in soleus or in EDL muscles, but we have detected a trend to increased activity. In addition, during muscle regeneration, the lack of innervation at an early stage (day 6 of regeneration) did not prevent the induction of GLUT4 protein, whereas denervation caused a marked down-regulation of GLUT4 expression later on. Nevertheless, regenerating denervated muscles did not display any alteration in the activity of the muscle-specific GLUT4 enhancer. Taken together, these data indicate that the muscle-specific GLUT4 enhancer does not play a role in the down-regulation of GLUT4 gene expression that occurs during muscle denervation or the down-regulation that occurs late during muscle regeneration. Tsunoda *et al.* (44) have mapped the region of the GLUT4 gene involved in the regulation of GLUT4 transcription by muscle denervation, which lies 3′ to position −423 (42) and is therefore outside of the muscle-specific GLUT4 enhancer.

In summary, our study indicates that the muscle-specific GLUT4 enhancer operates at a low level in intact adult muscle. In addition, it constitutes a switch that turns on GLUT4 transcription under conditions associated with GLUT4 induction, such as during muscle regeneration, possibly during myogenesis, and in the early phases of the development of skeletal muscle, *e.g*. in situations characterized by a high expression of myogenic factors, MEF2, and thyroid hormone receptors. In adult skeletal muscle and under normal conditions, the enhancer operates at a low rate, driven by MEF2 transcription factors and inhibited by the TRE, and participates in the differences in GLUT4 gene expression between oxidative and glycolytic muscle fibers. In regenerating muscle, the enhancer operates at a high rate, driven by MEF2, MRFs, and thyroid hormone receptors. Under all of these conditions, the activity of the enhancer is independent of muscle innervation.

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