GLYCEMIC CONTROL appears to be largely dependent on glucose disposal in skeletal muscle, inasmuch as ∼75% of the postprandial glucose load is deposited within this tissue (10). GLUT-4 and GLUT-1 are the predominant facilitative glucose transporters within skeletal muscle (27), although they are expressed and regulated differently. GLUT-4 is found in adipose tissue, heart, and skeletal muscle (21, 22, 30), and in response to insulin binding to its receptor a signal cascade is initiated that culminates in the recruitment of GLUT-4 protein from intracellular vesicles to the plasma membrane (9). On the other hand, GLUT-1 is expressed in most tissues (1), is constitutively found on the plasma membrane (34), and is responsible for the majority of basal glucose transport (34).

The expression of GLUT-4 and GLUT-1 proteins in skeletal muscle is altered after 72 h of denervation (2, 7). These latent changes in GLUT-4 protein, therefore, cannot explain the rapid onset of insulin resistance that occurs in skeletal muscle within 3 h of denervation (36). However, the severity of insulin resistance associated with denervation increases with time and reaches a maximum after 72 h (36) and is thought to be related to a reduction in GLUT-4 protein (7). According to Handberg et al. (15), the decrease in GLUT-4 protein is compensated for, in part, by an increase in GLUT-1, to offset the reduction in insulin-stimulated glucose transport.

GLUT-4 and GLUT-1 protein expression in denervated skeletal muscle appears to be dependent on changes in mRNA. Decremental changes in GLUT-4 protein in denervated muscle are paralleled by lower levels of GLUT-4 mRNA, whereas incremental changes in GLUT-1 protein are associated with higher levels of GLUT-1 mRNA (5, 28). However, it is not known whether these changes in GLUT-4 and GLUT-1 mRNA levels after denervation are due to altered rates of transcription. The purpose of this study was to determine whether the changes in GLUT-4 and GLUT-1 protein expression in denervated skeletal muscle are transcriptionally mediated. Results of this study demonstrated that denervation decreased GLUT-4 transcription but increased GLUT-1 transcription in rodent skeletal muscle.

METHODS

Materials. Radiolabeled UTP and dATP were obtained from DuPont-New England Nuclear. DNA polymerase I (Klenow fragment) and restriction enzymes were purchased from Promega (Madison, WI). RNasin and nonradiolabeled nucleotides CTP, GTP, ATP, and TTP were obtained from Pharmacia (Uppsala, Sweden). TRIzol reagent was purchased from Gibco-BRL (Gaithersburg, MD). Unless mentioned, all other reagents were of molecular biology grade and were obtained from Sigma Chemical (St. Louis, MO), Fischer Scientific (Springfield, NJ), or Pharmacia. The cDNA probes used in this study were as follows: GLUT-1, a 2.7-kb EcoRI fragment encoding the 3T3-L1 homolog of the HepG2/brain glucose transporter protein (20); β-galactosidase, a 1.3-kb SmaI/EcoR V fragment (Clonetech Laboratories, Palo Alto, CA); and pRibo (18S ribosomal RNA), a 2.0-kb Hind III fragment.

Mouse experiments. Transgenic mice were generated as previously described (26) using cDNA constructs from the plasmid containing 2,400 bp of the GLUT-4 5′-flanking DNA. The cDNA constructs used have identical 3′ ends (+163 bp) but contain sequential deletions (Fig. 1) from the 5′ end (−2,400, −1,639, −1,154, and −730 bp). Transgenic mice were used in the GLUT-4 study, because they provided a powerful tool to determine the effects of denervation on GLUT-4 transcription as well as the general location of a DNA sequence that is responsive to this perturbation. Transgenic mice were identified by Southern analysis of isolated tail DNA, as previously described (26). Male transgenic mice (≥8 wk) were anesthetized with a ketamine (18 mg/ml)-xylazine (2 mg/ml) mixture (0.05 ml/10 g body wt ip), then the right
hindlimb was denervated via sciatic nerve section (n = 4–7/group). The contralateral hindlimb was sham operated. Seventy-two hours after surgery, mice were stunned and killed by cervical dislocation. Gastrocnemius muscles were harvested and quick frozen to the temperature of liquid nitrogen for RNA isolation. Transgenic mice were provided food and water ad libitum. Room temperature (20–22°C) and nitrogen for RNA isolation. GLUT-4 and chloramphenicol acyl transferase.

RNA protection assay. The mouse GLUT-4-chloramphenicol acyl transferase (CAT) plasmid p469GLUT4.CAT (a gift from Dr. M. Daniel Lane, Johns Hopkins Medical School, Baltimore, MD) was linearized with Bsu 36I and then used to generate an antisense RNA probe. The synthesis of the radioactive (\(\alpha\}-32P\)UTP, 800 Ci/mmole) antisense RNA was done using Ambion’s T3 MAXscript in vitro transcription kit (Austin, TX). The RNase protection assay (RPA) was performed using the streamlined procedure of Ambion’s RPA II kit (Austin, TX). Briefly, 500,000 cpm of labeled probe in 3–15 µl of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, and 0.2% SDS) were hybridized to 10–20 µg of total RNA in 20 µl of hybridization buffer (80% deionized formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA). Samples were incubated overnight at 42–45°C. Nonhybridized RNA was digested using a 1:100 dilution of RNase A-T1 mix. The protected RNA fragments for CAT and GLUT-4 were 258 and 176 nucleotides, respectively. A 6% polyacrylamide gel containing 7 M urea (SequaGel 6, National Diagnostics, Atlanta, GA) was used to size fractionate RNAs.

Table 1. Effects of 72-h denervation on GLUT-4 mRNA and CAT mRNA expression in control and denervated gastrocnemius muscles of transgenic mice

<table>
<thead>
<tr>
<th>Construct</th>
<th>Control</th>
<th>Denervated</th>
</tr>
</thead>
<tbody>
<tr>
<td>−2,400 bp</td>
<td>GLUT-4 mRNA</td>
<td>426 ± 56.3</td>
</tr>
<tr>
<td>CAT mRNA</td>
<td>174 ± 45.0</td>
<td>33.1 ± 17.9*</td>
</tr>
<tr>
<td>−1,639 bp</td>
<td>GLUT-4 mRNA</td>
<td>326 ± 133</td>
</tr>
<tr>
<td>CAT mRNA</td>
<td>78.4 ± 32.8</td>
<td>17.5 ± 3.78*</td>
</tr>
<tr>
<td>−1,154 bp</td>
<td>GLUT-4 mRNA</td>
<td>840 ± 77.1</td>
</tr>
<tr>
<td>CAT mRNA</td>
<td>505 ± 156</td>
<td>108 ± 49.3</td>
</tr>
<tr>
<td>−730 bp</td>
<td>GLUT-4 mRNA</td>
<td>252 ± 71.4</td>
</tr>
<tr>
<td>CAT mRNA</td>
<td>575 ± 255</td>
<td>137 ± 72.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as arbitrary units. Male transgenic mice (8–12 weeks old) were anesthetized with ketamine (18 mg/ml)-xylazine (2 mg/ml) mixture (0.05 ml/10 g body wt ip), and right hindlimb was denervated via sciatic nerve section (n = 4–7/group). Contralateral hindlimb was sham operated. At 72 h after surgery, mice were stunned and killed by cervical dislocation. Gastrocnemius muscles were harvested and quick frozen to the temperature of liquid nitrogen for RNA isolation. GLUT-4 and chloramphenicol acyl transferase (CAT) mRNA levels were determined by RNase protection analysis. *Significantly different from control: P < 0.05; 1P = 0.063.
the RNA. Results were visualized by phosphor imaging and quantitated using Imagequant software.

Statistical analysis. To determine a significant difference between treatment means, a one-tailed paired t-test or a one-tailed Wilcoxon signed rank test was performed with significance set at $P < 0.05$.

RESULTS

The purpose of the first experiment was to determine whether GLUT-4 gene transcription is decreased in denervated skeletal muscle. RPA indicated that, after 72 h of denervation, GLUT-4 and CAT mRNA levels decreased 64–85% in gastrocnemius muscles of mice harboring −2,400, −1,639, −1,154, and −730 bp of the human GLUT-4 promoter (Fig. 2). This decrease in GLUT-4 mRNA levels is consistent with previously reported decrements in GLUT-4 mRNA after denervation (2, 7). The reduction in mRNA levels of the reporter gene, CAT, indicates that GLUT-4 transcription is decreased in denervated skeletal muscle and could therefore account for the lower levels of GLUT-4 mRNA (Fig. 2). Furthermore, these findings suggest that the DNA element(s) in skeletal muscle regulated by denervation may be within 730 bp of the 5’-flanking promoter region (Fig. 2). To substantiate the effects of denervation on the expression of the transgene, CAT mRNA-to-GLUT-4 mRNA ratios were compared between control and denervated samples of the respective constructs. Except for the −1,154-bp construct, where denervated samples had a lower ratio ($P < 0.05$), there were no differences between control and denervated samples (data not shown), indicating that the transgene was regulated by denervation like the endogenous GLUT-4 gene.

Previous studies showed that GLUT-1 mRNA and protein levels are altered in a fashion reciprocal to that of GLUT-4 mRNA and protein levels in denervated skeletal muscle (2, 7). In a second experiment, denervation of the right hindlimb skeletal muscles of male rats increased GLUT-1 gene transcription 94% (Fig. 3). Northern analysis indicated that GLUT-1 mRNA increased 213% in denervated mixed gastrocnemius muscles (Fig. 4). Although a 94% increase in transcription of the GLUT-1 gene contributes to the 213% increase in GLUT-1 mRNA levels, these results may suggest posttranscriptional control (e.g., mRNA stability) of GLUT-1 expression in denervated skeletal muscle.

DISCUSSION

The expression of GLUT-4 and GLUT-1 glucose transporters in rodent skeletal muscle is a complex and dynamic process dependent on exercise, age, and the hormonal/nutritional state of the animal. In addition, previous studies have demonstrated that denervation has many rapid and dramatic effects on GLUT-4 and GLUT-1 protein expression in skeletal muscle (2, 7). In response to exercise (32) and fasting (31), GLUT-4 protein is increased but is decreased in cases of streptozotocin-induced diabetes (31), eccentric exercise (23), and denervation (2, 7, 16, 29). All these perturbations have been shown to result in alterations in GLUT-4 gene transcription similar to changes in GLUT-4 protein, except for denervation, which has not been studied. Results of these experiments demonstrated that denervation decreased GLUT-4 transcription and increased GLUT-1 transcription in rodent skeletal muscle.
The expression of a gene product is a multistep process that includes transcription, nascent RNA processing, mRNA stability, mRNA translation, and protein processing, transport, and degradation. As described by Williams and Neufer (40), certain kinetic principles govern the flow of information from DNA to RNA to protein. Under steady-state conditions the abundance of a protein is determined by the rate of synthesis (translation) and the rate of degradation. The rate at which a protein is synthesized is determined, in part, by the abundance of the respective mRNA. The amount of mRNA is similarly dependent on the rate at which it is produced (transcription) and degraded (stability). Together, these synthetic and degradative processes determine the synthesis of a gene product, with the kinetics of the rate-limiting steps governing the rate of gene expression. Although transcription is often the rate-limiting step in the synthesis of proteins, other steps may be rate limiting, and such steps may change in response to different stimuli. By determining the effects of denervation on GLUT-4 and GLUT-1 gene transcription, a greater understanding of those processes (e.g., transcription, mRNA stability, translational control) that impact expression of these two genes is acquired.

The 65–85% reduction in transcription of the GLUT-4 gene could account for the 64–79% decline in GLUT-4 mRNA levels (Fig. 2). However, it is questionable that a 94% increase in transcription of the GLUT-1 gene (Fig. 3) could account for the entire 213% rise in GLUT-1 mRNA levels (Fig. 4). Previous studies have indicated that the stability of GLUT-4 and GLUT-1 transcripts is regulated differently in L6E9 myotubes and/or 3T3-L1 adipocytes. Treatment of these cells with 8-bromoadenosine 3',5'-cyclic monophosphate decreased transcription of the GLUT-4 gene but did not alter the half-life of the GLUT-4 transcript (19, 38). In contrast, GLUT-1 transcription rates and the GLUT-1 mRNA half-life are increased after treatment with 8-bromoadenosine 3',5'-cyclic monophosphate (19). Previous research has indicated that intracellular concentrations of cAMP are elevated in denervated skeletal muscle (4, 6, 17) and may influence expression of the GLUT-4 and GLUT-1 genes (18, 38).

The data presented here demonstrate that the transcription rates of GLUT-4 and GLUT-1 are altered in denervated skeletal muscle. The transcriptional mechanisms involved with denervation remain unknown; however, evidence suggests that the MyoD family, a
group of myogenic transcription factors, may be involved in regulating GLUT-4 and GLUT-1 expression. Myogenin and MyoD are two members of the MyoD family that bind to CANNTG elements of muscle genes (24, 25, 33, 39). Nonmuscle cells are committed to become myoblasts when transfected with the cDNA for myogenin or MyoD (11). It has been reported that mRNA levels of MyoD and myogenin decrease with postnatal development and increase with denervation (3, 12, 41). Electrical stimulation of denervated skeletal muscle represses the increase of MyoD and myogenin transcripts (3, 12). Recently, Vinals et al. (37) demonstrated that MyoD repression of Sp1 protein may explain the reduction of GLUT-1 expression during muscle cell maturation. Although we did not explore potential regulatory elements within the GLUT-1 promoter, results from this study suggest that a DNA element responsive to denervation exists within 730 bp of the 5′-flanking promoter region of the human GLUT-4 gene. Analysis of the human GLUT-4 gene reveals that three binding sites (CANNTG) for myogenin and MyoD exist within 730 bp of the 5′ flank. Whether MyoD and myogenin directly regulate GLUT-4 and GLUT-1 in skeletal muscle remains to be determined.

In summary, GLUT-4 transcription is decreased 65–85%, whereas GLUT-1 transcription is increased 94%, in denervated skeletal muscle. We believe that the 64–79% reduction in GLUT-4 mRNA levels is transcriptionally mediated, whereas the 213% increase in GLUT-1 mRNA levels suggests the involvement of posttranscriptional control. Furthermore, a DNA sequence that is regulated by denervation may lie within 730 bp of the 5′-flanking promoter region of the human GLUT-4 gene. We speculate that the reciprocal changes in GLUT-4 and GLUT-1 protein in denervated skeletal muscle involve transcriptional and posttranscriptional control.

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