Cytochrome c promoter activity in soleus and white vastus lateralis muscles in rats

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Yan, Zhen, and Frank W. Booth. Cytochrome c promoter activity in soleus and white vastus lateralis muscles in rats. J. Appl. Physiol. 85(3): 973–978, 1998.—Cytochrome c protein and mRNA are 300 and 100% higher, respectively, in the soleus muscle (predominantly slow-twitch oxidative) than the white vastus lateralis (predominantly fast-twitch glycolytic) muscles (W. W. Winder, K. M. Baldwin, and J. O. Holloszy. Eur. J. Biochem. 47: 461–467, 1974; M. M. Lai and F. W. Booth. J. Appl. Physiol. 69: 843–848, 1990). However, the mechanisms controlling these differences in cytochrome c mRNA are largely unknown. The present study employed direct plasmid injection techniques to determine whether the proximal promoter (−726 to +610) of the rat somatic cytochrome c gene was more active in the soleus than in white vastus lateralis muscles in rats. No difference between the soleus and white vastus lateralis muscles for the activities of the −726, −631, −489, −326, −215, −159 and −149 cytochrome c promoters was noted. The results of this study suggest that additional elements (outside of −726 to +610) in the cytochrome c gene may be required, or posttranscriptional regulation may account, for the higher cytochrome c mRNA in the slow-twitch oxidative muscle.

mitochondria; direct plasmid injections; muscle fiber types; oxidative capacity

SKELETAL MUSCLE FIBER diversity may arise from the commitment of distinct myoblast lineages during myogenesis (27). However, the phenotypes of adult muscles in living animals are very plastic because the quantity of muscle contraction regulates the expression of most contractile protein isoforms and metabolic enzymes (22, 34). Pette and Staron (22) have commented that future research should be directed at improving our understanding of the molecular basis for the functional specialization of phenotypic expression in skeletal muscle fibers. One approach to understanding the molecular mechanisms that control muscle diversification and plasticity is to identify the DNA-regulatory sequences that confer fiber type-specific expression of contractile proteins and metabolic enzymes. Extensive studies of troponin slow and fast genes have indicated that several core sequences in the promoters, such as E-box, CCAC-box, and MEF-2-like sequence, bind their cognate factors and these transcriptional factor-DNA interactions determine fiber type diversity (18). Others have found the E box and MEF-2 sequences in fiber type elements of troponin I slow (4) and myosin heavy chain IIB promoters (29). A more recent study showed that both the nuclear respiratory factor 1 (NRF-1)-binding site and CAMP-response element (CRE) in the −326 cytochrome c promoter are necessary and sufficient for the induction of its promoter activity in electrically stimulated cardiomyocytes in serum-free media (36). Because the same treatment (electrical stimulation) does not increase mitochondrial density in cultured myotubes (15, 25), it seemed necessary to learn whether the same promoter’s elements confer the fiber type-specific difference in cytochrome c gene expression in adult skeletal muscle. Moreover, because muscle cultures do not manifest the specific properties of muscles in living animals (9), the identification of the cis-acting sequences in the cytochrome c promoter that confer fiber specificity needs to be addressed by using a living animal.

In 1969, Gauthier (10) published electron micrographs that showed that red muscle fibers were richer in mitochondria than were white fibers in animals. The concentration of cytochrome c protein, a mitochondrial protein in the respiratory chain, has been used to determine the mitochondrial density and/or oxidative capacity in the skeletal muscle due to its parallel changes with other mitochondrial oxidative enzymes in animals (35) and its possible limiting role for electron transport (12). Winder et al. (35) reported that cytochrome c protein concentration was 3 and 12 nmol/g wet weight for the white vastus lateralis (predominantly fast-twitch glycolytic) and soleus (predominantly slow-twitch oxidative) muscles, respectively, in sedentary rats. The soleus and white vastus lateralis muscles are recruited ~7 h/day and ~2 min/day, respectively, in sedentary rats according to Hennig and Lomo (14). These data suggested that the cytochrome c level in skeletal muscle could be regulated, in part, by differences in the quantity of contractile activities among various fiber types in the animal. Mechanistically, the fourfold higher concentration of cytochrome c protein in slow-twitch oxidative muscle than in fast-twitch glycolytic muscles (17) may be contributed, in part, by the twofold difference in cytochrome c mRNA level. Therefore, it is very important to know whether the cytochrome c promoter’s activity is higher in the slow-twitch oxidative than in the fast-twitch glycolytic muscles because this information would be the basis for future experiments to generate transgenic mice lacking the fiber type-specific element in the cytochrome c promoter. A recent advance in gene delivery, the direct plasmid injection technique, has allowed the relatively rapid estimation of promoter activities in striated muscles of living animals (2, 4, 13, 16, 20, 21, 24, 29–31), and such results provide a more rational basis for future transgenic animal models (4). The present...
study was undertaken to determine whether the –726 promoter of the rat somatic cytochrome c gene was more active in the soleus muscle than in the white vastus lateralis muscle in the living animal (6, 22).

**METHODS**

**Animals.** Female Sprague-Dawley rats, weighing 100–149 g, were obtained from Harlan (Houston) and housed under temperature-controlled (21°C) conditions with a 12:12-h light-dark cycle. Rat chow and water were provided ad libitum. Protocols were approved by the University of Texas Health Science Center Institutional Animal Welfare Committee.

**Plasmids.** Rat somatic cytochrome c promoter (pRC)-luciferase plasmids were constructed from pRC4-chloramphenicol acetyltransferase (CAT) (8) and pGL2-Basic (Promega). Briefly, the Xho I-Hind III fragments containing the rat cytochrome c 5′-regions were inserted by T4 ligase into the 5.6-kb Xho I-Hind III fragment of pGL2-Basic. pRC-luciferase chimeric genes were constructed as follows: various deletion constructs of the 5′-rat somatic cytochrome c promoter (from either –726, –631, –491, –326, –215, –159, or –146 to +610) (8), the luciferase-coding region, and the SV40 polyadenylation site and splice signal at the 3′-end in pGL2 were ligated (Promega) (Fig. 1). Plasmid DNAs were transformed in JM109 bacteria (Promega). The bacteria were grown for 12 h in Luria-Bertani medium with moderate shaking at 37°C. Plasmid DNAs were isolated and purified by using alkaline lysis with differential polyethylene glycol precipitation and subsequent phenol extractions (26).

**Plasmid injections.** Rats were anesthetized with a mixture of ketamine (54 mg/ml), xylazine (2.2 mg/ml), and acepromazine (3.5 mg/ml) injected intramuscularly into the right gluteus muscle (1.4 ml/kg). All DNA deletion constructs were injected on the same day. Two-centimeter incisions were made lateral to the soleus muscle and to the white vastus lateralis muscles so that these muscles could be directly visualized. We selected these two muscles because they have a twofold difference in cytochrome c mRNA (17) and are visually accessible to verify the injections. To compare cytochrome c chimeric gene expression between the soleus and white vastus lateralis muscles, 20 µl of double-distilled water containing plasmid DNA cocktail [50 µg of chimeric reporter gene and 50 µg of constitutive reporter gene Rous sarcoma virus promoter driving CAT (pRSV-CAT)] were injected for each muscle. In preliminary experiments, luciferase and CAT activities were measured 4 days after injection of various amounts of DNA (0, 50, 100, or 200 µg for each construct; n = 4 for each dose) to determine linearity of injected plasmid. For the fiber type experiments, 200 µg of each plasmid were injected. Either 4 days (linearity experiments) or 7 days (fiber type experiments) after these injections, rats were again anesthetized. The entire soleus and white vastus lateralis muscles were dissected, weighed, and cut into seventeen 1-mm2 pieces, fast-frozen with a pair of Wollenberger tongs prechilled in liquid nitrogen, and held at –80°C until homogenized.

**Tissue homogenization.** Each sample was placed on ice and 5 ml/g (1 ml minimum) of ice-cold homogenization buffer [(in mM) 25 Tris-phosphate, pH 7.8, 2 trans-1,2-diaminocyclohexane-N,N′,N′-tetraacetic acid, 10% glycerol, 2,1,4-dithiothreitol, 1 phenylmethylsulfonyl fluoride, 1 EDTA, and 1 benzamidine as well as 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 µg/ml aprotinin] were added to each sample in a 15-ml polyethylene tube. The samples were homogenized at 4°C with a Polytron (Brinkman) at 70% of its maximal intensity with three 10-s intervals. After centrifugation at 4°C for 15 min at 15,000 g, the pellet fraction was discarded and the supernatant was used for luciferase and CAT assays. Assays for either CAT or luciferase were performed at the same time for all muscle samples.

**Luciferase assays.** Luciferase activity was assayed immediately after muscle homogenates were obtained by using a previously described procedure (32) with modification (2). Briefly, the reaction was started by addition of 20 µl of the homogenate to 100 µl luciferase reagent containing (in mM) 20 tricine (pH 7.8), 1.07 (MgCO3)·Mg(OH)2·5H2O, 2.67 MgSO4, 0.1 EDTA, 0.53 ATP, 0.27 CoA, and 33.3 1,4-dithiothreitol as well as 0.47 µM luciferin. Seventy seconds after the initiation of the reaction, light production becomes stable and it was measured by using a Monolight model 2010 luminometer (Analytical Luminescence Laboratory) and integrated for the next 10 s. Total luciferase activity was calculated in relative light units after correction for the background, which was the value from the un.injected control muscles.

**CAT assays.** Each muscle homogenate was assayed for CAT activity as previously described (11) with modification (1). Briefly, the reaction was initiated by adding 25 µl homogenate to 65 µl of reaction cocktail containing (in mM) 192.3 Tris·HCl

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**Fig. 1. Direct correlation between constitutive reporter gene activities and amount of DNA injected. Chloramphenicol acetyltransferase (CAT; A) and luciferase (Luc; B) activities from RSV and SV40 promoters, respectively, were measured 4 days after rat skeletal muscles were injected with 0, 50, 100, or 200 µg of each of pSV40-Luc and pRSV-CAT in 100 µl of normal saline. Values are means ± SE of 4 injections at each dose. %Conv., %conversion; RLU, relative luciferase light units.**
(pH 7.8), 1.85 acetyl-CoA, and 3.77 14C-labeled chloramphenicol (53 μCi/mmol). The promoter activity in each muscle sample was calculated as relative luciferase activity, i.e., the ratio of luciferase to CAT activity. For each animal, the mean value was taken from the left and right hindlimbs for the soleus and white vastus lateralis muscles.

Statistics. To determine the linear correlation between the constitutive reporter gene activities and the amount of DNA injected, regression with replication analysis was performed and the P value was calculated by analysis of variance. P < 0.05 was interpreted as a significant linear relationship between DNA-injection dose and the reporter gene activity. Cytochrome c promoter activity values are expressed as means ± SE. A paired Student’s t-test was used to determine whether differences between means from the soleus and white vastus lateralis muscles were significant at a P < 0.05 level.

RESULTS

Coinjection of pSV40-luciferase and pRSV-CAT into rat skeletal muscles was performed to determine the linearity of the injection procedure. Expression of luciferase driven by either the SV40 promoter or the RSV promoter was linear up to 200 µg of plasmid DNA injected into the tibialis anterior, lateral gastrocnemius, or quadriceps muscles (r = 0.74, P < 0.01 for luciferase and r = 0.65, P < 0.05 for CAT) (Fig. 1).

To determine the difference in the cytochrome c promoter activities among muscles with different inherent contractile activities, pRSV-CAT or plasmids containing various lengths of the cytochrome c promoter driving the luciferase reporter gene were coinjected into the more active soleus (predominantly slow-twitch oxidative fibers) and less active white vastus lateralis (predominantly fast-twitch glycolytic fibers) muscles. The CAT enzyme activities from the control plasmid, pRSV-CAT, were not significantly different between soleus and white vastus lateralis muscles (19.0 ± 2.3 vs. 14.9 ± 1.7% conversion/min, respectively; P > 0.05; n = 140). This result indicates that both the soleus and white vastus lateralis muscles have similar efficiency in taking up the pRSV-CAT plasmid DNA and expressing it, which, in addition to the above result of direct correlation between DNA dose and reporter gene activities, validated the use of pRSV-CAT as a control for DNA injection. There were variations in reporter gene activities from sample to sample, probably due to variations in plasmid DNA uptake by the muscle fibers (5). However, this error is diminished by correcting luciferase activity against its CAT activity from a constitutive promoter of a coinjected plasmid in the same muscle homogenate.

No differences in the −726, −631, −489, −326, −215, −159 and −149 cytochrome c promoter activities, expressed as relative luciferase activities, were noted between the soleus and white vastus lateralis muscles (Fig. 2). Because there were no significant differences in activity between the two muscles, we pooled the data from the soleus and white vastus lateralis muscles to examine the deletion effect on the promoter activity (Fig. 2, inset). Deletion from −726 to −489 did not markedly alter cytochrome c activity. Deletion from −489 to −159 progressively reduced promoter activity.

Deletion to −66 decreased promoter activity to the background of muscles injected with promoterless constructs (data not shown). The major difference with these data from a previous deletion study in cultured kidney cells was that deletion of the regions containing the 5′-CRE site (−326/−215) in the cytochrome c promoter region decreased the promoter activity in skeletal muscle of the living rat but not in cultured monkey kidney CV-1 cells (8). This difference might indicate that the regions containing the 5′-CRE site and region I are required for normal cytochrome c gene expression in adult skeletal muscles, not in cultured kidney cells.

DISCUSSION

No difference in the activities of the −726-bp cytochrome c promoter or of its deletion constructs has been found between rat skeletal muscles with greater and lesser cytochrome c mRNA concentrations. The following options could explain these negative results. The first possibility is that higher cytochrome c mRNA in the more active muscle is not a consequence of more promoter activity for the cytochrome c gene but is a result of greater mRNA stability. However, Connor et al. (3) were unable to detect a half-life for either cytochrome c oxidase subunit III or V1c mRNA in skeletal muscle of living rats, so determination of
cytochrome c mRNA half-life in muscles of living rats also seemed unfeasible. Furthermore, it is technically difficult to employ nuclear run-on (a more direct measurement of transcription rate) for cytochrome c gene expression in the skeletal muscles of living rats. For example, Neufer and Dohm (19) found that the measured transcription rates of the cytochrome c gene in skeletal muscle of control and treadmill-run rats were too close to background level to report. In the same muscles, GLUT-4 transcription rate was not only detectable but shown to be elevated transiently postexercise (19). Thus nuclear run-on assays for cytochrome c transcription rate in rat skeletal muscle were also deemed not feasible in the present study. If promoter activities are deemed as an index of transcription rate, observations from the present report imply that cytochrome c transcription is not different between the soleus and white vastus lateralis muscles.

The second possibility for the lack of a difference in cytochrome c promoter activity between muscles with different cytochrome c mRNA levels is that the methods employed in the present study failed to detect a difference in promoter activities. However, we believe this option is unlikely for the following reasons. Others have detected electrically responsive regulatory elements within −726 to +610 of this promoter in cultured cardiomyocytes. Two elements [the NRF-1 motif and CRE, which are located within −326 to +610 bp of the cytochrome c promoter] were shown to be necessary and sufficient for the induction of the cytochrome c promoter in electrically stimulated cardiomyocytes that had been cultured in serum-free medium (36). In contrast, our study showed that reporter genes bearing these same elements were not activated by the inherently higher contractile activity of the soleus muscle (compared with the white vastus lateralis muscle) in living rats. One potential interpretation is that this discrepancy could be the result of differences in gene regulation pathways between the cardiac myocytes and skeletal muscle cells. If this is true, then a signaling pathway of electrical stimulation to the activation of the cytochrome c promoter via NRF-1 and CRE sites in embryonic cardiomyocytes is missing in the skeletal muscle of young rats. Another potential interpretation for the differences between the previous and present study is that cytochrome c promoter regulation differs between cultured cells and living animals. Precedence for this possibility exists. For example, electrical stimulation of skeletal myotubes in tissue culture (15, 25) did not result in an increase in mitochondrial enzyme activities, whereas the indirect electrical stimulation of skeletal muscle in the living animal resulted in very large increases in mitochondrial proteins (33). Similar observations have been made in developmental studies; Firulli and Olson (9) wrote that it is becoming clear that significant discordances in gene regulation often exist between cell culture and whole animals. A final potential interpretation is that the fiber type and electrical activity elements for the cytochrome c gene differ.

The second methodological possibility for the lack of a difference in cytochrome c promoter activity between muscles with different cytochrome c mRNA levels is that the sensitivity of the technique employed is not suitable for detecting the difference in transcriptional rate. However, the standard error of the mean for promoter activity in the various cytochrome c deletion constructs in our present deletion experiments was 15 ± 2 relative luciferase light units (mean of 14 groups; 7 deletions tested in 2 muscles). Thus the methods employed appear to be sensitive enough to detect a difference between soleus and white vastus lateralis muscles when means differed by 35%, which is less than the twofold difference in cytochrome c mRNA. Moreover, others (2, 4, 13, 16, 20, 21, 24, 29–31) have been able to detect differences in promoter activities by using the direct injection of plasmids into striated muscles (Table 1).

Nevertheless, one limitation of the plasmid injection technique is that the expression of the injected reporter gene may be altered by the treatment independent of the reporter sequence. For example, if the activity in injected control plasmid increases with

Table 1. Studies that have employed plasmid injections into striated muscles

<table>
<thead>
<tr>
<th>Promoter (Ref. No.)</th>
<th>Species/Tissue</th>
<th>Change Observed</th>
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<tbody>
<tr>
<td>−613−to +32-bp α-Mysin heavy chain (16)</td>
<td>Rat heart Deletion of 700-bp M-isozyme and 295-bp promoter</td>
<td>100% Increase after thyroid treatment</td>
</tr>
<tr>
<td>−613−to +421-bp α-Mysin heavy chain (20)</td>
<td>Rat heart Deletion of 156 bp of 156-bp Cardiac troponin C</td>
<td>300% Increase after thyroid treatment</td>
</tr>
<tr>
<td>−3,542-bp β-Mysin heavy chain (13)</td>
<td>Rat heart Deletion of 613- to 1,267-bp</td>
<td>200% Increase in hypertrophy; mutation of GATA4 eliminated increase with hypertrophy</td>
</tr>
<tr>
<td>−667-bp β-Mysin heavy chain (31)</td>
<td>Dog heart Deletion of −70-bp</td>
<td>−354- or −215-bp promoters</td>
</tr>
<tr>
<td>−2,600-bp Mysin heavy chain II B (29)</td>
<td>Rat tibialis anterior and soleus muscles Deletion of −79-bp</td>
<td>Fiber type expression element is within the −295-bp promoter and doubles in hindlimb unloading</td>
</tr>
<tr>
<td>−156-bp Cardiac troponin C (21)</td>
<td>Rat heart Deletion of −124- to −79-bp abolished promoter activity</td>
<td>700 and −179 bps required for activity in heart and quadriceps, respectively</td>
</tr>
<tr>
<td>−700-bp M-isozyme of creatine kinase (30)</td>
<td>Rat heart and quadriceps muscles Deletion of −157-bp</td>
<td>100% Increase in hypertrophy</td>
</tr>
<tr>
<td>−4,200-bp Tropinin I slow promoter (4)</td>
<td>Rat extensor digitorum longus and soleus muscles Deletion of −2,090- to +318-bp</td>
<td>100% Increase in hypertrophy</td>
</tr>
<tr>
<td>−2,090−to +318-bp Skeletal α-actin (1, 2)</td>
<td>Rooster anterior latissimus dorsi Deletion of −81 bp</td>
<td>100% Increase in hypertrophy</td>
</tr>
</tbody>
</table>
treatment, the corrected value of the reporter gene of interest will be artificially lowered as a result. In earlier studies an effect of the stretch-induced hypertrophy (2) on the expression of the reporter gene was noted. This limitation is not applicable to the present study because we did not find a significant difference with a large sample size (n = 140) in the constitutive promoter activities between the soleus and white vastus lateralis muscles.

In summary, regulatory regions responsible for the fiber type differences in cytochrome c mRNA in rat skeletal muscle may reside outside of the −726 to +610 region of the gene, or it is posttranscriptional control with a large sample size (n noted. This limitation is not applicable to the present phy (2) on the expression of the reporter gene was earlier studies an effect of the stretch-induced hypertro-

interest will be artificially lowered as a result. In

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