Serum response factor mRNA induction in the hypertrophying chicken patagialis muscle

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Carson, James A., and Frank W. Booth. Serum response factor mRNA induction in the hypertrophying chicken patagialis muscle. J. Appl. Physiol. 86(1): 377–382, 1999.—Gene expression in the stretched chicken patagialis (Pat) muscle has not been extensively examined. This study's purpose was to determine the Pat muscle's expression pattern of serum response factor (SRF), skeletal α-actin, and MyoD mRNAs after 3 days (onset of stretch), 6 days (end of first week of rapid growth), and 14 days (slowed rate of stretch-induced growth) of stretch. SRF mRNA demonstrated two species (B1 and B2), with B2 being more prevalent in the predominantly fast-twitch Pat muscle, compared with the slow-tonic muscle. Stretch overload increased B1 and B2 SRF mRNA concentrations, and the increase in B1 SRF mRNA concentration was greater at day 6 compared with days 3 or 14. MyoD mRNA concentration was greater in 3-day-stretched Pat muscles, compared with days 6 or 14. Skeletal α-actin mRNA concentration was not changed during the study. Gel mobility shift assays demonstrated that SRF binding with serum response element 1 of the skeletal α-actin promoter had no altered binding patterns from 6-day-stretched Pat nuclear extracts. It appears that SRF and MyoD mRNAs are induced in the stretch-overloaded Pat muscle but at different time points.

WEIGHTING THE WING OF A CHICKEN produces rapid enlargement of the patagialis (Pat) and anterior latissimus dorsi (ALD) muscles (2, 3, 27). Seven days of wing weighting increases the chicken Pat muscle weight by 30–40% (2, 18). Stretch-induced hypertrophy of the Pat muscle plateaus after 14 days, and 80% of the Pat's stretch-induced growth at 2 wk occurs during the first week of stretch (12). Thus, during 2 wk of stretch overload, the chicken Pat muscle demonstrates periods of both rapid and slow muscle growth.

The Pat muscle has several characteristics that make the study of this muscle during stretch-induced hypertrophy interesting. These characteristics include the muscle's stretch-induced growth rate and morphological adaptation to stretch. The Pat muscle, a predominantly fast-twitch muscle, decreases its percentage of glycolytic fibers (α-white) and increases its percentage of oxidative fibers (α-red) (12). The stretch-induced growth rate of the Pat muscle is considerably less than that of the slow-twitch ALD muscle, which doubles its mass during 7 days of wing weighting (2, 18). Satellite cell activation and muscle fiber proliferation are not apparent in the hypertrophying Pat muscle (18). These are important components of stretch-induced hypertrophy to the ALD muscle (1, 25, 27). The Pat muscle's slow rate of stretch-induced growth, lack of fiber proliferation, and minimal satellite cell activation allow for an interesting paradigm to examine gene expression in a hypertrophying skeletal muscle.

Gene expression has not been extensively examined in the stretched Pat muscle. Insulin-like growth factor I mRNA expression in the Pat has been shown to follow a time course of stretch-induced expression, not being induced until after day 2 of stretch (10). Serum response factor (SRF) mRNA and MyoD mRNA are upregulated during stretch-induced hypertrophy of chicken ALD muscle (5, 6, 11). SRF and MyoD both have roles affecting myoblast proliferation and differentiation in cell culture (23, 28). SRF, a member of the MADS box family of transcription factors, binds to a consensus serum response element (SRE; GAG box) (26) and is essential for cultured myoblast proliferation and differentiation (28). MyoD is a member of the basic helix-loop-helix (bHLH) family of myogenic transcription factors. This bHLH family's sequential expression is involved in myoblast determination, proliferation, and differentiation in cell culture (23). It is not known whether the stretch induction of SRF and MyoD mRNA in the ALD muscle occurs in enlarging mature fibers or is mainly attributable to newly formed muscle fibers. However, there is a possible link between the induction of SRF and MyoD mRNA with the activation of satellite cells and subsequent formation of new fibers in the stretched ALD. The stretched Pat muscle allows the quantification of SRF and MyoD mRNA levels where fiber proliferation and satellite cell activation are thought to have a limited role during hypertrophy (18).

The purpose of this study was to examine the mRNA expression levels of two important transcription factors (SRF and MyoD) and one critical contractile protein gene (skeletal α-actin) during different phases of stretch-overload-induced growth in the fast-twitch Pat muscle. We hypothesized that MyoD and SRF mRNA would be induced during stretch of the Pat muscle, since these two transcription factors may be critical for stretch-induced hypertrophy of skeletal muscle. Pat mRNA expression was examined during different periods of stretch-induced enlargement: day 3 (onset of stretch), day 6 (end of the first week of rapid growth), and day 14 (slowed rate of stretch-induced growth) of stretch (12, 18). Additionally, SRF protein-binding interactions with SRE1 of the skeletal α-actin promoter were examined.

MATERIALS AND METHODS

Animal care and preparation. One-day-old roosters (White Leghorn males, Ideal-286, Ideal Hatcheries, Cameron, TX) were received and housed at the animal care facilities, University of Texas Health Science Center at Houston. The chickens were provided chicken chow and water ad libitum in...
a chicken brooder with a 12:12-h light-dark cycle. At ~3 wk of age, the chickens were group housed 6–10 per cage for the duration of the study. The chickens used in the study were between 5 and 7 wk of age (500–1,000 g). All animal protocols were approved by the Institutional Animal Welfare Committee, University of Texas Health Science Center at Houston.

Wing weighting. A weight corresponding to 10% of the chicken’s body weight was attached to the left wing while the unweighted contralateral wing served as the control. At the end of 14 days, this represented ~7–8% of body weight (5). Birds were wing weighted for 3, 6, or 14 days. At the time of death, Pat muscles were removed from anesthetized birds (ketamine 54 mg/ml, xylazine 2.2 mg/ml, and acepromazine 3.5 mg/ml; cocktail, 1.0 ml/kg), frozen in liquid N2, and stored at −80°C for total RNA determination (see below), and the remainder was used for RNA extraction. RNA was extracted by using the Trizol method (GIBCO). RNA concentration and purity were determined by ultraviolet spectrophotometry.

mRNA analysis. Total RNA was prepared as previously described (6, 7). Briefly, Pat muscles were powdered, 20–40 mg of powder were saved at −80°C for total RNA determination (see below), and the remainder was used for RNA extraction. RNA was extracted by using the Trizol method (GIBCO). RNA concentration and purity were determined by ultraviolet spectrophotometry.

Northern blot analysis was performed as previously described (6, 7). Briefly, 5–8 µg of nuclear protein were used for binding reactions. The faster migrating SRF mRNA species (B2) is more abundant in Pat compared with the ALD muscle. Northern analysis of 20 µg of total RNA from excised control or stretched Pat muscles were separately pooled for each nuclear extract (1–2 g of muscle). Nuclear protein extract concentration was determined by the DC protein assay (Bio-Rad). Total RNA and protein concentration per milligram of muscle. Total RNA was prepared as previously described (7). Briefly, the Pat muscle was powdered in liquid nitrogen, and a portion was weighed to the nearest milligram. This tissue was then processed for both RNA and protein concentration per milligram of muscle. Total RNA content and protein content were then calculated by multiplying the concentration by the wet weight of the muscle (concentration × muscle weight), as previously described (7).

Nuclei isolation and extraction. Nuclei isolation was performed as previously described (6). Four to five freshly excised control or stretched Pat muscles were separately pooled for each nuclear extract (1–2 g of muscle). Nuclear protein extraction was determined by the DC Lowry assay (Bio-Rad).

Mobility shift assay. Gel mobility shift assays (GMSA) were performed as previously described (6). Briefly, nuclear extracts were diluted to the same concentration (0.7 µg/µl), and 5–8 µg of nuclear protein were used for binding reactions. SRF and Yin Yang 1 (YY1) binding to the skeletal α-actin SRE1 were resolved from extracts incubated with 20 mM Tris, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol (5) and by nondenaturing polyacrylamide gel electrophoresis (5%, 29:1) with 0.5× Tris-glycine running buffer. Supershifted complexes were produced by adding 1 µl of supershift antibody to the binding reactions. The skeletal α-actin SRE1 double-stranded oligonucleotide for mobility shift experiments comprised nucleotides −100 to −73 (5′-GCCCGACACCCCAATATGGCGACGTCCG-3′) of the skeletal α-actin actin

Table 1. Patagialis muscle weight, protein content, total RNA concentration, and 18S rRNA concentration

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<table>
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<tr>
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<th>Day 3</th>
<th>Day 6</th>
<th>Day 14</th>
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<tr>
<td>Muscle weight, mg</td>
<td>Control 203 ± 7</td>
<td>Stretch 260 ± 7</td>
<td>Control 328 ± 8</td>
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<tr>
<td>% Difference (n)</td>
<td>28</td>
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<td>9</td>
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<td>Protein content, mg/muscle</td>
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<td>% Difference (n)</td>
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<td>31</td>
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<td>Total RNA concentration, µg/mg muscle</td>
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<td>% Difference (n)</td>
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<tr>
<td>Total RNA per muscle, mg/muscle</td>
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<td>% Difference (n)</td>
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<tr>
<td>18S rRNA concentration, IOD/µg total RNA</td>
<td>0.98 ± 0.06</td>
<td>1.00 ± 0.06</td>
<td>1.00 ± 0.05</td>
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<tr>
<td>% Difference (n)</td>
<td>4</td>
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*Values are means ± SE for n = no. of pairs of control and stretched patagialis muscle. IOD, integrated optical density; ND, not determined.

*Main effect of stretch (P < 0.05); †Significantly different from day 3.

Fig. 1. Patagialis (Pat) and anterior latissimus dorsi (ALD) muscles demonstrate 2 species of serum response factor (SRF) mRNA. The faster migrating SRF mRNA species (B2) is more abundant in Pat compared with the ALD muscle. Northern analysis of 20 µg of total RNA from control (C) and 14-day-stretched (S) Pat and ALD muscles taken from the same 5-wk-old White Leghorn chickens. Membranes containing Pat and ALD muscle were probed with the same SRF-labeled probe on the same day. The 14-day ALD SRF mRNA data are published elsewhere (5).
promoter. Oligonucleotides were end-labeled with \([\text{\textsuperscript{32}}P]\text{ATP}\) by using T4 polynucleotide kinase.

Statistics. The data are means ± SE. Differences were analyzed with a repeated-measures ANOVA (day and treatment) (4). Treatment was the dependent variable (left vs. right ALD of the same bird), and significant differences were determined at the \(P ≤ 0.05\) level. When significant interaction of main effects occurred, Fisher's pairwise comparison post hoc tests were used to assess significance.

RESULTS

Pat muscle mass, protein content, and total RNA content and concentration. The wet weight of the stretched Pat significantly increased (\(P = 0.001\); Table 1). Edema may account for a large portion of the increased wet weight at day 3 of stretch, since the protein content (mg protein/whole muscle) was not increased at this point. The stretched Pat muscle does not appear to accrue protein until after day 3 of stretch overload. The total RNA concentration (per gram of wet weight) of the stretched Pat was not affected during the study, but the total RNA content of the whole muscle increased (Table 1). The concentration of 18S rRNA per unit of total RNA was not affected by stretch at any day (Table 1).

SRF mRNA abundance. Two species (B1 and B2) of SRF mRNA templates were quantified in young chicken skeletal muscle (Fig. 1). Two SRF mRNA species have been previously reported in primary avian myoblast culture (9) and are thought to be due to differential 3’ polyadenylation (9). Polyadenylation has also been described for the human SRF (24). We report here that the Pat SRF mRNA pool from the same 5-wk old chickens has an increased ratio of B2/B1 SRF mRNA compared with the ALD (Fig. 1). However, the two SRF mRNAs responded similarly to stretch at 3, 6, and 14 days of stretch in the Pat muscle (Figs. 2–4).

The mRNA data are presented as the concentration of mRNA per microgram of total RNA and as the content mRNA per whole muscle. When examining a muscle that is changing mass, it is important to examine both the concentration and content of mRNA in the muscle. There was an effect of stretch on both B1 and B2 SRF mRNA concentrations in the stretch-overloaded Pat muscle (\(P = 0.001\)) (Figs. 2 and 3, B and C) during 2 wk of stretch overload. The induction of B1

![Fig. 2. SRF and MyoD mRNA levels in 3-and 6-day-stretched Pat muscle. Northern analysis of 20 µg of total RNA from control and 3- or 6-day-stretched Pat muscle. See Fig. 3 for tabulated results.](image1)

![Fig. 3. SRF, MyoD mRNA, and skeletal \(\alpha\)-actin concentrations per µg of total RNA during 2 wk of stretch overload in chicken Pat muscle. A: MyoD mRNA concentration per µg of total RNA. B: B1 SRF mRNA concentration per µg of total RNA. C: B2 SRF mRNA concentration per µg of total RNA. D: skeletal (Sk) \(\alpha\)-actin mRNA concentration per µg of total RNA. Values for control and stretched Pat muscles after 3 days (\(n = 5\) pairs), 6 days (\(n = 4\) pairs), or 14 days (\(n = 7\) pairs) of stretch overload. All mRNA values are corrected for loading by either 18S rRNA (SRF, MyoD) or 28S rRNA (skeletal \(\alpha\)-actin) (see MATERIALS AND METHODS). All mRNA values are corrected integrated optical density (IOD) (see MATERIALS AND METHODS). *Significantly different from day 3 (\(P ≤ 0.05\)). #Significant main effect of stretch across 3, 6, and 14 days (\(P = 0.05\)). @Significantly different from day 14 (\(P < 0.05\)).](image2)
SRF mRNA was greater at day 6 compared with day 3 or 14 (Fig. 3C). The stretch-overload-induced increase in B2 SRF mRNA concentration was greater at day 6 compared with day 14 (Fig. 3C). Stretch-induced changes in SRF mRNA content per muscle mirrored the changes reported for SRF mRNA concentration (Fig. 4, B and C). These results demonstrate that there is a transient induction in SRF mRNA during the first week of stretch overload in the Pat muscle.

MyoD mRNA abundance. There was a significant effect (P = 0.0008) of stretch on MyoD mRNA content per Pat muscle over 14 days of stretch overload (Figs. 2, 3A, and 4A). The stretch induction of MyoD mRNA concentration was significantly greater at day 3 compared with day 6 or 14 (Fig. 3A). The stretch induction of MyoD mRNA content per muscle was significantly greater at 3 days compared with 14 days. The stretched Pat muscle undergoes a transient induction of MyoD mRNA at the onset of hypertrophy, which occurs before increased SRF mRNA expression.

Skeletal α-actin mRNA abundance. Two weeks of stretch overload increased (P = 0.010) skeletal α-actin mRNA per Pat muscle (Fig. 4D); however, there was no interaction between the induction of mRNA and the duration of stretch. The concentration of skeletal α-actin mRNA was not altered during 14 days of stretch overload in the Pat muscle (Fig. 3D).

SRF:SRE1 interactions in GMSA. DNA-protein interactions were analyzed with GMSA. Binding was compared by using the chicken skeletal α-actin SRE1 with nuclear extracts from Pat muscles stretched for 6 days (n = 5 pooled pairs, 4–5 birds per point) and their contralateral control muscles. The SRE1 oligonucleotide formed binding complexes with both SRF and YY1 in both control and stretched nuclear extracts (Fig. 5).
The migration of the SRF or YY1 complex with SRE1 was not altered by 6 days of stretch.

**DISCUSSION**

This study's primary finding is that MyoD mRNA and SRF mRNA expression in the hypertrophying Pat muscle have distinct patterns of expression during 2 wk of stretch. There is the possibility that these patterns of gene expression are attributable to the hypertrophying Pat muscle's rate of enlargement. The Pat muscle responds to stretch with different rates of muscle growth during a 2-wk time course. It appears unlikely that the activation of quiescent satellite cells and subsequent proliferation of newly formed muscle fibers are a source of the stretch-induced gene expression pattern in the hypertrophying Pat. These events have not been found to occur during stretch-induced hypertrophy of the Pat muscle (18). SRF mRNA induction appears not to be required at the onset of stretch overload in the Pat muscle. SRF mRNA was not elevated until after the third day of stretch and has returned to control levels by the second week of stretch. The induction pattern of SRF mRNA corresponds to the stretch-induced growth of the Pat muscle, since no protein accretion occurs until after day 3, and 80% of the stretch-induced growth occurs during the first week. Insulin-like growth factor I mRNA is another mRNA showing an expression pattern similar to SRF mRNA at the onset of stretch in the Pat muscle, and it is not increased until after the second day of stretch (10).

The avian SRF mRNA template demonstrates two species, which can be delineated by Northern blot analysis (9). The two SRF transcripts adapt similarly to stretch overload in the Pat muscle. The B2 SRF mRNA template is less abundant in slow tonic ALD muscle compared with the mainly fast-twitch Pat muscle. Stretching of the Pat muscle increases the percentage of oxidative fibers and decreases the percentage of glycolytic fibers in the muscle (12); however, the distribution of the B2/B1 SRF mRNA ratio is not altered with stretch. It appears that the B1/B2 SRF mRNA ratio is not a marker of stretched muscle phenotype shifts.

SRF binding to skeletal α-actin SRE1 is necessary for actin promoter stretch responsiveness during in vivo chicken ALD muscle hypertrophy (6). During stretch-induced hypertrophy of the chicken ALD muscle, the SRF:SRE1 binding complex, in GMSA, migrates faster in 6-day stretched ALD nuclear extracts compared with the contralateral control (6). Nuclear extracts from 6-day stretched Pat muscle do not exhibit a faster migrating SRF:SRE1 binding complex. SRF can be posttranslationally modified in cell culture by phosphorylation, glycosylation, and dimerization with other transcription factors (16, 20, 26), and it remains a possibility that this could account for the altered mobility of the SRF:SRE1 binding complex from stretched ALD muscle. The differences between the stretched Pat and ALD in SRF:SRE1 binding complex mobility could be attributed to different signaling cascades being activated in the two muscles during stretch (5, 6). The difference between the two muscles may be related to the concentration of skeletal α-actin mRNA not being increased in stretched Pat muscle, as in the stretched ALD muscle (6).

The function of MyoD induction at the onset of stretch overload in the Pat is not certain, since activated satellite cells appear not to be the source. However, qmf1, an avian myogenic regulatory factor with homology to MyoD, has been found in ALD nuclei from preexisting fibers after 3–16 h of stretch (11). This early expression of qmf1 was thought to represent a recapitulation of developmental myogenic regulatory factors at the onset of stretch overload (11). Our present data help substantiate the findings of Eppley et al. (11), since MyoD was induced on the third day of stretch overload in the Pat muscle, even though muscle fiber number and satellite cell activation did not increase (18). This suggests that myonuclei from mature fibers were expressing MyoD mRNA. The lack of fiber proliferation in the hypertrophying Pat muscle may contribute to the narrower window of MyoD induction compared with the stretched ALD muscle. MyoD mRNA content per muscle is induced from 3 to 21 days in the stretched ALD (5, 6).

In summary, the stretch-overloaded Pat muscle undergoes a sequence of gene expression during the first 2 wk of stretch overload. These changes in gene expression include a transient induction of MyoD mRNA concentration at the onset of hypertrophy, which is prior to the transient increase in SRF mRNA concentration. These tightly regulated alterations in gene expression may influence the stretch-induced hypertrophy of the Pat muscle.

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SRF IN FAST-TWITCH MUSCLE HYPERTROPHY


