SRF protein is upregulated during stretch-induced hypertrophy of rooster ALD muscle

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Flück, Martin, James A. Carson, Robert J. Schwartz, and Frank W. Booth. SRF protein is upregulated during stretch-induced hypertrophy of rooster ALD muscle. *J. Appl. Physiol.* 86(6): 1793–1799, 1999.—Serum response element 1 has previously been reported to be necessary and sufficient for activation of the skeletal α-actin promoter during hypertrophy of the anterior latissimus dorsi (ALD) muscle of roosters [J. A. Carson, R. J. Schwartz, and F. W. Booth. *Am. J. Physiol.* 270 (*Cell Physiol.* 39): C1624–C1633, 1996]. Serum response factor (SRF) protein is the transcription factor that binds as a homodimer to serum response element 1 and activates the skeletal α-actin promoter. An increased expression of exogenous SRF protein in replicating C_2C_{12} myoblasts induced a three- to fourfold activation of the skeletal α-actin promoter (L. Wei, W. Zhou, J. D. Croissant, F.-E. Johansen, R. Prywes, A. Balasubramamyan, and R. J. Schwartz. *J. Biol. Chem.* 273: 30287–30294, 1998). Thus we hypothesized that SRF protein concentration would be increased during hypertrophy of skeletal muscle. In the present study, 10% of the rooster's body weight was attached to the left wing to induce enlargement of the ALD muscle compared with the contralateral muscle. With Western analysis, a significant increase in SRF protein per gram of wet weight of the ALD muscle was noted at 7 and 13 days of hypertrophy. Furthermore, the increase in SRF protein occurred in both crude nuclear protein and cytoplasmic fractions in 7-day stretched ALD muscles. This is the first report showing increased protein concentration for a transcription factor whose regulatory element in the skeletal α-actin promoter has previously been shown to be required for the transduction of a hypertrophy signal in overloaded skeletal muscle of an animal.

transcription factor; serum response factor; skeletal muscle; muscle enlargement

INCREASES IN TRANSLATION ACCOUNT for much of the increased rates of total protein synthesis rates during the first day of stretching the anterior latissimus dorsi (ALD) muscle of roosters (18). Thereafter, total RNA concentration increases so that total protein synthesized per unit of RNA returns to control values (18), whereas transcription of skeletal α-actin mRNA increases (4). Transcriptional processes that regulate increases in the promoter activity of the skeletal α-actin gene during hypertrophy of skeletal muscle in living animals have not been completely defined. However, stretch-induced activity of the skeletal α-actin promoter in ALD muscle of roosters was reported to be dependent on a functional serum response element (SRE) 1 [(SRE1) $CC(A/T)_6GG$, also know as CArG box (5)]. Two other SREs (SRE2 and SRE3) further upstream of SRE1 in the skeletal α-actin promoter were not required for stretch-induced transactivation of the skeletal α-actin gene (5). The expression pattern of serum response factor (SRF) protein, which binds to SRE as homodimer (32), is primarily restricted to striated and smooth muscle cell lineages (7). SREs are found in numerous other gene promoters, with c-*fos* the most frequently studied. The c-*fos* SRE contains a binding site for *ets* protein family, Elk-1 (13). Phosphorylation of Elk-1 by the mitogen-activated protein kinase cascade transactivates the c-*fos* promoter through SRF and SRE (12). The contextual sequence of SRE1 in the skeletal α-actin gene differs from c-*fos* by not having a binding site for *ets* proteins, but rather having a binding site for YY1 protein, whose binding to SRE1 represses the skeletal α-actin promoter (19). Transactivation of the skeletal α-actin promoter through SRE1 occurs through alterations in SRF and YY1 protein concentrations (21). Thus the presence of the common motif $CC(A/T)_6GG$ in the SRE/CArG family does not guarantee their functional equivalence (19). Nevertheless, a common occurrence for all SREs is transactivation by SRF binding. For example, overexpression of exogenous SRF protein in replicating C_2C_{12} myoblasts induced a three- to fourfold activation of the skeletal α-actin promoter (34). Thus we hypothesized that SRF protein concentration increases during stretch-induced hypertrophy of the ALD muscle in roosters.

MATERIALS AND METHODS

Production of SRF fusion proteins and generation of antiserum. SRF fusion constructs were described previously with modifications (6). In brief, after expression in *E. coli* strain BL21(DE3)pLysE, His-tagged SRF fusion proteins were isolated under denaturing conditions with the use of Ni^{2+}-affinity column (Qiagen) and were dialyzed twice against a solution of PBS, 0.5% TX-100, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride in a similar manner to the glutathione affinity-purified glutathione *S*-transferase fusion proteins. Two rabbits were each injected every 2 wk over a 6-wk period with 100 μg of His_6-$SRF_{1–508}$ fusion protein at Bethyl Laboratories (Montgomery, TX) with complete Freund's adjuvant. Two rabbits each initially received multiple subcutaneous injections of 100 μg of His_6-$SRF_{1–508}$ fusion protein in ~1 ml of complete Freund's adjuvant and then were immunized every 2 wk over a 6-wk period with the same amount of fusion protein in ~1 ml of incomplete Freund's adjuvant at Bethyl Laboratories. Before the initial injection, animals were prebled to collect preimmune sera (PI), and immunizing sera were collected at *weeks 5* and *7* after the initial injection. Batches of the immunizing sera were tested in our laboratory for affinity against His_6-$SRF_{1–508}$ fusion protein by using immunoprecipitation and immunodetection on Western blots.

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The sera of each rabbit with the best titer were finally affinity purified at Bethyl Laboratories. In brief, the sera were processed four times over an immunosorbent column that was constructed by conjugating His_6-SRF_{1-508} fusion protein to a gel matrix. Bound antibodies where eluted from the immunosorbent column with 0.1 M glycine (pH 2.5) and collected in citrate buffer to neutralize pH, and the titer of the affinity-purified sera was determined by enzyme immunoassay and immunoprecipitation. The titer of the best batch of affinity-purified antibody R86 of one rabbit was estimated to be 1:55,000.

Cell culture. Primary embryonic myoblast cultures were established from 11-day chicken embryos as previously described (21). Cell plates were periodically examined by using a phase-contrast microscope and were harvested at 24-h (myoblast stage) or 96-h postplating (myotube stage) by being washed twice with PBS (28) and scraped in cold Mueller buffer (50 mM HEPES, pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7 \cdot 10H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, 50 μg/ml leupeptin, 50 μg/ml pepstatin, 33 μg/ml aprotinin, and 5 ml buffer per 700 mg tissue). For selective depletion of replicating cells including fibroblasts, 10 mM cytosine arabinoside (CytA) was added 48-h postplating for 24 h (27).

Muscle loading. Young roosters (White Leghorn, Texas A&M Univ., College Station, TX) were received at 3–7 wk of age and were housed up to a dozen each at the animal care facilities, University of Texas Health Science Center at Houston, TX (as previously described in Ref. 4). The left wing was loaded with weight corresponding to 10% of the rooster's initial body weight for 1.5, 7, or 13 days, as previously described (4). The ALD muscle was harvested after anesthetization with a subcutaneous injection of a ketamine-xylazine-acepromazine cocktail (25:1:1.5 mg/kg), snap-frozen in liquid nitrogen, and stored in sealed tubes at −80°C until use. The animal protocols were approved by the Institutional Welfare Committee, University of Texas Health Science Center at Houston, TX.

Isolation of total protein. Frozen ALD muscles were homogenized in Mueller buffer with a Polytron mixer (Kinematica) for 3 × 20 s at a low setting on ice, frozen, and stored as aliquots (designated as total protein homogenates) at −80°C. Protein concentration was estimated by using a Lowry-based protein assay (Bio-Rad, DC protein assay). Total protein (50 μg) from an aliquot of each muscle sample was run on SDS-PAGE. The gel was stained with Coomassie blue to verify the validity of estimated protein concentration and to check the integrity of isolated proteins (28).

SDS-PAGE, Western blotting, and immunodetection. Protein samples that had been solubilized at 1 μg/μl in 1× SDS loading buffer (50 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.1% bromphenol blue) were separated by 8% SDS-PAGE (28) and were Western blotted in 25 mM Tris-base (pH ~8.3), 192 mM glycine, and 20% methanol onto a nitrocellulose membrane. Blotting efficiency (>80%) was verified by Coomassie blue staining of protein residual in the gel. Equal loading was checked by Ponceau S staining of the membrane. Immunodetection was achieved at room temperature (25°C) after blocking of the membrane [1 h in 2.5% nonfat dry milk, 1% BSA in TTBS (20 mM Tris-base, pH 7.5, 150 mM NaCl, 0.05% Tween-20)], probing with immune antiserum R86 or PI of the same rabbit (2 h at 1:1,000 dilution in blocking solution), serial washes in TTBS, incubation with horseradish-peroxidase-conjugated donkey anti-rabbit antibody (Amersham, 1 h at 1:7,500 in blocking solution), washes in TTBS, and finally visualization by enhanced chemiluminescence with recording on Kodak-XAR5 film. The intensity of SRF signals was quantified by densitometric scanning (Bio Image, Millipore, Ann Arbor, MI) as integrated optical density. Control integrated optical density values were set to one.

Isolation of crude nuclei. After determination of wet weights, ALD muscles were pooled until they exceeded 1 g in mass and were then processed with minor modifications, essentially as described previously (4). Additional inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 2.5 μg/ml aprotinin, 5 mM NaF, 0.5 mM Na_3VO_4) were added to all solutions. After the first homogenization with the use of a Polytron mixer, an aliquot (designated as the whole muscle homogenate) was removed, the insoluble matter (containing intact nuclei) pelleted (800 g, 10 min, 4°C), the supernatant saved (designated as crude cytoplasmic fraction), and the pellet resuspended and poured through a cheesecloth, after being sieved with a syringe through nylon filters of decreasing pore size (200 and 50 μm, Spectrum Medical). Nuclei (designated as crude nuclei) were pelleted by ultra-centrifugation through 2.0 M sucrose (100,000 g, 1 h, 4°C), resuspended, concentrated, and stored in 1 ml of cold nuclear storage buffer (20 mM Tris, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and inhibitors as stated above) at −80°C.

Statistical analysis. An ANOVA analysis was used to test for a significant effect ($P < 0.05$) of stretch. A paired Student's *t*-test was performed when a significant stretch effect was found.

RESULTS

SRF protein increases after fusion of chicken primary "embryonic" skeletal myoblasts. Our affinity-purified antiserum (R86) recognized various human SRF fusion-protein constructs (Fig. 1, *A* and *B*), in contrast to PI from the same animal that did not detect any protein (data not shown). Antibody R86 recognized epitopes in both the N- and C-terminal ends (data not shown). To further verify the antibody, it was shown to detect a 40-fold increase in SRF protein content per total cellular protein during fusion to myotubes of primary embryo chicken myoblasts (Fig. 1*C*), which supports previously published data (7). We extended this confirmation with the novel observation that treatment of myoblasts with CytA, which selectively depletes fibroblasts, augmented SRF protein by 80-fold after myoblast fusion (Fig. 1*C*).

SRF protein is upregulated in stretched ALD muscle during hypertrophy. SRF protein expression in ALD muscle was analyzed by Western blotting and immunodetection with the use of antiserum R86. SRF protein in 50 μg of total protein homogenate of ALD muscle was detected as a broad single band of ~53 kDa with the use of affinity-purified R86 SRF antiserum and is not detected with the use of the PI of the same animal (Fig. 2*A*). The addition of 10 μg of recombinant His_6-SRF_{1-508} protein (30 nM) to the first antibody solution competed away the 53-kDa band (Fig. 2*A*). Under the conditions employed, no protein other than the 53-kDa protein SRF was detected in Western blots of 8% SDS-PAGE gels loaded with 50 μg of total protein homogenate from ALD muscle.

Immunodetection of SRF protein on Western blots with 50 μg of total protein homogenates prepared from control or stretched ALD muscle showed a significant

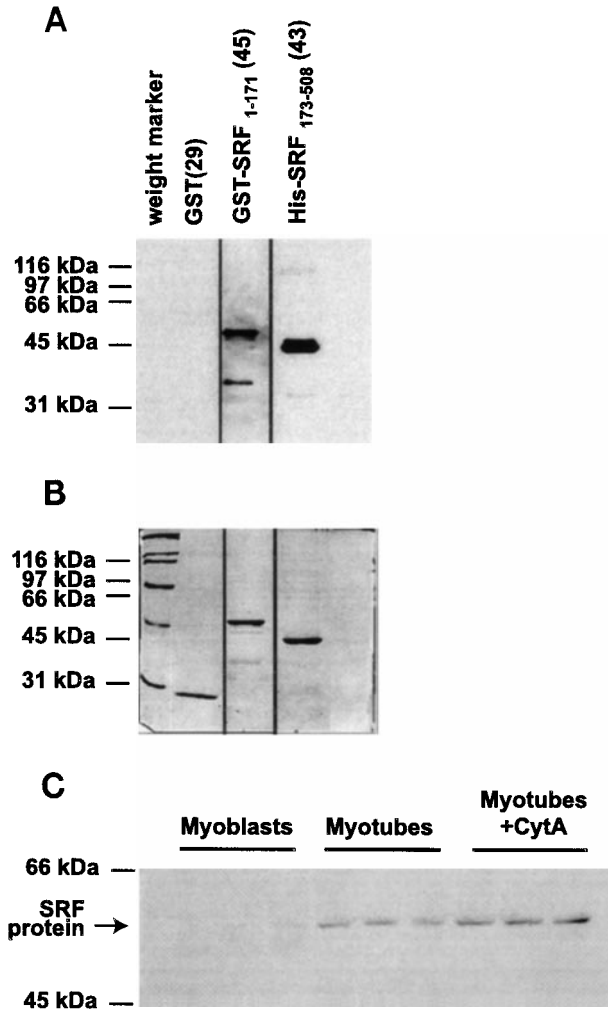

Fig. 1. Serum response factor (SRF)-specific antiserum demonstrates that increases of SRF protein in fused chicken myotubes are independent of fibroblasts. *A*: immunodetection with affinity-purified antiserum R86 on Western blots of SDS-PAGE gels run with ~50 ng of different SRF fusion proteins per lane. Immunodetection with preimmune serum (PI) was blank and is not shown. Sizes of full-length fusion proteins, some of which have a tendency to degrade, are indicated in parentheses above the name of the fusion protein. GST, glutathione *S*-transferase. *B*: loading control showing Coomassie blue-stained gel run with same amount of same fusion proteins as in parallel experiment shown in *A*. Detected bands appear similar in molecular size to ones detected on Ponceau S-stained membrane (data not shown). *C*: immunodetection of SRF protein at 53 kDa with affinity-purified antiserum R86 using 50 μg of total protein from multiple culture dishes that contained either chicken primary myoblasts, fused myotubes, or fused myotubes with addition of 10 mM cytosine arabinoside (CytA) to deplete replicating cells.

increase in the concentration of SRF protein per unit of total protein in stretched ALD muscle of 131 ± 29 and 115 ± 35% at 7 and 13 days of continuous stretch, respectively, compared with their contralateral control muscles (Fig. 2, *B* and *D*). SRF protein concentration was not significantly changed at 1.5 days of stretch.

The upregulation of SRF protein per whole muscle coincides with the previously reported increases in mRNAs for SRF, skeletal α-actin, and MyoD. From studies with avian and rodent skeletal muscle cultures, there is ample evidence that SRF protein is involved in the transcription of skeletal α-actin mRNA, MyoD, and its own message (1, 11, 20, 29, 30). Comparison of the present data with published reports (3, 4) on these gene transcripts in the hypertrophying ALD muscle indicates that SRF protein per total protein increases from the first to the seventh day of hypertrophy (Fig. 3). Total protein per whole ALD muscle was increased after 1.5, 7, and 13 days of stretch-induced overload (Table 1).

Increase in SRF protein is mainly in nuclei/satellite cells of ALD muscles. SRF protein has been described as a major nuclear localized protein (10). However, a recent study with primary chicken myoblast cultures indicates that SRF abundance in the cytoplasm is also enhanced, but only during the period of fusion into myotubes (7). Crude cytoplasmic and crude nuclei fractions were isolated from ALD muscles to identify by immunodetection the intracellular compartment in which the increasing SRF protein levels occur. These fractionation studies demonstrated that most SRF protein was found in the crude nuclear preparations from whole muscles. SRF protein per unit of extracted protein was highly associated with crude nuclei (~99%, $P < 0.005$, $n = 4$). After 7 days of continuous stretch, SRF protein per crude nuclear protein was 97 ± 38% ($P < 0.05$, $n = 3$) higher in stretched than in contralateral control ALD muscles (Fig. 4, *A* and *C*). SRF protein concentration per cytoplasmic protein becomes detectable in cytoplasmic fractions of 7-day stretched compared with their contralateral control ALD muscles (Fig. 4*C*). Thus the increase of SRF protein during stretch-induced hypertrophy is associated both with nuclei and cytoplasm.

DISCUSSION

A novel observation of the present study was the increased concentration of SRF protein in total muscle homogenates, cytoplasmic fractions, and nuclear-enriched fractions of stretched rooster ALD muscles undergoing hypertrophy. Although this is not the first report of an increase in a transcription factor in overload-induced hypertrophy of skeletal muscle (3, 8, 14, 22, 24, 31), it is the first report of an increase for the transcription factor SRF, which is a member of the MADS (MCM1-agamous-Arg-80-deficiens-SRF) box family that includes myocyte-enhancer binding factors, which share a common amino acid sequence for DNA binding and protein dimerization with SRF (32).

During overload-induced hypertrophy of skeletal muscle, increases in myogenic factor mRNAs (3, 22, 24) have been reported. Increases in mRNA levels do not always translate into additional protein when muscle size changes (9); however, the present findings extend the reports of increases in myogenic factor (myogenin, MyoD, myf-5, and myogenic regulatory factor 4) mRNAs in overload-induced hypertrophy (3, 22, 24) to the protein level for SRF. The expression of MyoD and myogenin appears to depend on the presence of SRF in differentiating myoblasts (11, 29). Another set of studies has shown that androgen and glucocorticoid receptor proteins increase (8, 14, 31) and transduce steroid

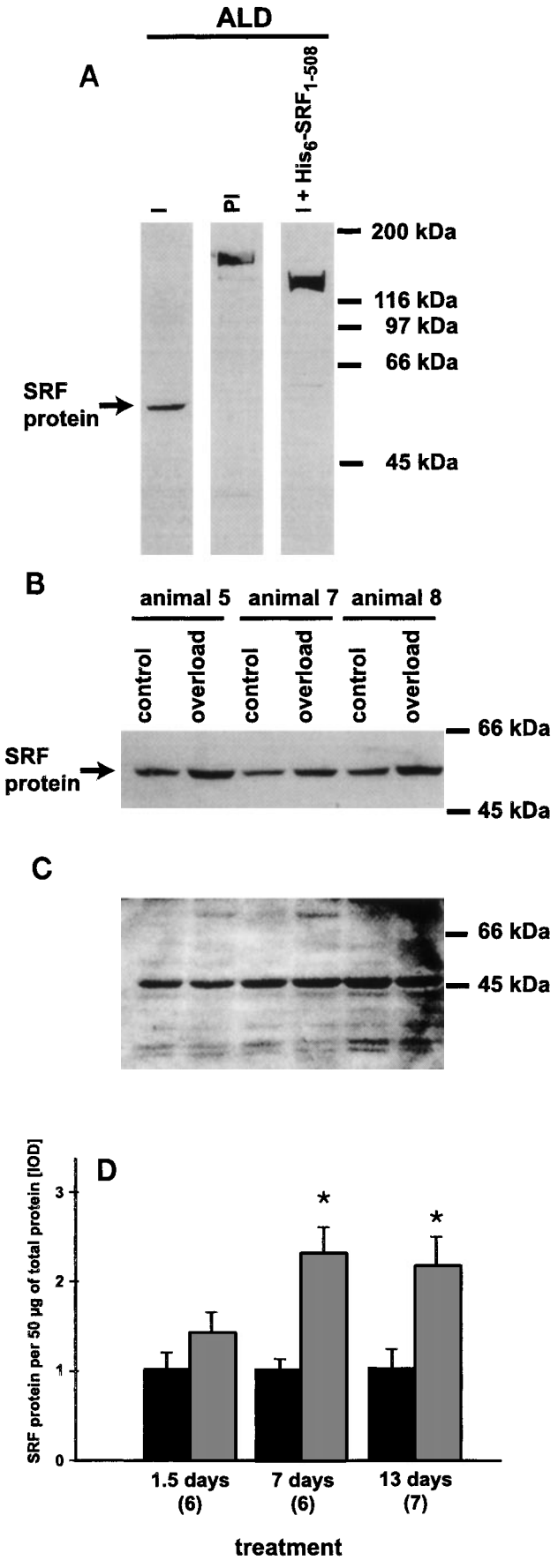

Fig. 2. SRF protein is upregulated in stretched anterior latissimus dorsi (ALD) muscle during hypertrophy. *A*: immunodetection with affinity-purified serum R86 (I), PI of same animal, and competition control (serum R86 + 10 µg His_6-$SRF_{1–508}$) on samples containing 50 µg of total protein from ALD muscle. SRF protein is specifically detected as broad single band with apparent weight of 53 kDa. *B*: representative immunodetection with SRF-antiserum R86 on Western blots of 50 µg total protein from either 7-day stretched (overload) or contralateral control ALD muscles. PI is not shown, and it did not detect a protein signal in chosen range. *C*: loading control showing Ponceau S-stained membrane before immundetection. *D*: representative data (means ± SE) given in *B* for integrated optical density (IOD) obtained from stretched (gray bar) or contralateral control (solid bar) ALD muscles of same animal for different durations of stretch. Total protein (50 µg) from each ALD muscle was analyzed by SDS-PAGE, Western blotting, and immunodetection with SRF antiserum R86. Intensity of detected SRF band was integrated, and average control level for each separate Western blot was set to value of 1. Control values for each treatment were normalized to 1.0. No. of animals (6 or 7) used per group is indicated in parentheses. *Significant difference between control and stretch values of same day ($P < 0.05$).

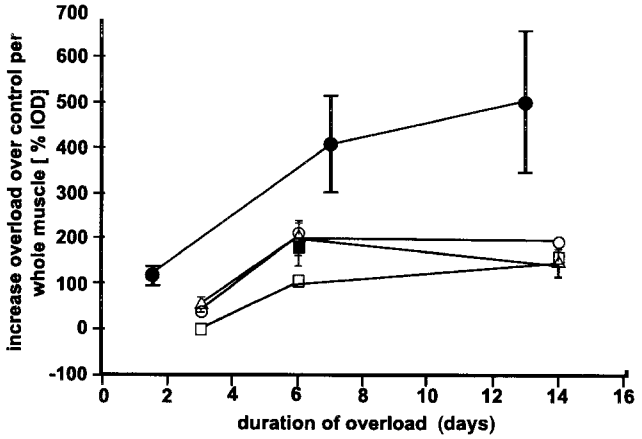

Fig. 3. Expression of SRF protein and downstream transgene targets. Data are presented as means ± SE of %increase per whole muscle in stretched relative to contralateral control ALD muscle as a function of duration of stretch. mRNA values for SRF (○), skeletal α-actin (□), MyoD (△), SRF protein data (●), and skeletal α-actin promoter activity (■) are from present and previous reports from our laboratory (3, 4).

hormone signals to alter gene promoter activity in overload-induced hypertrophy. The present study's results extend these findings to a transcription regulatory protein not known to bind steroids. The sum of previous and present observations supports the concept of muscle plasticity. Although muscle structural protein expression is altered in hypertrophying adult skeletal muscles, transcription-factor proteins can also exhibit plasticity.

A function for increased SRF protein concentration in stretch-induced hypertrophy of skeletal muscle in animals can be suggested from those studies of its interaction with the skeletal α-actin promoter in cultured myocytes and in in vitro studies. For example, overexpression of exogenous SRF protein in replicating C_2C_{12} myoblasts induced a modest activation of the skeletal α-actin promoter (34). Cotransfection of SRF with wild-type RhoA or constitutively active RhoA produced an additive effect on skeletal α-actin promoter activity (an ~3- or 4-fold activation by SRF or RhoA alone vs. an

Table 1. *Total milligrams of protein per ALD muscle*

Treatment	*Series 1*	*Series 2*	%Increase
1.5 Days			
Control	80.5 ± 7.4 (3)	40.3 ± 11.9 (4)	
Stretch	104.9 ± 7.2 (3)	59.7 ± 7.5 (4)	
Stretch/control	1.34 ± 0.15	1.63 ± 0.26	+46 ± 14%* (7)
7 Days			
Control	78.8 ± 5.2 (3)	28 ± 2.9 (3)	
Stretch	158.3 ± 9.5 (3)	47 ± 1.5 (3)	
Stretch/control	2.04 ± 0.18	1.73 ± 0.24	+88 ± 16%* (6)
13 Days			
Control	90 ± 12.1 (3)	35 ± 9.6 (4)	
Stretch	243.5 ± 31.6 (3)	55 ± 5 (4)	
Stretch/control	2.85 ± 0.59	1.72 ± 0.28	+137 ± 41%* (7)

Values are means ± SE in mg. Nos. in parentheses, no. of animals used per treatment. Total milligrams of protein per anterior latissimus dorsi (ALD) muscle increased continuously during first 13 days of hypertrophy as compared with contralateral control muscle. Shown are individual means ± SE and ratio of hypertrophy/hyperplasia (stretch) to contralateral control from two independent series of experiments (*Series 1* and *2*) for durations of 1.5, 7, or 13 days. Animals in *series 1* were older than those in *series 2*. *Significant difference between control and stretch values for pooled values ($P < 0.001$).

~10-fold activation by SRF and RhoA together) (34). On the other hand, overexpression of a dominant negative SRF protein blocked SRE-dependent skeletal α-actin promoter activation during both myogenesis (7) and RhoA overexpression (34). This mutant SRF protein dimerizes with wild-type SRF, preventing transcriptional activation (15). Myoblasts expressing the dominant negative mutant of SRF protein showed at least a 60% reduction in SRF-DNA interaction with a double-stranded skeletal α-actin SRE1 probe (34). Overexpression of SRF protein induces transcription from SRE-containing promoters of various other muscle-specific genes (1, 2, 16, 17, 19, 21, 23, 26, 33). SRF protein has previously been shown to be essential for expression of rodent MyoD, even though the MyoD gene lacks a SRE consensus sequence in its promoter (11, 29).

After the introduction of differentiation media to C_2C_{12} cell lines, induction both of endogenous skeletal α-actin and of genes not directly binding SRF, e.g., α-myosin heavy chain and myogenin, was suppressed when dominant negative SRF was being expressed (34). Thus in these studies, SRF protein activates the skeletal α-actin promoter in cultured myocytes. Although not within the scope of the present study, future studies should use transgenic technology to test the effects of dominant negative SRF on muscle growth when skeletal muscle in animals is overloaded.

ALD muscle mass doubled by the sixth day of stretch (the rapid growth phase of hypertrophy) but only increased another 20–50% during the second week of stretch (slow growth phase) in young roosters (Ref. 3; Table 1). Increased SRF protein concentration per milligram protein in homogenates (Fig. 2*D*), in cytoplasmic fractions, and in nuclear-enriched extracts (Fig. 4) was evident by the seventh day of stretch in the ALD muscle. This suggests that the increase in SRF protein concentration was initiated during the rapid phase of hypertrophy. Furthermore, it is possible that some of the increased nuclear SRF protein is associated with satellite cells after their fusion to muscle fibers, but such determinations were beyond the scope of the present study. In addition, increases in SRF protein paralleled increases in its mRNA/whole muscle (Fig. 3). The increase in SRF protein level after 2 wk is apparently increased a larger percent than the percent increase in its mRNA. On the sixth day of hypertrophy, the skeletal α-actin promoter activity is upregulated, presumably by SRF interaction with its SRE1 (4, 5). The increase of SRF protein during the first week of

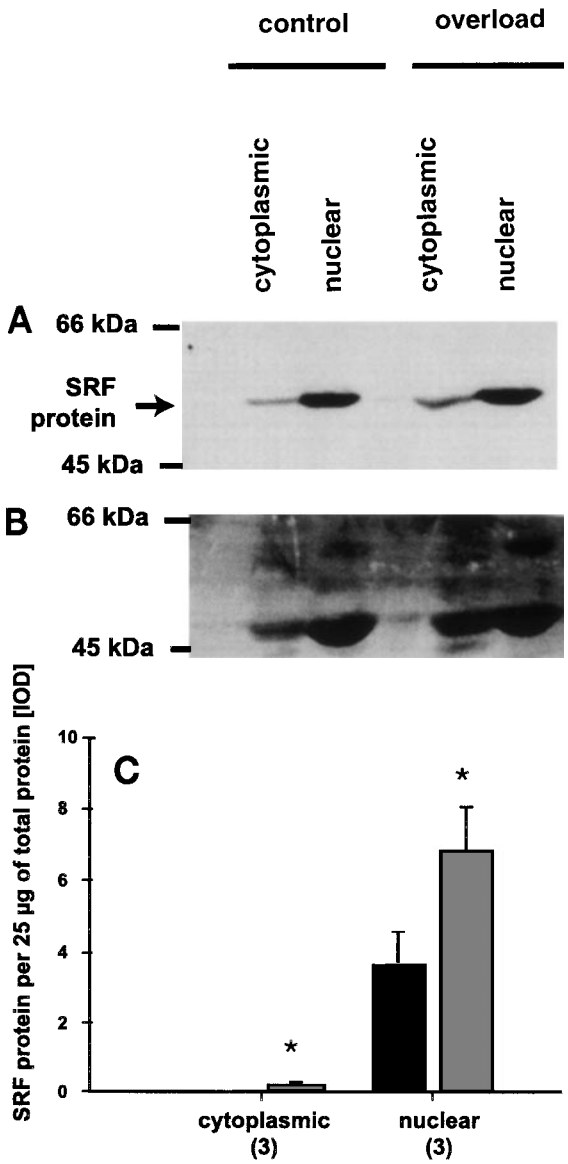

Fig. 4. SRF protein is localized to nuclei. *A*: representative immunodetection of SRF protein in Western blots of 25 µg protein from crude cytoplasmic and crude nuclear fractions from whole muscle homogenates of either 7-day stretched (overload) or contralateral control ALD muscles of same animals. *B*: loading control showing Ponceau S-stained membrane before immunodetection. *C*: IOD values (means ± SE) for SRF immunoreactivity in 25 µg protein of crude cytoplasmic and crude nuclear fractions from ALD muscles. Solid bars, contralateral control muscles; gray bars, 7-day stretched muscles. No. of animals is in parentheses. *Significant difference between control and stretch values for same day ($P < 0.05$).

stretch is paralleled by increases in the mRNAs for MyoD, skeletal α-actin, and SRF.

In summary, the present study adds to the growing body of literature as to the mechanism by which overload signals an increase in promoter activity of the skeletal α-actin gene. Previously, we have shown that SRE1 of the skeletal α-actin promoter is a regulatory element through which a signal for hypertrophy is transduced. We report that the quantity of transcription factor SRF is increased. Future studies now have a basis for hypothesizing that expression of a functionally inactive (dominant negative) SRF protein would lessen hypertrophy in overloaded skeletal muscle to test whether increases in SRF protein play some role in a hypertrophy-signaling pathway.

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