67th WEEKLY NEWSLETTER

USF INTERNAL MEDICINE & PEDIATRIC DIVISIONS OF ALLERGY AND IMMUNOLOGY

Next in the line of key historical articles of scientific impact in the medical literature is the paper on "Physiological control of smooth muscle-specific gene expression through regulated nuclear translocation of serum response factor" by Blanca Camoretti-Mercado, PhD, Assistant Professor of Medicine and Pediatrics, Division of Allergy and Immunology, Department of Internal Medicine.

From Blanca Camoretti-Mercado, PhD:

Camoretti-Mercado B, Liu HW, Halayko AJ, Forsythe SM, Kyle JW, Fu Y, McConville J, Kogut P, Vieira JE, Patel NM, Hershenson MB, Miano JM, Parmacek MS, Burkhardt JK, and Solway J. Physiological control of smooth muscle-specific gene expression through regulated nuclear translocation of serum response factor. *J Biol Chem* 2000; *275: 30387-30393*. PMID: 10866994.

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This seminal and highly cited publication showed that expression of specific smooth muscle genes requires the nuclear activity of the transcription factor Serum Response Factor (SRF). This was unexpected because SRF was cloned years earlier for its key role in mediating growth response to serum stimulation, which opposes cell differentiation. We explained this paradox (that SRF could be critical for both cell proliferation and differentiation) by showing physiological regulation of SRF subcellular localization. Prior to our discovery, the traffic in and out of the nucleus of proteins controlling gene expression was known for few transcription factors, including NF-kB, a master regulator of inflammation. Based on our study, other groups revealed an association of SRF localization with fibroproliferative disorders of the lung.

With warm regards,

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Physiological Control of Smooth Muscle-specific Gene Expression through Regulated Nuclear Translocation of Serum Response Factor*

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Prolonged serum deprivation induces a structurally and functionally contractile phenotype in about 1/6 of cultured airway myocytes, which exhibit morphological elongation and accumulate abundant contractile apparatus-associated proteins. We tested the hypothesis that transcriptional activation of genes encoding these proteins accounts for their accumulation during this phenotypic transition by measuring the transcriptional activities of the murine SM22 and human smooth muscle myosin heavy chain promoters during transient transfection in subconfluent, serum fed or 7 day serum-deprived cultured canine tracheal smooth muscle cells. Contrary to our expectation, SM22 and smooth muscle myosin heavy chain promoter activities (but not viral murine sarcoma virus-long terminal repeat promoter activity) were decreased in long term serum-deprived myocytes by at least 8-fold. Because serum response factor (SRF) is a required transcriptional activator of these and other smooth muscle-specific promoters, we evaluated the expression and function of SRF in subconfluent and long term serum-deprived cells. Whole cell SRF mRNA and protein were maintained at high levels in serum-deprived myocytes, but SRF transcription-promoting activity, nuclear SRF binding to consensus CArG sequences, and nuclear SRF protein were reduced. Furthermore, immunocytochemistry revealed extranuclear redistribution of SRF in serum-deprived myocytes; nuclear localization of SRF was restored after serum refeeding. These results uncover a novel mechanism for physiological control of smooth muscle-specific gene expression through extranuclear redistribution of SRF and consequent down-regulation of its transcription-promoting activity.

Confluent cultured, passaged canine tracheal myocytes exhibit divergent phenotypes when deprived of serum for 7 or more days. About 1/6 of these cells accumulate abundant contractile apparatus proteins, increasing whole culture contents of smooth muscle myosin heavy chain (smMHC)[1] and SM22 by 5–7-fold (1, 2). These myocytes acquire a contractile phenotype, characterized by morphological elongation, expression of functionally coupled muscarinic M_3 surface receptors, and substantial contraction (shortening) upon cholinergic stimulation. Presently, the mechanism responsible for this phenotypic differentiation is unknown.

In cultured skeletal muscle, differentiation of myoblasts into myotubes depends upon up-regulation of skeletal muscle-specific gene transcription, which results in abundant contractile apparatus protein accumulation (3–6). This precedent in skeletal muscle suggested that similarly enhanced transcription of contractile apparatus genes might account for the substantial smMHC and SM22 accumulation we observed in long term serum-deprived tracheal smooth muscle cells. To test this hypothesis, we assessed transcription from the smMHC and SM22 gene promoters in both preconfluent serum fed and post confluent long term serum-deprived tracheal myocytes. Contrary to our expectation, we found markedly reduced transcription from these promoters in serum-deprived myocytes, an effect mediated through reduced SRF binding activity attributable to reversible, extranuclear relocation of SRF. Our study discloses a novel mechanism for physiological regulation of smooth muscle gene transcription mediated through redistribution of SRF from nucleus to cytoplasm.

EXPERIMENTAL PROCEDURES

Cell Culture—Canine tracheal myocytes were grown on uncoated plastic dishes or glass coverslips (1) and were studied at passage 1 or 2. Serum fed myocytes were maintained in Dulbecco's modified Eagle's medium:F-12 (1:1) plus 10% FBS, 0.1 mM nonessential amino acids, 50 units/ml penicillin, and 50 μg/ml streptomycin. Serum-deprived cells were grown to confluence and then maintained for ≥7 days in serum-free Dulbecco's modified Eagle's medium:F-12 containing 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium (ITS), as well as nonessential amino acids and antibiotics as above. Fresh medium was provided every 48–72 h. Some myocytes deprived of serum for 7 days were refed with 20% FBS for 4 days. Long term serum-deprived myo-

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[1] The abbreviations used are: smMHC, smooth muscle myosin heavy chain; SRF, serum response factor; FBS, fetal bovine serum; bp, base pairs; AP, activator protein; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay.

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cytes from canine pulmonary artery myocytes or canine aorta were similarly prepared.

Plasmids—In pSM22luc, transcription of the luciferase cDNA in pGL2basic is directed by bp −445 to + 41 of the mouse SM22 gene (7). In p5xCArGluc, luciferase expression is directed by an artificial promoter containing five copies of the SRF binding site ($CC(A/T)_6GG$, or "CArG box") upstream of a minimal TATA box (Stratagene); p5xCArGluc does not contain the Ets binding site contained in the c-*fos* serum response element. We constructed psmMHCluc, in which the human smMHC promoter drives luciferase expression, as follows. We determined that human chromosome 16p13 BAC clone CIT987SK-972D3 (GenBank™ HSU91323; provided by Dr. Ung-Jin Kim) contains the 5′-end of the human smMHC gene, as evidenced by sequence homology with rat, mouse, and rabbit smMHC genes. BAC DNA was digested with *Kpn*I and *Spe*I, and the 3.3-kilobase fragment containing the smMHC promoter and all of exon 1 ligated into *Kpn*I/*Spe*I-digested pGL3basic. pMSVluc and pMSVβgal, in which the viral MSV-LTR promoter controls luciferase or β-galactosidase expression (8), and pSM22βgal, in which bp −445 to +41 of the mouse SM22 gene direct *lacZ* expression (9), were generated previously. To generate p4xAP2luc, four copies of the human keratin K14 promoter sequence from bp −257 to −211, which contains an AP-2 binding site (10), were cloned upstream of the minimal K14 promoter in the K14mpLuc vector (11), between the *Kpn*I and *Sac*I sites. In preliminary studies, transcription from this AP-2-dependent reporter was activated 5–10 times upon co-transfection with AP-2α expression plasmid in HepG2 and HeLa cells.[2] All plasmids were purified on CsCl gradients prior to transfection.

Transfections—Transient transfection of plasmid DNA was accomplished with cationic lipids. LipofectAMINE (Life Technologies, Inc.) provided efficient transfection of subconfluent, serum fed canine tracheal myocytes, whereas DOTAP (Roche Molecular Biochemicals) allowed for serum-free transfection of 7-day-old serum-deprived cells. Subconfluent myocytes in 6-well dishes were transfected in Optimem (Life Technologies, Inc.) with 14 μg of LipofectAMINE, 1.8 μg of luciferase reporter, and 0.6 μg of pMSVβgal (used to normalize transfection efficiency)/well. Myocytes were refed with serum 5 h later and harvested 48 h after transfection for measurement of luciferase and β-galactosidase activities (7, 8). Serum-deprived myocytes in 6-well dishes were transfected in Optimem containing 20 μg of DOTAP, 1.8 μg of luciferase reporter, and 0.6 μg of pMSVβgal. Myocytes were refed 5 h later with serum-free Dulbecco's modified Eagle's medium:F12/ITS and then harvested 48 h after transfection. Luciferase activity was measured for each sample and normalized to its β-galactosidase activity (7, 8). Results from three to four wells were averaged to provide the datum; experiments were repeated three to nine times, and the average (± S.E.) is shown. Additional serum-deprived myocytes were transfected with only pSM22βgal or pMSVβgal (2.5 μg of DNA plus 20 μg of DOTAP; $n = 3–4$), stained 48 h later with X-gal, and the fraction of blue-stained cells that are nonelongated determined by phase contrast microscopy. Discrimination of elongated *versus* nonelongated cells is easily made by visual inspection (*cf*. Fig. 2, *a* and *b*).

Transduction with Replication-deficient Adenovirus—The AdSM22-nlacZ virus was constructed by ligating a shuttle plasmid (pCA3, Microbix Inc.) containing 445 bp of mouse SM22 promoter fused to a nuclear-localizing *lacZ* reporter gene to *Cla*I-linearized dL327 adenovirus. Viral plaques were generated in HEK cells carrying the E1 gene of adenovirus. Putative recombinant plaques were purified twice in HEK cells. High titer virus ($1–2 \times 10^{10}$ pfu/ml) was analyzed for nuclear *lacZ* reporter expression in PAC SMC (12) at multiplicity of infection of 10–250; 50 multiplicity of infection virus resulted in 100% transduction. Serum-deprived cultured tracheal myocytes plated in 12-well dishes were transduced with AdSM22nlacZ in medium containing 2% FBS and 50 multiplicity of infection virus. After 1 h, cells were washed with phosphate-buffered saline and placed in serum-free medium for 24 h. Myocytes were then fixed and stained for β-galactosidase activity using X-gal reagent.

Nuclear Extracts—Nuclear extracts of 30–70% confluent, serum fed ,or 0–8 day serum-deprived canine tracheal myocytes were prepared at 4 °C using a modification of the method of Dignam *et al.* (13). Myocytes were trypsinized and rinsed twice with Dulbecco phosphate buffer, and then packed cells were incubated on ice for 10 min with 10 vol of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT) and then washed, and nuclei were pelleted in buffer A. Packed nuclei were gently resuspended in 1 vol of extraction buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT) and rocked for 30 min. After centrifugation, supernatants were dialyzed for 1 h against three changes of buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT) and then clarified by centrifugation at 14,000 rpm for 20 min. Protease inhibitors (leupeptin, antipain, chymostatin, and pepstatin A, 5 μg/ml each, Sigma) were added, and nuclear extracts were frozen in aliquots at −80 °C until use.

Electrophoretic Mobility Shift Assay (EMSA)—Double-stranded DNA fragments harboring the sequences of interest were prepared by annealing complementary synthetic oligonucleotides and were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. CArG-box-containing probes included those encompassing the 5′ (5′-GCTGCCCATAAAAGGTTTTTG-3′) or 3′ (5′-CTTTCCCCAAATATGGAGCCTG-3′) CArG boxes (underlined) of the mouse SM22 promoter. An oligonucleotide harboring the AP2 binding site (5′- TCGAACTGACCGCCCGCGGCCCGT-3′) was also used. 20,000 dpm (1–5 fmol) labeled oligonucleotide were preincubated for 15 min with 1.5 μl of binding buffer (50 mM Tris-HCl, pH 7.5, 20% Ficoll, 375 mM KCl, 5 mM EDTA, 5 mM DTT) and 1 μg of poly(dI-dC). When indicated, 200-fold molar excess of unlabeled competitor oligonucleotide containing an Sp1 binding site (5′-CCTGGCTAAAGGGGCGGGGCTTGGCCAGCC-3′) was added. For supershift experiments, 3 μg of antibody were added to the incubation mixture. Binding reactions (3–6 μg of nuclear extract protein) were performed at room temperature in 15 μl for 30 min. DNA·protein complex formation was analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels in TBE buffer (TBE, 40 mM Tris borate, 1 mM EDTA). Supershift antibodies included anti-SRF (gift of Dr. R. Prywes), anti-human IL-5 antibody (TRFK-5; gift of Searle, Inc.), anti-SAP-1a, anti-Elk-1, anti-YY1, anti-p300/CBP, and anti-C/EBP (Santa Cruz Biotechnology).

Western Blot Analysis—Protein lysates or nuclear extracts from subconfluent, serum fed, or postconfluent, serum-deprived myocytes were resolved using 8% SDS-polyacrylamide gel electrophoresis (1). Serum response factor was detected as a 67-kDa band using anti-SRF primary antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence reagents; AP-2α was similarly detected as a ~50-kDa band using anti-AP-2α antibody (Santa Cruz Biotechnology).

In Situ Hybridization—7-Day-old serum-deprived airway myocytes grown on glass coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), then washed with phosphate-buffered saline, treated serially with proteinase K (0.1 μg/ml in 100 mM Tris-HCl, 50 mM EDTA, pH 8.0) and acetic anhydride (0.25% v/v) in 0.1 M triethanolamine (pH 8.0), and then washed twice with SSC. Antisense and sense (for control comparison) cRNA probes labeled by incorporation of digoxigenin-UTP were synthesized by *in vitro* transcription from linearized pGEM3z containing the human SM22 cDNA (WS3–10 (14)), using the Dig RNA labeling kit (Roche Molecular Biochemicals) and T7 or SP6 polymerase. Hybridization was performed overnight at 55 °C in 75% formamide, 1.3X SSC, 1X Denhardt's solution, 200 μg/ml yeast tRNA, 50 mM Na_3PO_4 (pH 7.4), 0.1 gm/ml dextran sulfate, 5 mM EDTA, 10 mM DTT, 250 nM α-thio-ATP, and 1 ng/μl antisense or sense cRNA probe. Thereafter, slides were washed, digested with RNaseA (200 μg/ml at 37 °C for 45 min), rewashed (1X SSC, 55 °C, 10 min; 0.5X SSC, 55 °C, 1 h; 0.5X SSC, room temperature, 5 min), and then blocked with 5% nonfat milk in phosphate-buffered saline. Hybridized probe was detected with primary anti-digoxigenin antibody and secondary rhodamine-labeled antibody; cell nuclei were stained with Hoescht 33342.

Northern Blot Analysis—Total RNA isolated from subconfluent serum fed or 7-day serum-deprived tracheal myocytes was size fractionated (20 μg/lane) by electrophoresis in 1.2% formaldehyde-agarose and transferred to a Hybond plus membrane (Amersham Pharmacia Biotech). Prehybridization and hybridization were performed using ExpressHyb solution (CLONTECH) at 60 °C and an SRF-specific probe prepared by random primer labeling of the full-length human SRF cDNA.

Immunocytochemistry—Cellular localization of SRF was identified by immunocytochemistry performed as described previously (1), using primary anti-SRF antibody (Santa Cruz Biotechnology), secondary fluorescein isothiocyanate-labeled antibody, and propidium iodide or Hoescht 33342 nuclear counterstain. AP-2α was immunolocalized in additional cells using primary anti-AP-2α antibody (Santa Cruz Biotechnology). Samples were photographed on a Zeiss Axioskop microscope with a Photometrics PXL cooled CCD camera and Openlab V2 software (Improvision) or on a Nikon microscope with a Photometrics Sensys CCD camera and IPLab Spectrum software (Signal Analytics).

[2] S. Sinha, unpublished observation.

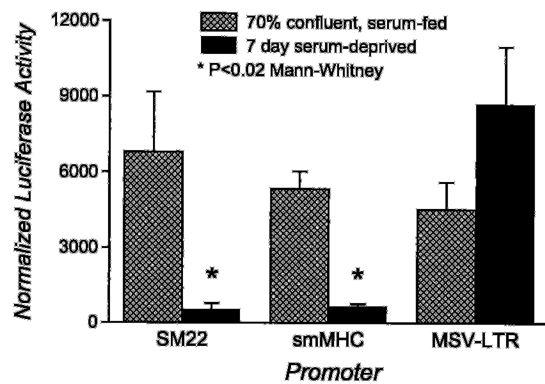

FIG. 1. **SM22 and smMHC promoter activities are down-regulated in long term serum-deprived airway myocytes.** Promoter activity is expressed as normalized luciferase activity (arbitrary units). Both smooth muscle-specific promoter activities are high and comparable to the MSV-LTR in serum fed cells. In serum-deprived myocytes, SM22 and smMHC promoter activities are markedly reduced, whereas MSV-LTR activity remains high.

RESULTS

SM22 and smMHC Promoter Activities Are Down-regulated in Long Term Serum-deprived Airway Myocytes—Fig. 1 shows the activities of the mouse SM22, human smMHC, and MSV-LTR promoters in subconfluent or 7-day-old serum-deprived myocytes. Transcription from both the SM22 and smMHC promoters was ≥8-fold greater in subconfluent myocytes than in 7-day-old serum-deprived cells. In contrast, transcription from the MSV-LTR promoter was greater in airway myocytes cultured under long term serum deprivation. Thus, the reduction in smooth muscle gene promoter activity in serum-deprived myocytes cannot be attributed to a generalized inhibition of gene transcription in such cells.

The Lower Activity of the Smooth Muscle Gene Promoters in Serum-deprived Myocytes Is Not Due to Selective Inactivity in Nonelongated Cells—Only 1/6 of serum-deprived tracheal smooth muscle cells become elongated and accumulate large quantities of contractile apparatus associated proteins (1). To determine whether the lower overall transcriptional activity of SM22 and smMHC promoters in serum-deprived cells was because of a differential activation of these promoters in elongated, but not nonelongated, myocytes, we performed three types of experiments.

First, we transfected serum-deprived myocytes with pMSVβgal or with pSM22βgal and counted the proportion of X-gal-positive cells that were nonelongated; Fig. 2, *a* and *b*, demonstrates the typical appearance of nonelongated and elongated myocytes. The fraction of nonelongated cells expressing the *lacZ* transgene under control of SM22 promoter was only slightly less than that found in cells expressing *lacZ* driven by the MSV-LTR promoter. In both cases, over 2/3 of β-galactosidase-expressing cells were nonelongated (Fig. 2*c*). Thus, when exogenously introduced, the smooth muscle-specific SM22 promoter is active in nonelongated as well as in elongated myocytes.

Second, we employed replication-deficient adenovirus to accomplish more uniform transfer of an SM22 promoter-driven nuclear localizing *lacZ* reporter gene to all myocytes in serum-deprived cultures. Almost all elongated and nonelongated myocytes (Fig. 2*d*) were transduced and expressed the SM22 promoter-driven reporter. This finding confirms that an exogenously introduced SM22 gene promoter is active in both elongated and flattened serum-deprived airway myocytes.

Finally, we performed *in situ* hybridization to evaluate whether the endogenous SM22 gene is transcribed in both elongated and nonelongated serum-deprived myocytes. A specific hybridization signal using the SM22 antisense probe reveals the presence of endogenous SM22 mRNA in all myocytes (Fig. 2*e*); no specific hybridization was seen with SM22 sense probe (not shown). Together, these three lines of evidence exclude restricted activation of smooth muscle-specific gene promoters to only elongated myocytes as a potential explanation for the reduced smooth muscle gene promoter activity seen in serum-deprived airway smooth muscle cells. These experiments demonstrate uniform distribution of promoter activation among serum-deprived airway myocytes and do not conflict with our demonstration above that the overall level of promoter activation is much reduced in these cells.

Long Term Serum-deprived Airway Myocytes Exhibit Decreased SRF Binding Activity without Reduction of Whole-cell SRF Abundance—Because of the central importance of SRF in activating smooth muscle gene transcription (7, 15–17), we analyzed SRF transcription-promoting activity in long term serum-deprived airway myocytes by quantifying transcription from the purely SRF-dependent promoter contained in p5xCArGluc. SRF-dependent luciferase expression was markedly reduced in long term serum-deprived airway myocytes *versus* the high level expression found in subconfluent serum fed cells transfected with p5xCArGluc (Fig. 3*a*). In contrast, transcription from the AP-2-dependent promoter contained in p4xAP2luc was relatively elevated in long term serum-deprived airway myocytes (Fig. 3*a*). Thus, SRF transcription-promoting activity is selectively diminished in serum-deprived myocytes.

Next we sought to determine whether this decrease in SRF transcription-promoting activity stems from reduction in SRF gene expression or from reduced SRF DNA binding activity. Northern blot analysis showed that SRF transcript levels remained high in both serum fed and serum-deprived myocytes (Fig. 3*b*). Likewise, SRF protein is abundant in both 70–100% confluent serum fed and multiday serum-deprived cells (Fig. 3*b*). In contrast, binding of SRF from nuclear extracts to oligonucleotides containing either CArG element from the mouse SM22 promoter was markedly lower in serum-deprived myocytes (Fig. 3*c*). This reduction of SRF binding activity was selective, in that binding of AP2 to its consensus site was similar in subconfluent or serum-deprived cells (Fig. 3*d*). Thus, a reduction of SRF binding activity, but not a reduction in SRF mRNA levels or SRF protein abundance, can explain its reduced transcriptional-promoting activity.

To evaluate whether other nuclear factors contribute to the formation of SRF-containing DNA complexes found in extracts from subconfluent myocytes, we performed additional EMSAs including antibodies directed against SAP-1a, Elk-1, YY-1, p300/CBP, and C/EBP (Fig. 3*e*). These antibodies neither prevented SRF-containing complex formation nor supershifted the SRF-containing DNA complex. Thus, none of these nuclear factors are contained within the SRF-containing complexes identified in this assay.

Extranuclear Localization of SRF in Long Term Serum-deprived Smooth Muscle Cells—Nuclear translocation of several transcription factors, including nuclear factor-κB (18, 19) and glucocorticoid-glucocorticoid receptor complexes (20–22), controls their transcription-regulating activity. A nuclear localization peptide has been identified in SRF protein (23), but regulated nuclear translocation of SRF has not yet been reported. To test whether cytoplasmic redistribution of nuclear SRF could account for the diminished nuclear SRF binding and transcription-promoting activities observed in long term serum-deprived cells, we analyzed nuclear SRF protein abundance by Western blot and immunostained smooth muscle cells to localize SRF. SRF protein is diminished in nuclear extracts

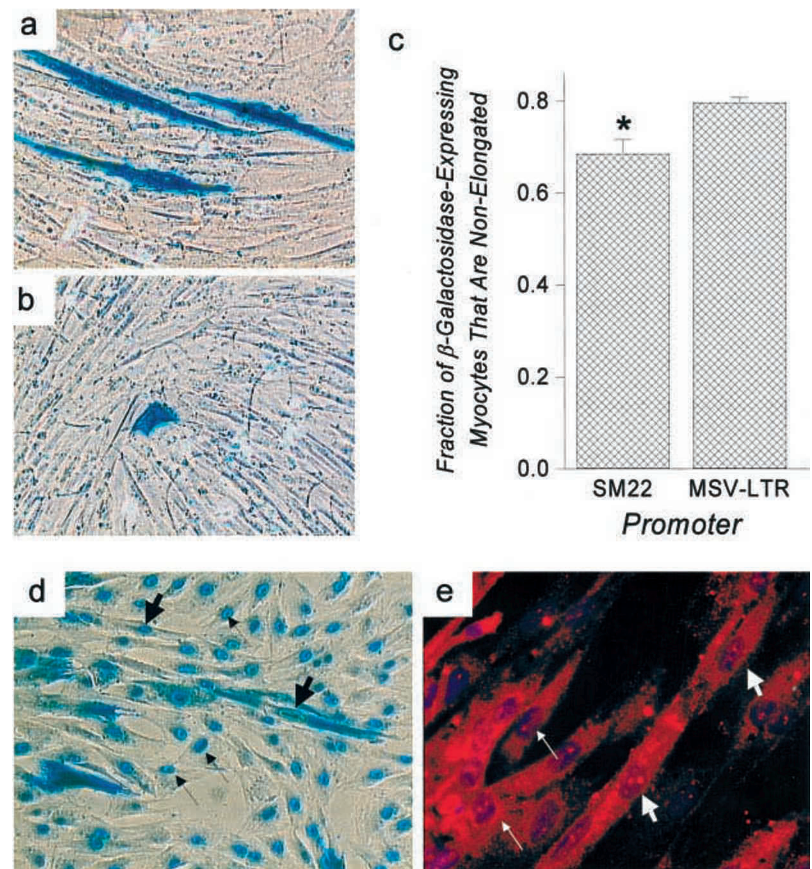

FIG. 2. **SM22 promoter activity is not restricted to elongated myocytes in serum-deprived cultures.** Typical appearance of serum-deprived elongated (*a*) or flattened (*b*) 7-day-old canine tracheal myocytes transfected with pSM22βgal. *c,* fraction of β-galactosidase-expressing myocytes transfected with pSM22βgal or pMSVβgal that are of nonelongated, flattened morphology; *, $p < 0.05$. *d,* transduction of 7-day-old serum-deprived airway myocytes with AdSM22nlacZ programs nuclear expression of β-galactosidase, detected by X-gal staining, in both elongated (*thick arrows*) and flattened (*thin arrows*) cells. *e, in situ* hybridization demonstrates that endogenous SM22 transcripts (*red* fluorescence) are present in elongated (*thick arrows*) as well as in flattened (*thin arrows*) cells; nuclei are visualized by *blue* fluorescence of Hoescht 33342.

from 7-day-old serum-deprived tracheal myocytes *versus* 70% confluent, serum fed cells (Fig. 3*f*). In contrast, nuclear AP-2 protein increases with serum deprivation (Fig. 3*f*). Furthermore, whereas SRF immunoreactivity is restricted to the nucleus of subconfluent, serum fed airway myocytes (Fig. 4, *a* and *b*), SRF is redistributed into the cytoplasm of long term serum-deprived cells, where it appears as a perinuclear cloud (Fig. 4, *c* and *d*) or as a polar extranuclear cap (Fig. 4, *g* and *h*). Refeeding of 7-day-old serum-deprived airway myocytes with 20% FBS for 4 days restores full nuclear localization of SRF (Fig. 4, *e* and *f*). Thus, SRF undergoes reversible nuclear cytoplasmic redistribution in response to external myocyte stimuli. Localization of SRF in an extranuclear polar cap was observed not only in 10-day-old serum-deprived canine pulmonary artery myocytes (shown in Fig. 4, *g* and *h*) but also in similarly treated aortic and tracheal myocytes (not shown). In contrast to the observed redistribution of SRF out of the nucleus in serum-deprived airway myocytes, AP-2 immunostaining demonstrated cytoplasmic predominance in serum fed subconfluent cells (Fig. 5, *a* and *b*), with heterogeneous (Fig. 5, *c* and *d*) or more uniform (Fig. 5, *e* and *f*) redistribution of AP-2 into the nucleus of 7-day-old serum-deprived myocytes. Together, these results confirm the specificity of intracellular SRF relocalization during long term serum deprivation.

DISCUSSION

This study was undertaken to identify mechanisms that lead to the abundant accumulation of the contractile apparatus-associated proteins SM22 and smMHC in long term serum-deprived cultured airway smooth muscle cells (1, 2). By analogy with the transcriptional up-regulation of muscle-specific genes that occurs when cultured skeletal myoblasts differentiate to myotubes (3–6), we hypothesized that similar transcriptional activation of smooth muscle-specific genes causes cultured airway smooth muscle cells to acquire the contractile phenotype during prolonged serum deprivation. However, our results soundly disprove this possibility and instead demonstrate that transcription from the SM22 and smMHC promoters is markedly reduced during long term serum deprivation (Fig. 1). This reduction is not because of restriction of promoter activity to the subset of myocytes that acquire the contractile phenotype (Fig. 2) but rather is attributable to reduction of SRF transcription promoting activity (Fig. 3), which in turn stems from extranuclear redistribution of SRF in all long term serum-deprived smooth muscle cells (Fig. 3*f* and 4). The potential physiological importance of these observations is considered below.

Much attention has been paid to mechanisms that restrict expression of tissue-specific genes to smooth muscle cells. Mutational analyses of transgene expression in cultured cells or in transgenic mice have proven that binding of SRF to its consensus CArG sequence is required for full transcriptional activation of smooth muscle-specific genes. Perhaps best studied has been the SM22 promoter, whose activity depends upon the binding of SRF to each of two CArG boxes within the 300 bp 5′ to the transcription start site. In cultured cells, mutations of either element that prevent SRF binding reduce SM22 promoter activity by half, and mutation of both CArG sites virtually ablates activity (24). *In vivo*, the more 3′ CArG box is required for SM22 promoter-driven tissue-specific reporter expression in transgenic mice (16, 24, 25), but a potential quantitative influence of the 5′-CArG site has not been tested. Two CArG sites within the rabbit and rat smMHC promoters positively regulate transcription during transient transfection of cultured smooth muscle (26–29), and CArG boxes within the smMHC (30) and smooth muscle α-actin (17) first introns are also required for smooth muscle-specific transgene expression *in vivo*. Despite the clear cut necessity of SRF binding for full transcriptional activation of smooth muscle-specific genes *in*

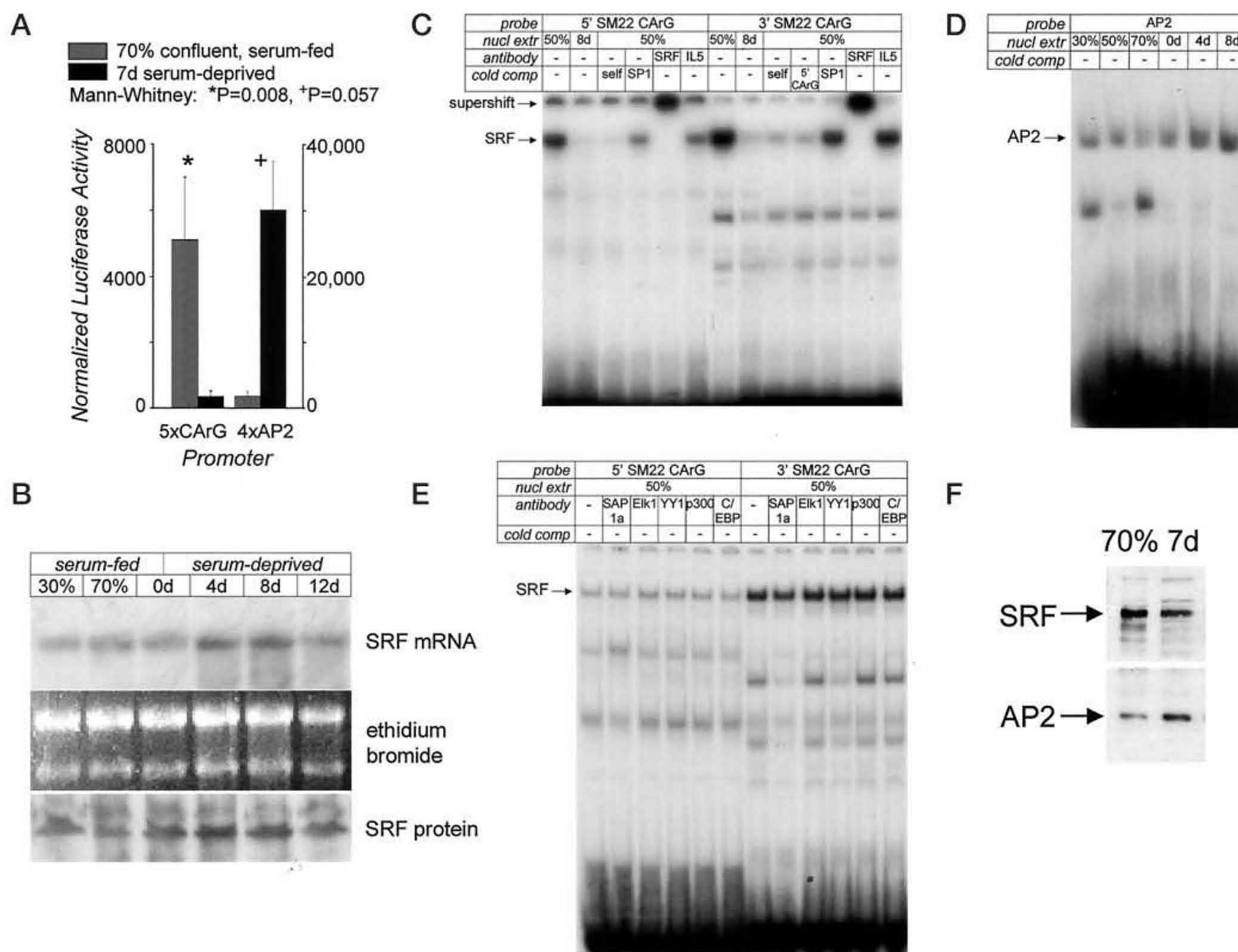

FIG. 3. **SRF-DNA binding and transcription promoting activity, but not mRNA or protein levels, are decreased in long term serum-deprived airway myocytes.** *a,* activity of a purely SRF-dependent artificial promoter (in p5xCArGluc), reflected in its normalized luciferase activity, is much greater in subconfluent serum fed than 7-day-old serum-deprived airway myocytes, whereas that of an AP-2 dependent promoter (in p4xAP2luc) displays greater activity in serum-deprived cells. *b,* Northern and Western blot analyses of total RNA and whole cell extracts prepared from 30 or 70% confluent serum fed and confluent myocytes deprived of serum for 0–12 days, demonstrating that expression of SRF is maintained at high levels in serum-deprived airway myocytes. *c,* EMSA showing that nuclear extracts from subconfluent (50%) airway myocytes contain SRF, which binds prominently to oligonucleotide probes containing the 5′- or 3′-CArG boxes from the murine SM22 promoter, whereas binding activity is markedly diminished in 8-day-old serum-deprived airway myocytes. Specificity of the SRF-containing DNA complex is demonstrated by selective supershift with anti-SRF antibody and by specific competition with CArG-containing unlabeled (cold) competitor oligonucleotides. *d,* EMSA demonstrating that nuclear extracts from subconfluent, serum fed (30–70%) or confluent airway myocytes deprived of serum for 0–8 days contain AP2 that binds to its consensus DNA sequence, without loss of DNA binding activity in serum-deprived myocytes. *e,* EMSA demonstrating that SRF-containing DNA complexes formed with nuclear extracts of 50% confluent serum fed airway myocytes are unaffected by co-incubation with antibodies to SAP-1a, Elk-1, YY1, p300/CBP, or C/EBP. *f,* Western blot demonstrating diminished SRF protein and elevated AP-2 protein within nuclear extracts of 7-day-old serum-deprived airway myocytes.

vitro or *in vivo*, the possibility that smooth muscle cells regulate SRF binding activity as a physiological mechanism to control smooth muscle gene transcription in response to changes in external environment has not been addressed thoroughly. Only one study suggested that increases in SRF binding partially up-regulate vascular smooth muscle gene transcription in angiotensin II-treated myocytes (31). Our results in cultured airway myocytes indicate that smooth muscle cells can employ this strategy more generally and dramatically and thereby extend this prior observation and a report that alterations in SRF abundance regulate smooth muscle gene transcription during embryogenesis (32) by revealing a novel mechanism through which SRF activity is regulated.

Several pathways controlling SRF transcription-promoting activity are already known. First, SRF can partner with other nuclear factors, as it does with Elk-1 or SAP-1 at the serum response element of the c-*fos* promoter to effect its full activation (33–37). However, DNA binding sites for ternary complex factors of the serum-responsive Ets family are not found adjacent to smooth muscle promoter CArG sites (15, 38), and we found no evidence for the presence of such factors in SRF-containing nuclear protein·DNA complexes (Fig. 3*e*). The non-histone chromosomal protein HMG-I(Y) can potentiate SRF binding to the SM22 promoter, even in the absence of direct HMG-I(Y) binding to DNA (39), and MHox can mediate increased SRF binding to the smooth muscle α-actin promoter in angiotensin II-stimulated vascular smooth muscle cells (31). Conceivably, reduction in HMG-I(Y) or MHox activities during long term serum deprivation could contribute to our observations. Second, SRF can interact with p300/CBP, whose histone acetyltransferase activity may regulate the availability of chromosomal DNA for transcription and promote SRF-dependent

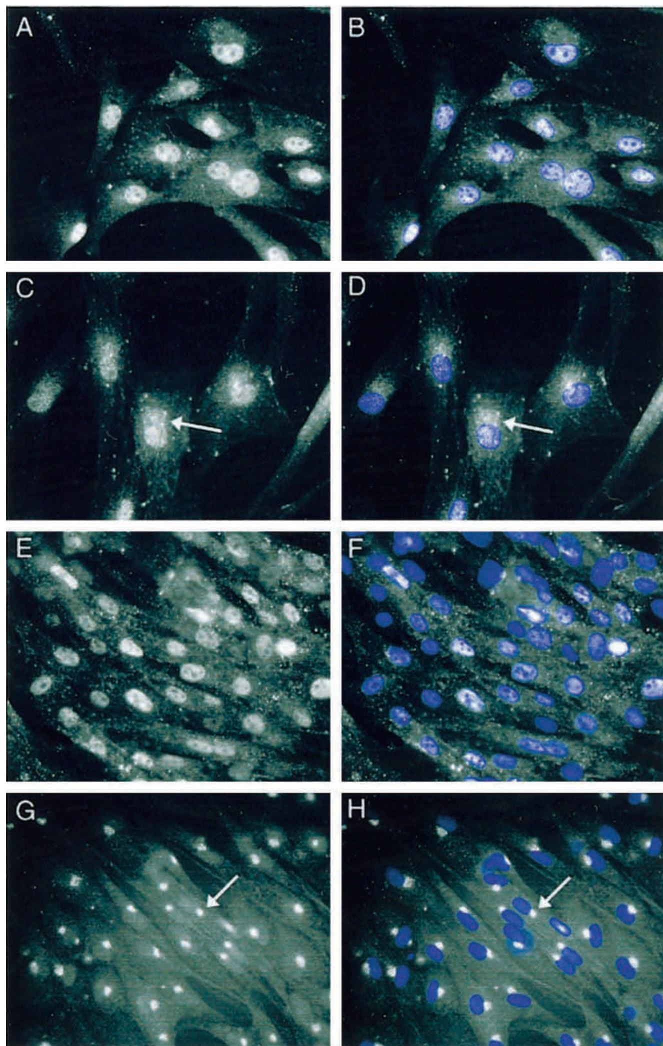

FIG. 4. **Immunocytochemical localization of SRF (fluorescein isothiocyanate fluorescence shown as *white*) within cultured canine tracheal (*A–F*) or pulmonary artery (*G* and *H*) myocytes.** *B*, *D*, *F*, and *H* are identical to *A*, *C*, *E*, and *G*, but include blue nuclear counterstain. SRF appears exclusively within the nucleus in subconfluent, serum fed airway myocytes (*A* and *B*) but is partially redistributed to a perinuclear cloud (*arrows*) in 8-day-old serum-deprived airway myocytes (*C* and *D*). 7-Day-old serum-deprived tracheal myocytes refed with 20% FBS again completely translocate SRF to the nucleus (*E* and *F*). In some serum-deprived cultures, nuclear exclusion of SRF was more complete than shown in *c* and *d*. For example, SRF was localized in a perinuclear cap in 10-day-old serum-deprived canine pulmonary artery myocytes (*G* and *H*). A similar appearance has also been observed in long term serum-deprived tracheal and aortic myocytes (data not shown).

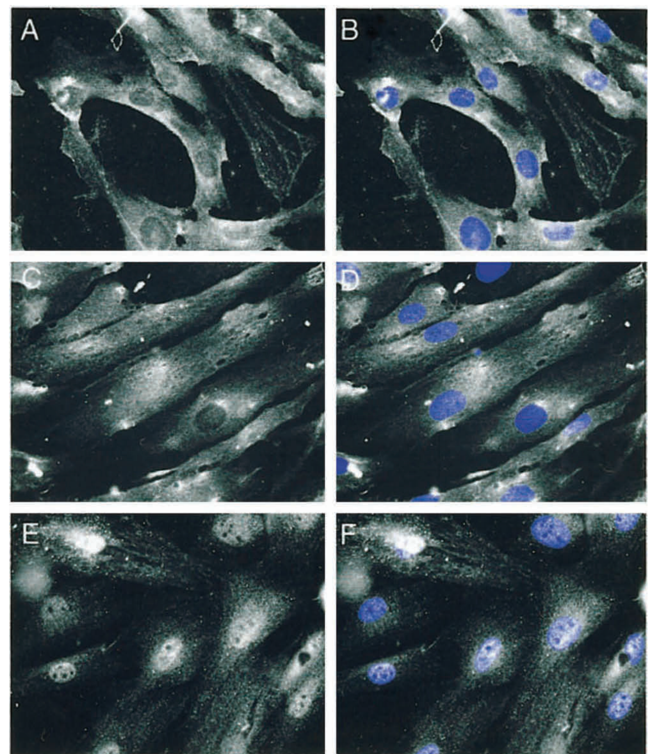

FIG. 5. **Immunocytochemical localization of AP-2α (fluorescein isothiocyanate fluorescence shown as *white*) within cultured canine tracheal myocytes.** *B*, *D*, and *F* are identical to *A*, *C*, and *E* but include blue nuclear counterstain. AP-2 appears primarily within the cytoplasm in subconfluent, serum fed airway myocytes (*A* and *B*) but is heterogenously (*C* and *D*) or more uniformly (*E* and *F*) redistributed to the nucleus in 7-day-old serum-deprived airway myocytes (*A–D*).

transcription (40). No evidence for p300 binding was revealed in our EMSA studies (Fig. 3*e*), however. Third, phosphorylation of SRF by the ribosomal S6 kinase pp90RSK, casein kinase II, or DNA-PK can enhance its transcription-promoting activity and DNA binding (37, 41–49). Whether differential phosphorylation of SRF contributes to the differences in binding activity observed in our subconfluent or long term serum-deprived cells requires further study. Fourth, activation of the Rho family GTPases can enhance the transcription-promoting activity of SRF (50) in skeletal and nonmuscle cells, in part by modulating nuclear factor-κB activity (51–55). RhoA can be activated during serum exposure, so reduction in its activity during prolonged serum deprivation might lower SRF activity and so reduce SM22 and smMHC promoter activation. Co-expression of constitutively active RhoA enhances transcription from the SM22 and smMHC promoters in tracheal myocytes,[3] but the physiological importance of changes in Rho family GTPase activities in our culture system, or in intact tissues *in vivo*, remains to be established. Fifth, SRFΔ5 is a naturally occurring dominant negative isoform of SRF (56), which interacts with full-length SRF and binds DNA, but cannot activate transcription. SRFΔ5 transcripts are more abundant in smooth muscle from proximal rather than distal aorta, and these levels correlate inversely with SM22 and smMHC mRNA levels in these areas. We do not think that increased SRFΔ5 abundance accounts for the diminished SM22 and smMHC promoter activities observed in serum-deprived myocytes. Had abundant SRFΔ5 been present in serum-deprived cells, we would have expected SRF·SRFΔ5 DNA complex formation to occur (56); this was not the case, as our experiments revealed almost complete absence of SRF-containing complex formation in serum-deprived myocytes.

Instead, our results disclose a previously unknown mechanism whereby SRF transcriptional activity is regulated through reversible translocation between cytoplasm and nucleus (Figs. 3*f* and 4). Gauthier-Rouviere *et al.* (23) identified a basic sequence in the N-terminal region of SRF responsible for its nuclear entry. Although basal protein kinase A activity was required for nuclear SRF translocation (23), regulation through this pathway was not specific, as SV40 nuclear localization also requires cAMP-dependent protein kinase activity. Moreover, cAMP-dependent protein kinase regulates extracellular signal-regulated kinase 2 and p38α translocation through control of their association with cytoplasmic protein tyrosine phospha-

[3] H.-W. Liu, B. Camoretti-Mercado, and J. Solway, unpublished observation.

tase, PTP-SL (57). Currently, the potential role of cAMP-dependent protein kinase in mediating the extranuclear relocation of SRF in long term serum-deprived smooth muscle cells, or the contribution of other signaling pathways, remains to be established.

Regulation of SRF transcription-promoting activity could provide a strategy for coordinated up- or down-regulation of expression of a complement of smooth muscle contractile apparatus-associated proteins in response to external environmental or internal cellular cues. Like the SM22 and smMHC promoters, transcription from h-caldesmon, h1-calponin, smooth muscle α-actin, and telokin promoter/enhancers depends on SRF binding for maximal activation (15, 38, 58). Furthermore, physiological regulation of smooth muscle-specific gene transcription through control of SRF activity might provide an alternative explanation for recently published observations in transgenic mice. Madsen *et al.* (30) found expression of an smMHC promoter/enhancer-driven lacZ reporter in transgenic mice in some, but not all, cells within individual smooth muscle tissues. These authors suggested that heterogeneous transgene activation might reflect diverse embryological origins of myocytes comprising these tissues, differences in local milieu, and/or episodic gene expression. Our results support the possibility that local factors might lead individual myocytes within a smooth muscle tissue to activate smooth muscle promoters through the same SRF-dependent transcriptional regulatory program but to differing degrees according to their individual states of SRF transcription-promoting activity. Conceivably, differences in SRF activation might also lead to intertissue differences in transgenic SM22 promoter activity, in which SM22 promoter-driven transgenes were expressed in vascular but not visceral smooth muscle tissues (24, 25, 59).

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