Binding of serum response factor to cystic fibrosis transmembrane conductance regulator CArG-like elements, as a new potential CFTR transcriptional regulation pathway

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ABSTRACT

CFTR expression is tightly controlled by a complex network of ubiquitous and tissue-specific cis-elements and trans-factors. To better understand mechanisms that regulate transcription of CFTR, we examined transcription factors that specifically bind a CFTR CArG-like motif we have previously shown to modulate CFTR expression. Gel mobility shift assays and chromatin immunoprecipitation analyses demonstrated the CFTR CArG-like motif binds serum response factor both in vitro and in vivo. Transient co-transfections with various SRF expression vector, including dominant-negative forms and small interfering RNA, demonstrated that SRF significantly increases CFTR transcriptional activity in bronchial epithelial cells. Mutagenesis studies suggested that in addition to SRF other cofactors, such as Yin Yang 1 (YY1) previously shown to bind the CFTR promoter, are potentially involved in the CFTR regulation. Here, we show that functional interplay between SRF and YY1 might provide interesting perspectives to further characterize the underlying molecular mechanism of the basal CFTR transcriptional activity. Furthermore, the identification of multiple CArG binding sites in highly conserved CFTR untranslated regions, which form specific SRF complexes, provides direct evidence for a considerable role of SRF in the CFTR transcriptional regulation into specialized epithelial lung cells.

INTRODUCTION

Expression of the cystic fibrosis transmembrane conductance regulator (CFTR) gene occurs in a subset of specialized cells of epithelial origin (1–3) and is tightly regulated both temporally and spatially (4,5). However, no clear mechanism responsible for this regulation has yet been reported in part due to the complexity of the non-coding regions structure of the CFTR gene. Despite the absence of a TATA element, the transcription of the CFTR gene may be initiated through several discrete start sites (6,7), including those that are tissue-specific (8). An intact CCAAT consensus is also required for accurate transcript initiation (9). Both basal and cAMP-mediated regulation of CFTR transcription involve a weak cAMP response element (CRE) nucleotide consensus (10,11) in tandem with a consensus inverted CCAAT element or Y box (9). In addition, CFTR transcription may be modulated by additional overlapping cis-acting elements, including a polymorphic YY1 site, located in the human minimal CFTR promoter (12). CFTR promoter activity may be enhanced by exogenous transfected NF-kB via its binding to the −1103 kB element (13). CFTR expression is also regulated through interactions with factors involved in remodeling of chromatin structure, such as CDP/cut and hGCN5 (14).

Because none of these cis-acting elements confers tissue-specific control of CFTR expression, several studies focused on identifying potential regulatory elements that lie mainly outside the coding region. Extensive analyses of the chromatin structure identified multiple clusters of DNase I-hypersensitive sites (DHSs) (15,16), some of which contain tissue-specific enhancer elements (17,18). Multiple binding sites for a tissue-specific transcription factor, called

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors

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HNF1α, recently identified in various DHS core, modulate likewise CFTR expression (19). Despite extensive studies, the transcriptional regulatory networks of CFTR expression remain to be unraveled.

Our current model for the regulation of human CFTR expression proposes that multiple transcription factors interact with previously reported polymorphic and composite cis-acting elements encompassing a CArG-like motif (12,20). The CArG box (CC(A/T)6GG), originally defined as the core component of the serum response element (SRE), is found in the 5′ region of immediate early response genes, such as c-fos (21), and in many muscle-specific gene promoters (22). This DNA element binds serum response factor (SRF), highly regulated and interactive transcription factor, which is phylogenetically conserved and belongs to the MADS box family of proteins, with MCM1, Agamous, Deficiens and SRF (23). Although SRF is particularly enriched in skeletal, cardiac and smooth muscle lineages (24,25), it is also expressed in a variety of human cell lines from distinct tissue origins (26), and, to a lesser extent, in ectoderm-derived tissue such as gastric epithelium (27). Interestingly, it has been shown that Drosophila SRF homolog is expressed in a subset of tracheal cells and regulates cytoplasmic outgrowth during terminal branching of the tracheal system (28). Hence, it has been suggested that SRF plays an essential role in the formation of the respiratory system. Moreover, it has been recently shown that SRF is involved in myofibroblast differentiation during lung damage (29). Therefore, we hypothesized that trans-acting factors interacting with CArG-like motif, such as SRF, could be important in CFTR expression regulation. We provided evidence that SRF is expressed in bronchial epithelial cell expressing endogenous CFTR protein. Through the use of combined gel mobility shift–chromatin immunoprecipitation (ChIP) assays, we first demonstrated that SRF protein binds, both in vitro and in vivo, the CFTR minimal promoter region spanning the CArG-like motif. Mutagenic studies and transient co-transfections experiments showed that SRF alone is not sufficient to transactivate the CFTR gene. Upon some mutations of the CFTR-CArG-like element, promoter activity is severely decreased, potentially implying YY1 as a co-repressor. Given the YY1 well-defined role in the transcriptional regulation of some SRE-dependent promoters (30–33), we explored the putative role of YY1 on the CFTR promoter activity and demonstrated functional interplay between this factor and SRF. Finally, by stringent in silico structural analysis, we identified additional multiple near consensus CArG sites within the highly conserved promoter and intronic regions of the CFTR gene. We also documented the existence of SRF-containing protein complexes on numerous predicted CFTR CArG-like elements. Taken together, the data obtained classify CFTR as a novel SRF target gene, subject to modest but significant SRF activation, and open the gate to further investigate how SRF may be involved in the regulation of CFTR gene expression.

MATERIALS AND METHODS

Cell culture

Human bronchial epithelial cells, Beas2B and A549 expressing endogenous CFTR were kindly provided by Dr Marc Chanson, Department of Pediatrics, Laboratory of Clinical Investigation, Switzerland. Beas2B and A549 cells were maintained in high glucose DMEM (4500 mg/l D-Glucose, Invitrogen Corporation) supplemented with 10% fetal bovine serum (FBS) (Eurobio, France) and 5 mM L-glutamine. C2C12 myoblasts were grown in DMEM supplemented with 10% FBS and 5 mM L-glutamine. All cell cultures, supplemented with 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin, were maintained at 37°C under 5% CO₂. As positive or negative controls for the different assays, other cells used in this study included colon adenocarcinoma Caco-2 cell line (ECACC) with functional CFTR (34,35) and normal monkey kidney fibroblast-like COS-7 cell line (ATCC) which does not express CFTR (36,37); all were maintained in DMEM supplemented with FBS and were incubated in the same conditions as above.

Plasmids constructs

The original luciferase expression vector pGL3-Basic containing the wild-type human CFTR minimal promoter was described previously (12). To further study the putative composite cis-acting element located at −108 from the major transcription initiation site of the human CFTR promoter (38), the CFTR-CArG-like motif was either mutated in a consensus CArG box or in a degenerated motif. Mutations were introduced into the WT-pGL3 plasmid using an oligonucleotide-directed mutagenesis system (QuickChange™Site-Directed Mutagenesis Kit, Stratagene) according to the manufacturer’s instructions. Mutagenesis primers are depicted in Table 1. The presence of mutations and sequence fidelity were verified by dideoxynucleotide sequencing. For each introduced mutation the loss or the improvement of transcription factor binding activity was checked by in vitro assays. The 3xSRE-fos TATA-luciferase reporter construct was kindly provided by R. A. Hipskind, Institut de Genetique Moleculaire, Montpellier, France. Expression vectors encoding either human wild-type SRF, the pHiv-SRF or SRF dominant-negative form deleted of an essential part of DNA-binding domain (DBD), the pHiv-DN, were generously given by D. Trouche, Laboratoire de Biologie Moleculaire Eucaryote, Toulouse, France. Another dominant-negative SRF mutant, pHiv-DBD, was constructed by PCR amplification of SRF fragment spanning the DBD flanked by HindIII and HincII sites to facilitate cloning into expression vector. The PCR was performed using pHiv-DBD-SRF primers (listed in Table 1), 10 ng of SRF expression vector, 200 μM of each dNTP, 0.5 μM of each primer and 1.5 U Taq DNA polymerase (Perkin Elmer, Inc.) in a 1× reaction buffer provided by the manufacturer, TranSilent human control siRNA vector and TranSilent™ human SRF siRNA vector (catalog no. SR1038) were purchased from Panomics. Expression vectors encoding either pCMV-YY1 or pcDNA3-YY1 were generously provided by E. Seto, E. Bonnefoy and A. Moustakas, respectively.

Transient transfections

Cells were seeded at a density of 200,000 cells/2 ml of medium and plated in 6-well dishes (NUNCclone®, Merck-Eurolab, Inc.). After a culturing period of 24 h, cells were transfected with PolyFect® transfection reagent (Qiagen Corporation,
confirm the specificity and level of SRF protein knockdown. Aliquots of cell lysates were used for western blotting and luciferase activity was determined as described above. For co-transfection assays, unless otherwise indicated, 1.8 g of each expression vector and 0.2 g of siRNA. The purity and concentration of protein samples were estimated by SDS–PAGE. To increase CFTR (39) and scraped into lysis buffer (50 mM sodium phenylbutyrate, which has been previously reported on the classical Bradford method. The pGEX-SRF plasmid, a generous gift from D. Trouche (Laboratoire de Biologie Moléculaire Eucaryote), was used for protein expression. Preparation of whole cell proteins, nuclear extracts and recombinant protein production

For the CFTR immunodetection analyses, Beas2B, A549, C2C12, COS-7 and Caco-2 cells were treated with 5 mM sodium phenylbutyrate, which has been previously reported to increase CFTR (39) and scraped into lysis buffer (50 mM Tris–HCl, 1% NP-40, 40 mM beta-glycerophosphate and 120 mM NaCl) supplemented with protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, and 1 µg/ml aprotinin, leupeptin and pepstatin]. Nuclear extracts were prepared using the Nuclear extraction kit according to the procedure recommended by the supplier (Panomics, Ozyme, France). Protein concentrations were determined using the Bio-Rad Protein Assay kit with BSA as a standard, based on the classical Bradford method. The pGEX-SRF plasmid, a generous gift from D. Trouche (Laboratoire de Biologie Moléculaire Eucaryote), was used for protein expression. GST–SRF fusion protein was purified from Escherichia coli as described previously (40). The purity and concentration of protein samples were estimated by SDS–PAGE.

Electrophoretic mobility shift assay (EMSA) and supershifts

Electrophoretic mobility shift assay (EMSA) and supershifts

| Primer sequence | Annealing temperature |
|-----------------|-----------------------|
| TGT TAT GAC CAG TCA ACA GGG | 52°C |
| TCA CCA AGG AAA GCA AAG TCT G | 52°C |
| CTA CCA GGT GTC GGA GTC TGA | 52°C |
| CCA GAT GAT GCT GTC AGG AAC A | 57°C |
| TTT CCT GGT GAT CCC TCC TT | 59°C |
| TCC ACG AAC CGC CAA CAA CT | 59°C |
| GCA CTC CTC ATG GCG CTA A | 59°C |
| TTT AGG ACA GGA GTC GTA CAA A | 59°C |
| AAG CTT ATG ACC GGG GCC AAG CCG G | 65°C |
| GTC AAC TCA GAA CGC CCG TTT AT | 64°C |
| ATG CTG CAC TGT GCC GGC AA | 70°C |
| TGC GGC CCG CTG GGT TTT AT | 70°C |
| GTC CGG TGG GTT CCC TTT G | 70°C |
| GAG CGC CGG CTT CAG TGT G | 70°C |
| GAA AGC CGC TAG ACC AAA TTT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |
| GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA | 54°C |

*Bold and italicized type indicates mutated nucleotides.

France) according to the manufacturer’s recommendations. For transient transfection, we used 1.8 µg of plasmid reporter and 0.2 µg of internal control pRL-SV40 containing Renilla luciferase (Promega Corporation). The pGL3-Basic vector lacking both eukaryotic promoter and enhancer sequences was used as a negative control. After 48 h, the luciferase activity was evaluated with the Dual Luciferase Reporter Assay System (Promega Corporation), as described previously (12). Luminescence measurements were performed on a Luminoskan Ascent luminometer (ThermoLabsystem Corporation, France). Firefly luciferase activity was normalized to Renilla luciferase activity. All luciferase activities represent at least three independent experiments with each construct tested in triplicate per experiment. Luciferase activity data are expressed as the means ± SD computed from the results obtained from each set of transfection experiments. To minimize the possibility of errors in DNA amplification, at least two independently constructed clones were tested. For co-transfection assays, unless otherwise indicated, 1.8 µg of luciferase reporter and 1–2 µg of each expression vector were used. Transfection with siRNA was performed using Lipofectamine 2000 (Invitrogen) with 2.5 µg of siRNA. The cells were transfected with CFTR promoter plasmid reporter, and luciferase activity was determined as described above. Aliquots of cell lysates were used for western blotting to confirm the specificity and level of SRF protein knockdown.

Table 1. Sequences of oligonucleotides

| RT–PCR primers (5′→3′)                      | Primer sequence | Annealing temperature |
|---------------------------------------------|-----------------|-----------------------|
| Hpt human forward                           | TGT TAT GAC CAG TCA ACA GGG | 52°C |
| Hpt human reverse                           | TCA CCA AGG AAA GCA AAG TCT G | 52°C |
| SRF forward                                 | CTA CCA GGT GTC GGA GTC TGA | 52°C |
| SRF reverse                                 | CCA GAT GAT GCT GTC AGG AAC A | 57°C |
| CFTR external primers forward 729           | TTT CCT GGT GAT CCC TCC TT | 59°C |
| CFTR external primers reverse 1487          | TCC ACG AAC CGC CAA CAA CT | 59°C |
| CFTR external primers forward 757           | GCA CTC CTC ATG GCG CTA A | 59°C |
| CFTR external primers reverse 1436          | TTT AGG ACA GGA GTC GTA CAA A | 59°C |

Expression vector construct primers (5′→3′)

- pHiv-DBD-SRF forward: AAG CTT ATG ACC GGG GCC AAG CCG G
- pHiv-DBD-SRF reverse: GTC AAC TCA GAA CGC CCG TTT AT

Chromatin immunoprecipitation primers (5′→3′)

- Negative control: GCT TAC CAA GCT GTG ATT CC
- β-Globin forward: AAG CAA TAG ATG GCT CTG CC
- Positive control: ATG CTG CAC TGT GCC GGC AA
- β-Actin forward: GTC GGC CCG CTG GGT TTT AT
- β-Actin reverse: TGC CGG TGG GTT CCC TTT G

Target sequence CFTR minimal promoter

- CFTR-CaR-like forward: GCC TCG AGG CTG GGA GTC ACA GTG G
- CFTR-CaR-like reverse: TTC CAT GGT CTC TCG GGC GGT GGT

Target sequence: CaR located at 2373 bp of the CFTR promoter

- CaR forward: GTT AAA GCC CTG ATG AAT GC
- CaR reverse: CAC ACA TGT ACA TAG GAA GA

Site-directed mutagenesis primers (5′→3′)

- C2: GAA AGC CGC TAG ACC AAA TTT GGG GCC GCA AA
- C4: GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA
- C5: GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA
- D1: GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA
- D2: GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA
- D3: GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA
- D4: GAA AGC CGC TAG ACC AAA TAT GGG GCC GCA AA

*Bold and italicized type indicates mutated nucleotides.

Electrophoretic mobility shift assay (EMSA) and supershifts

Oligonucleotides probes were synthesized corresponding to CFTR CaR-like element and mutant versions in which the
Table 2. Oligonucleotides used in EMSA analysis

| Oligonucleotides (5′−3′) | SREa | GATAb | WTc | Ci,d | C2e | C3e | C4 | C5e | D1f | D2f | D3f | D4f | CarG1 | CarG2 | CarG3 | CarG4 | CarG5 | CarG6 | CarG7 | CarG8 | CarG9 | CarG10 |
|-------------------------|------|-------|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|                         | GG ATG TCC ATA TTA GGA CAT CT | TGG TCA TAA TAA TGG CCA ATT TGG AAT GAC CAACAA | TTT ATT TCC ACC AAA ACA ATT TGG GCT TGT AAT GAT TTA | TTA ACT GAG CTT GGC CCA CAT ATG GTG TAA TCA GAT GCT | ATT TCT ATT TGG TCA TAT ATT GGC AAG AGG AGG TG | TGG AAG TCA ACT GAC CCA CAT TTA TGG GAA AAG TGA TCA GTG | ACC TGG GGG GAA GGT TTC ACC | AAA GGC TGG ACC CGT TCA ACC | ATT CCT ATT TGG CCA ATT TGG GCT TGT AAT GAT TTA | ATT GAG GAA GGA ACC CAA TGG GCA TGG ACC | CCA AAT GAG ACT TCA CGA AAT TGG CCT CTT GGG GCT |
| Bold and italicized type | indicates mutated nucleotides. | The CarG boxes in the CFTR minimal promoter are shaded. The underlined nucleotides indicate the in silico predicted CarG elements in the non-coding regions of the CFTR gene. | a-c-fos consensus SRE (56). | bOligonucleotide for competition assay, the GATA and E-box sequences are boxed (85). | cCarG-like box located in the human minimal CFTR promoter region. | dCarG-like box located in the human minimal CFTR promoter region containing the naturally occurring variant [−102ΔT-A1] (12). | eConsensus CarG box with minimal CFTR promoter flanking sequences. | fMutated CarG box with minimal CFTR promoter flanking sequences. |

CARG motif was either mutated in a consensus box or disrupted. The oligonucleotide sequences were listed in Table 2. Single-stranded complementary oligonucleotides containing the studied sequences were annealed, end-labeled with [γ-32P]ATP (5000 Ci/mmole) and purified as described previously (12). Mobility assay conditions were specifically optimized for binding of SRF. The binding reaction was carried out in a total volume of 20 μl containing 2 ng (~30,000 c.p.m.) of the labeled probe, 10 μl of binding buffer (2X), 0.25 μg poly(dI−dC) (Amersham Pharmacia) used as a non-specific competitor, 5−12 μl of nuclear extracts and 100−1000 ng of GST−SRF fusion chimera protein. For EMSA competition and antibody interference assays, proteins were incubated with either cold specific competitors (WT and SRE oligonucleotides, see Table 2), unspecific competitor (GATA oligonucleotide, see Table 2) or purified antibodies (anti-SRF: sc-335X; anti-YY1: sc-1703X or anti-HA, Santa Cruz, Biotechnology Inc., TEBU, France) for 20 min before addition of labeled probes. After incubation of 30 min at room temperature, complexes were resolved on 5% acrylamide: bisacrylamide (29:1) native gel containing 0.5x TBE at 26 mA for 1 h.

Formaldehyde cross-linking and chromatin immunoprecipitation

Beas2B and A549 cell proteins were cross-linked to DNA by adding formaldehyde to a final concentration of 1% for 10 min at 37°C. The fixed cells were prepared for immunoprecipitation using the protocol of ChIP assay kit (Upstate Biotechnology Inc., Euromedex, France) with minor modifications. Cells were resuspended in 1 ml cold phosphate-buffered saline (PBS), pelleted by centrifugation and lysed in 800 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1) containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml pepstatinA). After incubation on ice for 10 min, the cross-linked chromatin was sheared by sonication at the following conditions eight times for 10 s at 40% of maximum power and diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1 and 167 mM NaCl) with protease inhibitors. Aliquots containing 20 μl of each aliquot were used as a control to show total input DNA. The chromatin solution was pre-cleared with 80 μl of salmon sperm DNA/protein A agarose-50% slurry (Upstate Biotechnology Inc.) for 1 h at 4°C under agitation, and subsequently incubated at 4°C on a rotating stand overnight with either 3 μl of anti-SRF antibody (sc-335X, Santa Cruz, Biotechnology Inc.) or an irrelevant antibody (anti-HA). Immune complexes were precipitated by the addition of 60 μl of salmon sperm DNA/protein A agarose and incubated at 4°C for 1 h and low speed spinning. Precipitates were washed once with 1 ml of low salt buffer, once with high salt buffer, once with LiCl buffer and twice with 1X TE, according to the manufacturer’s recommendations (Upstate Biotechnology Inc.). The cross-links were reversed by the addition of 20 μl of 5 M NaCl and incubation at 65°C overnight. The samples were treated with proteinase K, phenol−chloroform extracted, ethanol precipitated and resuspended in 30 μl of sterile water. PCR analysis was carried out using primers from different regions of the human CFTR promoter and as additional controls from promoter regions of a number of genes that are either silent (β-globin) or having at least one functional CarG element (β-actin). The sequences of PCR primers are listed in Table 1. After 45 cycles of amplification, PCR products were separated on 1.8% agarose gels and visualized by ethidium bromide staining and UV transillumination.

Reverse transcriptase–PCR

Total RNA from epithelial cells (Beas2B and A549), myo-blasts (C2C12) and fibroblasts (COS-7) was isolated using the SV Total RNA Isolation System® (Promega, France). Reverse transcription was performed with 3 μg of total RNA, 300 ng of random hexamers (Invitrogen Corporation), 10 mM dNTPs, 1 μl of RNasin RNase inhibitor (Promega), 2 μl of DTT at 0.1 M, 4 μl of first strand buffer (Invitrogen), 200 U of M-MLV RT (Invitrogen) and nuclease-free water (Promega) in a final reaction volume of 20 μl. For each RNA template, a control reaction without RT was included. For each RT reaction condition, an H2O no-template reaction was included as an additional negative control. The reaction mixture was incubated first for 10 min at 25°C, then 40 min at 42°C; this was followed by a heat inactivation step of 72°C for 3 min. Aliquots containing 1 μl of the cDNA synthesis reaction mixture were used for PCR analyses. The expression level of SRF mRNA was analyzed by PCR amplification of the endogenous Hypoxanthine Phosphoribosyl-Transferase (HPRT) gene used as internal control. Amplification mixture
performed with an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 30 s of 95°C denaturation, 60 s of 52°C annealing and 2 min extension at 72°C. Amplification of CFTR was performed as described previously (41). The RT–PCR products were separated on an 8% non-denaturing polyacrylamide gel, stained with ethidium bromide. To ensure that the sequence of cDNA products derived from both SRF and CFTR RT–PCR analyses correspond to respective published sequences, the cDNA bands were purified and prepared for dideoxy DNA sequencing. Controls included RNA preparations from monkey kidney fibroblasts (COS-7), which are negative for CFTR expression (36,37).

Western blot analysis
Twenty-five microgram protein extracts (except where otherwise indicated) were resolved on a 10% (except where otherwise indicated) SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane (except where otherwise indicated). All membranes were Ponceau S stained to ensure sample integrity. Briefly, the membranes were blocked with 5% skim milk in PBS supplemented with 0.01% Tween-20 and incubated with diluted primary antibodies in 5% skim milk (1:5000 diluted anti-SRF Abs: sc-335X and sc-25290X purchased from Santa Cruz; 1:2500 diluted anti-β-actin antibody: AC-15 from Sigma, 1:2500 diluted anti-β-tubulin antibody: T6074 from Sigma) overnight. The membranes were then washed and incubated with an appropriate horseradish peroxidase (HRP) conjugated secondary antibody at 1:15 000 in PBS-5% milk. The membranes were reacted with chemiluminescence reagent ECL (Roche Diagnostic) as described by the manufacturer and subsequently exposed to Biomax photographic film (Kodak Corporation). The protein levels of the actin housekeeping gene (except where otherwise stated) were assayed for internal control of protein loading.

Indirect immunofluorescence
A549, Beas2B, C2C12 and COS-7 cells were plated on Lab-Tek™ II CC2™ Chamber Slide™ systems (Nalge Nunc) 1 day before immunohistochemical analysis. The cells were fixed and permeabilized in 4% paraformaldehyde and 0.1% Triton X-100, respectively, for 20 min durations at room temperature. Non-specific binding sites were blocked with 1% BSA in PBS for 1 h. The cells were subsequently incubated with primary antibodies diluted in 1:100 in PBS–BSA. Antibodies used included, the clone L12B4 (Upstate biotechnology), an anti-CFTR antibody known to recognize human, rat, mouse and bovine CFTRs (42) and the H-300 or the H-300 anti-CFTR antibody known to recognize human, cynomolgus, gibbon, squirrel monkey, rabbit, pig, sheep and cow sequences were aligned using the ClustalW global alignment tool (http://clustalw.genome.ad.jp/) (45). The gap open and extension penalties were set at 15 and 6.66, respectively, and sequence delay divergence of 30% with a DNA transition weight of 0.5 was used.

RESULTS

CFTR minimal promoter region encompassing the CFTR-CArG-like element is remarkably conserved
To search the cis-acting elements involved in the CFTR gene expression regulation, we previously performed an in silico search for transcription factor binding sites (46), and reported that the human minimal CFTR promoter contains a CArG-like motif, which is localized ~108 bp upstream of the major transcription initiation start site (38). Because so far all described CArG-box elements were found in the regulatory regions of immediate early genes (47) and in muscle-specific genes (22), we confirmed our first binding site prediction by using two other algorithms, AliBaba (http://www.alibaba2.com/) and ConSite (http://clus talw.genome.ad.jp/). As shown in Figure 1A, the CFTR-CArG-like element sequence
(GC(A/T)₆GG) diverges slightly at the 5' from the published SRF consensus sequence (CC(A/T)₆GG) (22,37). However, this divergence is similar to that observed in the smooth muscle-gamma-actin promoter which has been demonstrated to bind SRF (48).

To assess the putative functional importance of CFTR-CArG-like binding site, we used an orthogonal approach of deducing regulatory elements by considering orthologous CFTR minimal promoter regions from several mammalian species representing the Primates, Artiodactyla and
Lagomorpha orders. Comparison of human/species homologies was carried out using ClustalW multiple CPTFR sequence alignments according to the numerous alignment parameters described previously (49). Consistent with previous studies (50), we showed that the human minimal CPTFR promoter is remarkably conserved across the orders studied (Figure 1B, right panel). We found that the CPTFR-CarG-like element is conserved within all the Primates species studied and also within the pig while, within the sheep, cow and rabbit species, some substitutions occur in the middle of the CarG-like motif (Figure 1B). These data are consistent with previous CPTFR promoter phylogenetic work that identified two types of regulatory elements: some are conserved between species, such as a non-consensus CRE at positions –0.1 kb relative to TOFR and some are species-specific elements, such as a 300 bp purine-pyrimidine (Pu.Py) stretch that is present only in rodents (50).

**Bronchial epithelial expression of SRF**

Because we could not previously identify SRF binding to the CPTFR CarG-like element, even though an anti-SRF antibody had been used (12), we asked whether SRF, mainly described as highly expressed in muscle cells, was also present in epithelial cell lines. To measure the expression levels of SRF mRNAs, we used a RT–PCR-based procedure previously described by Davis et al. (52). As reverse-transcribed control, the Hypoxanthine Phosphoribosyl-Transferase (HPRT) housekeeping gene was amplified. Amplification of SRF from bronchial epithelial cells and myoblasts yielded a fragment of the same size, 651 bp (Figure 2A), as that of the full-length SRF transcript produced by RT–PCR from human hearts (52). The 453 bp band corresponding to SRF lacking exon 5 (SRF-Δ5) was only detected in the C2C12 myoblast line (Figure 2A). To ensure that these bands were not the result of a PCR artifact or genomic contamination of samples, negative controls such as exclusion of RT or RNA during cDNA synthesis were included in each assay. As expected, no PCR product appeared when either RT or the template was omitted (Figure 2A). Densitometric analysis revealed that bronchial epithelial cells contained an appreciable amount of SRF mRNA. Nuclear extracts from A549, Beas2B and C2C12 cells were electrophoresed and then assayed by western blotting with anti-SRF antibody reported to recognize all four isoforms of the protein (52). As shown in Figure 2B, we found that robust expression of a 67 kDa band, that based on molecular mass, corresponds to full-length SRF (SRF-FL) in all of the cell lines examined. Consistent with RT–PCR analysis, a 57 kDa band corresponding to SRF-Δ5 was only detected in the C2C12 myoblast line. As a control, we checked the β-actin levels (Figure 2B). Densitometric analysis of western blot revealed no apparent difference in the expression level of SRF proteins between the myoblasts and epithelial cells (Figure 2B). In subsequent western blot analyses in which parallel blots were probed with only the secondary antibody, no band was detected (data not shown), thus confirming that these bands are indeed specific for SRF. As further evidence, A549, Beas2B and C2C12 cells express relatively large amounts of SRF, the cell lines were assessed by indirect immunofluorescence labeling. A similar staining pattern was noted in the bronchial epithelial cells and myoblasts.  

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Bronchial epithelial expression of SRF. (A) Detection of SRF and HPRT transcripts from both bronchial epithelial and myoblast cell lines by RT–PCR analysis. Total RNAs isolated from A549, Beas2B and C2C12 cells, as indicated, were reverse transcribed with random hexamers and PCR amplified with SRF or HPRT primers. Amplified products were directly stained with bromide ethidium on non-denaturing polyacrylamide gel. Expected size of PCR products is given at left of each transcript. Negative controls are noted as follow: No RT for the absence of reverse transcriptase and No RNA for an H2O no-template reaction. Densitometric analysis of SRF bands intensity was performed with relative HPRT mRNA expression in C2C12 myoblasts. (B) Western blot analysis of nuclear extracts prepared from both epithelial and myoblast lines. Each extract (25 μg) was separated by SDS/PAGE (10%) and western blotted using an anti-SRF Ab. Relative mass, in kilodaltons, is indicated alongside. As a control for proteins loading in SDS–PAGE, an anti-β-actin Ab was used in western blot. Numbers below the β-actin panel indicate densitometric values of the total SRF bands normalized over the corresponding β-actin bands and expressed relative to the C2C12 controls bands, which are set to 1. (C) The A549, Beas2B and C2C12 cells (rows A, B and C, respectively) were grown on coverslips, fixed and subjected to indirect immunofluorescence with anti-SRF Ab. Left panel, cells were stained with FITC-conjugated secondary antibody (green). Middle panel, cells were counterstained with DAPI (blue) for nuclear staining. Right panel, shown is merge of SRF immunostaining and DAPI. Row D, background immunofluorescence obtained in the absence of the primary antibody.
incubated the 32P-labelled WT oligonucleotide corresponding to SRF and performed two series of bandshift experiments. First, we used a specific antibody against the SRF protein. Although the SRF protein contributes to the formation of nucleoprotein complexes (as indicated in Table 2), the absence of the SRF protein (Figure 3A, lane 7) or in the presence of an irrelevant antibody (Figure 3A, lane 8). These results demonstrate that the CArG-like DNA-binding site is a bona fide SRF-binding site. Second, we performed additional EMSA with the WT oligonucleotide, as main binding target, and nuclear extracts from both epithelial and muscle cells (Figure 3B). While three nucleoprotein complexes were formed from nuclear extracts of Beas2B and C2C12 cells on the WT probe, five complexes were detected from A549 cells. Each nuclear protein complex was designated by an upper case relative to nuclear lysate followed by a Roman numeral indicating its position into the gels. The binding specificity of the different complexes was examined by competition analyses with both specific and non-specific unlabeled oligonucleotides (Figure 3B, lanes 2–4). Although a few non-specific binding activities were observed, complexes BII, BIII, AIII, AV, CI, CII and CIII were efficiently competed with an excess of both WT and c-fos SRE unlabeled specific competitors indicating binding specificity (Figure 3B, lanes 2 and 3, respectively). Control experiments including an irrelevant oligonucleotide did not reveal any change in electrophoretic profiles (Figure 3B, lanes 4). To evaluate whether the SRF protein contributes to the formation of nucleoprotein complexes interacting with the CFTR-derived SRF site, we used a specific antibody against the SRF protein. Although the incubation with the anti-SRF antibody apparently failed to give any discrete supershift from various sources of nuclear proteins, a significant decrease of specific complexes was observed (Figure 3B, lanes 6). This effect seemed to be specific because none of these nuclear protein complexes was supershifted or abolished when a non-specific antibody was used (Figure 3B, lanes 7). Control experiments containing only the anti-SRF antiserum and the radiolabeled WT oligonucleotide did not reveal any binding activities (Figure 3B, lanes 8). In addition, supershift assays performed with the radiolabeled c-fos SRE oligonucleotide instead of the WT probe showed that bands corresponding to SRE–SRF complex disappeared and more slowly migrating bands appeared (Figure 3B, lane 10). The absence of supershift for specific WT binding complexes (Figure 3D, lanes 6) may be explained if the epitope recognized by the anti-SRF antibody is part of the SRE-binding site. Antibody binding would compete DNA binding, resulting in a significant decrease or total disappearance of CArG-like complexes rather than in supershifted complexes. Alternatively, if the interaction between the CArG-like DNA-binding site and the SRF protein is insufficiently strong, it could be not as stable as the one observed with the SRE probe (Figure 3B, lane 10) to be detected as a supershift in gel retardation assays. On the one hand, other DNA-binding proteins that are potentially part of this complex could interfere with the antibody’s access to SRF protein. Taken together, these EMSA studies establish SRF-binding CArG-like box within the minimal promoter of the human CFTR gene.

**SRF binds the CArG-like element**

Because the CArG box is not only found in the regulatory regions of many muscle-specific genes but also located at the center of other SREs (54) and forms the core binding site for SRF (55), we have attempted to determine whether or not SRF binds to the CArG-like element sequence. We performed two series of bandshift experiments. First, we incubated the 32P-labelled WT oligonucleotide corresponding to CArG-like element sequence (as indicated in Table 2) with bacterially expressed SRF. As shown in Figure 3A (lane 3), EMSA revealed a recombinant SRF–WT duplex. As shown in Figure 3A (lane 5), bands defined as the CArG-like-SRF complex disappeared when 100-fold molar excess of specific competitor containing the consensus SRE motif, derived from human c-fos promoter (56), was added. In contrast, competition by an irrelevant oligonucleotide (noted UC) did not appear to significantly alter the complex suggesting specificity of binding (lane 6). To confirm precisely the identity of protein binding in the minimal promoter (from –121 to –83 bp), bacterially expressed SRF was preincubated with anti-SRF antibody before incubation with 32P-labelled probe. As shown in Figure 3A (lane 4), bands corresponding to CArG-like-SRF complex disappeared, and more slowly migrating bands appeared when anti-SRF antibody was added. To verify the antibody specificity, control experiments were performed either in the absence of the SRF protein (Figure 3A, lane 7) or in the presence of a non-specific antibody (Figure 3A, lane 8). These results demonstrate that the CArG-like element is a bona fide SRF-binding site. Second, we performed additional EMSA with the WT oligonucleotide, as main binding target, and nuclear extracts from both epithelial and muscle cells (Figure 3B). While three nucleoprotein complexes were formed from nuclear extracts of Beas2B and C2C12 cells on the WT probe, five complexes were detected from A549 cells. Each nuclear protein complex was designated by an upper case relative to nuclear lysate followed by a Roman numeral indicating its position into the gels. The binding specificity of the different complexes was examined by competition analyses with both specific and non-specific unlabeled oligonucleotides (Figure 3B, lanes 2–4). Although a few non-specific binding activities were observed, complexes BII, BIII, AIII, AV, CI, CII and CIII were efficiently competed with an excess of both WT and c-fos SRE unlabeled specific competitors indicating binding specificity (Figure 3B, lanes 2 and 3, respectively). Control experiments including an irrelevant oligonucleotide did not reveal any change in electrophoretic profiles (Figure 3B, lanes 4). To evaluate whether the SRF protein contributes to the formation of nucleoprotein complexes interacting with the CFTR-derived SRF site, we used a specific antibody against the SRF protein. Although the incubation with the anti-SRF antibody apparently failed to give any discrete supershift from various sources of nuclear proteins, a significant decrease of specific complexes was observed (Figure 3B, lanes 6). This effect seemed to be specific because none of these nuclear protein complexes was supershifted or abolished when a non-specific antibody was used (Figure 3B, lanes 7). Control experiments containing only the anti-SRF antiserum and the radiolabeled WT oligonucleotide did not reveal any binding activities (Figure 3B, lanes 8). In addition, supershift assays performed with the radiolabeled c-fos SRE oligonucleotide instead of the WT probe showed that bands corresponding to SRE–SRF complex disappeared and more slowly migrating bands appeared (Figure 3B, lane 10). The absence of supershift for specific WT binding complexes (Figure 3D, lanes 6) may be explained if the epitope recognized by the anti-SRF antibody is part of the SRE-binding site. Antibody binding would compete DNA binding, resulting in a significant decrease or total disappearance of CArG-like complexes rather than in supershifted complexes. Alternatively, if the interaction between the CArG-like DNA-binding site and the SRF protein is insufficiently strong, it could be not as stable as the one observed with the SRE probe (Figure 3B, lane 10) to be detected as a supershift in gel retardation assays. On the one hand, other DNA-binding proteins that are potentially part of this complex could interfere with the antibody’s access to SRF protein. Taken together, these EMSA studies establish SRF-binding CArG-like box within the minimal promoter of the human CFTR gene.

**SRF binds the CArG-like motif of the CFTR minimal promoter within intact chromatin under physiological conditions**

Although there is extensive evidence that SRF can bind to CArG-like elements in *in vitro* assays and that it is involved in transcriptional regulation through CArG elements in reporter assays in cultured cells with a typical epithelial morphology (53), there is a lack of direct evidence for involvement of SRF in transcription of the endogenous CFTR gene within the context of intact chromatin. To directly address whether SRF also binds *in vivo*, ChIP was carried out in bronchial epithelial cells. For this purpose, DNA-binding proteins of Beas2B epithelial cells were covalently linked to genomic DNA by treatment of the cells with formaldehyde. Cross-linked chromatin was immunoprecipitated with either specific or irrelevant antibodies, anti-SRF and anti-HA, respectively. The precipitated chromatin DNA was then purified and amplified by PCR with specific primers of the target sequences (Table 1). The promoter of β-globin gene, which lacks CArG elements, was used as negative control (Figure 4, row A). The skeletal β-actin promoter sequence, which contains at least a consensus SRF-binding site, was used as positive control (Figure 4, row B). As expected, PCR signals were obtained when the DNA/protein adducts were immunoprecipitated with an anti-SRF antibody from both Beas2B (Figure 4, rows B and C, lane 1) and A549 (data not shown) epithelial cell lines. In contrast, the negative controls, in which immunoprecipitation was performed with an irrelevant antibody (Figure 4, rows A–C, lane 3) or without antibody (Figure 4, rows A–C, lane 2), did not show any PCR signal. These results were reproduced in several independently
Figure 3. SRF is a component of CFTR-CArG-like binding complexes. (A) EMSAs were performed with a radiolabeled oligonucleotide encompassing the studied CFTR-CArG-like motif (noted WT, see Table 2) and bacterially expressed SRF (lane 3). Competition assays were carried out using a 100-fold molar excess of two specific competitors, noted WT and SRE (lanes 2 and 5, respectively). UC (lane 6) indicates unspecific competitor that is used as negative control. Supershift assays were carried out using either a specific antibody (lane 4) or an irrelevant antibody (lane 8). The arrow SS indicates the Ab-supershifted complex. Neither the retarded nor the supershifted complexes were found when the 32P-labeled WT double-stranded oligonucleotide was incubated either only with the binding buffer (lane 1) or with specific anti-SRF antibody in the absence of purified SRF protein (lane 7). (B) EMSAs were performed with WT 32P-labeled probe in the absence of proteins (lanes 1) or with nuclear extracts (lanes 5) isolated from Beas2B (upper panel), A549 (middle panel) and C2C12 cells (lower panel). Three or five nuclear protein complexes were detected and designated by a cell type-dependent upper-case to the left of autoradiograms. Some binding reaction mixtures included a 100-fold molar excess of the indicated cold probes (lanes 2–4). Immunologic assays were performed with either anti-SRF Ab (lanes 6), or anti-HA as irrelevant antiserum (lanes 7). As a control, the 32P-labeled WT probe was incubated with anti-SRF Ab without nuclear extracts (lanes 8). In additional control experiments, radiolabeled SRE double-stranded oligonucleotide corresponding to the SRE binding site of the c-fos promoter was subjected to EMSAs with nuclear extracts from Beas2B cells (upper panel) in the absence (lane 9) or presence of anti-SRF antiserum (lane 10). Nuclear protein complexes that were significantly decreased or supershifted with anti-SRF Ab are indicated.
isolated ChIP populations. These data show that the CFTR-CaR-like binding site identified within the CFTR minimal promoter binds SRF in vivo, in bronchial cell lines.

The human minimal CFTR promoter shows activity in both epithelial and muscular cells

As the SRF transcription factor is thought to mainly mediate the tissue-specific transcription in muscular cells, we first assessed the activity of the minimal CFTR promoter in both epithelial and muscular cell types. We therefore addressed the ability of the WT-pGL3 construct, previously described (12) to drive the expression in Beas2B, A549 and C2C12 cells. As shown in Figure 5A, an efficient transcription of the CFTR promoter could be readily observed in all three cell lines shown in Figure 5A, an efficient transcription of the minimal promoter. Chromatin immunoprecipitation was carried out as described in Materials and Methods. Sheared DNA/protein complexes were immunoprecipitated by using either an anti-SRF Ab or an irrelevant anti-HA Ab. Then, PCR was carried out to detect the endogenous CaR-like regions in immunoprecipitated chromatin fragments. Target promoter sequences are indicated as follow: row A, β-globin; row B, β-actin; row C, CFTR. Lane 1 shows amplification of target sequences in immunoprecipitated chromatin fragments with anti-SRF Ab. Lanes 2 and 3: PCR amplification of control samples, without Ab or with an irrelevant Ab. Lane 4 shows amplification of 1:100 dilution samples of total input DNA for immunoprecipitation.

Figure 4. ChIP analysis of SRF binding to the endogenous CFTR minimal promoter. Chromatin immunoprecipitation was carried out as described in Materials and Methods. Sheared DNA/protein complexes were immunoprecipitated by using either an anti-SRF Ab or an irrelevant anti-HA Ab. Then, PCR was carried out to detect the endogenous CaR-like regions in immunoprecipitated chromatin fragments. Target promoter sequences are indicated as follow: row A, β-globin; row B, β-actin; row C, CFTR. Lane 1 shows amplification of target sequences in immunoprecipitated chromatin fragments with anti-SRF Ab. Lanes 2 and 3: PCR amplification of control samples, without Ab or with an irrelevant Ab. Lane 4 shows amplification of 1:100 dilution samples of total input DNA for immunoprecipitation.

Effects of SRF on the proximal CFTR promoter

To assess whether SRF protein might affect CFTR transcriptional regulation, we took two related approaches. First, we co-transfected either exogenous full-length SRF or truncated SRF mutants with the wild-type CFTR minimal promoter. Second, we utilized a siRNA approach to ask whether a reduction in endogenous SRF levels could affect basal CFTR activation. Luciferase assays were carried out in Beas2B cells using the indicated SRF expression vectors and the WT-pGL3-luciferase reporter construct (Figure 6B, left panel). The 3XSRE-fos TATA-Luciferase reporter gene was used as positive control because its transcriptional activity is highly dependent on SRF binding (Figure 6B, right panel). As expected, while this synthetic promoter containing several consensus SREs was markedly and significantly activated (>2-fold) by forced expression of full-length SRF protein (pHiv-SRF) (Figure 6B, right panel), truncated SRF mutants either containing only the amino acids 133–264 corresponding to DNA-binding domain of SRF (pHiv-DBD-SRF), or deleted of amino acids 153–165, an essential part for the DNA binding (pHiv-DN-SRF) induced modest but significant decrease of SRE activity (~10% and ~30%, respectively). When these SRF expression vectors were transiently co-transfected with the WT-pGL3 luciferase reporter construct, the trans-activator effect of SRF was notably reduced. Indeed, overexpression of full-length and dominant-negative (pHiv-DN-SRF) SRF proteins resulted in ~50% increased and ~30% decreased
of luciferase activity, respectively (Figure 6B, left panel). Unexpectedly and inconsistent with previous study (60), the pHiv-DBD-SRF construct induced ~40% increase of the WT-pGL3 luciferase activity. This results disparity may be explained by the fact that the c-fos promoter study (60) assessed the DBD–SRF domain activity via microinjection of corresponding truncated SRF polypeptide. Perhaps, the best explanation for this increased activity is that the DNA-binding domain of SRF, also called MADS box, is sufficient for transcriptional activation of some SRF-dependent genes because it mediates interactions with accessory co-activators (61). These results suggest that neither of the regions outside the MADS box, N- or C-terminal, appear essential for trans-activation of the CFTR promoter by SRF.

To further establish the role of SRF in mediating the increase in CFTR gene transcription, we performed siRNA experiments (Figure 6C). To demonstrate the efficacy of specific SRF siRNA construct, Beas2B cells were co-transfected with either control or SRF siRNA plasmids and western blots were performed to analyze levels of SRF in these cells. Densitometric analysis of western blot showed that endogenous SRF levels were strongly reduced in the presence of the SRF siRNA plasmid (Figure 6C, left panel). Then, control and SRF siRNA plasmids were co-transfected into Beas2B cells with either the WT-pGL3 or the 3XSRE luciferase reporter construct. As shown in Figure 6C (right panel), reduction of endogenous SRF protein level decreased the activity of both wild-type CFTR promoter and 3XSRE-fos TATA-Luciferase reporter constructs by ~25%.

Taken together, these findings indicate that endogenous SRF into bronchial epithelial cells normally function as transcriptional coactivator for the CARG-mediated transactivation. However, we found that SRF activated the CFTR promoter less well than the SRF-containing synthetic promoter, suggesting that the CARG-like element mediated CFTR transcriptional activation probably required other bridging or co-activating factors.

The CARG-like element contained in the human minimal CFTR promoter is important but not sufficient for basal transcriptional activity in Beas2B cells

To assess the transcriptional relevance of the CARG-like binding sequence of CFTR minimal promoter, mutants were
**Figure 6.** Effect of SRF on the activity of CArG-containing CFTR minimal promoter. (A) Schematic representation of different SRF constructs analyzed. The SRF fragments shown were cloned into the pHiv expression vector as indicated in ‘Materials and Methods’. The position of the MADS box containing the DNA-binding domain and dimerization domain of SRF is depicted by black rectangle. WT SRF construct encodes a human full-length SRF open reading frame. DBD SRF construct encodes only the DNA-binding domain of SRF. DN SRF deletion construct encodes full-length SRF deleted of an essential part for the DNA binding. (B) Expression of exogenous SRF in Beas2B cells. Cells were co-transfected with the WT-pGL3 luciferase reporter plasmid (left panel) together with the empty pHiv vector, pHiv-SRF, pHiv-DBD-SRF or pHiv-DN-SRF constructs, as indicated below the bar diagram. As a positive control, 3XSRE luciferase reporter plasmid was used. In each experiment, a Renilla luciferase expression plasmid was included to normalize for transfection efficiency. For each reporter plasmid, luciferase activity (mean ± SE) is derived from 4 to 7 different transfection experiments performed in triplicate. The errors bars indicate the standard deviations. The luciferase activity value 100 was assigned to the samples co-transfected with the indicated reporter plasmid and the empty pHiv expression vector. Statistical significance is referred to differences with the activity obtained with empty expression vector. *, *P* < 0.05 versus WT or 3XSRE. (C) Effects of SRF siRNA on CFTR promoter activity and expression in Beas2B epithelial cells. Left panel: Beas2B cells were co-transfected with either human control siRNA vector or plasmid encoding SRF siRNA as indicated. Forty-eight hours following transfection, total proteins were harvested and analyzed by western blotting. Densitometric analysis showed a substantial decrease of endogenous SRF. Right panel: the luciferase activity value 100 was assigned to the samples co-transfected with the control siRNA. *, *P* < 0.05 versus WT or 3XSRE.
performed to create either more consensus CArG element or degenerated motif (Table 1). We used the D upper-case and the C upper-case to designate the Degenerated CArG motifs and the Consensus CArG elements, respectively. To ensure the efficacy of mutations in abrogating transcription factor binding, we first performed a series of EMSA experiments with each CArG mutant and the WT probe (Table 2). As expected, based on the sequence analysis, the mutations resulting in more degenerated CArG motifs (D1, D2, D3 and D4 mutants) completely abolished SRF-binding activity (Figure 7A, lanes 1–4), when we compared with SRF-WT complexes (Figure 7A, lane 5). In addition, the probes containing more consensus CArG boxes (C1, C2, C3, C4 and C5 mutants) bound SRF more strongly in nuclear extracts prepared from Beas2B (Figure 7A, lanes 6–10). Inclusion of an anti-SRF antibody partially abolished SRF-binding activity and resulted in a slower mobility band, confirming the identity of the binding protein (Figure 7A, lane 11). Then, minimal CFTR promoter constructs bearing the same consensus and disrupted CArG boxes were tested in a transient transfection assays in Beas2B cells (Figure 7B). Compared with luciferase activity resulting from the WT-pGL3 construct, variable increases in reporter activity are observed when D1, D2, D3 and D4 mutant constructs, with disrupted CArG motif, were transiently transfected. On the other hand, mutations that create more consensus CArG element result in either reducing or increasing in the transcriptional activity (Figure 7B). Consistent with the preceding gel-shift results

![Figure 7. The CFTR-CArG-like element is not sufficient for basal transcriptional activity in Beas2B cells. (A) EMSA analysis with nuclear extracts from SRF protein-enriched Beas2B cells using mutated labeled oligonucleotide probes (sequences listed in Table 2). Ab against SRF was included as indicated (lane 10). The arrow SS indicates the supershifted complexes. (B) Basal transcriptional activity of CArG variants of the CFTR promoter. Luciferase activity obtained with the WT-pGL3 luciferase construct was defined as 100%, and relative luciferase activities from mutant constructs are expressed as a percentage of this value. Firefly luciferase activity was normalized with respect to Renilla luciferase activity. The errors bars indicate the standard deviations. *, P < 0.05 versus WT.]
**A**

Labeled probes

|          | WT | C1 | C3 | C4 | D2 |
|----------|----|----|----|----|----|
| Antibody| -YY1| -YY1| -YY1| -YY1| -YY1|

**B**

Probes

|          | WT | C1 | C3 | C4 |
|----------|----|----|----|----|
| YY1-transfected Nuclear Extract GST-SRF | + | + | + | + |

**C**

Relative luciferase activity to 100% control

|          | WT | C1 | C3 | C4 |
|----------|----|----|----|----|
| control | 0  | 50 | 100 | 150 |
| SRF     | 200| 250| 300 | 350 |
| YY1     | 400| 450| 500 | 550 |
| SRF/YY1 | 600| 650| 700 | 750 |
revealing an uncharacterized protein/DNA complex with slightly slower mobility and variable intensity compared with the SRF–DNA complex, these finer mutagenesis studies suggest that both co-activators and co-repressors might interact with the studied promoter sequences. Taken together, these data demonstrated that the CFTR-CArG-like element alone is not sufficient to confer basal transcriptional activation of the CFTR gene.

**Functional antagonism between SRF and YY1 through competition for the CFTR-CArG-like binding**

As a further means of establishing the role for SRF-binding CArG boxes in mediating either the CFTR-enhancer or silencer activity observed with the different minimal CFTR promoter mutants, we performed additional computational analyses. Sequence analysis of CArG boxes and their immediate flanking sequences showed that C1, C3 and C4 constructs contain consensus binding sites for a number of transcription factors, in addition to SRF, including the transcription factor YY1. These findings with previous work (12) have particularly evoked our interest to examine the role of YY1 in SRF-mediated CFTR transcriptional activity.

In a first set of experiments, we evaluated whether the YY1 factor contributes to the formation of nucleoprotein complexes interacting with the WT, C1, C3 and C4 radiolabeled probes. As shown in Figure 8A, incubation with specific anti-YY1 antibody resulted in either a significant decrease or total disappearance of some complexes normally formed between the CArG boxes and a component of Beas2B nuclear extracts, establishing DNA binding between YY1 and the sequences tested. As expected, control experiment performed with radiolabeled probe, D2, devoid of any YY1 binding site and the anti-YY1 antiserum did not reveal any change in electrophoretic profiles indicating specificity of the YY1 binding. Thus, these EMSAs demonstrated that the CFTR-CArG-like (WT probe) and more consensus CArG elements C1 < C3 < C4 served as binding site for at least two distinct nuclear factors, namely SRF and YY1 from bronchial epithelial cells.

Then, to determine whether the interaction of these two factors with their target DNA (CFTR-CArG-like or more consensus CArG sequence) was either mutually inclusive or exclusive, EMSAs were conducted using bacterially expressed SRF and nuclear extracts prepared from Beas2B cells transfected with YY1 expression vector as a source of YY1 DNA-binding activity. As shown in Figure 8B, the YY1-binding complex was significantly reduced by increasing SRF binding activity, suggesting their mutually exclusive binding to CArG sites.

Consistent with other studies (32,62), these EMSAs results suggested as a working hypothesis that YY1 and SRF might work together as functional competitors in the CFTR promoter activity. Since the minimal CFTR promoter is activated by SRF and knowing from previous studies (30–33) that YY1 negatively regulates some promoter containing CArG elements, it seemed logical to first assess whether YY1 could also repress the CFTR promoter. We asked whether CArG-like-binding site in the CFTR promoter acted as negative regulatory elements. Several CFTR promoter reporter constructs containing either CArG-like (namely, WT) or consensus CArG element (C1, C3 and C4) were co-transfected with SRF and/or YY1 expression vectors into Beas2B cells. As shown in Figure 8C, while co-transfections with SRF alone resulted in 1.5- to 2-fold luciferase activation, forced expression of YY1 protein caused a strong decrease in reporter activities (~50–75% of the control luciferase value). Therefore, we wanted to determine the functional relationship of both factors in the CFTR activity regulation. Transient co-transfections analysis of combination of protein expression vectors showed that over-expression of SRF either did not restore (Figure 8C, panel with the WT and C4 reporter constructs) or only very slightly raised the YY1-repressed reporter activities (Figure 8C, panel with the C1 and C3 reporter constructs). These findings are consistent with results obtained from co-transfections performed with the YY1 expression vector alone and previous alpha-actin promoter studies (30–33), in which YY1 acted as a strong repressor. Taken together, these data suggested functional antagonism between SRF and YY1 through DNA-binding competition.

**Identification of multiple CArG binding sites in the non-coding regions of the human CFTR gene and assessment of their putative functional importance**

To assess the putative importance of CArG motifs in the human CFTR expression regulation, we performed an in silico inspection of additional sequences upstream of the minimal promoter and in the intronic regions. Four CArG binding sites were found in the CFTR promoter regions, and seven in the intronic regions. The distribution and the positions of these putative binding sites for SRF are shown in Figure 9A. Then, to confirm the computational predictions, we used several approaches. First, the nucleotide regions harboring these predicted cis-acting elements were analyzed for conservation in the cow, as physiologically relevant transcription factor binding sites are frequently conserved in the non-coding regions of orthologous genes (49,63). Second, the SRF-binding sites were tested by EMSAs and also for one of them by ChIP.

We showed that all the predicted CArG elements occur in highly conserved CFTR non-coding regions (Figure 9B), which may contain tissue-specific transcription factors (5). We provided evidence that the majority of in silico predicted CArG motifs form specific DNA–protein complexes with
bacterially expressed SRF protein (Figure 9C, left panel). We also demonstrated by using Beas2B nuclear extracts and super-shift assays that some of these CArG elements bound \textit{in vitro} SRF protein (Figure 9C, right panel, lanes 1, 3, 7 and 9). Finally, additional ChIP experiments indicated that the only tested CArG element, located at –2373 bp in the \textit{CFTR} promoter, bound SRF in chromatin from intact cultured Beas2B cells (Figure 9D, lane 1). Collectively, these findings support the concept that SRF may play a key role in the \textit{CFTR} expression regulation via binding to multiple \textit{CFTR} CArG elements.

**DISCUSSION**

While great strides have been made in \textit{CFTR} research with emphasis on the structure and function of this protein, only incremental advances have been achieved in the field of transcriptional regulation. In our previous works (12,20), we have identified a polymorphic CArG-like site and showed that a naturally occurring sequence variation in this motif may enhance the basal \textit{CFTR} transcriptional activity. The goal of the present study was to better characterize the human \textit{CFTR} promoter region encompassing the CArG-like element.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{CArG Number} & \textbf{Location of CArG in human} & \textbf{Human clone} & \textbf{Human-cow Homology} & \textbf{Cow clone} \\
\hline
1 & Upstream exon 1 & AC000111 & 79% & AC089992 \\
 & -10.7 kb & 8846-9031 & & 79372-79554 \\
2 & -2.7 kb & AC000111 & 70% & AC089992 \\
 & 16861-17034 & & & 88539-88711 \\
3 & -2.4 kb & AC000111 & 80% & AC089992 \\
 & 17241-17491 & & & 88821-89081 \\
4 & Intron 1 & AC000111 & 80% & AC089992 \\
 & 185 + 9.5 kb & 29167-29430 & & 105768-106034 \\
5 & 185 + 9.9 kb & AC000111 & 72% & AC089992 \\
 & 29699-29846 & & & 106311-106457 \\
6 & 185 + 14.7 kb & AC000111 & 73% & AC089992 \\
 & 34514-34681 & & & 111573-111738 \\
7 & 185 + 20.8 kb & AC000111 & 76% & AC089992 \\
 & 40563-40861 & & & 126635-126931 \\
8 & Intron 3 & AC000111 & 80% & AC089992 \\
 & 405 + 1 kb & 49422-49657 & & 13843-14064 \\
9 & Intron 10 & AC000111 & 75% & AC089993 \\
 & 1716 + 23 kb & 122060-122341 & & 66259-66531 \\
10 & Intron 17a & AC000061 & 73% & AC089993 \\
 & 3271 + 0.7 kb & 1141-1494 & & 100451-100785 \\
\hline
\end{tabular}
\caption{CArG boxes of the human \textit{CFTR} gene and the corresponding regions of homology in the cow}
\end{table}
Comparisons of nucleotide sequences of this CFTR promoter region in eight mammalian species representing three different orders (Primates, Artiodactyla and Lagomorpha) revealed high levels of conservation of the minimal promoter region, as described previously (64). However, the CArG-like element (GC(T/A)$_6$G), which does not conform to the authentic CArG box (CC(T/A)$_6$G), is absent from three species (sheep, cow and rabbit). Even though this motif occurs in only a subset of the input sequences, it does not necessarily mean that it is of no functional importance. Instances of cis-regulatory elements being species-specific have been already reported for the CFTR gene (50). More generally, it is well known that some transcription factors can tolerate more than one type of nucleotide at a given position of the binding site. Instances of substitutions in both the central core of the CArG element and the highly conserved contact points for SRF, at the terminal ends, not impairing SRF binding have been previously reported (48,65–68). Taken together, these data support the notion that the CFTR-CArG-like element has possible important implication in the CFTR transcriptional regulation.

Interestingly, analysis of SRF expression has revealed relatively high expression levels of SRF protein in the bronchial epithelial cells (Figure 2), as shown in other differentiated epithelial cells (27). Accumulating evidence supports the concept that SRF could contribute to the CFTR expression regulation. In addition to regulating growth-responsive genes and numerous muscle-specific genes (22,69), SRF is also described as a key trans-binding factor in various important physiological events. For example, SRF has been shown to regulate pulmonary development in Drosophila (28), to promote both re-epithelialization and muscular structure restoration during gastric ulcer healing (70) and more recently to be involved in the regulation of endoplasmic reticulum-targeted gene (67). Therefore, it was relevant to examine the potential role of SRF on the CFTR transcriptional activity.

In the present study, we demonstrated that CFTR-CArG-like element forms specific DNA–protein complexes that include SRF protein as part of the complex, in vitro and in vivo (Figures 3 and 4). We have investigated the expression driven by the proximal human CFTR promoter in a pGL3 construct in both epithelial and myoblast lines. Results of our transient transfections of 3XSRE-fos TATA and WT-pGL3 luciferase reporter genes showed that the human minimal CFTR promoter encompassing a CArG-like motif was sufficient to drive the expression in both cell types (Figure 5). Consistent with other studies showing CFTR expression in smooth muscle tissue (71,72), our results demonstrated that the human CFTR minimal promoter had the ability to drive the expression in muscle lineages. Our transient co-transfections analyses performed with full-length and dominant-negative SRF forms expression vectors, and also specific SRF siRNA constructs (Figure 6, panels B and C) provide the first evidence for a positive role of the SRF protein in the regulation of the human CFTR promoter. However, the SRF-induced CFTR transactivation, though significant, is relatively modest. These data with the results of finer mutagenesis studies presented in Figure 7 support the concept that SRF alone is not sufficient to drive the basal expression of the CFTR gene. As suggested by other previous studies (73–75), it is likely that additional factors might be required to optimally drive
the CFTR promoter, as well as to specify the cell-type-restricted expression. Since YY1 was previously shown to bind the CFTR promoter (12) and a subset of SREs serve as binding sites for YY1 (30–33), we first evaluated this candidate in the SRF-mediated CFTR transcriptional activity. The data analysis suggests a functional antagonism between SRF and YY1 through binding competition for the CFTR-CArG-like box. However, the analysis of combinatorial co-transfection studies, presented in Figure 8C, suggests that the functional interplay between SRF and YY1, alone, is not sufficient to account for basal CFTR transcriptional activation. Further studies will be necessary to determine whether another player might be involved in this functional interplay and accounted for the basal transcriptional CFTR activity observed in the Beas2B bronchial epithelial cells.

Interestingly, we showed that, in addition to the CArG-like element defined in the minimal promoter of the CFTR gene, there are three CArG motifs located at the 5’ end of the gene and seven are present in the intronic regions (Figure 9). We also evidenced SRF binding to CArG elements of the endogenous CFTR gene in the context of intact chromatin (Figures 4 and 9D). Although the role of these more distal CArG boxes has yet to be assessed, these findings are of major importance, since it has been shown that optimal activity of the SRF-dependent target genes may require a number of CArG boxes, including those that are defined in the intronic region (66,67,76). Moreover, as already suggested for the α-actin promoters (32,74), perhaps the presence multiple CArG elements in the CFTR gene with cooperative binding events might be essential for SRF to prevent YY1 from binding to the CFTR promoter. Clearly, further studies are needed to directly investigate the role of these multiple CArG elements in transcriptional regulation of the CFTR gene.

Taken together, our results demonstrated for the first time that the human CFTR promoter is a novel SRF target gene, subject to modest but significant SRF activation partially due to functional antagonism between YY1 and SRF through mutually exclusive DNA-binding activities of YY1 versus SRF to the CFTR-CArG-like site.

A critical question is, thus, how might SRF contribute to orchestrating cell-restricted and context-dependent programs of CFTR gene expression? Our findings and investigations to date clearly indicate the presence of several different underlying mechanisms of SRF-dependent transcriptional activation, as represented in Figure 10. These might include association of SRF with a variety of cell-restricted co-factors (noted in gray bubbles, Figure 10), such as MRTF-A highly expressed in epithelial cells of the lung, kidney, colon and testis, and MRTF-B also expressed in smooth muscle cells (77), ternary complex factors, such as p62 and SAP-1 (25,78) and remodeling chromatin co-factors in an HAT-dependent manner, such as the CREB-binding protein (79). In addition, it is well known that binding of SRF to the CArG box induces an acute bend in the DNA and that this bending may vary with changing base compositions across the CArG box (80). Such bending can probably facilitate interactions with other proteins already involved in the CFTR expression regulation, such as NF-kB (13) and C/EBP (9) and described as interacting with SRF (81). Finally, the Ras-related GT-Pases family (rac, RhoA and cdc42) might have a critical role in the activation of SRF (82). Indeed, it is reported that RhoA-induced SRF activation occurs through a phenomenon of ‘actin treadmilling’ restricted to CArG-dependent genes that do not have adjacent ETS-binding sites (e.g. smooth muscle cell restricted CArG genes) (83,84). Moreover, it has been shown that the activation of SRF may be mediated by the NF-kB and C/EBP transcription factors (81).

These data provide the foundation for further studies on the regulation of CFTR and will enable the rational design of further functional studies, including various strategies aiming to further dissect the different regulation pathways.

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REFERENCES

1. Crawford, L., Maloney, P.C., Zeitlin, P.L., Guggino, W.B., Hyde, S.C., Turley, H., Gatter, K.C., Harris-A, and Higgins, C.F. (1991) Immunocytochemical localization of the cystic fibrosis gene product CFTR. Proc. Natl Acad. Sci. USA, 88, 9262–9266.

2. Denning, G.M., Ostgaard, L.S., Cheng, S.H., Smith, A.E. and Welsh, M.J. (1992) Localization of cystic fibrosis transmembrane conductance regulator in chloride secretory epithelia. J. Clin. Invest., 89, 339–349.
3. Engelhardt,J.F., Yankaskas,J.R., Ernst,S.A., Yang,Y., Marino,C.R., Boucher,R.C., Cohn,J.A. and Wilson,J.M. (1992) Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nature Genet.*, 2, 240–248.

4. Trezise,A.E., Linder,C.C., Grieger,D., Thompson,E.W., Meunier,H., Grisswood,M.D. and Buchwald,M. (1993) CFTR expression is regulated during both the cycle of the seminiferous epithelium and the oestrous cycle of rodents. *Nature Genet.*, 3, 157–164.

5. Mouchel,N., Broackes-Carter,F. and Harris,A. (2003) Alternative S' exons of the CFTR gene show developmental regulation. *Hum. Mol. Genet.*, 12, 759–769.

6. Yoshimura,K., Nakamura,H., Trapnell,B.C., Dalemans,W., Pavirani,A., Trouet,M., Vanmechelen,E., Moyna,M., Pawitan,M., Sch cuenta,E., Yanez,M., Vaseille,M., Denai,E. and Ghanem,M. (1999) The cystic fibrosis gene has a ‘housekeeping’-type promoter and is expressed at low levels in epithelial origin. *J. Biol. Chem.*, 266, 9140–9144.

7. Koh,J., Sferra,T.J. and Collins,F.S. (1993) Characterization of the cystic fibrosis transmembrane conductance regulator promoter region. *Chromatin context and tissue-specificity. J. Biol. Chem.*, 268, 15912–15921.

8. White,N.L., Higgins,C.F. and Trezise,A.E. (1998) Tissue-specific in vivo transcription start sites of the human and murine cystic fibrosis genes. *Hum. Mol. Genet.*, 7, 363–369.

9. Pittman,N., Shue,G., LeLeiko,N.S. and Walsh,M.J. (1995) Transcription of cystic fibrosis transmembrane conductance regulator requires a CCAAT-like element for both basal and cAMP-mediated regulation. *J. Biol. Chem.*, 270, 28846–28857.

10. Matthews,R.P. and McKechnie,G.S. (1996) Characterization of the cAMP response element of the cystic fibrosis transmembrane conductance regulator gene promoter. *J. Biol. Chem.*, 271, 31809–31877.

11. McDonald,R.A., Matthews,R.P., Iizerda,R.L. and McKechnie,G.S. (1995) Basal expression of the cystic fibrosis transmembrane conductance regulator gene is dependent on protein kinase A activity. *Proc. Natl Acad. Sci. USA.*, 92, 7560–7564.

12. Romero,M.C., Pallares-Ruiz,N., Mange,A., Mettling,C., Peytavi,R., Loebeer,L., Playfer,R.J. and Mettling,C. (1995) Mutation analysis of 3′ flanking regions of vertebrate actin genes. *J. Mol. Evol.*, 45, 597–598.

13. Chen,C.Y. and Schwartz,R.J. (1997) Competition between negative acting YY1 versus positive acting serum response factor and trimmer homologue Nkx-2.5 regulates cardiac alpha-actin promoter activity. *Mol. Endocrinol.*, 11, 812–822.

14. Martin,K.A., Gualberto,A., Colman,M.F., Lowry,J. and Walsh,K. (1997) A competitive mechanism by which CREB is recruited to the cystic fibrosis transmembrane conductance regulator promoter in MDCK I cells. *Biochem. J.*, 322, 259–265.

15. Bradbury,N.A., Clark,J.A., Watkins,S.C., Widnell,C.C. and Smith,H.S. (1999) Characterization of the internalization pathways for the cystic fibrosis transmembrane conductance regulator. *Am. J. Physiol.*, 276, L659–L668.

16. Tousson,A., Van Tine,B.A., Naren,A.P., Shaw,G.M. and Schwartz,L.M. (1998) Characterization of CFTR expression and chloride channel activity in human endothelia. *Am. J. Physiol.*, 275, C1555–C1564.

17. Peter,K., Varga,Z., Bebok,Z., Mcnicholas-Bevensee,C.M., Schwartz,L., Sorscher,E.J., Schwartz,E.M. and Collawn,J.F. (2002) Ablation of internalization signals in the carboxyl-terminal tail of the cystic fibrosis transmembrane conductance regulator enhances cell surface expression. *J. Biol. Chem.*, 277, 49952–49957.

18. Chou,J.L., Rozmahel,R. and Tsui,L.C. (1991) Characterization of the promoter region of the cystic fibrosis transmembrane conductance regulator gene. *J. Biol. Chem.*, 266, 24471–24476.

19. Cheng,S.H., Fang,S.L., Zubner,J., Marshall,J., Piraino,S., Schiavi,S.C., Jefferson,D.M., Welsh,M.J. and Smith,A.E. (1995) Functional activation of the cystic fibrosis trafficking mutant delta F508-CFTR by overexpression. *Am. J. Physiol.*, 268, L615–L624.

20. Shore,P. and Schwartz,L. (1994) The transcription factors Elk-1 and serum response factor interact by direct protein-protein contacts mediated by a short region of Elk-1. *Mol. Cell. Biol.*, 14, 3283–3291.

21. Romey,M.C., Tuffery,S., Desgeorges,M., Bienvenu,T., Demaille,J. and Claudres,M. (1996) Transcript analysis of CFTR gene expression. *FEBS Lett.*, 391, 247–251.

22. Miano,J.M. (2003) Serum response factor: toggling between disparate programs of gene expression. *J. Mol. Cell Cardiol.*, 35, 577–593.

23. Shore,P. and Sharrocks,A.D. (1995) The MADS-box family of transcription factors. *Eur. J. Biochem.*, 229, 1–13.

24. Browning,C.L., Cubлерson,D.E., Aragon,L.V., Fillmore,R.A., Croissant,J.D., Schwartz,R.J. and Zimmer,W.E. (1998) The developmentally regulated expression of serum response factor plays a key role in the control of smooth muscle-specific genes. *Dev. Biol.*, 194, 18–37.

25. Camoreti-Mercado,B., Dulin,N.O. and Solway,J. (2003) Serum response factor function and dysfunction in smooth muscle. *Respir. Physiol. Neurobiol.*, 137, 223–235.

26. Managlia-Jaulin,L., Masutani,H., Lipinski,M. and Harel-Bellan,A. (1996) Analysis of SRF, SAP-1 and ELK-1 transcripts and proteins in human cell lines. *FEBS Lett.*, 391, 247–251.

27. Chai,J., Baatar,D., Moon,W. and Tarnawski,A. (2002) Expression of serum response factor in normal rat gastric mucosa. *J. Physiol. Pharmacol.*, 53, 289–294.

28. Gillemin,K., Groppe,J., Ducker,K., Treisman,R., Hafen,E., Affolter,M. and Krasnow,M.A. (1996) The pruned gene encodes the Drosophila serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system. *Development*, 122, 1353–1362.

29. Yang,Y., Zhe,X., Phan,S.H., Ullenbruch,M. and Schuger,L. (2003) Involvement of serum response factor isoforms in myofibroblast differentiation during blemycin-induced lung injury. *Am. J. Respir. Cell Mol. Biol.*, 29, 583–590.

30. Liu,T., Wu,J. and He,F. (2000) Evolution of cis-acting elements in 5' flanking regions of vertebrate actin genes. *J. Mol. Evol.*, 50, 22–30.

31. Natesan,S. and Gilmam,M. (1995) YY1 facilitates the association of serum response factor with the c-fos serum response element. *Mol. Cell. Biol.*, 15, 5975–5982.

32. Natesan,S. and Gilmam,M. (1995) YY1 facilitates the association of serum response factor with the c-fos serum response element. *Mol. Cell. Biol.*, 15, 5975–5982.

33. Natesan,S. and Gilmam,M. (1995) YY1 facilitates the association of serum response factor with the c-fos serum response element. *Mol. Cell. Biol.*, 15, 5975–5982.
