Cooperative Transcriptional Activation by Serum Response Factor and the High Mobility Group Protein SSRP1*

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Jeffrey A. Spencer‡, Margaret H. Baron§, and Eric N. Olson‡¶

From the ‡Department of Molecular Biology and Oncology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9148 and the §SUNY Mount Sinai School of Medicine, New York, New York 10028

Serum response factor (SRF) is a MADS box transcription factor that controls a wide range of genes involved in cell proliferation and differentiation. The MADS box mediates homodimerization and binding of SRF to the consensus sequence CC(A/T)G, known as a CArG box, which is found in the control regions of numerous serum-inducible and muscle-specific genes. Using a modified yeast one-hybrid screen to identify potential SRF cofactors, we found that SRF interacts with the high mobility group factor SSRP1 (structure-specific recognition protein). This interaction, which occurs in yeast and mammalian cells, is mediated through the MADS box of SRF and a basic region of SSRP1 encompassing amino acids 489–542, immediately adjacent to the high mobility group factor SRF (1). The MADS box mediates homodimerization and binding of SRF to the consensus sequence CC(A/T)G, known as a CArG box, which is found in the control regions of numerous serum-inducible and muscle-specific genes. Using a modified yeast one-hybrid screen to identify potential SRF cofactors, we found that SRF interacts with the high mobility group factor SSRP1 (structure-specific recognition protein). This interaction, which occurs in yeast and mammalian cells, is mediated through the MADS box of SRF and a basic region of SSRP1 encompassing amino acids 489–542, immediately adjacent to the high mobility group domain. SSRP1 does not bind the CArG box, but interaction of SSRP1 with SRF dramatically increases the DNA binding activity of SRF, resulting in synergistic transcriptional activation of native and artificial SRF-dependent promoters. These results reveal an important role for SSRP1 as a coregulator of SRF-dependent transcription in mammalian cells.

Among the transcription factors that influence SRF activity are Ying Yang 1 (YY1) (14), the homeodomain proteins phoxI/MHox (15, 16) and Nkx2.5 (9), NF-κB (17), ATF6 (18), myogenic basic helix loop helix factors (19), and members of the ETS family of proto-oncogene products (reviewed in Ref. 20). Most recently, two members of the HMG-I family of nonhistone nuclear proteins, HMG-I(Y) and HMG-I(C), have also been demonstrated to associate with SRF (21). Physical association of SRF and HMG-I proteins leads to enhanced SRF-DNA association and increased SRF-dependent transcription in yeast and Drosophila cells.

HMG transcription factors are the most abundant nonhistone proteins in the nucleus. These proteins fall into three distinct classes: HMG-I, HMG-14/17, and HMG-12/22 (22). There are three known members of the HMG-I family. HMG-I and HMG-Y are derived from alternatively spliced transcripts from the same gene and show indistinguishable biologic activities and HMG-I-C is derived from a separate gene (22). The HMG-I factors bind AT-rich DNA in the minor groove through an AT-hook protein motif (22, 23). These factors influence the DNA binding and transcriptional activity of other transcription factors, but they lack intrinsic transcriptional activity. The HMG-1/2 class of transcription factors is divided into two subfamilies according to the number of HMG domains, DNA sequence recognition, and evolutionary conservation (22). One subfamily, which includes yeast ARS-binding factor, ABF-2, RNA polymerase II upstream binding factor, UBF, and the mitochondrial transcription factor, mTF-1, consists of proteins with multiple HMG domains that bind to structurally modified DNA with little or no sequence specificity (22). Members of the other subfamily, which includes the lymphoid enhancer-binding factor, LEF-1, the sex-determining factor, SRY, the related Sox factors (23), and structure-specific recognition protein, SSRP1 (24), possess a single HMG box and bind DNA with moderate sequence specificity. By themselves, members of the HMG-1/2 family of factors do not activate transcription, but are postulated to facilitate DNA binding of other transcription factors (reviewed in Ref. 23). Specifically, HMG-1/2 factors have been shown to enhance the transcriptional activity of Oct-1, Oct-2, and Oct-6 (25), the homeotic protein HOXD9 (26), MLT2 (27), p53 (28), and a variety of steroid hormone receptors (29). Genetic evidence for a role of HMG-1/2 factors in transcriptional regulation comes from gene disruption experiments in yeast. Disruption of the yeast genes encoding the HMG-like factors NHP-6A and -6B results in diminished expression of a subset of genes in response to stimuli (30). This effect seems to be transcription factor-specific since only certain genes are affected.

In the present study, using a modified yeast one-hybrid screen to identify SRF-interacting factors, we demonstrate that the HMG-1/2 protein SSRP1 physically interacts with SRF.
Transfection assays reveal that SSRP1 enhances SRF-dependent transcription in mammalian cells and that this is mediated by an increase in DNA binding by SRF in the absence of detectable SSRP1-DNA interaction. These results suggest that SSRP1 is a positive coregulatory protein involved in modulating SRF-dependent gene expression in mammalian cells.

MATERIALS AND METHODS

Yeast Interactive Screen—A modified yeast one-hybrid screen was performed using full-length human SRF as bait to identify potential interacting factors. The SRF bait plasmid encoded amino acids 1–508 under control of the alcohol dehydrogenase promoter. For this screen, we created a yeast strain, 4×CarGnear-LacZ, by integration of an SRF-dependent lacZ reporter into the URA3 locus. This reporter contains the lacZ gene under control of 4 tandem copies of the promoter-proximal CARG box, referred to as CarGnear, from the mouse SM22 promoter (6) plus 15 flanking nucleotides on each side, in the vector pLacZ. Each copy of CarGnear contained the sequence (+ strand): 5′-ACTTGGTGTCTTTCCGAAAAATATGGACCTGTGCAGTG-3′. In addition, this strain contains a plasmid expressing the histidine marker under control of 4 tandem copies of CarGnear as described above. This was integrated into the HIS4 locus allowing for growth on selective media upon activation of the reporter.

pADH-SRF was co-transformed into the 4xCarGnear-LacZ yeast strain with adult rat lung cDNA library (CLONTECH) that contained the GAL4 transcription activation domain (TAD) fused to random cDNA. Greater than 4 million primary colonies were screened for positive interactions and 62 LacZ-positive yeast colonies were initially identified. From each positive colony, the activating plasmid was rescued, transformed into Escherichia coli DH5α, plasmid DNA was isolated and the cDNA insert sequenced. Clones containing inserts in the antisense orientation or out-of-frame with the GAL4 TAD were discarded. The 2 remaining clones were retransformed back into yeast to verify the interaction with SRF. Both clones (48 and 62) encoded amino acids 489–587 of SSRP1.

Plasmid Construction—Deletions of clone 48 were made using unique restriction sites contained within clone 48 that allowed for in-frame fusions to the carboxyl terminus of the Gal4 activation domain in plasmid pGADT7 (CLONTECH). Flag-clone 48 was constructed by fusing the cDNA fragment isolated in the initial one-hybrid screen in-frame downstream of a flag epitope in vector pCITE. Myc-SSRP1 expression plasmid was provided by M. Baron.2 For plasmids used for in vitro transcription/translation, the SRF1 cDNA (amino acids 22–709) plus the NH2-terminal Myc epitope was excised from vector pMT2-SSRP1 by digestion with NotI and ligated in-frame into vector pCITE2A creating pCITE-SSRP1. The cDNA insert of clone 48 was excised with EcoRI and ligated in-frame into vector pCITE2A, creating pCITE-clone 48.

Cell Culture, Transfection, and Luciferase Assays—COS cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 0.01% penicillin, and 0.01% streptomycin. Cells were seeded at approximately 50% confluence in 6-well plates and grown for 18 h before transfection. Cells were transfected using Fugene 6 as recommended by the manufacturer (Roche Molecular Biochemicals) and harvested 24–48 h later. Typically, greater than 40% of the cells were transfected as detected by LacZ staining (not shown). Approximately 200 ng of luciferase reporters under control of the following SRF-dependent promoters were used: the 322-base pair SRF promoter (6), the 322-base pair SM22 promoter (6), and the 600-base pair α-myosin heavy chain promoter (32). A luciferase reporter under control of three tandem copies of the muscle creatine kinase MEP2 site and the E1b promoter (33) was also used. Transactivation assays also included 100 ng of pCGB-SRF or pML1 (34), 200 ng of near full-length SSRP1 (amino acids 22–709) or flag-clone 48 (amino acids 489–587), 100 ng of pDNA-MEF2C (33), and 50 ng of pHis68-LacZ were used in the transfections where indicated. In transfections not receiving the full complement of expression plasmids, total plasmid was adjusted to 1 μg with parental expression plasmid lacking insert. Luciferase assays were performed as described by the manufacturer (Promega). In each instance, luciferase data were normalized to β-galactosidase activity to correct for transfection efficiency.

Nuclear Extract Preparation, In Vitro Transcription/Translation and Gel Mobility Shift Assays—Nuclear extracts were prepared as described in Ref. 31. In vitro transcription/translation was performed as recommended by the manufacturer (Promega). SSRP1 products were generated from pcITE-SSRP1 and pcITE-clone 48. SRF was generated from plasmid pTDAATG (2). Gel mobility shift assays were performed using 32P-labeled complementary oligonucleotides spanning nucleotides 489–587 of the SM22 promoter. The sequence of the top strand was 5′-ACCTTGGTGTCTTTCCGAAAAATATGGACCTGTGCAGTG-3′. In gel mobility shift reactions using nuclear extract, approximately 5 μg of total protein was used. Approximately 5 μl of in vitro translated SSRP1 or clone 48 were used in the binding reaction with or without increasing concentrations of in vitro translated SRF. The gel mobility shift buffers, binding conditions, and electrophoresis conditions are described elsewhere (31).

Coimmunoprecipitation and Western Analysis—COS cells were transfected as described above with the following modifications. Cells were seeded onto 10-cm plates and transfection mixtures (volumes and amounts of DNA) were increased 10-fold. Two μg of plasmids expressing Myc-SSRP1 (amino acids 22–709) or flag-clone 48 (amino acids 489–587) were included in the transfections. Nuclear extracts were prepared as described above. Approximately 500 μg of nuclear extract was diluted to 1 ml in nondeaturing immunoprecipitation buffer (25 mM NaPO4, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 1 mM diethiothreitol, 1% glycerol, 1× EDTA-free Complete Protease Inhibitor (Roche Molecular Biochemicals)). Five μg of anti-SRF antiserum (Santa Cruz) and 50 μl of protein A/G beads (Santa Cruz) were added and incubated at 4 °C on a rotating wheel for 50 min. Reactions were spun at 2500 rpm for 3 min and pellets were washed three times with 500 μl of coimmunoprecipitation buffer. After the final wash, pellets were resuspended in 50 μl of 2× SDS sample buffer and boiled for 5 min. Samples were spun briefly and denaturing electrophoresis performed. Electrophoresis conditions and transfer to membrane were performed using standard protocols (35). Western blot analyses were performed using the ECL-PLUS detection kit (Amersham), anti-Myc (Santa Cruz), anti-flag (Kodak), and anti-SRF (Santa Cruz) antibodies. Quantitation was performed by densitometry of x-ray films.

Immunocytochemistry—COS cells were transfected as described above with the following modifications. Cells were plated onto sterile glass coverslips before transfection. Thirty-six hours after transfection, cells were fixed with 1× PBS, fixed in 3.7% formaldehyde for 5 min at room temperature, washed three times in 1× PBS, and then pre-blocked in 1× PBS containing 2% horse serum, 2% bovine serum albumin (fraction V), and 0.1% Nonidet P-40 for 30 min at room temperature. Anti-Myc or anti-flag antibody was added at dilutions of 1:200 and 1:400, respectively, in fresh pre-block solution and incubated an additional 30 min. Cells were then washed three times in 1× PBS. Anti-rabbit or anti-mouse fluorescein isothiocyanate-conjugated secondary antibody was then added at a dilution of 1:250 for 30 min in pre-block solution and cells washed again three times with 1× PBS before visualization.

RESULTS

Identification of SSRP1 as an SRF-Interacting Factor in the Yeast Modified One-hybrid System—Our initial goal was to identify factors from smooth muscle cells that interact with SRF, using a rat lung cDNA library in a yeast one-hybrid screen. Adult lung was used as the source of the cDNA library due to its high degree of vascularization and smooth muscle content. The SRF “bait” consisted of the entire 508-amino acid human SRF protein. Expression of SRF was verified by Western analysis of yeast extracts using an anti-SRF antibody (data not shown). As a reporter, we used a lacZ gene under control of 4 tandem copies of the proximal CarG box from the SM22 promoter, referred to as CarGnear (6), plus 30 nucleotides of 5′- and 3′-flanking sequence. CarGnear has been shown to bind SRF and to be essential for SM22 expression in subsets of smooth muscle in transgenic mice (6). Although the SRF bait used in the screen contained the SRF transcription activation domain (36), it failed to activate transcription of the lacZ reporter on its own in yeast. The inability of full-length SRF to activate transcription in yeast has been previously reported (37).

Screening of approximately 4 million primary yeast colonies for lacZ expression following transformation with the rat lung

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2 J. A. Spencer, M. H. Baron, and E. N. Olson, unpublished data.

3 L. Li and E. Olson, unpublished observations.
SSRP1 Interacts with SRF

FIG. 1. Interaction of clone 48 with SRF in yeast. A, schematic diagram of full-length human SSRP1. The SSRP1 protein is 709 amino acids and contains several regions of clustered charged residues. The acidic region spans amino acids 440–496. The HMG box spans amino acids 539–614 and is flanked by two short basic regions. B, clone 48 and amino- and carboxyl-terminal deletions fused to the GAL4 transactivation domain were expressed in yeast with SRF to determine the region of clone 48 that mediates the interaction with SRF. Residues 489–542 interact with SRF as detected by growth on selective media (minus histidine, minus leucine, minus tryptophan (~HLT)) and activation of the lacZ reporter gene, whereas residues 543–587 do not interact with SRF. No growth on selective media nor activation of the lacZ reporter was observed in the absence of SRF. C, comparison of the amino acids of SSRP1 and HMG-I(Y) shown to interact with SRF. Significant homology is observed between the two regions of SSRP1 and HMG-I(Y), particularly in the charged residues.

cDNA library resulted in identification of two clones, referred to as 48 and 62, that strongly activated lacZ and fulfilled all criteria for specificity (see “Materials and Methods”). Both clones, which were isolated from independent yeast transformations, contained cDNA inserts encoding amino acids 489–587 of the HMG protein SSRP1. This region encompasses eight amino acids of the acidic region, a basic region, and approximately the first two-thirds of the HMG box (Fig. 1A).

SSRP1-SRF Interaction Is Mediated by Amino Acids 489–542 of SSRP1—The modified yeast one-hybrid screen indicated that a 99-amino acid portion of SSRP1, residues 489–587, physically associated with SRF. To dissect this region further, we divided clone 48 into two parts, residues 489–542 and 543–587, and tested the ability of each region, when fused to the GAL4 TAD, to interact with SRF and activate the integrated CArG box-dependent reporter genes in yeast. As seen in Fig. 1B, residues 489–542 retained the ability to interact with SRF, while residues 543–587 were unable to interact with SRF, as measured by growth on selective media and LacZ activity. Neither clone 48 nor the two deletion derivatives activated expression of the reporter in yeast in the absence of SRF. These results demonstrate that activation of CArG-dependent transcription in yeast by clone 48-GAL-TAD is SRF-dependent and that this interaction is mediated by amino acids 489–542 of SSRP1. This region includes the basic region, but excludes the HMG domain.

Recently, residues 50–81 of HMG-I(Y), which include the third AT-hook DNA-binding domain, were shown to interact with SRF (21). To determine whether all the SRF-binding domain of SSRP1 might share homology with this region of HMG-I(Y), we compared the two domains using the MacVector Clustal Alignment program. Indeed, there was substantial homology between the two regions, particularly within the charged residues (Fig. 1C).

Clone 48 and Full-length SSRP1 Are Localized to the Nucleus in Mammalian Cells—To determine whether clone 48 and full-length SSRP1 colocalize to the nucleus, COS cells were transfected with expression plasmids encoding clone 48 with a flag-epitope tag or SSRP1 with a Myc-epitope tag and immunocytochemistry was performed. Full-length SSRP1 was localized to the nucleus of COS cells, as was the portion of SSRP1 encoded by clone 48 (Fig. 2). The nuclear localization of SSRP1 is consistent with its ability to interact with SRF and enhance SRF-dependent transcription (38). These data indicate that the nuclear localization of SSRP1 is contained within amino acids 489–587. This signal is probably contained within the basic region amino-terminal to the HMG domain of clone 48 and SSRP1 (Fig. 1A).

SSRP1 Activates SRF-dependent Promoter Activity in Mammalian Cells—Our one-hybrid data demonstrated that SSRP1 was capable of interacting with SRF in yeast. However, because SSRP1 was fused to the GAL4 transactivation domain, it was unclear whether native SSRP1 might influence SRF-dependent transcription in mammalian cells. We therefore transfected COS cells with a plasmid expressing near-full-length SSRP1 with or without SRF, and the effect on SRF-dependent transcription was determined using a variety of promoters linked to luciferase. Three SRF-dependent luciferase reporters were used in the analysis: SRF-luciferase containing the SRF promoter with two functionally redundant CArG boxes (31), SM22-luciferase containing 282 nucleotides of promoter sequence including CARGnear and CARGfar (6), and α-myosin heavy chain-luciferase containing approximately 600 nucleotides of promoter sequence with a single CARG box shown to bind SRF (32). As shown in Fig. 3, SSRP1 weakly activated these three promoters. Similar results were obtained with clone 48 (not shown). SRF was also able to activate transcription from all three promoters in COS cells. However, co-transfection of SSRP1 and SRF resulted in synergistic activation of all three promoters, suggesting a functional role for the SSRP1-SRF interaction identified in yeast. Since SSRP1 does not contain a transcription activation domain (24), the ability of SSRP1 to enhance activity of these promoters in the absence of exogenous SRF is likely to reflect synergy with endogenous SRF.

To determine if SRF-binding sites are required for SSRP1-dependent activation of transcription, an SRF promoter-lucif-
erases reporter containing in-context mutations of both CArG boxes was used. These mutations have been demonstrated to disrupt SRF DNA binding and ablate serum-inducible expression of the SRF promoter in fibroblasts (31). As shown in Fig. 3A, mutation of the CArG box sequences completely abolished SRF- and SSRP1-dependent activation. This demonstrates that SSRP1-dependent activation is mediated by the CArG boxes and not simply by a general up-regulation of transcription due to SSRP1 recruitment of other regulatory factors to the promoter.

To further investigate the requirement of SRF in SSRP1-mediated activation of transcription through the CArG box, we used a dominant negative SRF mutant (pM1) that lacks DNA binding activity but possesses the ability to dimerize with endogenous SRF (34). Transfection of pM1-SRF reduced expression of all three luciferase reporters, consistent with a primary role of SRF in regulating these promoters. More importantly, co-transfection of SSRP1 with pM1-SRF severely hampered the ability of SSRP1 to enhance CArG box-dependent expression, reflecting interference with endogenous SRF. These data demonstrate that SSRP1 is able to enhance CArG box-dependent transcription specifically through SRF and not through other mammalian CArG box-binding factors.

We also examined the role of the MADS box of SRF in SSRP1-dependent transcriptional activation. The MADS box of SRF, contained in amino acids 90–222 of SRF and referred to as SRFcore, was fused to the activation domain of VP16, creating the chimeric protein SRF-VP16. This chimeric protein also synergized with SSRP1 to activate SRF-dependent transcription (data not shown). These data demonstrate that SSRP1 enhances SRF-dependent transcription from a variety of promoters and suggest that the MADS box-DNA binding/dimerization domain of SRF is required to observe the effect.

SSRP1-enhanced Gene Expression Is Not a Generalized MADS Box-dependent Effect—Given the ability of SSRP1 to enhance the transactivation potential of a SRFcore-VP16 chimeric protein containing the MADS box of SRF, we considered the possibility that a similar effect might be observed with other MADS box-containing transcription factors. To test this, we transfected COS cells with a MEF2-dependent reporter gene in the presence or absence of SSRP1 and MEF2C. MEF2 contains a MADS box related to that of SRF, but it binds a different DNA sequence (CTA(T/A)TAG). As shown in Fig. 3D, MEF2C activated this reporter, whereas SSRP1 had no effect either alone or with MEF2C. This demonstrates that SSRP1 is not simply a MADS box-dependent transcriptional activator.

SSRP1 Increases Binding of SRF to the CArG Box—To determine whether SSRP1 increased transcriptional activity of SRF by enhancing SRF-DNA binding activity, we performed gel mobility shift assays with in vitro-translated SRF and SSRP1 and a labeled probe corresponding to the CArGnear sequence from the SM22 promoter. In the presence of SRF, a single DNA-protein complex was observed (Fig. 4A). SSRP1 did not show detectable binding to the probe, but it enhanced the binding of SRF. The SRF-containing complex exhibited the same mobility in the presence and absence of SSRP1, suggesting that SSRP1 enhanced SRF-DNA binding activity without forming a stable complex with SRF on DNA. The same results were observed in the presence of clone 48 protein (data not shown).

We next examined whether SSRP1 could enhance the DNA binding activity of SRF in vivo (Fig. 4B). Nuclear extracts from COS cells gave rise to a DNA-protein complex with the CArGnear probe that comigrated with the complex formed with in vitro translated SRF (lane 2 and data not shown). This complex was supershifted in the presence of anti-SRF antibody (compare lanes 2 and 3). By transfecting COS cells with an expression vector encoding Myc-SSRP1, we were able to assess the affects on DNA binding activity of SRF. No binding of either SSRP1 or clone 48 protein to the probe was observed (data not shown), consistent with the one-hybrid data demonstrating that SSRP1 fused to the GAL4 transactivation domain was incapable of activating CArG box-dependent transcription in the absence of SRF in yeast. However, in the presence of Myc-SSRP1 (lanes 4–6), there was a dramatic enhancement in SRF-DNA binding. Similar enhancement of SRF DNA binding activity was observed in the presence of clone 48 (lanes 7–9). Consistent with the conclusions from in vitro translation products, Myc-SSRP1 was not detected as forming a ternary complex with SRF (lanes 4–6). These data suggest that the enhancement of SRF-dependent transcription by SSRP1 is due, at least in part, to increased association of SRF with its binding site via an SSRP1-dependent mechanism.

SRF and SSRP1 Physically Associate in Vivo—Our results demonstrated that SSRP1 can enhance the ability of SRF to interact with the CArG box. However, we were unable to detect ternary complex formation between SRF and SSRP1 on the CArG box nor SSRP1-DNA interaction. To investigate whether SSRP1 could interact directly with SRF in the absence of DNA, nuclear extracts prepared from SSRP1-transfected COS cells were subjected to coimmunoprecipitation using an anti-SRF antibody followed by Western blot analysis with antibody against the epitope contained on SSRP1(Myc) and clone 48/Flag (Fig. 5A). As shown in Fig. 5B, lane 3, SSRP1 was readily detectable in transfected COS cells and was coimmunoprecipitated with SRF (lane 2), demonstrating a physical association between the two factors in vivo. Although clone 48 was expressed at appreciable levels in COS cells (Fig. 5B, lane 3), clone 48 failed to coimmunoprecipitate with SRF. In light of the functional data indicating that clone 48 could interact with SRF in yeast and enhance SRF-dependent transcription in mammalian cells, the inability to detect interaction of clone 48 and SRF in coimmunoprecipitation assays may suggest that the interaction of these proteins is less stable or more transient.
than for full-length SSRP1 and is therefore unable to be detected by coimmunoprecipitation.

To control for similar levels of SRF in each sample, immunoprecipitates were probed with anti-SRF antibody. Interestingly, as seen in Fig. 5C, approximately 3-fold more SRF protein was detected in SSRP1- and clone 48-transfected cells. Similar increases in SRF protein were confirmed by Western analysis using extracts from SSRP1-transfected cells before coimmunoprecipitation (not shown). These coimmunoprecipitations appear to be quantitative because a second round of immunoprecipitation failed to recover additional SRF (not shown). These results suggest that SSRP1 is able to enhance SRF-dependent transcription from the SRF promoter as it exists within chromatin. This is consistent with our transfection
Our results show the HMG factor SSRP1 interacts with SRF in yeast and mammalian cells and that SSRP1 enhances SRF-dependent transcription from a variety of SRF-dependent promoters, including the SRF promoter itself. The increased transcriptional activity of SRF in the presence of SSRP1 is accompanied by an increase in SRF-DNA binding activity in the absence of stable SSRP1 interaction with the CArG box or SRF bound to DNA. The enhanced transcriptional activity of SSRP1 in the presence of SSRP1 is not a general property of MADS box proteins because the myogenic MADS box factor MEF2C was not responsive to SSRP1. SSRP1 was also unable to activate transcription in yeast from a CArG box-dependent lacZ reporter gene. This suggests that SSRP1 cannot cooperate with MCM1, the yeast SRF-like MADS box factor. In addition, Irx1, a yeast SRF-like protein that confers sensitivity to cisplatin (40), does not interact with SRF, as evidenced by a lack of autonomous SRF-dependent activation of the reporter. This suggests that SSRP1-SRF interaction is highly specific.

SSRP1 is an HMG box protein identified initially by its ability to recognize and bind cisplatin-modified DNA (39). The functions of SSRP1-like proteins are only beginning to be elucidated, however, the evolutionary conservation of these proteins from plants to humans suggests a role in fundamental

**DISCUSSION**

Our results show the HMG factor SSRP1 interacts with SRF in yeast and mammalian cells and that SSRP1 enhances SRF-dependent transcription from a variety of SRF-dependent promoters, including the SRF promoter itself. The increased transcriptional activity of SRF in the presence of SSRP1 is accompanied by an increase in SRF-DNA binding activity in the absence of stable SSRP1 interaction with the CArG box or SRF bound to DNA. The enhanced transcriptional activity of SSRP1 in the presence of SSRP1 is not a general property of MADS box proteins because the myogenic MADS box factor MEF2C was not responsive to SSRP1. SSRP1 was also unable to activate transcription in yeast from a CArG box-dependent lacZ reporter gene. This suggests that SSRP1 cannot cooperate with MCM1, the yeast SRF-like MADS box factor. In addition, Irx1, a yeast SRF-like protein that confers sensitivity to cisplatin (40), does not interact with SRF, as evidenced by a lack of autonomous SRF-dependent activation of the reporter. This suggests that SSRP1-SRF interaction is highly specific.

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cellular processes. In this regard, recent studies have implicated SSRP1 in cell proliferation. Decreased expression of SSRP1 by antisense RNA impairs growth of fibroblasts in culture (41). SSRP1 has also been demonstrated to be upregulated in proliferating fetal kidney cells and iron-nitrotetroacetate-induced renal carcinomas (42), but is not readily detectable in normal adult kidney.

Based on its ability to recognize and bind cisplatin-modified DNA, SSRP1 was postulated to bind specific DNA structures targeting them for repair or altering their structure and thereby affecting DNA replication and transcription (39). SSRP1 also binds the positive regulatory element, PREII, from the human embryonic-like β-globin gene (24), resulting in an increase in transcription. However, SSRP1 does not contain a transcriptional activation domain (24). Thus, it has been proposed that SSRP1, like other HMG proteins, may play an architectural role in the control of gene expression by coordinating the assembly of multiprotein transcriptional complexes.

Given the ability of SSRP1 to recognize and manipulate DNA structure and its lack of a transactivation domain, two general models can be envisioned whereby SSRP1 enhances SRF-dependent transcription. One possibility is that the CArG box is a combination of the binding site for SRF and a structure recognized by SSRP1. In this case, SSRP1 might act as a molecular chaperone, transiently manipulating DNA structure and facilitating DNA binding of SRF. According to this model, SSRP1 would associate upon binding of SRF to DNA and would not be an essential component of the final complex. However, because SSRP1 does not bind detectably to the CArG box sequence and can be co-precipitated with SRF in the absence of DNA, we feel it is unlikely that the potentiation of SRF activity by SSRP1 requires interaction of SSRP1 with DNA. A second possibility is association of SRF and SSRP1 in the absence of DNA results in a change in conformation of SRF that increases its DNA binding activity, with a consequent increase in transcriptional potency. Again, SSRP1 would not be an essential component of the final complex. Based on our results, we believe this second model best explains the mechanism for SSRP1-mediated enhancement of SRF-dependent transcription. The ability of SSRP1 to increase DNA binding by SRF without forming a stable complex with SRF on DNA is reminiscent of the activity of the homeodomain protein phox/MHox (15). However, the possibility exists that SSRP1 physically associates with the CArG box but is not detected under the conditions of the assay system used.

Whether the observed stimulatory effect of SSRP1 on SRF-dependent transcription is entirely due to increased DNA binding by SRF remains unknown. Like other HMG proteins (22, 23), SSRP1 may also facilitate the formation of enhancesome complexes by altering DNA conformation, juxtaposing components of the transcriptional machinery in a more favorable orientation for transcription. Consistent with this possibility, the yeast SSRP1-like factor Poh3 has been implicated in forming a protein complex with Cdc68, manipulating chromatin structure, thereby enhancing transcription (43). It would be interesting to know if SSRP1 increases SRF association not only to DNA but to components of the transcriptional machinery previously shown to associate with SRF (44).

In SSRP1-transfected cells, we observed an increase in SRF expression. Given the ability of SSRP1 to increase expression of the SRF promoter, these results suggest that enhancement of SRF-dependent gene expression by SSRP1 can occur in the context of chromatin, as well as from reporter genes. An alternative explanation for the increase in SRF protein in SSRP1 cells would be an increase in stability of SRF in the presence of SSRP1. However, we do not favor this interpretation since SSRP1 enhances SRF promoter activity likely leading to increased SRF protein expression.

Recently, HMG-I(Y) was shown to interact with SRF and to potentiate SRF-dependent transcription (21). Although HMG-I(Y) interacts with the CArG box, enhancement or SRF-dependent transcription does not require interaction of HMG-I(Y) with DNA. Deletion mapping of HMG-I(Y) showed that amino acids 50–81, which encompass the third A/T-hook DNA-binding domain, mediate interaction with SRF. Intriguingly, the region of SSRP1 (amino acids 489–542) that interacts with SRF shows significant amino acid homology to this region of HMG-I(Y), suggesting that the interaction of these two HMG factors with SRF may be mediated by a common functional domain. However, it should be pointed out that although the SRF-interacting region of HMG-I(Y) maps to the AT-hook domain, SSRP1 does not contain an AT-hook.

SRF integrates growth factor signals and coordinates programs for muscle gene expression. These diverse roles in gene regulation are dependent on interactions with a wide range of signal-dependent and cell-specific cofactors. While our initial goal was to identify smooth muscle-specific cofactors for SRF, SSRP1 is expressed in numerous cell types in addition to smooth muscle cells and is therefore unlikely to confer smooth muscle specificity to SRF. Rather, given the ubiquitous expression of SSRP1, we believe we have identified a more general mechanism for potentiation of SRF-dependent transcription. The potential involvement of SSRP1 in the control of SRF-dependent genes during proliferation and differentiation is currently being investigated.

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