Physical Interaction Between the MADS box of Serum Response Factor and the TEA/ATTS DNA-Binding Domain of Transcription Enhancer Factor-1.

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ABSTRACT

Serum response factor is a MADS box transcription factor which binds to consensus sequences CC(A/T)_nGG found in the promoter region of several serum-inducible and muscle-specific genes. In skeletal myocytes SRF has been shown to heterodimerize with the myogenic basic helix-loop-helix family of factors related to MyoD, for control of muscle gene regulation. Here we report that SRF binds to another myogenic factor, TEF-1 that has been implicated in the regulation of a variety of cardiac muscle genes. By using different biochemical assays such as affinity precipitation of protein, GST-pull down assay and co-immunoprecipitation of proteins, we showed that SRF binds to TEF-1 both in vitro and in vivo assay conditions. A strong interaction of SRF with TEF-1 was seen even when one protein was denatured and immobilized on nitrocellulose membrane, indicating a direct and stable interaction between SRF and TEF-1 which occurs without a cofactor. This interaction is mediated through c-terminal subdomain of MADS box of SRF encompassing amino acids 204-244 and the putative 2nd and 3rd a-helix/β-sheet configuration of the TEA/ATTS DNA-binding domain of TEF-1. In the transient transfection assay, a positive cooperative effect of SRF and TEF-1 was observed when DNA-binding sites for both factors, SRE and M-CAT respectively, were intact; mutation of either site abolished their synergistic effect. Similarly a SRF mutant, SRFpm-1, defective in DNA binding failed to collaborate with TEF-1 for gene regulation, indicating that the synergistic trans-activation function of SRF and TEF-1 occurs via their binding to cognate DNA-binding sites. Our results demonstrate a novel association between SRF and TEF-1 for cardiac muscle gene regulation, and disclose a general mechanism by which these two super families of factors are likely to control diversified biological functions.
INTRODUCTION

Serum response factor (SRF) belongs to a super family of transcription factors which contain a highly conserved DNA-binding and dimerization domain termed as MADS-box, because of its homology among yeast (MCM1, Agamous) plant (Deficiens) and vertebrate (SRF) proteins (1,2). MADS proteins play a wide range of functions from development in plants to muscle cell differentiation and growth response in mammalian cells (reviewed in ref 2). SRF is a 67kDa molecular weight phosphoprotein that was first identified as a major regulatory protein controlling the serum-activated expression of the c-fos gene. SRF regulates gene transcription through binding to a consensus DNA sequence, the serum response element (SRE), CC(A/T)6GG also known as CArG box (2-4). Functional SREs are found in the promoter region of many immediate early genes involved in the mitogenic response of proliferative cells (2-4). Paradoxically, SREs are also found in the promoters of several muscle genes expressed in post-replicative myocytes, where they are involved in controlling the gene tissue-specificity as well as their response to hypertrophic growth stimuli (5-8). SREs between muscle and non-muscle gene promoters can be interchanged without loss of their function (9), thus suggesting that in addition of SRF binding to SRE in both types of promoters, its activity is very much governed by other cell-type and promoter specific factors interacting with SRF.

Several transcription factors have been identified that either interact directly with SRF or bind DNA sequences adjacent to SRE, in order to influence the SRF activity. These include Ying Yang 1 (YY-1) (10,11), homeodomain protein phox-1/MHox (4,12) and Nkx 2.5 (6), NF-kB (13), ATF6 (14), TCFs (tertiary complex factors of Ets family) (2), myogenic basic helix-loop-helix proteins (15) and high mobility group factor, SSRP1 (16). In skeletal myoblasts SRF interacts with myogenin-E12 (or MyoD-E-12) heterodimer, and this interaction has been implicated by which SRF activity is
modulated from proliferative myoblast to control muscle gene expression in differentiated myocytes (15). However, there are many other muscle genes which have a functional SRE, but do not require myogenic basic helix-loop-helix family of factors for their tissue-specific regulation. For example, genes expressed in cardiac myocytes such as cardiac a-actin (7), skeletal a-actin (17), MLC-2 (18), a- MHC (19), ANF (20), MCK (21) as well as cardiac troponin T (22,23) all possess SREs that are critical for their activation in cardiac muscle cell context, but are independent of MyoD regulatory mechanisms. Therefore, it is likely that the SRE of these promoters could be interacting with other muscle-specific regulatory elements for gene trans-activation. Recently, a considerable amount of evidence has indicated that SREs of skeletal a-actin and a-MHC gene promoters cooperate positively with another muscle-specific element M-CAT which is recognized by transcription enhancer factor-1 (TEF-1) (17,19,24). A combinatorial interaction between both these elements are found necessary for a1-adrenergic, TGFß and stretch induced activation of skeletal a-actin gene expression in cardiac myocytes and slow twitch skeletal muscle fibers (17,24,25). These findings raised a possibility of a mutual cooperation between SRF and TEF-1 proteins.

TEF-1 is a member of a new family of transcription factors that are characterized by a structurally conserved DNA binding domain, TEA/ATTS (26,27). Proteins containing TEA domain have been shown to control function in a variety of animals and plant phyla (for review see ref. 26). In humans, at least four different TEF-1 genes have been identified which encode hTEF-1, hTEF-3, hTEF-4, and hTEF-5 isoforms (26). Homologues of these isoforms have been also isolated from mice (28) and chicks (29). Members of TEF-1 family have been found to be important for muscle-specific expression of several cardiac and smooth muscle genes, and are targets of hypertrophic stimuli (23,24,29-31). A TEF-1 homologue, scalloped, in Drosophila, has been shown to play an important role in the lineage progression of sensory neuronal development (32). In mammals, functional TEF-1 is found as early as 2-8 cell stage in the mouse zygote development, and
inactivation of TEF-1 gene has been shown to result in embryonic lethality due to myocardial defects (33,34).

In this paper we tested the hypothesis whether SRF can physically interact with TEF-1. We demonstrate that SRF forms a stable complex with TEF-1 both in vitro and in cultured cells. The formation of this complex requires MADS and TEA DNA-binding domains of SRF and TEF-1, respectively. This interaction discloses a novel mechanism by which SRF activity could be modulated to control the expression of muscle genes in differentiated cells where MyoD-dependent regulatory mechanisms are not in play.
MATERIAL AND METHODS

Over-expression and purification of GST fusion proteins: The GST fusion proteins were expressed in bacteria and purified as described previously (31,35). In brief, bacteria harboring plasmids GST-SRF and GST-TEF-1 were grown overnight in LB-ampicillin medium. The next morning, cells were diluted 1:10 with fresh medium, grown to an OD of 0.6-0.75 and induced with 0.1 mM IPTG to direct expression of fusion proteins. After 3-7 hrs of expression of the GST-fusion proteins, cells were harvested and the fusion proteins isolated as follows. Cells were pelleted at 4,000 g at 4°C, re-suspended in phosphate-buffered saline (PBS) containing protease inhibitors and sonicated for a total elapsed time of 120 seconds. The bacterial lysate was solubilized by the addition of Triton X-100 to a final concentration of 1% and centrifuged at 13,000 g at 4°C to remove insoluble material. Glutathione-agarose beads were added to the soluble supernatant fraction, and the binding of GST fusion proteins was allowed to occur at 4°C for 30 minutes. The beads were pelleted in an Eppendorf centrifuge at 4,000 g for 2 minutes and the GST fusion proteins bound to the glutathione-agarose beads were washed thoroughly with PBS containing 0.1% Triton X-100. The integrity of the GST-fusion proteins bound to the beads was analyzed by resolving of proteins on SDS-PAGE and coomassie blue staining, along with known amounts of bovine serum albumin on the same gel to calculate the yield of full-length fusion proteins.

Preparation of nuclear extract and electro-mobility gel shift assay: Nuclear extract was prepared from neonatal rat hearts by the method of Dignam et al. (36), with slight modifications as described previously (35). For the electro-mobility gel shift assay (EMSA), double-stranded oligos were 5’ end-labeled with T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD) and [γ-32P]ATP. The analytical binding reaction was carried out in a total volume of 25 μl containing approximately 10,000
cpm (0.1 to 0.5 ng) of the labeled DNA probe, 2-5 μg of the nuclear extracts, and 1 μg of poly(dI-dC) (Sigma, Inc., St. Louis, MO) as a non-specific competitor. The binding buffer consisted of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1 mM EGTA [ethylene glycol-bis (β-aminoethylether)-N,N',N",N"-tetraacetic acid], 0.5 mM dithiothreitol, 0.3 mM MgCl₂, 8% glycerol, and 0.5 mM PMSF.

After incubation at room temperature for 20 minutes, the reaction mixtures were loaded on 5% native polyacrylamide gels (44:1, acrylamide/bisacrylamide), and electrophoresis was carried out at 150 V in a 0.5 X TBE buffer, in a cold room. For competition and antibody experiments, unlabeled competitor DNAs or the antibody were pre-incubated with nuclear extracts at room temperature for 15-20 minutes in the reaction buffers prior to addition of the labeled DNA probe. The Jurkat T cell nuclear extract, anti-SRF and anti-Flag antibodies used in this study were obtained commercially from The Stratagene Inc, LaJolla, CA and a monoclonal anti-TEF-1 antibody was purchased from The Transduction Laboratories, Lexington, KY. Sense-strand sequence of double-stranded oligos used in EMSA were:

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\text{a-MHC C(Ar)G,} \quad 5'\text{GTCCCAGCAGATGACTCCAAATTTAGGCAGCAGGCA}^3' \\
\text{cTNT M-CAT,} \quad 5'\text{AGTGGTGCATTCTCTCTGG}^3'
\]

Affinity-precipitation of SRF with the GST-TEF-1 protein: Five micrograms of GST or GST-TEF-1 bound to glutathione-agarose beads were incubated with 40 μg of neonatal rat heart or Jurkat T cell nuclear extract in 1x DNA-binding buffer for 3 hours at 4°C with continuous rocking. Glutathione-agarose beads without GST fusion protein were also incubated with nuclear extract to serve as a negative control. After 3 hours of incubation, beads were pelleted at 14,000 g for 2 minutes, the supernatant was collected and used directly for the mobility gel shift assay. To detect the interaction of SRF with GST-TEF-1 protein, the pelleted beads were washed five times with 1 ml of 1x DNA-binding buffer, suspended in 2x Laemelli's buffer and subjected to subsequent Western blot analysis.
In-vitro characterization of SRF binding to TEF-1: The TNT-coupled rabbit reticulocyte lysate system (Promega, Inc. Madison, WI) was used to translate pBS-Max, pBS-SRF, pBS-MyoD and pBS-myogenin plasmids. After translation, the specific incorporation of $^{35}$S-methionine into proteins was determined by TCA precipitation and the integrity of translated-proteins was checked by SDS-PAGE and autoradiography. For *in vitro* binding assay, $^{35}$S-labeled proteins were incubated with 2-3 µg of GST or GST-fusion proteins on glutathione-agarose beads in 1 x protein-interaction-buffer (PIB) (20 mM HEPES pH7.5, 75 mM KCl, 1 mM EDTA, 2 mM MgCl$_2$, 2 mM DTT and 0.5% NP-40) for 2 hrs at 4°C with continuous rocking. The beads were pelleted and washed five times with 1 X PIB. The bound proteins were eluted with Laemelli's sample buffer and analyzed on SDS-PAGE.

Co-immunoprecipitation of SRF with TEF-1: Jurkat T cells at a density of 1X10$^6$ cells/tube were transfected with the expression plasmid pCMV.Flag-TEF-1 (35) by using the electroporation procedure (BTX, Inc. Ca). Following 36 hrs of transfection, cells were collected, washed with ice cold PBS and lysed in a high salt (100 mM NaCl) lysis buffer. The cells were allowed to lyse on ice for 15 minutes and then were further disrupted by forcing them through 22 gauge needle several times. The lysed cell extract was spun to remove cell debries, and the supernatant (whole cell extract) was transferred to a fresh tube. It was checked for the expression of ectopically expressed Flag-TEF-1 protein by Western-blot analysis. For immunoprecipitation of proteins the whole-cell extract was incubated with 2µl of anti-Flag or anti-SRF antibodies (Santa Cruz Biotechnology) conjugated with agarose beads (1.8 mg antibody/ml) in a total volume of 1 ml for an hour with continuous rocking at 4°C. The beads were pelleted, washed 5 times in the lysis buffer, suspended in 2X Laemlli’s buffer and subsequently subjected to Western-blot analysis using either anti-Flag or anti-SRF antibodies.

In order to determine SRF-TEF-1 interaction in cardiac myocytes, the neonatal rat heart
nuclear extract (300-500 μg protein) was incubated with 0.5 μg of control mouse or rabbit IgG (species depends upon the source of the primary antibody) together with 20 ul of protein A/G-agarose (Santa Cruz Biotechnology, Inc., Ca) at 4°C, for 30 minutes. The pre-cleared nuclear extract (supernatant) obtained by centrifugation of reaction mixes was incubated with 20 ug of either anti-TEF-1 antibody or anti-SRF antibody at 4°C for 60 minutes. Protein A/G-agarose (20ul) was then added to each sample and incubated for 4 to 6 hours at 4°C, on a rotating plateform. The Absence of primary antibody in a parallel reaction mix served as negative control. Agarose beads were pelleted by centrifugation, washed four times in PBS (phosphate buffered saline) and reconstituted in 50ul of PBS. Proteins were removed from the beads by boiling in a 2X Laemllii's sample buffer and analyzed by Western-blot analyses using either anti-TEF-1 or anti-SRF-antibodies.

Far-Western analysis: Far-Western analysis was performed essentially according to the protocol as described before (37). A bacterial expression plasmid, pGEX-KG-cAMP-TEF-1 was generated to synthesize GST-cAMP-TEF-1 fusion protein. A double stranded oligonucleotide that encodes two cAMP-dependent protein-kinase-A (PK-A) sites was introduced into BamH1-EcoR1 sites of pGEX-KG to create the pGEX-KG-cAMP plasmid. Subsequently, an Nco1-Sac1 fragment containing full-length TEF-1 cDNA was subcloned into the Nco1-Sac1 sites of the plasmid pGEX-KG-cAMP to direct expression of GST-cAMP-TEF-1. The GST-cAMP-TEF-1 fusion protein was over expressed in DH5a cells and then it was isolated and labeled with catalytic subunit of the bovine cAMP-dependent PK-A as described before (37). The labeled GST-cAMP-TEF-1 fusion protein was eluted from beads and 2X 5×10^10 cpm of protein probe per ml was added to the nitrocellulose (NC) blot that contained 50 ng of each protein (GST-SRF, SRF, GST-Max, Max and GST) in 1x hybridization buffer (20 mM HEPES [pH7.7], 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 1 mM DTT, and 0.05% NP-40). The NC blot was incubated with the labeled GST-cAMP-TEF-1 protein overnight in a cold
The unbound labeled protein was removed by four washes with 1x hybridization buffer at room temperature. The NC-blot was wrapped in Saran wrap and subjected to autoradiography at -70°C for 4 to 12 hours.

Cell culture and transfection: Primary myocytes were cultured from 18 day old fetal rat hearts (see ref. 41). After differential plating to eliminate non-muscle cells, myocytes were plated at a density of 2 x 10⁶ cells/100 mm culture dish (Falcon brand, Becton Dickinson Labware) pre-coated with 0.1% gelatin in Ham's F-12 medium (Gibco BRL) with 5% calf serum. Cultures generally consisted of more than 90% myocytes, as measured by immunocytofluorescence with anti-myosin antibody. More than 90% of the cells began to contract spontaneously within 24 hours after plating. Jurkat and Cos1 cells were grown in growth medium containing Dulbecco's modified Eagle's medium (Gibco, BRL) supplemented with 10% fetal bovine serum in an atmosphere of 5% CO₂. All culture media contained penicillin (5 mg/ml), streptomycin (5 mg/ml) and neomycin (100 mg/ml).

Primary cultures of cardiac myocytes were transfected after 48 hours in culture with 5 µg of DNA/plate by use of a lipotaxi reagent (Stratagene, Inc, Ca.). Cos1 cells were transfected by using Calcium phosphate procedure. Typically cells were plated in 100mm plates and after reaching at 50% confluency they were transfected with 5µg of total DNA. All transfections contained 1 µg of the pCMV-βgal reference plasmid. The next morning (~18 hrs after transfection) medium was changed and, after an additional 48hrs cells were harvested, cell-lysate prepared, and assayed for luciferase, and β-galactosidase activities and protein content. The luciferase activity for each construct was corrected for protein content of each extract and normalized to the activity of β-galactosidase activity in the same cell extract.

Construction of plasmids: The bacterial expression plasmid pGEX-KG was utilized to direct over-
expression of full-length TEF-1 or TEF-1 mutants. Briefly, the full length TEF-1, truncated or point mutated versions of TEF-1 were amplified from pBS-TEF-1 (28) by PCR, digested with Xba1/Xho1, and subcloned into the Xba1/Xho1 sites of the pGEX-KG vector. The GST-TEF-1 expression plasmids were subsequently transformed into HB101 cells for inducible production of the protein. The skeletal a-actin reporter, -394/+24 Sk-a-actin-luc was constructed by subcloning nucleotides -394 to +24 bp Rsal-HindIII fragment of chicken Sk-a-actin into pXP1 vector. Linker-scanning bgIII mutants of the Sk-a-actin promoter have been described before (39). The p5xSREluc reporter plasmid is directed by an artificial promoter containing five copies of the SRF binding sites (no Ets binding sites) upstream of a minimal TATA box (provided by Dr. J. Solway, University of Chicago).

The 5x M-CAT-luc reporter plasmid containing five copies of cTNT M-CAT-1 element was generated by amplifying five M-CAT-1 sites from an artificial 5x M-CAT promoter/reporter plasmid (supplied by Dr. C.P. Ordahl University of California, San Francisco, ref. 40) and subsequently cloning into PGL-3 vector containing luciferase reporter gene (Promega, Inc. Madison, WI).

Oligonucleotide-primers used for generating TEF-1 mutants:
Forward primer containing Xba1 site, 5'TCTAGAGATTGAGCCCAGCAGCTGGAGCGGC 3',
Reverse primers with or without Xho1 site;
for TEF(1-210), 5' CTGAGTCACCAGGCAGGAACTGAGGGGGC 3'
for TEF (1-123) 5' CTGAGTCACAGGGCCTTGTCCTTGGCAGT 3'
for TEF(1-113) 5' CTGAGTCAGCTTGTTACCTTCAGCTTGGA3'
for TEF (1-103) 5'CTGAGTCAGCAGATTTTCCTCTGGAACACCTGAATGTGACTAGAC
ACCTGCTT3'.
for TEF (1-88) 5'CTGAGTCACCTTGTCTTTCCCGTCCTGAGTTGATGTATCTGGCTAT3'
for TEF (Helix-3mt) 5'CTGAGTCACCGAGATTTTCCTCTTGCAAGAACCCTGAATGGGGAC
TAGACACCCGGCTT3'.
for TEF (Helix-2mt) 5' CTTGGTTTTCCGAGTCCGATGTATCTGCTATCAA 3'.
For TEF (114-430) a forward primer, 5' TCTAGAGATGGATCAGACTGCAAGGAC 3' and reverse primer, 5' GGC CGGCTCGAGTCAGTCCTTCACAAGCCTGTAGATATGTTG 3' were used.
For TEF (27-113) the forward primer 5'TCTAGAGGTCTGGATGCTCTGATATTGAGCAG3' and the reverse primer same as used for TEF (1-113) construct.
For TEF (Helix-1mt) the forward primer 5' TCTAGAGGTCTGGATGCTCTGATATTGGCAGAGTTTCGGGGAG 3' and the reverse primer same as used for TEF (1-103) construct.
RESULTS

Physical interaction of TEF-1 with SRF: Our initial goal was to determine whether TEF-1 can physically associate with SRF. To test this possibility we first attempted to deplete the nuclear extract of SRF by affinity precipitation of the protein with GST-TEF-1 beads. The neonatal rat heart nuclear extract or Jurkat T cell nuclear extract, containing high levels of SRF, was incubated with GST-TEF-1 or GST proteins that were immobilized on the glutathione-agarose beads. After removal of GST beads from nuclear extract, the supernatant was analyzed by an EMSA using C(Ar)G oligo as a labeled probe. As shown in figure 1A, the specific SRF/DNA complex that could be supershifted by SRF antibody, was found to be completely abolished in nuclear-extract samples treated with GST-TEF-1 beads, but not with GST beads alone. These results suggested that a protein physically interacting with TEF-1 segment of the GST fusion protein must be involved in generating the C(Ar)G/protein complex. To find out whether SRF was indeed removed from the nuclear samples, we analyzed the presence of SRF on glutathione-agarose beads by Western blot analysis using an anti-SRF antibody. As shown in figure 1B, SRF was present on GST-TEF-1 beads but not on GST beads alone, thus indicating a direct interaction between SRF and TEF-1 proteins.

These results were confirmed in-vivo by co-immunoprecipitation of SRF with TEF-1 from both Jurkat T cells as well as cardiac myocytes. TEF-1 expression has been shown to be undetectable in lymphocytes (27). Therefore, in order to see TEF-1/SRF interaction in Jurkat T cells we over-expressed TEF-1 in these cells by transfecting them with an expression plasmid (pCMV.Flag.TEF-1) encoding the full-length TEF-1 tagged with a Flag peptide. For the control, cells were transfected with a parental plasmid (pCMV.Flag) lacking the TEF-1 cDNA. The 2nd day after transfection, cells were harvested and cell-lysate examined for expression of the Flag.TEF-1 protein by Western-blot analysis using an anti-Flag antibody. Cell-lysate that showed a high level expression of Flag.TEF-1 was then...
incubated with anti-Flag affinity gel beads. These beads were separated by centrifugation, washed repeatedly and analyzed by Western-blot analysis using an anti-SRF antibody to examine whether SRF was co-immunoprecipitated with the Flag.TEF-1 protein. As shown in figure 2, a ~67kDa SRF protein was pulled-down from cells transfected with the pCMV.Flag.TEF-1, but not from control cells that received the plasmid pCMV.Flag. In order to confirm these results we performed an inverse experiment, in which the cell-lysate was subjected to immunoprecipitation with an anti-SRF antibody linked agarose beads, and beads were analyzed by Western-blot analysis using an anti-Flag antibody. As expected a band of ~56 kda of the Flag.TEF-1 protein was found precipitated with SRF antibody (Fig. 2). In order to determine interaction of TEF-1 with SRF in cardiac myocytes, cardiac nuclear extract was first pre-cleared with IgG and resulting supernatant was subjected to immunoprecipitataion with either anti-TEF-1 or anti-SRF antibodies conjugated with agarose beads. Beads were pelleted, washed repeatedly and analyzed by Western-blot analysis. As shown in figure 3, while an expected band of ~ 54kda of TEF-1 was pull-down by an anti-SRF antibody, two bands of ~67kda and ~53kda of SRF were precipitated by an anti-TEF-1 antibody, whereas, neither protein was precipitated with IgG or agarose beads alone. This lower band of SRF is likely to be an alternatively spliced variant of SRF that has been shown highly expressed in the heart (57). These results strongly demonstrate an in-vivo TEF-1/SRF association.

Myogenic b-HLH proteins, myogenin and MyoD, have been shown to heterodimerize with E-12 proteins, in order to bind to SRF (15). Therefore, we were interested to test whether TEF-1 also requires a cofactor to interact with SRF. For this purpose we carried out another protein-protein interaction assay (far-western analysis) in which proteins were resolved by SDS-PAGE, transferred to NC membrane and then hybridized with 32P-labeled GST-TEF-1 fusion protein. In this assay protein-protein interaction takes place only when they are binding directly to each other without a cofactor. Previously, using the same strategy we have shown that N-terminal region of TEF-1 binds
to Max protein (35). Therefore, in this experiment we utilized interaction of TEF-1 with Max and no interaction with GST as positive and negative controls, respectively. As shown in figure 4, by this analysis also we found that TEF-1 strongly binds to SRF even when the protein is denatured and immobilized on the NC membrane. Together, these experiments demonstrate a direct association between TEF-1 and SRF both \textit{in vitro} and \textit{in vivo} assay conditions.

**Mapping regions of SRF and TEF-1 necessary for their interaction:**

In order to map the region of SRF required for binding to TEF-1 we examined the ability of different in-vitro translated SRF segments to interact with bacterially expressed GST-TEF-1 fusion protein. In vitro translation reaction was programmed with plasmids encoding either full length or different deletion mutants of SRF. After translation, glutathione-agarose beads bound with GST or GST-TEF-1 fusion protein were incubated with translated proteins. Beads were removed, washed extensively and analyzed by SDS-PAGE. As shown in figure 5C, the $^{35}$S-methionine labeled full length SRF (wt.) was retained on beads that contained GST-TEF-1, but not on beads with GST alone, thus further confirming a physical association between SRF and TEF-1 in \textit{in-vitro} conditions.

Removal of C-terminal 337 to 508 amino acids of SRF retained the ability of the protein to bind to GST-TEF-1. However, when N-terminal 1 to 244 amino acids region which contains MADS-box of SRF, was deleted, no TEF-1 binding was observed. To test whether MADS-box sequences are involved in TEF-1 binding another SRF mutant was analyzed which contains 144 to 244 amino acids region consisting of MADS-box. Results showed that these sequences are sufficient to bind to TEF-1. To further narrow down the TEF-1 binding region of SRF we analyzed an additional SRF mutant containing amino acids 1-204 region. This SRF mutant was incapable of binding to TEF-1, thus indicating that within the MADS-box amino-acids from 204 to 244 position are obligatory for SRF/TEF-1 association. Results of this analysis are summarized in the figure 5A.
To define the region of TEF-1 that was necessary for SRF binding, we analyzed several TEF-1 deletion mutants for their ability to interact with in-vitro translated SRF (Fig. 6). Each TEF-1 mutant was synthesized as a GST recombinant protein and only those preparations that showed more than fifty percent of the expected size of GST-fusion protein molecules were used for a further study. As shown in figure 6C, GST-TEF-1 mutants in which carboxy terminal region was deleted up to 113 amino acids retained the ability to bind to $^{35}$S-labeled SRF. Likewise, when 113 amino acids N-terminal region of TEF-1 was omitted (GST-TEF 114-430), no binding of SRF was observed. Another deletion mutation was generated in which N-terminus first 27 amino acids were deleted. This GST-TEF (27-113) mutant was also able to retain successfully in-vitro translated SRF. Thus, these data indicated that the 27 to 113 amino acids segment of TEF-1 consisting of the TEA/ATTS DNA-binding domain of TEF-1 is required for TEF-1/SRF association. In this assay conditions we utilized interaction of TEF-1 with Max, and an absence of interaction with b-HLH myogenic factors, MyoD or myogenin; as a positive and negative controls, respectively (31).

The TEA/ATTS DNA-binding domain of TEF-1 is located between 28 to 97 amino acids. It is predicted to consist of either three a-helices or one a-helix and two $\beta$ sheet structures (42). To determine the role of these regions and their potential a-helicity in SRF binding we generated additional TEF-1 mutants, in which amino acids were either deleted or those with high probability of a-helicity were replaced by either proline (P) or glycine (G), having very low probability of a-helix structure (43). Each mutant was synthesized as GST-fusion protein in a comparable amount and used to test for their ability to bind to SRF. As shown in figure 7, the N-terminal 103 amino acids region of TEF-1 was sufficient to bind to in vitro translated $^{35}$S-labeled SRF, and changing two amino acids, Glu (E) and Gln (Q) to G, in the first putative a-helix structure had no effect on their SRF binding ability. In contrast, replacement of two amino acids Ala (A) and Lys (K) to G in the putative 2nd a-helix, and Gln (Q) and His (H) to P in the 3rd a-helix completely abolished their ability
to bind to SRF. A TEF-1 mutant with deletion of amino acids corresponding to 3rd putative a-helix structure was also unable to bind to SRF, even when an excess amount of protein was used (Fig. 7C). These results revealed that amino acids of 2nd and 3rd a-helix/ß sheet, but not 1st a-helix, of the TEA/ATTS domain of TEF-1 mediate interaction with SRF. We also analyzed DNA-binding ability of these TEF-1 mutants using a M-CAT oligo as a labeled probe. As shown in figure 7B, TEF-1 mutant having only 1-103 amino acid region has ability to bind to DNA, albeit several fold lower than the binding ability of the full-length TEF-1. Substitution mutation of two amino acids in the putative 1st and 3rd a-helices configuration completely abolished their DNA binding ability (Fig. 7B). However, replacement of two amino acids in the putative 2nd α-helix/ß sheet structure resulted in weak but detectable DNA-binding activity, consistent with a previous report (42). From these results we conclude that while amino acids comprising 1st a-helix and 3rd a-helix/ß sheet of TEA/AATTS domain are absolutely required for DNA binding, the 2nd and 3rd a-helices/ß sheet portions are sufficient to bind to SRF.

Positive cooperation between TEF/SRF interaction:

In order to determine the physiological significance of TEF-1/ SRF interaction, we carried out a transient transfection analysis with primary cultures of cardiac myocytes or Cos1 cells by using reporter plasmids containing either -394/+24 bp promoter region of the skeletal a-actin gene consisting of both SRE and M-CAT sites or artificial promoters containing multiple copies of SREs or M-CAT sites. Cells were transfected with various combinations of the reporter plasmids and expression vectors encoding either full-length SRF or TEF-1 (Fig. 8). When an SRF expression vector was cotransfected with the reporter plasmid a modest increase (1.5 fold) in activity was observed. While the over expression of TEF-1 tended to suppress the activity of the reporter plasmid, with combination of both TEF-1 and SRF expression vectors the luciferase activity was
found to be elevated by 5-6 fold. This combinatorial effect of TEF-1 and SRF was concentration sensitive and maximal effect could be observed when equal amounts of both expression plasmids were used; raising the concentration of one over the other had a negative effect. In order to see whether this effect was mediated through binding of SRF and TEF-1 to their cognate DNA-binding sites, we utilized a reporter plasmid in which SRE1 sequences of Sk-a-actin gene were mutated. As shown in figure 8B, mutation of SRE-1 that can no longer bind to SRF (39) significantly reduced the synergistic effect of SRF and TEF-1. Mutation of an adjacent YY-1 binding site that has been shown before to be exerting a negative effect on SRE1 (17) further enhanced trans-activation activity of SRF and TEF-1 (data not shown). When a reporter plasmid was used in which the M-CAT site of the skeletal a-actin gene was mutared the positive cooperative effect of TEF-1/SRF was found to be completely abolished. These results indicated involvement of both SRE and M-CAT sites of the Sk-a-actin gene in this synergistic effect of TEF-1 and SRF. To further confirm these results, we analyzed two other reporter plasmids containing either multiple SREs or M-CAT sites. As shown in figure 8, neither SREs nor M-CAT sites alone could support the synergistic effect of the SRF and TEF-1. In order to see that a lack of synergistic effect of SRF and TEF-1 was not contributed by their inability to bind to each other, we also co-immunoprecipitated both proteins from these cells. And results indicated that SRF and TEF-1 efficiently associated with each other in these transfections (Fig. 8C). Next we asked whether DNA-binding by SRF was necessary for a cooperative effect with TEF-1. To address this point we examined the activity of a SRF mutant, SRF-pm1, which is defective in DNA-binding because of mutations in DNA recognition sequences (44), but it still retains the ability to bind to GST-TEF-1 (data not shown). As shown in figure 8, SRF-pm-1 plasmid alone showed a negative effect on sk-a-actin gene transcription, and when combined with TEF-1 this mutant was found incapable of supporting the synergistic effect of SRF and TEF-1 on gene regulation, even when a large range of concentrations of SRF-pm-1 and TEF-1 plasmids were tested. Collectively,
these results indicated that both SRE and M-CAT sites together are required for the positive cooperative effect between TEF-1 and SRF for gene regulation.

The requirement of DNA-binding sites of TEF-1 and SRF for their synergistic effect indicated that these two factors may co occupy M-CAT and/or SRE sites. To test this possibility an EMSA was performed in which labeled M-CAT oligo was incubated with either GST-TEF-1 or GST-TEF-1 combined with in vitro translated cold SRF. As shown in figure 9A, while SRF alone was incapable of binding to M-CAT oligo, in the presence of GST-TEF-1 it did produce a concentration-dependent slow migrating complex, and that was accompanied with disappearance of fast migrating complexes generated by smaller fragments of GST-TEF-1 proteins (Fig. 9A, arrow), thus suggesting that SRF has the ability to associate with TEF-1/M-CAT complex. Since in this experiment we did not see reduction of the complex generated by the full-length GST-TEF-1 we performed another experiment to test whether full-length TEF-1 and SRF have ability to co occupy a single M-CAT site. In a scaled up binding reaction in vitro translated cold TEF-1 was incubated with the labeled M-CAT oligo. After completion of reaction the TEF-1/M-CAT complex was separated out from the unincorporated radioactivity by a gel-shift analysis. The complex was visualized by autoradiography, eluted from the gel by electrophoresis and counted for the M-CAT radioactivity. In a protein binding buffer the equal counts of M-CAT/complex were then incubated with resin-beads linked either with GST-SRF, GST-MyoD, GST-myogenin or GST for 2 hrs in a cold room. The beads were separated by centrifugation, washed four times with 1x binding buffer and analyzed for the trapped M-CAT oligo radioactivity with beads. As shown in figure 9B, the GST-SRF beads retained tremendously greater amount of the radioactivity than any other beads tested, thus indicating that TEF-1/M-CAT complex is being retained on beads through binding to the SRF segment of the GST-SRF resin. We also analyzed the ability of SRFpm-1 mutant to retain the TEF-1/MCAT complex and results were positive (data not shown). These results provided further support that SRF was capable of binding
to M-CAT bound TEF-1 protein, and such an interaction may be partly important for the synergistic activation of gene transcription. An identical experiment to test whether SRF/SRE complex could be retained by GST-TEF-1 beads remained unsuccessful in these assay conditions.
DISCUSSION

A considerable amount of evidence has indicated an essential role of the SRF protein in muscle cell differentiation. For instance, (i) an SRF-related protein (SL-1) was found expressed in the presumptive heart region of the Xenopus early-bud embryo prior to detection of any marker of cardiac muscle differentiation (45). (ii) SRF transcripts are found enriched in the developing myocardium during mouse embryogenesis (7). (iii) SRF binding activity is increased almost 40 fold during avian myoblast differentiation that correlated with the appearance of the sarcomeric a-actin transcripts (8). (iv) Ectopic expression of SRF together with Nkx2.5 was found sufficient to induce expression of the endogenous cardiac a-actin gene in the 10T1/2 pluripotent fibroblast (6). (v) Neutralizing the SRF activity by micro-injection of SRF antibodies or SRF antisense oligonucleotides prevents differentiation of skeletal myoblast (46). These studies strongly suggest an obligate role of SRF in myogenic differentiation and muscle gene expression. During skeletal muscle cell development SRF has been reported to bind to DNA-binding domain of myogenic basic helix loop helix factors related to MyoD (15). Since cardiac myogenic program develops independently of MyoD related protein it was postulated that SRF must be interacting with other cardiac myogenic factors. In this study we tested this hypothesis and demonstrated that identical to SRF/MyoD interaction in skeletal muscle cells, SRF associates with TEF-1 in cardiac myocytes utilizing again DNA-binding domains of both proteins, and this collaboration activates transcription of a cardiac muscle gene. This type of collaboration can also provide an explanation why TEF-1 null mice shows defect only in the heart while TEF-1 is expressed both in cardiac and skeletal muscle cells (34).

Several lines of evidence obtained from our protein-protein interaction assays have shown that binding of SRF to TEF-1 occurs independently of their DNA-binding activity. For instance SRF was pulled down from solution by GST-TEF-1 when DNA-binding of either factor was not possible,
both proteins were co-immunoprecipitated by antibodies against either protein and SRF/TEF-1 mutants defective in DNA-binding interacted as efficiently as their wild-type counterparts. Furthermore, data obtained by the far-western analysis, in which radio-labeled GST-TEF-1 was found to interact with SRF and GST-SRF even when these proteins were denatured and immobilized on the NC membrane indicated that this is a direct and stable interaction between two proteins, and secondary structures of SRF preserved in this assay are sufficient for SRF-TEF-1 association. Results of this assay also demonstrate that SRF/TEF-1 interaction occurs without need of any cofactor, which is in contrast to requirement of E-12-MyoD heterodimer formation prior to binding to SRF (15). By analyzing different deletion mutants of SRF we have found that C-terminal half portion (amino acids 204-244) of the MADS-box is sufficient to bind to TEF-1. Previously, the core region of SRF comprising MADS-box has been found to be necessary for binding to several other transcription factors including bHLH myogenic factors, TCFs, Nkx2.5, and Phox-1 (Mhox) as well as for the response to certain signal transduction pathways, thus suggesting multiple roles of this region in SRF function. The homeodomain proteins Nkx2.5 (and perhaps Phox-1) has been shown to interact with N-terminal subdomain of the MADS-box (6). However, the region of SRF necessary for binding to tertiary complex factors of Ets family (Elk-1) has been mapped to C-terminal half of MADS (aa 175-to 217), in which dimerization of SRF was found to be a prerequisite for the formation of the Elk-SRF complex (47). Previously, Shore and Sharrocks have suggested that Elk-1 interacts with SRF either with a composite surface involving portions from both halves of the SRF dimer or with a motif which is exposed only upon dimerization (48). Our data presented in this study would indicate that dimerization of SRF was not a prerequisite for its binding to TEF-1, as we could observe TEF-1/SRF association even when SRF was denatured and immobilized on the NC membrane. These findings could also explain why SRF binding to TEF-1/M-CAT complex could be seen, while under identical conditions we were unable to detect binding of TEF-1 to SRF/SRE
complex. We believe that a single SRF molecule directly binds to TEF-1 to co-occupy the M-CAT site. However, when SRF is bound to SRE as a dimer it is accessible to make tertiary complex with Ets members, as reported before, but not to TEF-1. It is also possible that binding of TEF-1 to SRF reduces its affinity to SRE site and thus both factors could not be seen occupying SRE simultaneously in the conditions applied here. Yet, in vivo they may co-occupy SRE site with a lower affinity and that may be important for their coordinated trans-activation function. Recently, a reduced affinity of SRE to SRF has been shown to facilitate ANF gene transcription in cardiac myocytes (20).

TEF-1 is a prototype member of a large family of transcription factors containing a highly conserved TEA/ATTS DNA-binding domain. Amino acids sequences of the TEA/ATTS domain have been predicted to form three α-helices, or one α-helix and two β-sheets configuration (26,42). Previously, by mutagenesis, amino acids of helix1 and helix3 have been documented to be essential for protein-DNA contact; however, the role of the 2nd helix amino acids remained unknown (42). In the present study, by using deletion and amino-acid substitutions, we have shown that amino acids of 2nd and 3rd α-helix/β-sheet configuration of TEA/ATTS domain are necessary for SRF binding. Recently certain cellular proteins that bind to TEF-1 have been identified these include, bHLH-LZ protein, Max (35); TATA-binding protein, TBP (49); PARP (poly ADP-ribose polymerase) (50) and Drosophila vestigial (Vg) (51). However, until now no cellular protein binding directly to the TEA/ATTS domain has been found (except this study). A viral protein, SV40 TAg has been shown to directly bind to TEA/ATTS domain of TEF-1, and that leads to inhibition of TEF-1 DNA-binding activity, yet it activates transcription of the target viral gene (52). However, this does not appear to be the case with TEF-1/SRF interaction as we did not observe inhibition of TEF-1 DNA binding activity upon SRF binding. These findings may indicate that binding of SRF to TEA/ATTS domain of TEF-1 follows a different mechanism of gene activation than that exerted by TEF-1/TAg.
interaction for SV_{40} late promoter (52).

How does TEF-1/SRF association activate gene transcription? Results obtained from our transfection analysis have shown that both SRE and M-CAT sites were required for a cooperative trans-activation effect of these two factors, either site alone was not sufficient to yield synergistic effect. This is in agreement with previous reports where a coordination between SRE (or MEF-2 site) and M-CAT sites was shown important for the tissue specific and signal transduction induced expression of the troponinT (22) and skeletal a-actin genes (17,24,25). Several possibilities could be envisioned for the synergistic effect of TEF-1/SRF interaction. First; binding of SRF to TEF-1 might reduce affinity of the factor to its cognate DNA binding site, and a weaker and transient binding to DNA may allow more rapid transcriptional initiation to take place. There are examples for both SRF and TEF-1 where a lower affinity to DNA-binding site has been implicated for the activation of target gene transcription (20,31,38,53). Second; over expression of both TEF-1 and SRF has been shown to result in repression of gene transcription due to squelching phenomenon, and this negative phenotype could be relieved by over-expression of the sub-units of TATA-box binding proteins (49,54). Thus, the negative phenotype of TEF-1 and SRF is likely to be a result of their binding to TATA box associated proteins, which might lead to inhibition of transcription due to disruption of pre-initiation complex formation. In this context SRF/TEF-1 association might serve as a co-activator for each other and thus by antagonizing each other's inhibitory effect on the basal transcription-complex formation they may lead to gene activation. Third; binding of TEF-1 to SRF might exclude binding of negative acting factors such as Ets members, ERP/Net/Sep2 and/or YY-1 factors to SRF and thus allowing the DNA site to be occupied by more positive acting SRF complex to activate gene transcription (11,55). Recently a dominant negative isoform of SRF (\textsuperscript{?} 5 SRF) that possess MADS-box sequences but lacks most of the c-terminal portion of the protein has been shown to be expressed in skeletal and cardiac myocytes (57). This isoform has the ability to heterodimerize with
full-length SRF and suppress its gene activation potential. It is likely that TEF-1 through binding to the MADS-box of ?S5RF prevents it from binding to SRF and that this results in gene activation.

Fourth; SRF has been shown to bind to other cardiac myogenic factor, Nkx2.5 and GATA-4 via interaction with N-terminal domain of the MADS box (7,56). Thus, a possibility exist that binding of TEF-1 to C-terminal half of the MADS box may influence the binding of NKx2.5 and/or GATA-4 to other half of the MADS-box, and such a Nkx2.5/GATA4-SRF-TEF1 ternary complex activates gene transcription. This type of ternary complex could be analogous to the Phox1-SRF-Elk1 complex that has been implicated in controlling the c-fos gene expression in proliferating cells (47). Future studies directed towards determining exact amino acids of TEF-1 and SRF involved in their synergistic effect should be able to make a distinction among these possibilities.

In summary, in this study we have demonstrated that SRF and TEF-1 collaborate to activate muscle gene expression and that this cooperativity is mediated by direct protein-protein interaction between MADS and TEA/ATTS DNA-binding domains of these two heterologous family of transcription factors. Since MADS and TEA/ATTS domains are highly conserved such an interaction indicates that it would be a common mechanism for these two groups of members to control diversified biological functions, and specificity of this interaction may lie with tissue specific TEF-1 isoforms, other factors binding to these proteins and/or signaling mechanisms that influence their mutual interaction.
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Fig. 1: Affinity precipitation of SRF with GST-TEF-1. (A) Forty micrograms of nuclear extract (N.E.) was incubated in a binding buffer with 5 μg of GST or GST-TEF-1 linked glutathione-agarose beads for 3 hrs at 4°C. Beads were pelleted and EMSA was performed with an equal volume of supernatant from each tube. In lanes 3 and 7, nuclear extract was pre-incubated with 3 μl of anti-SRF antibody. SS represents super-shifted complex. In lane 2, a 100 fold excess unlabeled oligo (same as probe) was used as a competitor. (B) Pelleted beads from Jurket T cell nuclear extract were subjected to Western-blot analysis using an anti-SRF antibody.
Fig. 2: Characterization of *in vivo* TEF-1-SRF interaction as determined by co-immunoprecipitation of proteins from Jurkat T cells. Cells were transfected either with pCMV.Flag-TEF-1 encoding a Flag-TEF-1 fusion protein or parental vector, pCMV.Flag. The whole cell-lysate was subjected to immuno-precipitation (IP) with either anti-Flag or anti-SRF antibodies conjugated agarose beads. Beads were pelleted, washed, and subsequently analyzed by Western-blot analysis using anti-Flag or anti-SRF antibodies. For SRF blot 5µg nuclear extract (N.E.) of Jurkat T cells was used as a positive control.
Fig. 3. Co-immunoprecipitation of TEF-1 and SRF from cardiac myocytes: Neonatal rat hearts nuclear extracts (500 µg protein) was incubated with either 0.5 µg of control mouse IgG for TEF-1 immunoprecipitation, or rabbit IgG for SRF immunoprecipitation, together with 20 µl of protein A/G-agarose beads at 4°C for 30 minutes. The pre-cleared nuclear extract (supernatant) obtained by centrifugation was incubated with 20 µg of either anti-TEF-1 or anti-SRF antibodies at 4°C for 60 minutes for immunoprecipitations of proteins. Absence of primary antibody in parallel reaction mixes served as negative controls (indicated by control IgG). Resuspended protein A/G-agarose (20 µl) was then added to each sample and incubated at 4°C for 6 hours, on a rotating device. Beads were pelleted by centrifugation, washed four times in PBS and reconstituted in 50 µl of PBS. Proteins were removed from the beads by boiling in a Laemli’s sample buffer and subsequently analyzed by Western analyses using anti TEF-1 or anti SRF antibodies. Samples incubated with protein A/G agarose alone were utilized as an additional negative control (beads).
Fig. 4: TEF-1-SRF interaction as determined by a Far-Western analysis. Fifty nanogram amount of different proteins, as indicated above each lane, were resolved by SDS-PAGE, transferred to nitrocellulose membrane and hybridized with $^{32}$P-labeled GST-TEF-1 fusion protein. Membrane was washed and exposed to X-ray film at $-70^\circ$C for 6 hrs for autoradiography.
Fig. 5: Mapping the region of SRF necessary for TEF-1 binding. (A) Schematic representation of different SRF constructs analyzed and summary of their interaction ability with GST-TEF-1. The position of MADS-box containing DNA-binding and dimerization domain of SRF are depicted by different shades. (B) In the rabbit reticulocyte-lysate (R.R.) pBS-SRF constructs were transcribed/translated with $^{35}$S-methionine and 20% input of labeled proteins was resolved by SDS-PAGE. (C) The remaining portion of labeled-SRF was divided into two equal halves and incubated with either GST-TEF-1 or GST linked glutathione-agarose beads. Beads were pelleted, washed and proteins bound to beads were analyzed on SDS-PAGE in the lanes indicated. Wt = wild-type full-length SRF.
**Fig. 6:** Identification of TEF-1 interacting domain with SRF  

(A) Schematic diagram of different TEF-1 mutants and a summary of their observed binding activity with SRF. The hatched box indicates the position of TEA/ATTS domain of TEF-1.  

(B) In the rabbit reticulocyte lysate pBS-SRF, -MyoD, -myogenin and -Max plasmids were transcribed and translated with $^{35}$S-methionine.  

(C and D) The $^{35}$S-labeled SRF (C), MyoD or max (D) were incubated with GST or GST-TEF-1 fusion peptides on beads as indicated above each lane, and proteins bound to beads were analyzed on SDS-polyacrylamide gels.
Fig. 7: Fine mapping of the region with in TEA/ATTS domain of TEF-1 that binds to SRF. (A) Diagrammatic representation of TEA/ATTS domain of TEF-1 containing three α-helices or one α-helix and two β-sheet structures. Amino acids that have been mutated are shown with single letter symbols with their positions in the protein. (B) EMSA showing the DNA-binding ability of different TEF-1 mutants as indicated above each lane. (C) Interaction of different GST-TEF-1 mutants with in vitro translated 35S-labeled SRF is shown.
Fig. 8: Positive co-operation between SRF and TEF-1 for gene activation  (A) Schematic representation of different reporter plasmids used in this study. (B) Cos1 cells were co-transfected with 3µg of reporter plasmid and 0.1µg of each expression plasmid (pCGN-SRF, pCGN-SRF-pm-1 and pCMV-TEF-1) either alone or in combination as given below the bar diagram. In each instance a β-gal expression plasmid was included to normalize for transfection efficiency. For each reporter plasmid luciferase activity (mean ± SE) is derived from 6 to 10 different transfection experiments. (C) Co-immunoprecipitation of TEF-1 and SRF from Cos1 cells transfected with 5xSRE-luc reporter plasmid and expression vectors encoding TEF-1 and SRF. Nuclear extracts (100 µg protein) pre-cleared with rabbit IgG was incubated with 10 µg of anti-SRF antibody and protein A/G-agarose beads (20 µl) for immunoprecipitations of proteins, as described in legend of figure 3. Beads were pelleted, washed in PBS and boiled in 2x Laemmli's sample buffer and subsequently analyzed by Western blot analyses using an monoclonal anti TEF-1 antibody. Samples cleared with normal rabbit IgG (no SRF antibody) or incubated only with protein A/G agarose were utilized as negative controls.
Fig. 9: Co-occupation of SRF-TEF-1 complex on a single M-CAT site. (A) EMSA was performed using M-CAT oligo as a labeled probe and different combinations of proteins as indicated above each lane. In 3rd lane 50x unlabeled M-CAT oligo was included as competitor. Arrow indicates position of fast migrating TEF-1 complex that disappears upon inclusion of SRF in the binding reaction. (B) In a protein binding buffer the radio-labeled M-CAT oligo was incubated with in-vitro translated TEF-1, the unincorporated probe was separated from the DNA-protein complex by a gel-shift analysis. The radioactive M-CAT/TEF-1 complex was eluted from the gel and incubated with beads linked either to GST, GST-SRF, GST-MyoD or GST-myogenin. Beads were separated, washed extensively and counted for the trapped M-CAT radioactivity.
Physical interaction between the MADS box of serum response factor and the TEA/ATTS DNA-binding domain of transcription enhancer factor-1
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