Increased actin polymerization reduces the inhibition of serum response factor activity by Yin Yang 1

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Recent evidence has implicated CC(A/T)nCGG (CArG) boxes, binding sites for serum response factor (SRF), in the regulation of expression of a number of genes in response to changes in the actin cytoskeleton. In many cases, the activity of SRF at CArG boxes is modulated by transcription factors binding to overlapping (e.g. Yin Yang 1, YY1) or adjacent (e.g. ets) binding sites. However, the mechanisms by which SRF activity is regulated by the cytoskeleton have not been determined. To investigate these mechanisms, we screened for cells that did or did not increase the activity of a fragment of the promoter for a smooth-muscle (SM)-specific gene SM22α, in response to changes in actin cytoskeletal polymerization induced by LIM kinase. These experiments showed that vascular SM cells (VSMCs) and C2C12 cells increased the activity of promoters containing at least one of the SM22α CArG boxes (CArG near) in response to LIM kinase, whereas P19 cells did not. Bandshift assays using a probe to CArG near showed that P19 cells lacked detectable YY1 DNA binding to the CArG box in contrast with the other two cell types. Expression of YY1 in P19 cells inhibited SM22α promoter activity and conferred responsiveness to LIM kinase. Mutation of the CArG box to inhibit YY1 or SRF binding indicated that both factors were required for the LIM kinase response in VSMCs and C2C12 cells. The data indicate that changes in the actin cytoskeletal organization modify SRF activity at CArG boxes by modulating YY1-dependent inhibition.

Key words: CArG box, cytoskeleton, jasplakinolide, LIM kinase, transcription factor.

INTRODUCTION

The promoter regions of genes encoding many muscle-specific contractile proteins contain CC(A/T)nCGG (CArG) boxes. These sequences are the binding sites for the transcription factor serum response factor (SRF), which was first identified as an activator of the proto-oncogene Fos [1, 2]. Analysis of the promoters of many muscle-specific genes in vitro and in vivo has shown that these CArG boxes are important in the regulation of promoter activity in the three major types of muscle cells. For example, the CArG boxes present in the promoter of the smooth-muscle (SM)-specific gene SM22α are required for high-level activity of this promoter in vitro [3] and are essential for activity of the promoter in vascular SM cells (VSMCs) in vivo [4, 5]. Similarly, the CArG boxes present in the cardiac α-actin promoter are required for the expression of cardiac α-actin in both the cardiac- and skeletal-muscle cell lineages [6].

Although SRF is recognized as a major regulator acting through CArG boxes, many of these boxes and/or their immediate flanking sequences contain consensus sites for other DNA-binding proteins that are important in the regulation of SRF activity. For example, in skeletal muscle, SRF has been shown to interact with the tissue-restricted transcription factor MyoD to promote transcription from some skeletal muscle-specific promoters [7], whereas in cardiac-muscle SRF interacts with the cardiac-restricted transcription factor Nkx2.5, resulting in increased transcription from cardiac muscle-specific promoters [8, 9]. These interactions of SRF with other transcription factors may account for the ability of SRF to regulate tissue-restricted gene expression. SRF activity is also regulated by transcription factors that are widely expressed. The CArG boxes present in the promoters of many genes contain binding sites for the transcription factor Yin Yang 1 (YY1) [10] that can increase [11] or inhibit [8, 12] SRF activity. The mechanisms by which YY1 regulates SRF activity may depend on the specific CArG-box sequence or context. For example, analysis of the CArG box in the Fos promoter has indicated that YY1 increases SRF binding and SRF-dependent promoter activity [11]. Conversely, YY1 has been shown to inhibit SRF-dependent activity of the skeletal-muscle α-actin promoter and to compete with SRF for binding to a CArG box within this promoter [8, 12].

SM-actin RNA levels are regulated by changes in the organization of the actin cytoskeleton [13], but the mechanisms by which this occurs are unknown, although recent experiments have implicated CArG boxes and SRF in this response [14, 15]. Using a mammalian two-hybrid screen that was designed to identify partners for SRF, Sotiropoulos et al. [14] found that LIM kinase increased the activity of a SRF-dependent reporter gene. The only known function of LIM kinase is phosphorylation and inactivation of the actin-depolymerizing protein cofillin to promote actin filament formation, suggesting that the polymerization of the actin cytoskeleton may modify SRF activity. Further studies by Sotiropoulos et al. showed that drugs that reduced the G-actin pool (e.g. jasplakinolide, which binds to and stabilizes F-actin [16] or swarmholide A, which sequesters G-actin dimers) increased the activity of the SRF-dependent reporter. Conversely, agents that increased the monomeric G-actin pool (e.g. latrunculin B, which sequesters monomeric G-actin or C2 toxin, which inhibits actin polymerization) reduced the activity of the reporter gene [14].

The coupling of actin cytoskeletal organization to the modulation of CArG-box activity provides a potential mechanism for the control of muscle gene expression by many factors. These factors include interaction with the extracellular matrix and...
mechanical stretch, both of which modify the polymerization of the actin cytoskeleton [17–20]. However, the molecular mechanisms by which the actin cytoskeleton regulates SRF activity have not been identified. For example, it is not known whether control is exerted by an increase in the intrinsic activity of SRF or by inhibition of a repressor of SRF. Furthermore, the effects of the actin cytoskeleton on the activity of promoters containing CArG boxes are dependent on the specific promoter [14] (e.g. the vinculin and SRF promoters are activated by the polymerization of the cytoskeleton but the Fos promoter is not). It is therefore probable that the mechanism by which SRF activity is modulated involves additional DNA-binding factors.

To investigate the mechanisms by which the actin cytoskeleton modifies SRF activity, we screened for cells that did not increase the activity of a chloramphenicol acetyltransferase (CAT) reporter gene under the control of a SM22α promoter. In a previous study, we showed that the activity was regulated by SRF in a number of cell types [21]. We compared the proteins that bound to the CArG box in these transfection experiments with those in cells in which the activity of the same reporter gene construct was increased in response to modification of the actin cytoskeleton. These studies identified YY1 as a candidate regulator of the SM22α promoter by Dr J. Sinclair (University of Cambridge, U.K.) and Professor P. Caroni (Friedrich Miescher Institute, Basel, Switzerland).

EXPERIMENTAL

Vectors

The promoter constructs pSM22-319/+65, pSM22-197/+65 and pSM22-118/+65 contain the regions -319 to +65, -197 to +65 and -118 to +65 of the rat SM22α promoter [3] cloned into pCAT basic (Promega). The mutant forms of the promoter pSM22AY1, pSM22ASRF, pSM22AY1ASRF and pSM22YY1rev were generated by PCR using the forward primers CCGACAGACTGTCACTAGGTGTCTTTCCCAATTAAGGGAGGCTGTGTGG, CCGACAGACTGTCACTAGGTGTCTTTCCCAATTAAGGGAGGCTGTGTGG, CCGACAGACTGTCACTAGGTGTCTTTCCCAATTAAGGGAGGCTGTGTGG and CCGACAGACTGTCACTAGGTGTCTTTCCCAATTAAGGGAGGCTGTGTGG together with the reverse primer AAGGCTTGGTCGTTTGCTTTCTCCATATTTGGAGCCTGTGTGG to generate pCAT basic and sequenced. pcDSRFL [21] has been described previously.

Expression vectors for YY1 and LIM kinase-1 were donated by Dr J. Sinclair (University of Cambridge, U.K.) and Professor P. Caroni (Friedrich Miescher Institute, Basel, Switzerland).

Cell culture and transient transfection

P19 embryonic carcinoma cells and C2C12 myoblasts were grown and transfected as described previously [21,22]. For P19 cells, the DNA used in each transfection was made up of 1 µg CAT vector, 0.5 µg pCD/lgal, 0.25 µg of each test vector (as indicated in the Figure legends) and pCDNA3 was added as required to a total DNA content of 600 ng. Rat aortic VSMCs were derived and grown as described previously [23]. For transfection, VSMCs were seeded into 6-well plates at 2 x 10⁴ cells/well and grown for 24 h. A 3 µl aliquot of Fugene (Roche) and 97 µl of OptiMEM (Gibco) was mixed with 1.6 µg DNA [made up of 0.5 µg CAT vector, 0.5 µg pCD/lgal, 0.3 µg of each test vector (as indicated in the Figure legends) and pCDNA3 was added as required] and incubated at room temperature for 15 min. The medium was replaced with Dulbecco’s modified Eagle’s medium supplemented with 10% FCS and the transfection mixture added. Cells were harvested 48 h after transfection, lysed and assayed for CAT and LacZ activity as described previously [21,22]. The protein content was measured using Bradford assay. All transfections were set up in triplicate and repeated 2–4 times.

Nuclear extraction and electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared as described in [21] from VSMCs, P19 cells or C2C12 cells grown to confluence, washed twice in ice-cold PBS and scraped into 0.4 ml of cell lysis buffer (10 mM Hepes, pH 7.9/0.1 mM EGTA/0.1 mM EDTA/1 mM dithiothreitol/10 mM KCl/proteinase inhibitors; Complete Boehringer). The cells were allowed to swell for 15 min before 25 µl of Nonidet P40 (10%) was added and the cells were vortexed vigorously for 10 s and centrifuged for 5 min in a microcentrifuge. The nuclear pellet was resuspended in 50 µl of nuclear extraction buffer (20 mM Hepes, pH 7.9/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/400 mM NaCl) by pipetting and incubated on ice for 30 min. The samples were centrifuged for 5 min in a microcentrifuge, the supernatants removed and stored at −80 °C. EMSA was performed using the CArG-near oligonucleotides as described previously [21]. Anti-SRF (Santa Cruz SC335) and anti-YY1 (Santa Cruz SC281) were added to the binding reactions prior to the addition of nuclear extract.

Northern-blot analysis

RNA was extracted, as described previously, from VSMCs grown to confluence, then treated with jasplakinolide (Molecular Probes) for 2, 4, 8 and 24 h. RNA (10 µg) was separated on a 1% agarose formaldehyde gel, blotted and probed with an SMα-actin or SM22α probe then stripped and re-probed for GAPDH as described previously [24].

Western blotting

Nuclear extracts from P19, C2C12 cells and VSMCs were run on SDS/PAGE (10% gel). The proteins were transferred to a PVDF membrane at 0.8 mA/cm² of membrane for 1 h using a semi-dry blotter (LKB). The membrane was rinsed in PBS and blocked for 30 min in block buffer (1 X TBS/5% dried milk powder/0.1% Tween 20) at room temperature before being incubated with 2 µg/ml anti-YY1 antibody (Santa Cruz) for 3 h in block buffer. The membrane was washed with five changes of TBS/0.05% v/v Tween 20 (3 min per wash) before being incubated with a 1:5000 dilution of horseradish peroxidase-linked anti-rabbit IgG (Sigma) for 1 h in block buffer. After washing, the proteins were visualized by SuperSignal (Pierce, Rockford, IL, U.S.A.) in accordance with the manufacturer’s instructions.

RESULTS

Actin polymerization increases SM gene expression

Actin cytoskeletal polymerization is promoted by jasplakinolide and so its effects on the expression of the endogenous SM22α and
SMα-actin genes in VSMCs were determined. RNA was extracted from VSMCs 2, 4, 8 and 24 h after addition of jasplakinolide. Northern-blot analysis showed that expression of both genes had increased by 2 h (2-fold compared with GAPDH; Figure 1) and continued to increase up to 8 h (3-fold in SM22α and 2.5-fold in SMα-actin, compared with GAPDH; Figure 1). It is therefore assumed that the effects on SM-specific genes of modulating the actin cytoskeleton with jasplakinolide or, because it has similar effects on the polymerization state of the actin cytoskeleton, LIM kinase can be analysed using reporter genes under the control of SM22α promoter constructs.

LIM kinase increases SM22α promoter activity in C2C12 cells and VSMCs but not in P19 cells

To identify cells in which alteration of the actin cytoskeleton did not modulate SM22α promoter activity, the effect of LIM kinase on the activity of a fragment of this promoter (pSM22α – 319/+65) was determined in a range of cell types. These experiments showed that LIM kinase did not affect the activity of the SM22α promoter in P19 cells (a pluripotent cell type derived from an embryonic carcinoma) but increased the activity by 3-fold in C2C12 cells (a myoblast line that can be differentiated into skeletal-muscle myotubes) and approx. 2-fold in VSMCs (Figure 2a).

The pSM22α – 319/+65 promoter construct contains two CARG boxes (CARG near and CARG far). To determine whether either or both of these boxes were required for the response to LIM kinase its effects on two shorter fragments of the SM22α promoter, pSM22α – 197/+65 (1 CARG box; CARG near) and pSM22α – 118/+65 (no CARG box), were determined. Cotransfection of LIM kinase with pSM22α – 197/+65 did not affect the reporter gene expression in the P19 cells, but increased the activity of pSM22α – 197/+65 by approx. 3-fold in C2C12 cells and 2-fold in VSMCs (Figure 2b). However, there was no effect of LIM kinase on the activity of pSM22α – 118/+65 (no CARG box) in any of the three cell types (Figure 2c). These data indicate that the response of the SM22α promoter to LIM kinase in VSMCs and C2C12 cells requires only one CARG box.

Further analysis of the control of SM22α promoter activity was therefore focused on the pSM22α – 197/+65 construct.

Analysis of CARG box-binding proteins in the three cell types

The absence of an effect of LIM kinase on the activity of the SM22α promoter in P19 cells can be explained by at least three mechanisms: (i) the SM22α promoter may be maximally activated by endogenous SRF; (ii) an essential component of the signalling pathway from the cytoskeleton to the CARG box may be missing (or inoperative) or (iii) the pathway may be repressed. Bandshift experiments were therefore performed to compare the proteins present in nuclear extracts from the three cell types that bound to the CARG near-box sequence. P19 nuclear extracts contained only one major binding protein, whereas C2C12 extracts contained three binding proteins and VSMC extracts contained at least five (Figure 3). A nuclear protein complex of the same mobility was observed in all three cell types. Each experiment was repeated twice and the data presented are from a representative experiment.
Figure 3  CArG near-binding proteins present in the nuclear extracts from the three cell types

(a) Nuclear extracts were prepared as described in the Experimental section. Extracts were incubated with a 32P-labelled oligonucleotide probe containing the CArG-near box in the presence or absence of antibodies raised against SRF or YY1 as indicated. Arrows indicate the position of SRF and YY1. (b) Each nuclear extract (20 μg) was separated on SDS/PAGE (10% gel) and analysed by Western blotting for YY1 as described in the Experimental section.

cell types and this complex was supershifted by an antibody raised against SRF, consistent with the previous observations in P19 cells and C2C12 cells (Figure 3). Two complexes of the same mobility as those in the C2C12 cells were also observed in VSMCs.

Sequence analysis of the CArG-near box and its immediate flanking sites showed that it contained consensus binding sites for a number of transcription factors, in addition to SRF, including the transcription factor YY1. Addition of an anti-YY1 antibody inhibited the formation of a complex, normally formed between the CArG-near box and a component of C2C12 and VSMC nuclear extracts (Figure 3a), indicating the presence of YY1 in these extracts. These results are consistent with the observations of Strobeck et al. [25], who showed that YY1 present in nuclear extracts from the VSMC cell line A7R5 could bind to CArG near. The absence of any other similar complex in the P19 cell extracts indicates that either these cells do not express YY1 or that the YY1 present in the cells is not capable of binding to the DNA. Western blotting was used to determine whether the P19 cells expressed YY1 protein. This analysis showed that all the three cell types contained a protein of ~68 kDa that was recognized by the anti-YY1 antibody (Figure 3b). The C2C12 cells and P19 cells also contained a 43 kDa protein that was recognized by the anti-YY1 antibody (Figure 3b). Proteins of these sizes have been shown previously by in vitro translation [26] and Western blotting [27] to be YY1 and a truncated form of YY1 containing the C-terminus. Together these observations indicate that in P19 cells this protein does not bind to CArG near. These results suggested as a working hypothesis that both SRF and YY1 act as regulators of SM22α promoter activity and that YY1 conferred sensitivity to changes in polymerization of the actin cytoskeleton. In this hypothesis, the absence of YY1 DNA-binding activity in P19 cells accounts for the insensitivity towards LIM kinase.

Exogenous YY1 conferred responsiveness of pSM22 −197/+65 to LIM kinase

The hypothesis was tested first in P19 cells by transfection of the cells with the pSM22 −197/+65 promoter construct and expression vectors for SRF, YY1 or LIM kinase. Co-transfection with SRF showed a significant increase in promoter activity, indicating that the absence of a response to LIM kinase in these cells was not due to maximal activation of the promoter by endogenous SRF (Figure 4a). These data also indicate that SRF alone is not sufficient to confer sensitivity to LIM kinase. However, transfection with YY1 resulted in a 50% inhibition of SM22α promoter activity. Furthermore, co-transfection of YY1 with SRF inhibited activation of the promoter by SRF (Figure 4a).

To determine whether YY1 conferred responsiveness of pSM22 −197/+65 to LIM kinase, P19 cells were transfected with YY1 with or without LIM kinase. In these experiments, LIM kinase relieved 60% of the inhibition caused by YY1 (Figure 4b). YY1 also inhibited the activity of pSM22 −319/+65 (containing two
Cytoskeletal regulation of YY1 activity

Figure 5 Effect of SRF, YY1 and LIM kinase on the activity of pSM22 \( -197/+65 \) in C2C12 cells

C2C12 cells were transfected as described in the Experimental section with pSM22 \(-197/+65\) and (a) pCDSRF-L or pYY1, (b) pCDSRF-L or pLimK or (c) pLimK or YY1. Cells were harvested 48 h after transfection, CAT and LacZ activity were determined and CAT activity was normalized to LacZ activity for the same sample. Data are presented as fold activation relative to transfection without test constructs (see the Experimental section) for the same experiment. Each experiment was repeated three times and the data presented are from a representative experiment.

CArG boxes) and this effect was partially relieved by the addition of LIM kinase (results not shown).

These results indicate that in P19 cells, YY1 inhibits SRF activation of the SM22\(\alpha\) promoter. The results are consistent with the proposed hypothesis for regulation of activity by LIM kinase via YY1.

Regulation of the SM22\(\alpha\) promoter in C2C12 cells and VSMCs

C2C12 cells contain endogenous YY1 that bind to the SM22\(\alpha\) CArG-near box, in addition to SRF. Experiments were performed to determine whether the responses of these cells to SRF, YY1 and LIM kinase were consistent with the model derived from the P19 cell data. Transfection studies to determine the effect on the pSM22 \(-197/+65\) promoter of each of these regulators separately and in combination showed that SRF activated the promoter, whereas YY1 had little effect on its own but completely inhibited the activation by exogenous SRF (Figure 5). SRF did not further increase the activation obtained with LIM kinase, but YY1 reduced the activation by LIM kinase (Figure 5). These data for C2C12 cells are consistent with the simple hypothesis derived from the P19 cell data and suggest that in the absence of exogenous LIM kinase the SM22\(\alpha\) promoter activity is repressed by endogenous YY1.

Experiments similar to those on C2C12 cells were also performed on VSMCs (Figure 6). In these cells, LIM kinase activation was antagonized by co-transfection with YY1 as for P19 and C2C12 cells. However, a striking difference was observed in the response to SRF which did not activate the pSM22 \(-197/+65\) promoter and which substantially reduced the activation by LIM kinase (see the Discussion section).

Mutation of either the SRF or YY1-binding site inhibits promoter activation by LIM kinase

To analyse the role of SRF and YY1 in LIM kinase-mediated control of the SM22\(\alpha\) promoter, a set of mutant forms of pSM22 \(-197/+65\) were generated in which the SRF-binding site (pSM22ASRF), the YY1-binding site (pSM22AYY1) or both sites (pSM22ASRFAYY1) were mutated. A mutant was also made (pSM22YY1rev) in which the orientation of the CArG box/YY1-binding site was reversed, as the activity of YY1 has been shown to be dependent on the phase and orientation of the binding site. None of the mutant forms of the promoter was activated above its basal activity by LIM kinase in any of the cell types (Figure 7).
Jasplakinolide modifies SM22α promoter activity through an SRF/YY1-dependent mechanism

The role of SRF and YY1 in the activation of the SM22α promoter by increased actin polymerization was also examined by determining the effects of jasplakinolide on pSM22 -197/+65 in the presence or absence of exogenous SRF and YY1 in VSMCs. Jasplakinolide caused an increase in the activity of pSM22 -197/+65 and this increase was inhibited by expression of YY1 and SRF, consistent with the result for LIM kinase (Figure 8a). Similarly, the effect of jasplakinolide on the mutant SM22α promoters was determined in VSMCs. In these experiments, jasplakinolide did not increase the activity of the SM22α promoters in which the SRF- or YY1-binding sites had been mutated, similar to the effects of LIM kinase on these promoters (Figure 8b). Taken together these results imply that both SRF and YY1 are required to permit activation of the promoter by the actin cytoskeleton.

To determine whether jasplakinolide modified the binding of SRF and YY1 to the CArG-near box, nuclear extracts were made from C2C12 cells and VSMCs that had been treated with jasplakinolide for 6 h and from cells treated with ethanol vehicle only. Bandshift assay using these extracts showed that treatment of the cells with jasplakinolide increased the binding of SRF to the CArG box and reduced the binding of YY1 (Figure 9a). These results support the hypothesis that increased polymerization of the actin cytoskeleton reduces YY1-mediated repression of SRF activity.

DISCUSSION

The results suggest a model for the control of SRF activity by the actin cytoskeleton depicted in Figure 9(b). In this model when the actin cytoskeleton is disorganized, YY1 acts as a repressor of SRF activity at the CArG box. Increased polymerization of the actin cytoskeleton results in release of this repression and activation of transcription by SRF.

The model accounts for the lack of activation of the SM22α promoter by LIM kinase in P19 cells as these cells lack detectable YY1 DNA binding and explains all of the responses of C2C12 cells. In particular, the model explains the inability of LIM kinase to activate reporter gene expression in the C2C12 cells from CArG boxes in which the YY1-binding site is mutated. Furthermore, the bandshift result showing a reduction in YY1 and an increase in SRF binding to the CArG-near box in response to jasplakinolide suggests that actin cytoskeletal polymerization modifies the ability of these proteins to bind to DNA.
The effect of mutating the CaRG-near box on the response to the reorganization of the actin cytoskeleton in VSMCs was consistent with a requirement for both SRF and YY1, as in the other cell types examined. However, although VSMCs contained SRF and YY1 that bound to the CaRG-near box, exogenous SRF did not activate the CaRG-near box in these cells. Indeed, exogenous SRF inhibited LIM kinase activation of the promoter. Consequently, the response of VSMCs to actin polymerization is likely to involve factors (possibly those detected in the bandshift assay; Figure 3) in addition to SRF and YY1 that modulate the activity of SRF in these cells.

The mechanism proposed is consistent with several previous studies on the activities of both SRF and YY1, in which YY1 has been shown to be an inhibitor of SRF-dependent transactivation. For example, the CaRG boxes present in the SM22α-actin gene are responsible for the repression of SM22α-actin gene expression in Ras-transformed fibroblasts. The activity of the SM22α-actin promoter in these cells is repressed by YY1 but restored by over-expression of SRF [28,29]. There is also evidence showing that YY1 represses skeletal-muscle actin expression in myoblasts and YY1 has been shown to act as a competitive inhibitor of SRF in the activation of the cardiac α-actin promoter [8,12].

The data demonstrating that reversing the orientation of the CaRG box (which reverses the YY1-binding site) prevented YY1-dependent inhibition of reporter gene expression in PI9 cells are consistent with known activities of YY1. For example, YY1 is known to either activate or inhibit transcription from promoters depending on the sequence and context of the site through which it acts. Studies by two groups have shown that reversing the orientation or changing the phase of a YY1-binding site can convert the effect of bound YY1 from inhibition to activation [30,31]. Therefore, the inability of LIM kinase to activate reporter gene expression from a promoter containing this reversed YY1-binding site supports a role for YY1 in the modulation of CaRG-box activity by the actin cytoskeleton.

The model indicating a role for YY1 in the regulation of CaRG-box activity by the actin cytoskeleton may also explain the observation of Sotiropoulos et al. [14] that a promoter containing a multimerized Fos CaRG box responds to changes in the actin cytoskeleton, but the endogenous Fos gene is unaffected by changes in cytoskeletal polymerization. As described above, the activity of YY1 is phase- and orientation-sensitive. Fusing a multimer of the Fos CaRG box to a heterologous promoter is likely to result in a different phase and context compared with that of the CaRG box in the endogenous gene resulting in the observation that this CaRG box was regulated by the actin cytoskeleton.

In conclusion, our results indicate that changes in the polymerization of the actin cytoskeleton may alter the activities of a positive-acting transcription factor SRF and an inhibitory transcription factor YY1.

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