MOLECULAR AND CELLULAR BIOLOGY, Sept. 1992, p. 4209–4214
0270-7306/92/094209-06$02.00/0

Vol. 12, No. 9

Functional Antagonism Between YY1 and the Serum Response Factor

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Received 12 May 1992/Returned for modification 29 May 1992/Accepted 30 June 1992

The rapid, transient induction of the c-*fos* proto-oncogene by serum growth factors is mediated by the serum response element (SRE). The SRE shares homology with the muscle regulatory element (MRE) of the skeletal α-actin promoter. It is not known how these elements respond to proliferative and cell-type-specific signals, but the response appears to involve the binding of the serum response factor (SRF) and other proteins. Here, we report that YY1, a multifunctional transcription factor, binds to SRE and MRE sequences in vitro. The methylation interference footprint of YY1 overlaps with that of the SRF, and YY1 competes with the SRF for binding to these DNA elements. Overexpression of YY1 repressed serum-inducible and basal expression from the c-*fos* promoter and repressed basal expression from the skeletal α-actin promoter. YY1 also repressed expression from the individual SRE and MRE sequences upstream from a TATA element. Unlike that of YY1, SRF overexpression alone did not influence the transcriptional activity of the target sequence, but SRF overexpression could reverse YY1-mediated *trans* repression. These data suggest that YY1 and the SRF have antagonistic functions in vivo.

The $CC(A/T)_6GG$ sequence, or CArG motif, is the core of a family of DNA regulatory elements that occur in the promoters and enhancers of genes which are subject to different regulatory controls (12, 23). Included in this family of regulatory elements are the c-*fos* serum response element (SRE), which confers serum-inducible expression, and the skeletal α-actin muscle regulatory element (MRE), which is sufficient for muscle-specific expression when it is placed upstream from a TATA element (22, 24). The serum response factor (SRF) binds to the core CArG motif of both elements, while other proteins directly bind to the sequences that flank the CArG motif (9, 11, 16, 22, 24, 25). In addition, *ets*-related proteins bind to the c-*fos* SRE as part of a ternary complex with the SRF (1, 7). Complex protein-nucleic acid interactions presumably allow these elements to respond to diverse intracellular signals, but the functions of the individual factors are generally not known and contradictory findings have been reported (4, 8, 10, 17, 20, 26).

Here, we report that the transcription factor YY1, also referred to as NF-E1 and δ (2, 6, 14, 19, 21), specifically binds to the c-*fos* SRE and the skeletal actin MRE in vitro and that the binding of YY1 will inhibit the binding of the SRF transcription factor. YY1 overexpression represses transcription from the c-*fos* and skeletal actin promoters, and it appears that the repression is mediated, at least in part, by the CArG regulatory elements within these promoters. In contrast to that of YY1, SRF overexpression did not detectably alter expression from these elements; however, SRF overexpression could reverse YY1-mediated *trans* repression. These data suggest that YY1 and the SRF have antagonistic functions that may result from a competition for binding to DNA.

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MATERIALS AND METHODS

Probe preparation and DNA-binding assays. Oligonucleotide duplexes were end labeled with polynucleotide kinase and [γ-^{32}P]ATP and were purified with Elutip-d columns (Schleicher & Schuell) according to the directions of the manufacturer. The γ-actin SRE sequence is from positions −94 to −75 of the *Xenopus laevis* (type 5) cytoskeletal actin promoter. The c-*fos* SRE sequence is from the human c-*fos* promoter (positions −296 to −323). The MRE sequence is from the chicken skeletal α-actin promoter (positions −73 to −100). The MRE CC and GG mutants result from the substitution of A's at positions −74 and −75 and that of T's at positions −82 and −83, respectively, in the MRE sequence. The κ E3′ μE1 probe corresponds to 28 bp of the μE1 site in the immunoglobulin κ 3′ enhancer. YY1 was expressed in *Escherichia coli* as a histidine tag (H6) fusion protein and purified by nickel chelate chromatography in the presence of 6 M guanidine hydrochloride (Qiagen) (14). Following chromatography the fractions were dialyzed in phosphate-buffered saline (PBS) and stored in the same buffer plus 5% glycerol. The SRF was prepared by in vitro transcription and translation. Linear SRF plasmid pT7ΔATG (4 μg) was transcribed for 2 h at 37°C with T7 polymerase in the presence of RNasin (13). In vitro translation was done at 25°C for 1 h with a rabbit reticulocyte lysate (Promega) in a 50-μl reaction mixture with 10% of the synthesized RNA as substrate under conditions recommended by the manufacturer. Electrophoretic mobility shift reaction mixtures contained the indicated amounts of recombinant YY1, SRF, and DNA probe and 10 mM Tris (pH 7.5), 30 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 8% glycerol, and 0.1 to 1.0 μg of poly(dI-dC) · poly(dI-dC) in a 10-μl total volume. Following a 30-min incubation at room temperature, binding mixes were loading onto a 5% polyacrylamide gel and electrophoresed at 20 V cm^{-1} in 22 mM Tris-borate buffer with 0.5 mM EDTA. A longer incubation time did not change the protein-DNA complex formation. Gels were dried and ex-

posed to film for 8 h at −70°C with an intensifying screen. Dissociation constants were determined by electrophoretic mobility shift assays with the recombinant YY1 and SRF proteins. A constant amount of protein was incubated with increasing amounts of MRE or SRE probes for 30 min at 20°C. Probes were varied from 0.5 to 40 nM. Electrophoresis was performed under standard conditions, and the extent of complex formation was quantified with a PhosphorImager (Molecular Dynamics). Binding constants were calculated from Scatchard plots.

The YY1 methylation interference footprints on the c-*fos* SRE and the α-actin MRE were performed on ^{32}P-labeled oligonucleotide probes that were partially methylated in 0.2 ml of 50 mM sodium cacodylate (pH 6.5)–1 mM EDTA with 1 μl of fresh dimethyl sulfate for 20 min at room temperature. The reactions were stopped by the addition of 50 μl of 1.5 M sodium acetate (pH 6.5)–9 M 2-mercaptoethanol. The modified DNA sequences were used as probes in 20-fold scaled-up electrophoretic mobility shift assays. The nucleoprotein complex and free DNA bands were excised and eluted overnight into 20 mM Tris HCl (pH 7.5)–1 mM EDTA–200 mM NaCl. This material was purified with Elutip-d columns and ethanol precipitated. Specific cleavage at the modified guanine residues was performed with 100 μl of 1 M piperidine at 90°C for 30 min. The unused methylated probe was also cleaved with piperidine to provide a G ladder. The samples were dried repeatedly under vacuum, dissolved in deionized formamide, and loaded onto a denaturing 20% polyacrylamide gel. Following electrophoresis, the gel was exposed to film for 4 days at −70°C.

Cell culture and transfections. Primary cultures of pectoralis skeletal muscle, skin fibroblasts, and liver were prepared from 11-day-old chicken embryos. Cells were transfected by the calcium phosphate method (17). The plasmid p356fosCAT contains the mouse c-*fos* promoter (positions −356 to +109) upstream from the CAT reporter gene, the full-length chicken skeletal α-actin promoter is from positions and is −2.0 kbp to +300 bp upstream from the CAT reporter gene, and the MRE/Δ56CAT and SRE/Δ56CAT plasmid constructs have the 28-bp CArG elements inserted at a unique *SalI* site that is directly upstream from the truncated mouse c-*fos* promoter, positions −56 to +109, and the CAT reporter gene (17, 24). The YY1 expression vector had the full-length coding sequence of human YY1 under control of the cytomegalovirus (CMV) enhancer-promoter. All plasmids were purified by Qiagen columns according to the directions of the manufacturer. Transfection mixes typically included 1 μg of CMV-luciferase, which has the firefly luciferase gene under the control of the CMV enhancer, to control for transfection efficiency. Luciferase assays were performed in 20 mM Tricine–1.07 mM $(MgCO_3)_4Mg(OH)_2$–2.67 mM $MgSO_4$–0.1 mM EDTA–33.3 mM dithiothreitol–270 μM coenzyme A–470 μM luciferin–530 μM ATP, and activity was recorded by an LB 9501 Lumat luminometer (EG & G Berthold). CAT activity was quantified by exposure to phosphor screens and analysis on a Molecular Dynamics PhosporImager.

Serum induction experiments were performed with primary cultures of embryonic chick pectoralis muscle that were plated on 100-mm-diameter plates 24 to 36 h prior to transfection. Cells were grown in minimal essential medium (MEM) plus 10% horse serum supplemented with 4% chicken embryo extract (GIBCO). Cultures were transfected with 4 μg of p356fosCAT and increasing amounts of a YY1 expression vector and 1 μg of pXGH5, a growth hormone expression vector. Cell cultures were transfected in duplicate, and the amount of DNA was kept constant by the addition of vector without insert. After 8 h of transfection, the medium was removed, the plates were washed twice with PBS, and the cultures were serum starved in MEM with 0.5% horse serum for 36 to 48 h. Transfection efficiency was determined by radioimmune assay for secreted growth hormone (Nichols Diagnostics). One set of duplicate plates was then induced for 30 min by the addition of MEM plus 10% horse serum. All cells were then washed with PBS, scraped, and washed twice more with PBS. RNA was harvested in 4 M guanidinium thiocyanate–25 mM sodium citrate (pH 7.0)–0.5% sarcosyl–0.1 M 2-mercaptoethanol and then extracted by phenol and chloroform and precipitated twice with ethanol. Following treatment with DNase I, the RNA was separated on 1% agarose–2.2 M formaldehyde gels and subjected to Northern (RNA) blot analysis with a radiolabeled probe to the CAT sequence (c-*fos* CAT). Probes were stripped and then hybridized to a full-length cDNA probe to rat 3-phosphoglyceraldehyde dehydrogenase to check the quality of the extracted RNAs.

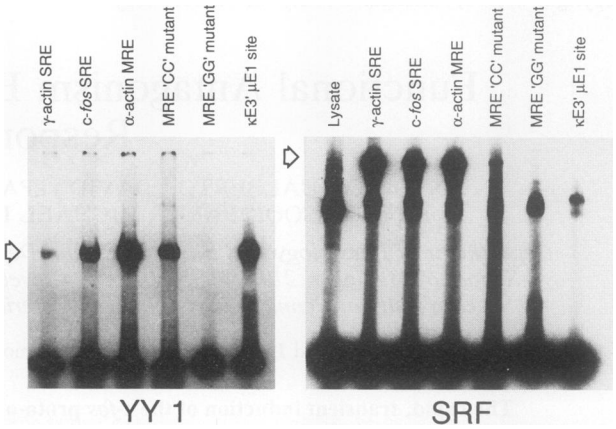

FIG. 1. Comparison of recombinant YY1 and SRF binding to DNA regulatory elements. The γ-actin SRE probe is from the *X. laevis* (type 5) cytoskeletal actin promoter, the c-*fos* SRE probe is from the human c-*fos* promoter, the κ E3′ μE1 probe is from the immunoglobulin κ 3′ enhancer, and the MRE probe is from the chicken skeletal α-actin promoter. The MRE CC and GG mutants have substitutions at the upstream and downstream portions of the $CC(A/T)_6GG$ motif, respectively. YY1 was expressed in *E. coli* as a histidine tag fusion protein and purified by nickel chelate chromatography. SRF was prepared by in vitro transcription and translation. Electrophoretic mobility shift assays contained 1 nM ^{32}P-labeled DNA probe and 2 μg of YY1 or 0.1 nM SRF. Nonprogrammed lysate with the c-*fos* SRE was included as a control.

RESULTS

We tested the binding of YY1 to duplex oligonucleotide probes to CArG box elements because they contain nucleotide sequence identities that are similar to those of the reported YY1-binding sites. Electrophoretic mobility shift assays with bacterially produced YY1 revealed prominent nucleoprotein complexes with the α-actin MRE and the c-*fos* SRE, but the interaction with the *Xenopus* γ-actin SRE appeared weaker (Fig. 1). These complexes had the same mobility as that of the bound immunoglobulin κ 3′ enhancer μE1 sequence, a known YY1-binding site that was originally used to isolate molecular clones for this protein. Transversion mutations in the MRE at positions −83 and −82 (GG mutant) abolished binding, but transversion mutations at

positions −91 and −90 (CC mutant) did not, providing an initial indication that YY1 binds asymmetrically to this element. On the basis of quantitative competition experiments, the relative rank of YY1 affinity for these probes was MRE and μE1 > c-*fos* SRE > *Xenopus* γ-actin SRE.

The binding of the SRF to CArG elements differed from that of YY1. The SRF formed prominent complexes with the *Xenopus* γ-actin SRE, the c-*fos* SRE, and the α-actin MRE, but no binding to the immunoglobulin κ 3′ enhancer μE1 site was detected (Fig. 1). Furthermore, both sets of MRE transversion mutations significantly diminished SRF binding, as would be anticipated from the symmetrical methylation interference footprint produced by the SRF on this site (24). Equilibrium-binding measurements revealed that the affinities of YY1 and SRF for the MRE were similar (K_d = 6.8 and 5.8 nM, respectively), but the YY1-SRE complex was less stable than the SRF-SRE complex (K_d = 40 and 2.5 nM, respectively). Previously, the SRF-SRE dissociation constant was reported as 0.5 nM by using purified nuclear factor (18). The discrepancy between our findings and those of this previous report may result from differences in the assay conditions or from differences in the thermodynamic properties of the recombinant and endogenous SRF proteins.

The YY1 nucleoprotein complexes were analyzed in greater detail by methylation interference footprinting (Fig. 2). The N-7 methylation of any one of 5 guanine residues from positions −78 to −83 in the MRE interfered with the binding of YY1. The methylation interference footprint of the c-*fos* SRE-YY1 complex identified 2 sensitive guanine residues at positions −313 and −314 in the core CArG motif and partial interference from the methylation of position −316. We note that the asymmetric footprint of YY1 markedly differs from the symmetrical footprint of the SRF on these elements, and these proteins have overlapping contacts in the major grooves of each element (Fig. 2C). On the basis of similarities in binding specificities and methylation interference footprint patterns, YY1 appears similar, if not identical, to the nuclear protein previously referred to as MAPF or $p62^{DBF}$ (16, 24, 25).

The combined binding of YY1 and SRF to these elements was analyzed (Fig. 3). Increasing amounts of recombinant YY1 protein were added to a fixed amount of the SRF and the c-*fos* SRE or the skeletal actin MRE. The addition of YY1 to the binding mixture led to a diminution of the SRF-DNA complex with both probes. The overlapping footprints produced by these proteins in the major groove suggest that the binding of YY1 would exclude the binding of the SRF to DNA elements because of steric hindrance. This apparent competition was not detected when the YY1 fraction was heat inactivated prior to its inclusion in the binding mix. YY1 was a more potent inhibitor of SRF binding to the α-actin MRE than to the c-*fos* SRE, which is consistent with the finding that YY1 has a greater affinity for the muscle element. These properties also functionally distinguish YY1 from the *ets*-related proteins that bind to the c-*fos* SRE as a ternary complex with the SRF (1, 7).

The c-*fos* gene is rapidly and transiently activated when quiescent, serum-deprived cells are exposed to serum growth factors. This regulation is conferred by the SRE (22, 23). The effect of YY1 on serum induction was tested by transiently cotransfecting increasing amounts of a YY1 expression vector with a fixed amount of a c-*fos* promoter-CAT construct into primary myocytes (Fig. 4). YY1 overexpression diminished both basal and serum-induced expression from the c-*fos* promoter. We also assessed the effect of

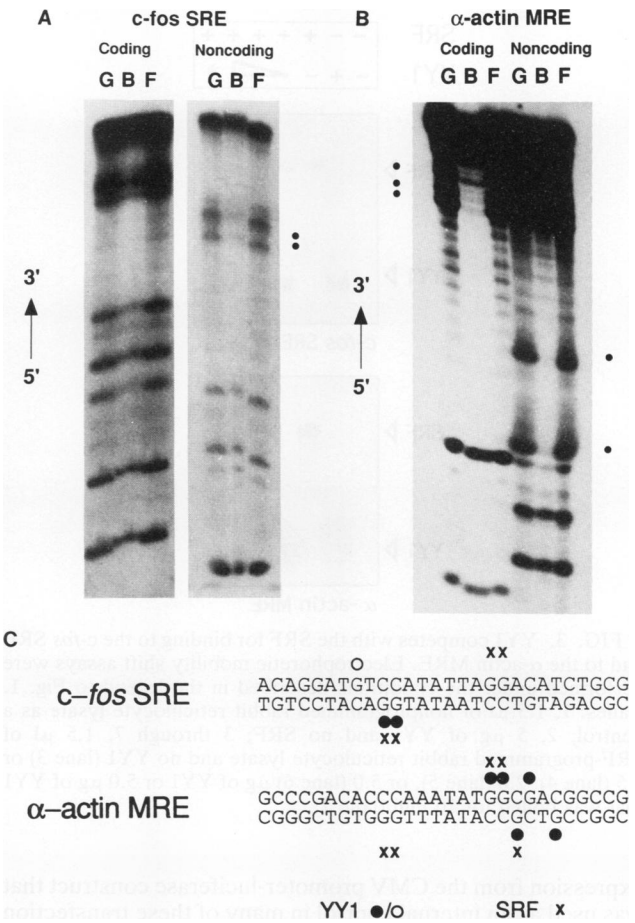

FIG. 2. YY1 methylation interference footprint on the c-*fos* SRE (A) or the α-actin MRE (B). Partially methylated oligonucleotide probes were used in 20-fold scaled-up electrophoretic mobility shift assays. The nucleoprotein complex (lane B) and free DNA (lane F) bands were excised and treated as described in Materials and Methods. The unused methylated probe was utilized for the G ladder. (C) Comparison of YY1 (●) and SRF (X) (22, 24) methylation interference footprints. Open circles indicate partial interference with binding.

YY1 overexpression on skeletal α-actin transcription in transfected myocytes. Basal expression from the skeletal actin promoter was significantly more sensitive to repression by YY1 than the c-*fos* promoter (Fig. 5A). Unlike α-actin, the c-*fos* construct was active in fibroblasts as well as in myocytes, and YY1 was equally effective at repressing c-*fos* transcription in either cell type (not shown). YY1 overexpression also repressed transcription from the individual 28-bp α-actin MRE and c-*fos* SRE sequences when they were placed upstream from the TATA element of the truncated c-*fos* promoter (Fig. 5B). The effect of YY1 on expression from the MRE and the SRE was similar to that of their cognate full-length promoters (in that the muscle sequence was more sensitive to repression). The differential effects of YY1 on expression from the MRE- and SRE-test promoter constructs demonstrate that YY1 exerts its effect, at least in part, through CArG elements and that the repression cannot solely result from general effects at TATA or initiator sequences. YY1 specificity for these CArG elements is further demonstrated by its inability to *trans* repress

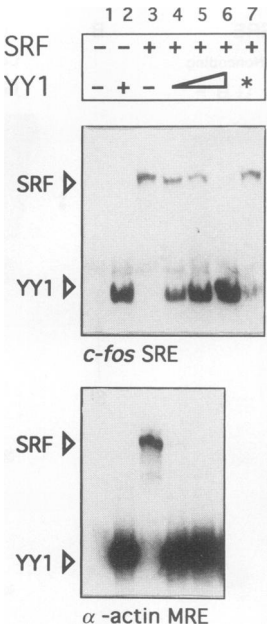

FIG. 3. YY1 competes with the SRF for binding to the c-*fos* SRE and to the α-actin MRE. Electrophoretic mobility shift assays were performed under the conditions described in the legend to Fig. 1. Lanes: 1, 1.5 μl of nonprogrammed rabbit reticulocyte lysate as a control; 2, 5 μg of YY1 and no SRF; 3 through 7, 1.5 μl of SRF-programmed rabbit reticulocyte lysate and no YY1 (lane 3) or 0.5 (lane 4), 2.0 (lane 5), or 5.0 (lane 6) μg of YY1 or 5.0 μg of YY1 that was boiled for 10 min (lane 7).

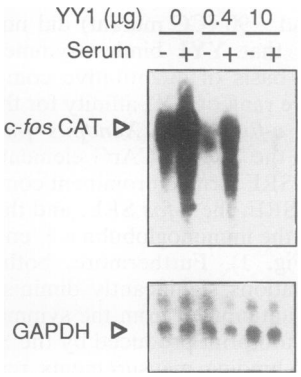

FIG. 4. YY1 *trans* represses the serum induction of c-*fos* expression. Primary cultures of embryonic chick pectoralis muscle were transfected with 4 μg of p356fosCAT containing the mouse c-*fos* promoter upstream from the CAT reporter gene, 1 μg of pXGH5, and increasing amounts of the YY1 expression vector. Eight hours after being transfected, the cultures were serum starved in MEM with 0.5% horse serum for 2 days. One set of duplicate plates was then serum induced by the replacement of media with MEM containing 10% horse serum. Control cells received fresh media containing no serum. Following incubation for 30 min, RNA was prepared and subjected to Northern blot analysis with a radiolabeled probe to the CAT sequence (c-*fos* CAT). Blots were rehybridized with a 3-phosphoglyceraldehyde dehydrogenase probe to check RNA quality. Transfection efficiency was determined by a radioimmune assay for secreted growth hormone (Nichols Diagnostics).

expression from the CMV promoter-luciferase construct that was used as an internal control in many of these transfection assays. Furthermore, since these CArG elements are essential for basal and regulated expression (15, 26), it appears that these elements are likely targets for YY1 regulation within the full-length c-*fos* and α-actin promoters.

The combined effects of SRF and YY1 overexpression on transcription were examined in order to explore potential interactions between these proteins in vivo. YY1 overexpression decreased transcription, but overexpression of the SRF had no detectable effect on the minimal MRE/Δ56CAT construct (Fig. 6). However, the SRF could fully restore transcriptional activity when it was coexpressed with YY1. A similar expression pattern was also observed with the c-*fos* promoter sequence, but higher levels of the YY1 and SRF expression plasmids were required for repression and its reversal (not shown). These cotransfection experiments suggest that YY1 and SRF have antagonistic effects on expression.

DISCUSSION

YY1 is a multifunctional transcription factor that can act at promoters, enhancers, and initiator elements (2, 6, 14, 19, 21). Here, we report that YY1 is a potential regulator of the c-*fos* and skeletal actin genes and that it exerts its effect through their CArG motif promoter elements. In the c-*fos* gene, this motif, the SRE, mediates the rapid and transient activation of expression by serum growth factors. In the skeletal actin promoter, this motif, the MRE, confers muscle-specific expression. Multiple lines of evidence suggest that YY1 is a regulator of these CArG elements. YY1 specifically binds to these sequences in vitro (Fig. 1), and the overexpression of YY1 will repress basal and regulated expression from the c-*fos* SRE and the skeletal actin MRE (Fig. 4 and 5). This YY1-mediated *trans* repression can be reversed by overexpression of the SRF (Fig. 6). These data further suggest that the SRF is a positive transcription factor and that the SRF and YY1 have antagonistic functions in vivo. This functional antagonism may result from the competition for binding to the DNA regulatory element. YY1 and SRF have overlapping methylation interference footprints on both of these CArG elements (Fig. 2), and the binding of YY1 will exclude the binding of the SRF in vitro (Fig. 3).

YY1 binds asymmetrically to the c-*fos* SRE and the skeletal actin MRE. The CArG motifs within these elements contain the sequence 5′-ATGG-3′, which comprises the core of YY1-binding sites found in other promoters and enhancers (2, 6, 14, 21). In these other genes, YY1 can function as an activator or a repressor of transcription, but the repression does not appear to result from the steric hindrance or competition between YY1 and other positive transcription factors. YY1 has properties that are most similar to the MAPF1 or $p62^{DBF}$ nuclear protein that was previously reported to bind to the c-*fos* SRE and the skeletal actin MRE (16, 24, 25). The MAPF/$p62^{DBF}$ nuclear protein and recombinant YY1 produce the same methylation interference footprints, and they have the same sequence requirements for binding. Also, the MAPF/$p62^{DBF}$ activity is ubiquitous, as is the YY1 transcript (6, 25). YY1 migrates with an apparent molecular mass of 62 to 68 kDa during sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, which is similar to the migration pattern of MAPF/$p62^{DBF}$ (16, 21). We note that the predicted molecular mass of YY1 is 45 kDa, but the aberrantly slow mobility in SDS gels may result from peptide sequences in YY1 that give rise to an unusually stable secondary structure.

The mechanisms by which proteins regulate expression from the c-*fos* SRE and the skeletal actin MRE are poorly

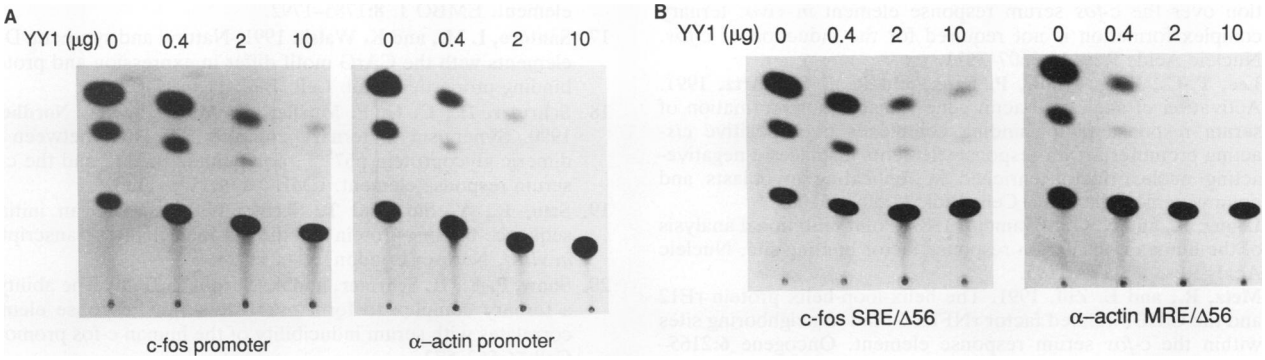

FIG. 5. Repression of basal transcription by YY1. The effects of YY1 overexpression on the full-length c-*fos* and skeletal α-actin promoters (A) and individual c-*fos* SRE and α-actin MRE elements upstream from the truncated c-*fos* promoter (B) are shown. Primary myocyte cultures were transfected with 4 μg of the respective target sequences upstream from the CAT reporter gene and the indicated amounts of YY1 expression plasmid. Cells were harvested 2 days posttransfection and assayed for CAT activity.

understood. Correlations between expression patterns and protein binding in vitro have not provided clear insights about the roles of the individual proteins, and contradictory findings have been reported previously (4, 8, 10, 17, 20, 26). Similarly, mutations that block YY1 (or MAPF/p62^{DBF}) binding do not give rise to a particular expression pattern, and for this reason we tested for function by overexpressing YY1 and the SRF. These data suggest that the nucleoprotein complex is more complicated than has been indicated by in vitro DNA-binding assays. In this regard, we note that the ternary complex nuclear activity, which binds to a subset of CArG elements, was recently shown to represent at least three *ets*-related proteins (1, 7). Furthermore, the A/T-rich cores of CArG elements resemble homeodomain protein-binding sites, and members of this large family of cell-type-specific factors may participate in the regulation of these elements.

In summary, we demonstrate that YY1 binds to CArG elements and negatively regulates expression from the c-*fos* and α-actin promoters. We also demonstrate that YY1 can inhibit the binding of the SRF to promoter sequences in vitro and that overexpression of the SRF can reverse YY1-mediated *trans* repression in vivo. These findings are consistent with the hypothesis that YY1 and SRF are negative and positive transcription factors, respectively, which compete for binding to key regulatory elements within the c-*fos* and α-actin promoters. CArG elements occur in many genes, and they mediate the response to diverse cell-type- and signaling pathway-specific signals (3, 5, 24). The identification of YY1 as a potential regulator of CArG elements will permit further studies of how complex protein-nucleic acid interactions control expression from this family of DNA regulatory elements.

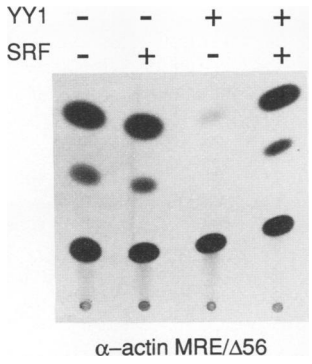

FIG. 6. SRF overexpression reverses YY1-mediated *trans* repression. Cultures were transfected with 4 μg of the target MRE/Δ56CAT plasmid alone, 5 μg of the human SRF under the control of the CMV enhancer, 2 μg of YY1 expression plasmid, or 5 and 2 μg of the SRF and YY1 expression vectors, respectively. Transfections also included 1 μg of CMV-luciferase to control for transfection efficiency.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH (AR40197 and HL45345) and the Life and Health Insurance Medical Research Fund to K.W., grants from the NIH (GM42415), the Markey Charitable Trust, and the Jesse B. Cox Charitable Trust to M.L.A., and a grant from the NIH (AG00387) to S.L.M. A.G. is a fellow of the Ministerio de Educacion y Ciencia, Spain, and K.W. is an established investigator of the American Heart Association.

We thank M. Gilman and J. Swain for the SRF and luciferase expression plasmids, respectively. We thank R. M. Patrick for preparing recombinant YY1 protein.

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