Enhancement of Serum-response Factor-dependent Transcription and DNA Binding by the Architectural Transcription Factor HMG-I(Y)*

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The mechanisms by which HMG-I proteins regulate cell growth are unknown, and their effects on gene expression have only been partially elucidated. We explored the potential interaction between HMG-I proteins and serum-response factor (SRF), a member of the MADS-box family of transcription factors. In cotransfection experiments, HMG-I(Y) potentiated SRF-dependent activation (by more than 5-fold) of two distinct SRF-responsive promoters, c-fos and the smooth muscle-specific gene SM22α. This effect was also observed with a heterologous promoter containing multiple copies of the CC(A/T)6GG (CArG) box. HMG-I proteins bound specifically to the CArG boxes of c-fos and SM22α in gel mobility shift analysis and enhanced binding of SRF to these CArG boxes. By chelating peptide-immobilized metal affinity chromatography, we mapped the domain of HMG-I(Y) that interacts with SRF to amino acids 50–81, a region that does not bind specifically to DNA in electrophoretic mobility shift assays even though it includes the third AT-hook DNA-binding domain. Surprisingly, HMG-I(Y) mutants that failed to bind DNA still enhanced SRF binding to DNA and SRF-dependent transcription. In contrast, deletion of the HMG-I(Y) 50–81 domain that bound SRF prevented enhancement of transcription. To our knowledge, this is the first report of an HMG-I protein interacting with a MADS-box transcription factor. Our observations suggest that members of the HMG-I family play an important role in SRF-dependent transcription and that their effect is mediated primarily by a protein-protein interaction.

Serum-response factor (SRF),1 a member of the MADS-box family of transcription factors, activates a variety of genes through binding to the sequence CC(A/T)6GG (the CArG box). SRF is also an important integrator of intracellular signal transduction pathways through its interaction with accessory factors (members of the ets, homeodomain, and zinc finger families) (1, 2). SRF activates transcription in genes of two classes: muscle-specific genes (3–5) and immediate-early genes expressed after serum stimulation (2), of which c-fos is the prototype.

Architectural transcription factors of the HMG-I family are important in gene expression and growth regulation (6). There are currently three members: HMG-I, HMG-Y, and HMGI-C. HMG-I (107 amino acids) and HMG-Y (96 amino acids) derive from alternatively spliced transcripts of the same gene. Because their biological properties are indistinguishable, they are referred to as HMG-I(Y). HMGI-C is related to them structurally but derives from a distinct gene. The HMG-I transcription factors bind broadly to AT-rich DNA in the minor groove through the AT-hook peptide motif (7). Although HMG-I proteins have no intrinsic ability to activate transcription, they regulate the affinity and activity of other transcription factors by altering local chromatin structure.

The best example of a regulatory region responsive to HMG-I(Y) is the β-interferon promoter. HMG-I(Y) binds to the PRDII element of the promoter and recruits the transcription factor NF-xB (8). HMG-I(Y) also binds the PRDIV element of the promoter and recruits ATF-2/c-Jun, producing synergy between the PRDII and PRDIV elements (9). In the absence of HMG-I(Y) binding, neither NF-xB nor ATF-2 confers viral inducibility on this promoter. HMG-I(Y) seems to enhance β-interferon transcription by facilitating formation of a stereospecific complex referred to as an enhancerosome (10, 11), which reverses an intrinsic bend in the DNA (12). Other examples of HMG-I(Y)-responsive genes include the endothelial cell adhesion molecule E-selectin (13), the chemokine MGSA/GROα (14), and the IgE locus (15). In the case of the IgE promoter, HMG-I(Y) represses basal transcription until it is phosphorylated by an interleukin 4-dependent pathway, which decreases its affinity for DNA in vitro. Thus, HMG-I(Y) can be a positive and a negative regulator of transcription. Recently, HMG-I(Y) has been shown to repress the T cell-receptor gene enhancer by influencing DNA topology (16).

HMG-I proteins are thought to play critical roles in cell growth and transformation. They are expressed at low or undetectable levels in adult tissues but are expressed highly in embryonic and neoplastic tissues (17, 18). There are many data in support of a causal role for HMGI-C in cell growth and transformation. Overexpression of HMGI-C antisense RNA prevents retrovirally induced transformation (19). Also, the HMGI-C gene on human chromosome 12q15 is often rearranged in benign mesenchymal tumors (20, 21). Analysis of some of the chimeric transcripts resulting from these rearrangements shows that the AT-hook DNA-binding motifs are fused with a LIM domain in one case and an acidic transcrip-
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We have found that the HMG-I proteins are induced dramatically in proliferating vascular smooth muscle. Because the role of SRF in cell proliferation and expression of vascular smooth muscle-specific genes is well established, and its DNA-binding element (the CArG box) is rich in adenine and thymine residues, we hypothesized that HMG-I proteins may bind to the CArG box and affect the ability of SRF to bind to DNA and activate transcription.

We show in this report that HMG-I(Y) enhances SRF-dependent activation of the c-fos and SM22α promoters in eukaryotic cells, an effect that is also observed for a heterologous promoter containing a CArG box. HMG-I(Y) binds specifically to the CArG box and enhances binding of SRF in vitro, and the SRF-interacting domain maps to HMG-I(Y) amino acids 50–81. Surprisingly, mutations that interfere with DNA binding by HMG-I(Y) but preserve its interaction with SRF retain the ability to enhance binding of SRF to DNA in vitro and augment SRF-dependent transcription in vivo.

MATERIALS AND METHODS

Plasmids—Using rat genomic DNA as a template, we cloned the rat SM22α promoter by amplifying nucleotides 1507 to +32 by the polymerase chain reaction (PCR). The PCR fragment was then inserted into the XhoI site of pGLO2-Basic (Promega). Nucleotides –711 to +97 of the human c-fos promoter were amplified by PCR from pF4 (24) and inserted into the XhoI and HindIII sites of pGLO2-Basic. A DNA fragment containing a single copy of the proximal SM22α CArG box (5′-CATCTTGGGTCTTCCCCCCTGAATGGGCACTTGTCGTTGCTGA-3′) was inserted into the EcoRI site of standard methods (25).

The Drosophila expression plasmids pPAC, pPACHMGI, and pPCHMGI were made by cloning the full-length cDNA into the EcoRI and XhoI sites of the yeast expression vector pJG4–5. The HMG-I(Y) 50–96 mutant was constructed by cloning two additional copies of a DNA fragment containing the SM22α CArG box (described above) and the c-fos CArG box sequence element CArG box (5′-GTACGATGTCTCAGATGACCTCTTTT-3′) and 5′-GTACGATGTCTCAGATGACCTCTTTT-3′, encoding a vector-derived polyhistidine tag and an enterokinase cleavage site in addition to the cloned insert protein, were transfected into the prostate-deficient E. coli strain BL21(DE3)pLysS expression. Expression was induced in 500-mL cultures (mid-log phase) by the addition of isopropyl β-D-thiogalactopyranoside to 1 mM. Three hours after induction, the bacterial cells were lysed and purified by cobalt affinity chromatography under denaturing conditions according to the instructions of the manufacturer (Clontech). Proteins were renatured by dialysis overnight against 20 mM HEPES (pH 7.8), 20 mM KCl, and 0.2% Tween 20 at 4 °C. The dialysate was stored in 50-μl aliquots at –80 °C.

Electrophoretic Mobility Shift Assays—Annealed oligonucleotides for the SM22α CArG box (described above) and the c-fos CArG box sequence element CArG box (5′-GTACGATGTCTCAGATGACCTCTTTT-3′) and 5′-GTACGATGTCTCAGATGACCTCTTTT-3′, encoding a vector-derived polyhistidine tag and an enterokinase cleavage site in addition to the cloned insert protein, were transfected into the prostate-deficient E. coli strain BL21(DE3)pLysS expression. Expression was induced in 500-mL cultures (mid-log phase) by the addition of isopropyl β-D-thiogalactopyranoside to 1 mM. Three hours after induction, the bacterial cells were lysed and purified by cobalt affinity chromatography under denaturing conditions according to the instructions of the manufacturer (Clontech). Proteins were renatured by dialysis overnight against 20 mM HEPES (pH 7.8), 20 mM KCl, and 0.2% Tween 20 at 4 °C. The dialysate was stored in 50-μl aliquots at –80 °C.

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Cell Culture, Transfections, Yeast Strains, and Reporter Assays—Drosophila SL2 cells were cultured and transfected essentially as described (10). In brief, SL2 cells were transfected by the calcium phosphate method on 6-well trays at approximately 50% confluence, transfected with plasmids encoding proteins as outlined in Figs. 1A and 5. Plasmid pbsp2LacZ (100 ng), which encodes β-galactosidase transcribed from a Drosophila heat shock promoter, was cotransfected in all experiments to correct for differences in transfection efficiency. The ratio of luciferase to β-galactosidase from each plasmid was calculated and expressed as relative luciferase activity, with the value for pPAC set arbitrarily at 1.

The yeast strain MCY-1, a derivative of EGY48 (29), was created by transforming EGY48 with the integrating vector pCarG-LacZ. The cells were transformed by the lithium acetate procedure as described (30). Luciferase and β-galactosidase assays on transfected SL2 cell lysates were performed as described (31). Yeast β-galactosidase assays were also performed as described (32).

Chelating Peptide-Immolated Metal Affinity Chromatography (CP-IMAC)—CP-IMAC was performed as described (33), with some modification. In brief, [35]Smethionine-labeled SRF was prepared from pDNA3SRF with an in vitro transcription/translation kit according to the instructions of the manufacturer (Promega). A 20-μl bed volume of cobalt affinity resin (Clontech) was equilibrated in a 1.7 mL microfuge tube with the following buffer, pH 8.0: 50 mM Tris-HCl (pH 8.0), 50 mM NaPO4 (pH 8.0), and 100 mM NaCl. The resin was then washed three times with 1 mL of ice-cold binding buffer.

The mixture was incubated at 4 °C for 2 h with rotary shaking.

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buffer and collected by centrifugation as above. Bound protein was eluted with 20 µl of 100 mM EDTA and analyzed on 10% Tricine-SDS gels as described (34).

RESULTS

HMG-I(Y) Enhances SRF-dependent Transcriptional Activation in Eukaryotic Cells—Given that the HMG-I proteins bind to AT-rich sequences and enhance transcription by recruiting other factors, we tested the hypothesis that HMG-I(Y) might bind CArG boxes and enhance SRF-dependent transcription. Transient transfection assays were performed in Drosophila SL2 cells because they do not express high levels of SRF or HMG-I(Y) and are thus ideally suited for studying the effects of these proteins on transcription (10, 26). Transfection of the pPAC, HMG-I(Y), or SRF expression plasmids alone had little effect on the activity of the SM22α promoter (Fig. 1A). Transfection of the SRF and the HMG-I(Y) expression plasmids together, however, increased SM22α promoter activity by ~20-fold. HMG-I(Y) and SRF had a similar synergistic effect on the c-fos promoter (Fig. 1A).

To determine whether this effect was mediated by the CArG box alone, we introduced three copies of the SM22α CArG box into a yeast β-galactosidase expression vector and created a stable yeast strain with this construct integrated into the genome. This approach would also reveal whether the HMG-I proteins affected genetic signals incorporated into genomic chromatin. Neither HMG-I(Y) nor HMGI-C had any effect on β-galactosidase expression (Fig. 1B). SRF alone also had no significant effect in yeast (Fig. 1B), consistent with data published elsewhere (35). When HMG-I(Y) or HMGI-C was coexpressed with SRF, β-galactosidase expression increased by ~15-fold (Fig. 1B). These results indicate that HMG-I proteins activate transcription by interacting with SRF through the CArG box.

HMG-I(Y) Binds Specifically to the CArG Boxes of SM22α and c-fos—As a preliminary exploration of how HMG-I proteins enhance SRF-dependent transcription, we determined whether HMG-I proteins bound specifically to SRF-binding sites through their AT-rich consensus sequence. Recombinant polyhistidine-tagged HMG-I(Y) and HMGI-C were expressed in E. coli, purified by cobalt affinity chromatography, and used in electrophoretic mobility shift assays. A DNA-protein complex formed when HMG-I(Y) was incubated with a labeled, SM22α CArG box probe (Fig. 2, left panel, arrow). The binding was specific because a 500-fold molar excess of unlabeled oligonucleotide probe encoding the SM22α CArG box or the c-fos CArG box, but not one encoding an unrelated sequence, competed for HMG-I(Y) binding. Results from a similar analysis with a labeled c-fos CArG box probe were identical (Fig. 2, right panel). Results obtained with recombinant HMGI-C were similar (data not shown).

HMG-I(Y) Enhances Binding of SRF to the CArG Box—To explore the functional significance of HMG-I(Y) binding to the CArG box, we investigated the effect of HMG-I(Y) on the binding of SRF. Electrophoretic mobility shift assays were performed with increasing amounts of purified, recombinant polyhistidine-tagged SRF in the presence or absence of HMG-I(Y). SRF binding to the c-fos CArG box probe resulted in a slowly migrating DNA-protein complex (Fig. 3), whereas HMG-I(Y) binding produced a more rapidly migrating complex. In the presence of HMG-I(Y), SRF binding increased markedly. The presence of SRF in the slowly migrating complex was verified by its supershifting in the presence of anti-SRF antibody (Fig. 3, last lane). Similar results were obtained with an SM22α CArG box probe and with recombinant HMGI-C (data not shown). These results suggest an important role for HMG-I proteins in enhancing the binding of SRF to DNA and promoting the assembly of DNA-protein complexes.

HMG-I(Y) Interacts with SRF in the Absence of DNA through HMG-I(Y) Amino Acids 50–81—We determined whether HMG-I(Y) interacted with SRF in the absence of DNA and identified the HMG-I(Y) domain required for this interaction by a CP-IMAC method (33). In this experiment (summarized in Fig. 4A), recombinant polyhistidine-tagged mutant and wild-
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**Fig. 2.** HMG-I(Y) binds specifically to CArG boxes. Electrophoretic mobility shift assays were performed as described under "Materials and Methods." Oligonucleotides containing a single CArG box from the SM22α promoter or the c-fos promoter were radiolabeled, and 35 fmol of radiolabeled probe was incubated with 15 pmol of recombinant HMG-I(Y) as outlined under "Materials and Methods." Unlabeled competitors were added at a 500-fold molar excess as indicated. NS, nonspecific competitor; arrow, DNA-protein complex.

**Fig. 3.** HMG-I(Y) enhances binding of SRF to the c-fos CArG box promoter. Electrophoretic mobility shift assays were performed with increasing amounts of SRF (0, 0.16, 0.32, 0.48, 0.64, and 0.80 pmol) in the presence or absence of recombinant HMG-I(Y) (7 pmol). Antibody to SRF (α-SRF) was used to verify the presence of SRF in the slowly migrating complex.

Type HMG-I(Y) proteins were incubated in the presence of a cobalt-Sepharose resin with radiolabeled, in vitro translated SRF. Because proteins that interacted with labeled SRF would retain SRF on the resin, labeled SRF would appear on the protein gel after elution from the resin. Deletion of HMG-I(Y) C-terminal amino acids 82–96 (containing the acidic domain) had no effect on its interaction with SRF (Fig. 4B, lane 1–81). Further deletion to amino acid 45, however, abolished the ability of HMG-I(Y) to retain SRF on the resin (Fig. 4B, lane 1–81 versus lane 1–45). Because deletion of amino-terminal residues 1–50 also had no effect on the interaction with SRF (Fig. 4B, lane 50–96), the region of HMG-I(Y) required for SRF binding appeared to map to amino acids 50–81. Indeed, this position was confirmed by lane 50–81 of Fig. 4B, which shows that this domain of HMG-I(Y) interacts directly with SRF.

Enhancement of SRF Binding Does Not Require HMG-I(Y) Binding to DNA—To see if HMG-I(Y) had to bind to DNA to augment SRF binding, we used the mutant HMG-I(Y) proteins shown in Fig. 4A in electrophoretic mobility shift assays with the c-fos CArG box probe. Consistent with our data shown in Fig. 3, wild-type HMG-I(Y) bound to DNA and augmented SRF binding (Fig. 4C, lane 1–96). Similar results were obtained for HMG-I(Y)1–81 (data not shown), HMG-I(Y)50–96, HMG-I(Y)50–81, and HMG-I(Y)1–45 did not bind DNA (Fig. 4C, SRF lanes), consistent with results published by others (36). Surprisingly, HMG-I(Y)50–96 and HMG-I(Y)50–81 were able to enhance binding of SRF to the CArG box despite their inability to bind DNA (Fig. 4C, SRF lanes). Because the effect of HMG-I(Y) on SRF was independent of DNA binding, it probably involved protein-protein interactions.

**Fig. 4.** Map of HMG-I(Y) domains important for interaction with SRF. A, structure of HMG-I(Y) mutations. Recombinant polyhistidine-tagged proteins containing portions of HMG-I(Y) were synthesized as indicated. Their ability to bind SRF, to bind DNA, and to enhance SRF binding to DNA are shown on the right. B, HMG-I(Y) amino acids 50–81 are required for interaction with SRF. CP-IMAC assays were performed as described under "Materials and Methods." Radiolabeled SRF was incubated with 1 µg of recombinant protein and 20 µl of cobalt affinity resin for 2 h at 4 °C. Bound proteins were eluted with 100 mM EDTA and resolved on 10% Tricine-SDS gels. Recombinant polyhistidine-tagged SRF was included as a positive control. C, DNA binding by HMG-I(Y) is not required for enhancement of SRF binding. Electrophoretic mobility shift assays were performed with a radiolabeled CArG box oligonucleotide from the c-fos promoter and recombinant proteins containing the indicated HMG-I(Y) mutations in the presence or absence of SRF (0.8 pmol). Equimolar amounts (25 pmol) of recombinant mutant protein were used.

HMG-I(Y) Binding to DNA Is Not Required for Enhancement of SRF-dependent Transcription—Because HMG-I(Y)50–81 augmented SRF binding in the absence of DNA binding, we studied its effect on SRF-dependent transcription. HMG-I(Y)50–81, HMG-I(Y)1–81, and HMG-I(Y)1–45 were cloned into the Dro sophila expression vector pPAC (27). The activity of HMG-I(Y)50–81 was similar to that of the wild-type protein (Fig. 5), a result consistent with the ability of HMG-I(Y)1–81 to bind SRF and enhance SRF binding to DNA in vitro (Figs. 3 and 4, B and C, and data not shown). HMG-I(Y)1–45, a protein unable to interact with SRF or enhance SRF binding to DNA in vitro, had no significant activity. In contrast, HMG-I(Y)50–81, which does not bind DNA but clearly enhances SRF binding to DNA in vitro, was able to stimulate SRF-dependent transcription to a
FIG. 5. DNA binding by HMG-I(Y) is not required for enhancement of SRF-dependent transcription. A luciferase construct containing the SM22e promoter was transfected with the indicated expression plasmids, and activity was normalized to β-galactosidase as described for Fig. 1. Results represent the average (± S.E.) of at least three independent experiments.

level 10-fold above baseline (Fig. 5). These data indicate that the effect of HMG-I(Y) on SRF-dependent transcription is independent of the ability of HMG-I(Y) to bind DNA.

DISCUSSION

Little is known about the mechanisms by which HMG-I proteins affect cell growth and differentiation. In contrast, SRF is known to regulate a number of immediate-early genes, such as c-fos, thought to be important in cell proliferation. The presence of an AT-rich core within the SRF-binding site (a CArG box) led us to hypothesize that the HMG-I proteins and SRF may interact at this element.

We found that HMG-I(Y) facilitates activation of the c-fos promoter by SRF (Fig. 1A) and that transcriptional activation by HMG-I(Y) could be mediated by the CArG box alone when it was linked to a heterologous promoter in the context of genomic chromatin (Fig. 1B). Our findings are consistent with previous reports that HMG-I(Y) itself does not have an intrinsic ability to activate promoters but that it can alter chromatin structure and regulate the activity of other transcription factors (12). Our findings suggest that HMG-I proteins may be involved in the regulation of a variety of SRF-responsive genes. The recent observation that expression of junB, an SRF-responsive gene, is essential for neoplastic transformation in rat thyroid cells, and that it requires concurrent expression of HMGI-C (37), is consistent with our data. Our findings also suggest a potential mechanism by which HMG-I proteins interact with SRF and participate in the regulation of growth-related genes. To our knowledge, this is the first description of an interaction between a MADS-box transcription factor such as SRF and the HMG-I proteins. Given that the MADS-box protein MEF2 also has AT-rich recognition sequences, our results raise the possibility that HMGI-C involves a different domain.

Our results from in vitro binding studies of wild-type HMG-I(Y) and SRF are consistent with results from transfection assays; however, the results from the former are somewhat surprising given that each protein is expected to bind in the minor groove (7, 42). We found that mutations that removed the first and second or second and third AT-hook domains block DNA binding (Fig. 4), consistent with published findings that the second AT-hook domain is important for sequence-specific binding (36). HMG-I(Y)50–81 and HMG-I(Y)50–81 are not able to bind DNA in electrophoretic mobility shift assays, although they are able to enhance the binding of SRF to DNA, at a level similar to that obtained with wild-type HMG-I(Y). This observation is in contrast to those obtained in other studies which show that DNA binding by HMG-I(Y) is critical to enhancement of Oct-6 binding to the JC virus promoter (43) and activation of the β-interferon promoter by NF-κB (36). One explanation for our observations is that the interaction of HMG-I(Y) with SRF induces a conformational change in SRF that enhances its ability to bind to DNA. Further studies will be necessary to test this hypothesis.

Although our data (Fig. 4) and data reported by Yie et al. (36) indicate that the third AT hook does not bind specifically to DNA in gel mobility shift assays, it remains possible that the third AT-hook domain present in their and our HMG-I(Y) mutants may bind DNA in vivo. In a recent NMR study, Huth et al. (45) show that the third AT hook makes specific contacts with DNA when bound as part of a molecule containing the second AT hook. A previous NMR study also showed that a peptide containing the core AT-hook motif PRGRP binds specifically to the minor groove of AT-rich DNA, but with low affinity (46).

In the transient transfection analysis (Fig. 5), all mutations that preserve the interaction with SRF in vitro enhance SRF-dependent transcription in vivo. HMG-I(Y)50–81, which does

being explored, is that the effect of HMG-I(Y) proteins on smooth muscle may be important after hypertrophic stimuli.

To understand the mechanism by which HMG-I proteins enhance SRF-dependent transcription, we analyzed the binding of HMG-I proteins to the CArG box and assessed their effect on SRF binding to DNA. As expected, given its preference for AT-rich DNA, HMG-I(Y) binds to CArG boxes specifically (Fig. 2). Also, SRF binding to the CArG box is enhanced dramatically in the presence of HMG-I(Y) (Fig. 3). Although little change was seen in the mobility of the SRF-containing complex generated in the presence of HMG-I(Y), this was probably due to the low molecular weight of HMG-I(Y) relative to SRF and to the marked change in DNA conformation already induced in the presence of SRF (42). The sharp bend in the DNA induced by SRF binding alters electrophoretic mobility markedly so that an additional effect of HMG-I(Y) may not be apparent.

By a CP-IMAC assay, we found that HMG-I(Y) and SRF interact in the absence of DNA (Fig. 4B). Mutational analysis indicated that the region of interaction comprises amino acids 50–81, an area surrounding the third AT-hook domain. It has been shown that HMG-I(Y) amino acids 46–56 contain the domain that binds Oct-6, a POU-domain transcription factor (43). We do not know whether the Oct-6 and SRF interaction domains are distinct, and we know little about the region of HMG-I(Y) required for binding to other transcription factors. Computer data base searching with the BLAST algorithm (44) revealed no significant homology between this peptide interaction domain and a domain in any protein other than HMG-I(Y). Fig. 1B shows that the effect of wild-type HMGI-C on SRF is similar to that of HMG-I(Y). Other than the AT hooks in this region, however, these two proteins share little homology. This lack of homology raises the possibility that the effect of HMGI-C involves a different domain.

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not bind DNA, still augments transcription though to a lesser degree than does wild-type HMG-I(Y). The reasons for the slight disparity between these in vivo data and the in vitro electrophoretic mobility shift assay data are not clear. It is noteworthy that deletion of the C-terminal acidic tail (Figs. 4A and 5) does not affect the ability of HMG-I(Y) to enhance SRF-dependent transcription. Our results are in contrast with results from studies by others on the SRF-dependent transcription. We thank Matthew Layne and Chung-Ming Hsieh for many useful comments and suggestions, Thomas McVarish for editorial support, and Bonna Ith for expert technical assistance. We thank Raymond Reeves for the mouse HMG-Y cDNA, Alfredo Fusco for the mouse HMG-I(Y) cDNA, Tom Maniatis for the Drosophila expression plasmids pPACHMGI, pPAC, and pshp62LacZ, and Michael Gilman for the human SRF cDNA. We also thank Cheeptip Benyajati for the Drosophila SL2 cells.

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