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YY1 Facilitates the Association of Serum Response Factor with the c-*fos* Serum Response Element

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YY1 is a multifunctional transcription factor that acts as an activator or repressor in different contexts. YY1 binds to multiple sites in the mouse c-*fos* promoter, inducing at each site a sharp DNA bend. Binding of YY1 to a site situated between the cyclic AMP response element (CRE) and the TATA box bends the DNA in a way that interferes with the interaction of proteins bound at the CRE and TATA elements, resulting in repression of transcription. Here, we show that binding of YY1 to a different site in the c-*fos* promoter has a different result. Binding of YY1 to the c-*fos* serum response element (SRE) enhances the binding of serum response factor (SRF). This enhancement requires the binding of YY1 to SRE DNA. YY1 and SRF can cooccupy the SRE at least transiently. In the region of overlapping contact, YY1 contacts DNA in the major groove, while SRF contacts DNA in the minor groove. YY1 also enhances the association of SRF with the SRE in transfected insect cells. Thus, although YY1 induces similar structural changes in DNA at different binding sites, it can have distinct local effects on protein-DNA and protein-protein interactions. These data support a general role for YY1 in the building of highly organized promoter complexes.

The promoter regions of eukaryotic genes generally contain multiple binding sites for different transcription factors. The spacing and relative phasing of these binding sites are crucial for the stereospecific interactions among the proteins bound in the promoter region (for a review, see reference 50). These interactions can be regulated by structural, or architectural, transcription factors (8, 33, 49, 50). These factors bind to DNA in a sequence-specific manner, but rather than directly controlling the activity of the transcription machinery, they facilitate or disrupt interactions among other, more conventional transcription factors. This activity is associated with the ability of these factors to alter local DNA structure.

We have shown previously that in the mouse c-*fos* promoter, the transcription factor YY1 appears to function as such a structural transcription factor (33). YY1 is a zinc finger protein with multiple reported functions, including transcriptional activation and repression and the ability to act as an initiator of transcription (19, 20, 35, 45, 54). YY1 binding sites are found in the promoter regions of many genes, and mutational analyses indicate that these sites are often essential for the correct regulation of these genes (2, 7, 17–19). YY1 interacts with several cellular and viral proteins, including the adenovirus E1A protein, transcription factor Sp1, and the c-*myc* oncoprotein (39, 42, 45, 46). The mechanism of action of YY1 in these contexts is not fully understood, nor is it clear whether these diverse functions reflect a single underlying biochemical activity.

YY1 binds to at least three sites in the mouse c-*fos* promoter (16, 27, 33). YY1 binding induces DNA bends of approximately 80° in each of these sites and therefore can introduce significant curvature into promoter DNA (33). YY1-induced DNA bending at a site between the cyclic AMP response element and the TATA box in the c-*fos* promoter results in repression of promoter activity. However, this repression is not an intrinsic activity of YY1. Rather, YY1 acts to bend the DNA in a way that interferes with the productive interaction of proteins bound to the two flanking elements. When the orientation of the YY1 site is reversed or the phasing of the sites is changed, YY1 becomes an activator of the same promoter. Thus, at this site, YY1 acts as a structural factor to physically organize the DNA-protein complex that forms on the promoter.

In this study, we have analyzed the role of YY1 DNA binding at a second site in the c-*fos* promoter, the serum response element (SRE). The SRE is an essential element for the rapid induction of c-*fos* transcription in response to growth factors, cytokines, and other extracellular stimuli (53). This element is bound by a 67-kDa phosphoprotein termed serum response factor (SRF) (10, 13, 38, 51). Several lines of evidence suggest that the binding of SRF to the SRE is necessary but not sufficient for all of the functions of the SRE (12, 21, 26, 29, 44). This has led to a search for other SRE-binding proteins that are involved either directly or indirectly in the activation of the c-*fos* gene in response to extracellular signals. One such protein, $p62^{DBF}$, binds asymmetrically to the 5′ side of the SRE, and its binding site overlaps that of SRF (41). Several lines of evidence suggest that $p62^{DBF}$ is identical to YY1 (16, 32, 33). Here, we show that YY1 enhances the binding of SRF to the SRE in vitro and in vivo. This process requires the binding of YY1 to the SRE. We show that even though the binding sites for SRF and YY1 overlap, the two proteins can cooccupy this element by binding to the minor and major grooves of the DNA, respectively. We propose that the YY1-induced bend in SRE DNA enhances the kinetics of SRF binding, suggesting that a single biochemical activity of YY1, DNA bending, can result in distinct functional outcomes at two different sites in the c-*fos* promoter.

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MATERIALS AND METHODS

Plasmid constructions. Plasmids DHYY1 and DLacZ were described previously (33). Plasmid DHSRF was made by cloning the full-length human SRF as an *Xba*I-*Bam*HI fragment into the pDAC5 vector as described previously (33).

Plasmid DHSRF-VP16 was constructed as follows. The VP16 activation domain was amplified by PCR using oligonucleotides 5′-ACTGACTAGTGTCAGCCT GGGGGACGAG-3′ and 5′- GCATGGATCCGATTCATCTAGACCCACCG TACTCGTCAATTCC-3′. The PCR fragment was digested with *Spe*I and *Bam*HI and cloned into the pDAC5 vector digested with *Xba*I and *Bam*HI. This vector, pDACVP16, was further digested with *Xba*I and *Bam*HI to clone the *Xba*I-*Bam*HI fragment of full-length human SRF. Bacterial expression vectors p6XHis-YY1, p6XHis-SRF, and p6XHis-ZBP (expressing YY1, SRF, and ZBP, respectively) were described previously (1, 33).

Protein purification. Bacterially synthesized histidine-tagged recombinant SRF and YY1 proteins were affinity purified over a nickel chelate column as described previously (33). GST-Phox1 and histidine-tagged SRE-ZBP were prepared as described previously (references 1 and 14, respectively). Human $p62^{DBF}$ was prepared from HeLa cells as follows. HeLa nuclear extract was fractionated on an S-300 column, and $p62^{DBF}$ activity was determined by mobility shift assay. Active fractions were pooled and applied to a heparin-agarose column. $p62^{DBF}$ activity eluted at 0.4 M KCl. Active fractions were pooled and passed over an oligonucleotide affinity column. The oligonucleotide consisted of the following complementary strands:

5′-TCGACCACAGGATCGCCATATTACCACATCTGCG-3′

5′-TCGACGCAGATGTGGTAATATGGCGATCCTGTGG-3′

Active fractions eluting at 0.6 M KCl were pooled.

DNase I footprinting. DNase I footprint assays were done essentially as described previously (14). Probes for DNase I footprinting were prepared by PCR, except that only a single primer was labeled with [γ-^{32}P]ATP. Proteins were incubated on ice for 30 min in a 50-μl reaction mixture containing 50 mM Tris (pH 7.9), 1 mM dithiothreitol, 80 mM KCl, 1 mM EDTA, 10% polyvinyl alcohol, 5% glycerol, 100 ng of poly(dI-dC), 20 μg of bovine serum albumin (BSA), and 10,000 cpm of SRE probe fragment. After the incubation, 50 μl of an ice-cold solution containing 10 mM $MgCl_2$, 5 mM $CaCl_2$, and DNase I (10 ng) was added to the reaction mixture. The reaction mixture was incubated on ice for 1 min. The reaction was stopped by the addition of 90 μl of stop buffer containing 20 mM EDTA (pH 8.0), 1% sodium dodecyl sulfate, 0.2 M NaCl, and 100 μg of glycogen per ml. The DNase I-treated probe was purified by extraction with phenol and chloroform and precipitated with ethanol and analyzed on an 8% polyacrylamide–7 M urea gel.

Mobility shift assays. All mobility shift reactions were performed with 20-μl reaction mixtures that contained 10 mM Tris-Cl (pH 7.9), 1 mM dithiothreitol, 1 mM EDTA, 20% glycerol, 0.05% Nonidet P-40, 60 mM KCl, 20 μg of BSA, and various amounts of recombinant SRF and YY1 as indicated below. The proteins were incubated for 10 min at room temperature before and after the addition of the probe. Standard assays were analyzed on 5% (39:1) polyacrylamide gels run in 0.5× Tris-borate-EDTA buffer. When competitor oligonucleotide or antibody was used, it was added 10 min after the addition of the probe and the mixture was incubated at room temperature for another 10 min. In the experiments whose results are shown in Fig. 5, the reactions were analyzed on 6% (39:1) polyacrylamide gels run in 0.5× Tris-glycine-EDTA containing 5 mM magnesium chloride. Monoclonal antibody against YY1 was a gift from Tom Shenk (Princeton University), and polyclonal antiserum against SRF was a gift from Michael Greenberg (Harvard Medical School).

Transfection assays. *Drosophila melanogaster* SL2 cells were grown in Schneider's insect medium supplemented with 10% heat-inactivated fetal bovine serum. All transfections were performed by a calcium phosphate coprecipitation procedure as described previously (33). Transfections were carried out with 3 μg of reporter plasmids, 1 μg of pACLacZ internal control plasmid, and the amounts of effector plasmids indicated below. In all cases, the total amount of DNA was adjusted to 20 μg with pUC119 DNA. Chloramphenicol acetyltransferase (CAT) assays were done as described elsewhere (11). CAT activities were normalized to the β-galactosidase activity measured for the extracts. In all cases, results are expressed as mean percent conversion of chloramphenicol to acetylated forms ± standard deviation, on the basis of three independent transfections.

RESULTS

$p62^{DBF}$ and YY1 enhance the binding of SRF to the SRE in vitro. Comparison of the sequences of YY1 binding sites from a number of promoters shows that YY1 binding sites frequently contain a core motif of CCAT (2, 19, 33). This motif is found in the SRE, and it is bound by factors $p62^{DBF}$ and MAPF1 (41, 55), which are now known to be YY1 (16, 33). This sequence is also contacted by SRF (10, 51), and substitution of the C · G base pairs in this sequence abolishes binding of both proteins (41). Since this sequence is essential for the binding of both SRF and YY1 to the SRE, it has been proposed that these proteins bind competitively to the SRE (16, 28).

To test this idea, we examined whether $p62^{DBF}$ affinity purified from HeLa cells influenced the binding of bacterially expressed, affinity-purified SRF, as assayed by DNase I footprinting assays with an SRE probe. Surprisingly, we found that $p62^{DBF}$ enhanced rather than inhibited the binding of SRF to the SRE. The results of a representative experiment are shown in Fig. 1A. At amounts between 10 and 40 ng, SRF alone failed to generate a footprint on the SRE (lanes 1 to 3). In contrast, when 20 ng of affinity-purified HeLa $p62^{DBF}$ was included in the binding reaction mixture, 10 ng of SRF was sufficient to give a complete footprint (Fig. 1A, lanes 4 to 6). $p62^{DBF}$ alone was unable to generate a footprint at amounts between 20 and 100 ng (Fig. 1A, lanes 7 to 9). The inability of $p62^{DBF}$-YY1 to form a footprint is due to inhibition of $p62^{DBF}$-YY1 activity by the divalent cations added during the DNase I digestion (data not shown); DNA binding by similar concentrations of $p62^{DBF}$-YY1 is readily demonstrated in a mobility shift assay (see below). The footprint generated by SRF in the presence of $p62^{DBF}$ is indistinguishable from that observed with SRF alone at a 10-fold-higher concentration (Fig. 1A, compare lanes 4 to 6 with lane 10). Because the binding site of $p62^{DBF}$ is entirely within the limits of the SRF footprint, this observation does not permit us to determine whether $p62^{DBF}$ is present in the footprinting complex. Thus, despite overlapping binding sites that would suggest competitive binding, $p62^{DBF}$ enhances the binding of SRF to the SRE.

To determine whether recombinant YY1 behaved similarly, we produced YY1 in *Escherichia coli*. Figure 1B shows that affinity-purified, bacterially expressed YY1 also enhanced the binding of SRF to the SRE (compare lanes 1 to 5 with lanes 6 to 10). Therefore, affinity-purified HeLa $p62^{DBF}$ and recombinant YY1 share the ability to enhance the binding of SRF to the SRE. This observation supports the idea that $p62^{DBF}$ is YY1. Furthermore, that this activity is observed with both native protein purified from human cells and recombinant protein purified from bacteria strongly suggests that it represents an authentic biochemical activity of YY1.

To establish further that the effect on SRF binding was due to an intrinsic activity of YY1 and not, for example, buffer components or the N-terminal histidine tag on the recombinant protein, we produced and purified another histidine-tagged zinc finger SRE-binding protein, SRE-ZBP (1). Figure 2 shows the results of mobility shift assays of bacterially produced SRF (10 to 50 ng) alone or in the presence of 20 ng of recombinant YY1 or SRE-ZBP. In the presence of YY1, SRF binding to the SRE was enhanced at all SRF concentrations, consistent with the results of footprinting assays. In contrast, SRF activity was unaffected by the presence of SRE-ZBP. Although the affinity of SRE-ZBP for the SRE is lower than that of YY1, the concentrations of protein, histidine tag, and other buffer components were very similar in these reaction mixtures. Thus, the effect of YY1 in these experiments is not due to nonspecific effects of buffer conditions or protein concentrations and likely reflects an authentic biochemical activity of the protein.

YY1 enhances occupancy of the SRE by SRF in *D. melanogaster* SL2 cells. To test whether YY1 can enhance the association of SRF with the SRE in vivo, we performed transient-transfection assays with *D. melanogaster* SL2 tissue culture cells, which lack detectable YY1 activity (33, 42). We transfected the cells with plasmids expressing YY1 and an SRF-VP16 fusion protein together with a simple CAT reporter gene carrying a single SRE. Association of SRF-VP16 with the SRE should result in activation of the reporter gene because of the transcriptional stimulatory activity of the VP16 domain. Thus, this assay provides an indirect measure of occupancy of the

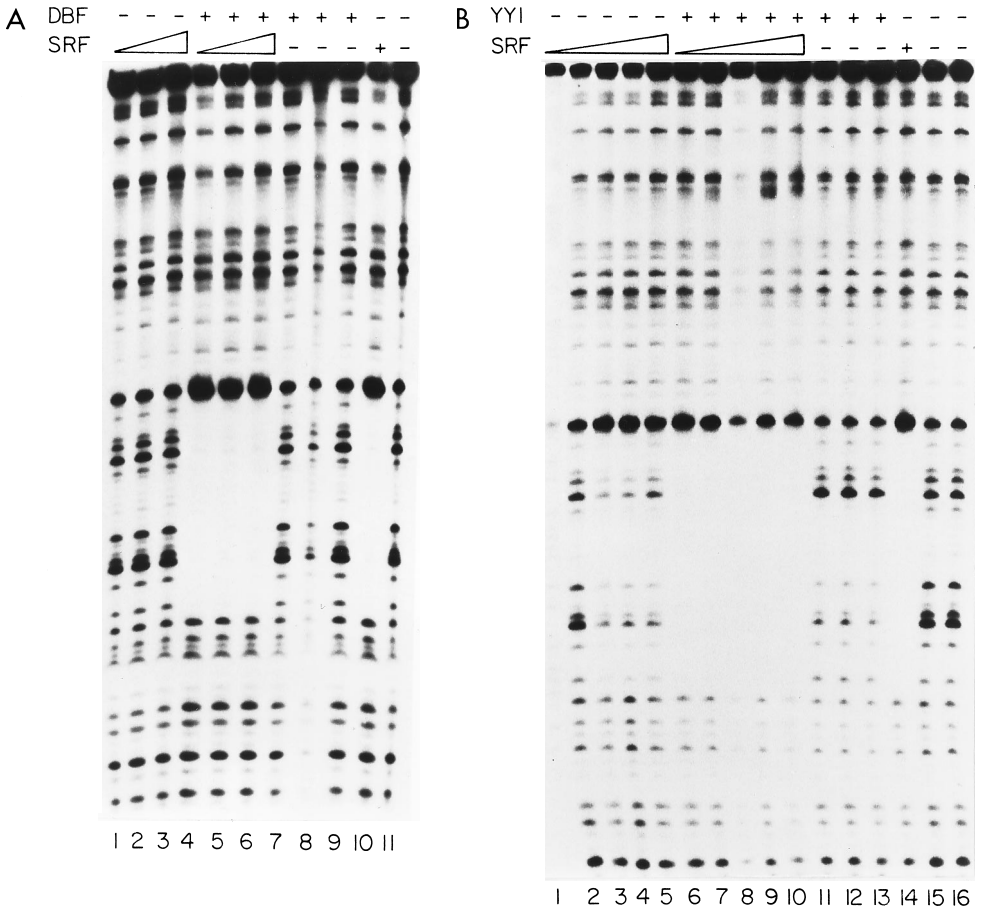


FIG. 1. $p62^{DBF}$ and YY1 enhance the binding of SRF to the SRE. (A) Results of DNase I footprinting assay showing that affinity-purified $p62^{DBF}$ from HeLa cells can enhance the binding of bacterially expressed SRF to the SRE. The singly end-labeled probe used in this assay contains mouse c-*fos* promoter sequences between −356 and −248. Reaction mixtures contained the following: lanes 1 to 3, 10, 20, and 40 ng of SRF, respectively; lanes 4 to 6, 10, 20, and 40 ng of SRF, respectively, with 20 ng of $p62^{DBF}$; lanes 7 to 9, 20, 40, and 100 ng of $p62^{DBF}$, respectively; lanes 10 and 11, 200 ng of SRF alone and without any protein, respectively. (B) Results of DNase footprinting assay showing that recombinant YY1 can enhance the binding of bacterially expressed SRF to the SRE. Reaction mixtures contained the following: lanes 1 to 5, 10, 15, 20, 25, and 50 ng of SRF, respectively; lanes 6 to 10, 10, 15, 20, 25, and 50 ng of SRF, respectively, in addition to 25 ng of YY1; lanes 11 to 13, 25, 50, and 100 ng of YY1, respectively; lane 14, 200 ng of SRF; lanes 15 and 16, no protein.

SRE by SRF-VP16 in vivo. Figure 3A shows that SRF-VP16 alone elicited an approximately twofold induction of the SRE reporter (lane 2), presumably reflecting the baseline interaction of SRF with the SRE in the transfected cells. Addition of increasing amounts of YY1 expression plasmid resulted in a dose-dependent increase in reporter gene activity (Fig. 3A, lanes 3 to 5). This enhancement was not observed with a reporter carrying a mutant SRE that binds neither SRF nor YY1 (Fig. 3A, lanes 11 to 13). YY1 alone modestly activated both reporter genes, but this effect was not dose dependent, and we regard it as nonspecific. We conclude that YY1 is able to enhance the association of SRF with its binding site in vivo.

YY1 binding to the SRE is essential for enhancement of SRF binding. Binding of YY1 to the SRE induces a DNA bend of approximately 80° (33). If this structural change in the DNA is required for enhancement of binding of SRF to the SRE, then binding of YY1 to the SRE must be required for this effect. To test this hypothesis, we prevented the association of YY1 with the SRE by the inclusion of an unlabeled oligonucleotide carrying a YY1 binding site from the adeno-associated virus P5 promoter (45). Figure 4 shows the results of mobility shift assays of 10 to 50 ng of SRF in the absence or presence of 20 ng of recombinant YY1. As seen previously, the inclusion of YY1 resulted in a clear enhancement of SRF binding activity on the SRE (compare lanes 4 to 6 with lanes 1 to 3). However, this enhancement was abolished in the presence of the adeno-associated virus P5 oligonucleotide, which binds YY1 but not SRF (Fig. 4, lanes 7 to 9). In contrast, inclusion of an oligonucleotide that binds neither YY1 nor SRF (pm12 [9]) did not affect the enhancement activity of YY1 (Fig. 4, lanes 10 to 13). These observations suggest that YY1 must bind to the SRE to enhance the association of SRF with this element. This result contrasts with our previous analysis of enhancement of SRF-SRE association by the homeodomain protein Phox1, which does not require DNA binding for its enhancement activity (14).

YY1 and SRF can cooccupy the SRE. Since YY1 must bind to the SRE to enhance SRF binding, the structure of the YY1-SRE complex must kinetically favor the binding of SRF to the SRE. If so, then at some point during the association of SRF with the SRE, YY1 and SRF must cooccupy the SRE. However, our standard mobility shift and footprinting protocols were unable to detect a ternary complex containing both proteins. Therefore, we experimented with gel electrophoresis conditions to find a protocol that stabilized this complex. We found that gels run in Tris-glycine buffer containing 5 mM $MgCl_2$ allowed the detection of a novel complex with a lower

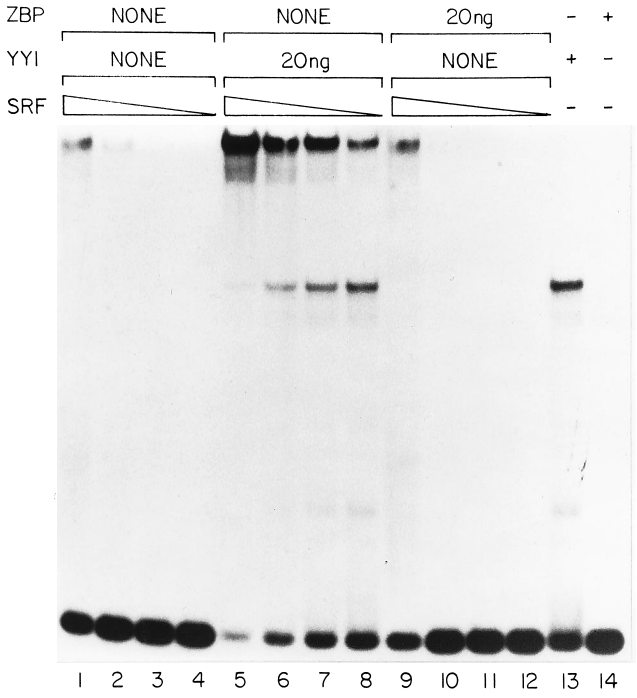


FIG. 2. The effect of YY1 on the binding of SRF to the SRE is specific. Results of mobility shift assays of bacterially expressed SRF, YY1, and ZBP either alone or in combination are shown. Lanes 1 to 4, SRF alone; lanes 5 to 8, SRF and 20 ng of YY1; lanes 9 to 12, SRF and 20 ng of ZBP; lanes 13 and 14, YY1 alone and ZBP alone, respectively. The amounts of SRF in the titrations were 100, 50, 25, and 10 ng.

mobility (Fig. 5A). Incubation of 10 ng of SRF with the SRE probe resulted in the detection of SRF-SRE complexes under these gel conditions (Fig. 5A, lane 1). In the presence of 100 ng of YY1, a novel complex with a lower mobility was observed (Fig. 5A, lane 2). The formation of this complex was abolished by the inclusion of an unlabeled oligonucleotide that binds YY1 (Fig. 5A, lane 3) but not by an oligonucleotide that does not bind YY1 (lane 4). Thus, the formation of this complex correlates with the ability of YY1 to enhance formation of the binary SRF-SRE complex in Tris-borate gels.

To confirm that this complex contained both SRF and YY1, we challenged binding reaction mixtures with antibodies to each protein. Because the YY1 antibody apparently cross-reacts with the histidine tag in bacterially produced SRF, for this experiment it was necessary to prepare SRF as a glutathione *S*-transferase (GST) fusion protein. Figure 5B shows that the complex formed by GST-SRF is further retarded in mobility by the inclusion of YY1, as seen with histidine-tagged SRF (compare lanes 1 and 2). Addition of antibody to YY1, but not a control antibody, restores the mobility of the complex to that of GST-SRF alone (Fig. 5B, lanes 3 and 4). This observation shows directly that YY1 is required for the formation of the high-mobility complex formed in the presence of both proteins, although it does not formally show that YY1 is present in the complex. Addition of antibody to SRF to reaction mixtures containing both proteins results in a further reduction of complex mobility (Fig. 5B, lane 5), showing that SRF is present in the complex. On the basis of the dependence on YY1 for formation of this complex and its sensitivity to both YY1 antibody and YY1 binding sites, we conclude that it is most likely a ternary complex containing SRF, YY1, and DNA.

That SRF and YY1 can simultaneously occupy the SRE is surprising, since mutagenesis of the SRE and modification-interference assays suggest that the two proteins interact with the same nucleotides in the SRE (16, 41). Substitutions in the C · G base pairs of the CCAT motif on the 5′ side of the SRE abolish binding of both proteins (16, 41), and certain mutations in the A · T base pairs also affect binding (32). In addition, methylation of the guanine bases opposite the cytosine residues on the bottom strand of this sequence prevents binding of both proteins (41), and ethylation of adenine bases in this sequence prevents binding of SRF (3, 52). Thus, these data suggest that each protein recognizes these base pairs independently, and the methylation and ethylation experiments suggest that contacts occur in the major groove. How could the two proteins simultaneously occupy the same major groove?

Structural studies of zinc finger proteins related to YY1 show that proteins of this class bind DNA in the major groove (6, 36, 37). In contrast, the structure of the SRF-SRE complex is not reported, although major-groove binding has been inferred on the basis of the modification-interference results and modeling studies (3, 43, 48). That we can observe simultaneous binding of YY1 and SRF to the DNA suggests that SRF may recognize the minor groove of the SRE in the region of overlap. To test this hypothesis, we synthesized SRE oligonucleotides in which the A · T base pairs in the core of the SRE were substituted with inosine · cytosine base pairs (Fig. 6). Inosine · cytosine base pairs resemble adenine · thymine base pairs in the minor groove and guanine · cytosine base pairs in the major groove. Thus, this substitution effectively mutates the major groove only. A similar approach has been used to demonstrate minor-groove interactions for a number of DNA-binding proteins, including TBP (23, 24, 47).

Radiolabeled oligonucleotides containing wild-type and I · C-substituted SREs were incubated separately with SRF and YY1. As a control, we also tested the homeodomain protein Phox1, which binds also to the A · T core of the SRE (14). Structural studies of homeodomain-DNA interactions indicate that the major homeodomain-DNA contacts are made in the major groove (25, 34, 56). As expected on the basis of the predominantly major-groove interaction of closely related proteins, neither YY1 nor Phox1 bound the I · C-substituted SRE probe (Fig. 6, lanes 4 and 6). In contrast, SRF bound as well to this probe as to the wild-type SRE (Fig. 6, compare lanes 1 and 2). This result suggests that SRF does not make significant major-groove contacts in the A · T core of the SRE. It may instead contact the DNA solely in the minor groove, or, alternatively, it may not contact the DNA at all in this region.

To distinguish between these alternatives, we made use of the drug distamycin, a nonintercalating DNA-binding molecule that interacts with the minor groove of DNA sequences containing at least five consecutive A · T base pairs. Several studies have shown that distamycin acts as a simple competitive inhibitor of DNA binding for proteins that recognize DNA in the minor groove (4). Thus, if SRF recognizes the A · T core of the SRE in the minor groove, we would expect distamycin to inhibit SRF binding. Figure 7 shows that distamycin indeed inhibited SRF binding in a dose-dependent fashion (lanes 1 to 6). In contrast, distamycin had no effect on binding of the SRE by YY1 (lanes 7 to 12), consistent with the idea that YY1 recognizes the major groove of the A · T core. Taken together, these data suggest that in the SRF-YY1-SRE ternary complex, YY1 recognizes the major groove of the A · T core, while SRF simultaneously occupies the minor groove on the opposite face of the DNA helix.

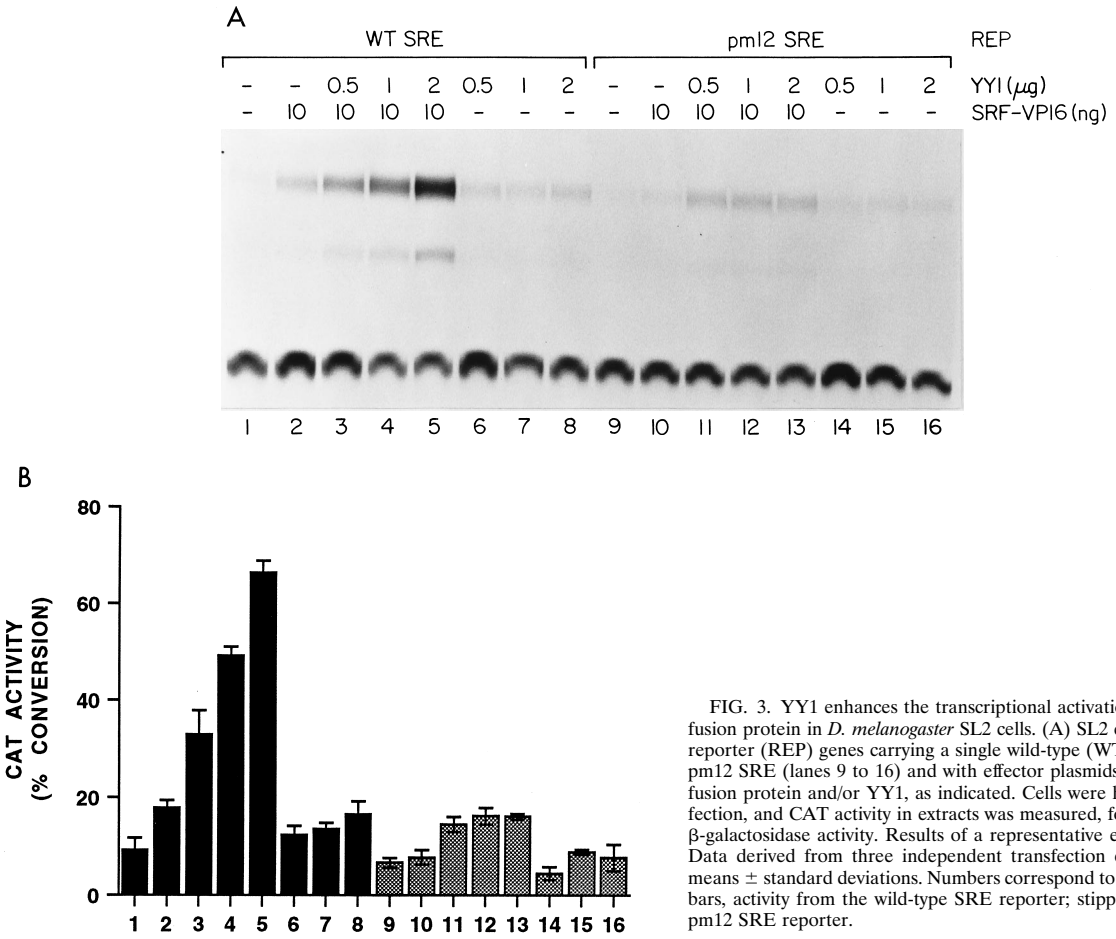


FIG. 3. YY1 enhances the transcriptional activation potential of SRF-VP16 fusion protein in *D. melanogaster* SL2 cells. (A) SL2 cells were transfected with reporter (REP) genes carrying a single wild-type (WT) SRE (lanes 1 to 8) or a pm12 SRE (lanes 9 to 16) and with effector plasmids expressing an SRF-VP16 fusion protein and/or YY1, as indicated. Cells were harvested 36 h after transfection, and CAT activity in extracts was measured, following normalization for β-galactosidase activity. Results of a representative experiment are shown. (B) Data derived from three independent transfection experiments, expressed as means ± standard deviations. Numbers correspond to the lanes in panel A. Solid bars, activity from the wild-type SRE reporter; stippled bars, activity from the pm12 SRE reporter.

DISCUSSION

Here, we have shown that YY1 enhances the binding of SRF to the SRE in vitro and in vivo. Binding of YY1 to the SRE is essential for the enhancement of SRF binding activity, and during this process SRF and YY1 cooccupy the SRE. Although SRF and YY1 recognize overlapping sequences within the SRE, we find that their binding is not mutually exclusive. We can account for simultaneous binding of SRF and YY1 to the SRE, because in overlapping areas of recognition, SRF binds to the minor groove and YY1 binds to the major groove.

Previous reports suggested that the interaction of SRF and YY1 on the c-*fos* SRE and related sites is competitive (16, 28). One group has suggested that SRF can displace YY1 on an SRE-like site in a muscle actin gene (28). These data are not inconsistent with ours, in that under conditions in which a high level of SRF-DNA complex formation is promoted, formation of a simple YY1-DNA binary complex is inhibited (Fig. 2 and 4). We interpret this phenomenon as a competition between the SRF-DNA complex and free DNA for YY1; increased SRF concentrations direct YY1 toward the ternary complex and away from the simple binary complex with DNA. This is distinct from simple competition of two proteins for a common binding site, in which the competition must be reciprocal. Indeed, we do not observe the reciprocal phenomenon, reduction of SRF-DNA complex formation in the presence of YY1. In this regard, our data differ from those of an earlier report in which such competition is clearly observed (16). We ascribe this discrepancy to significant differences in experimental protocols. We note, however, that we observe enhancement of SRF-DNA interaction by YY1 and/or formation of a ternary complex in multiple experimental formats, using SRF prepared and purified either as histidine-tagged or GST fusion proteins and YY1 expressed in bacteria or purified from HeLa cells. Moreover, we observe these effects with nanogram quantities of purified proteins.

The binding of SRF is an essential step in the assembly of a growth factor-responsive protein-DNA complex on the SRE (53). Several lines of evidence suggest that the binding activity of SRF is regulated, by both covalent and noncovalent modification. For example, phosphorylation of SRF by several different protein kinases regulates its DNA binding activity in vitro (22, 30, 31, 40). In addition, SRF binding can be enhanced by noncovalent interaction with the human homeodomain protein Phox1 and other homeodomains of the paired class (14) and, as we show here, by YY1. For both Phox1 and YY1, enhancement can be demonstrated in vivo as well (reference 15 and this study).

Indeed, one of the striking features of SRF is that in the absence of such modifications or cofactors, interaction with the SRE is extremely slow (22, 30, 31, 40). Equilibrium binding in vitro with bacterially produced or dephosphorylated eukaryotic cell-derived SRF takes hours. Because the initial collision of SRF and its binding site under typical assay conditions in vitro is diffusion limited and therefore occurs on the order of milliseconds, stable binding of SRF to the SRE must require a rate-limiting isomerization of the SRF-SRE complex. Because

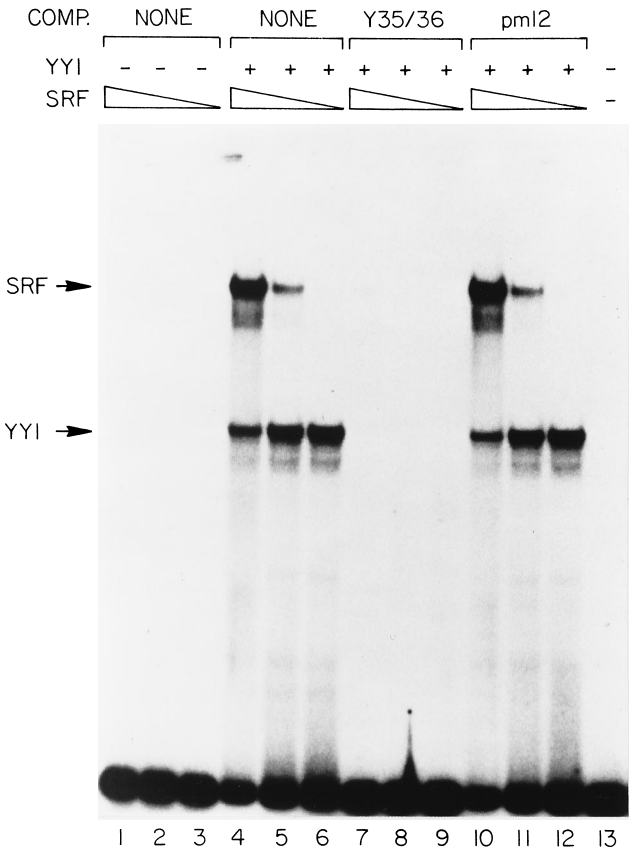


FIG. 4. YY1 must bind to the SRE to enhance SRF binding. Results of mobility shift assays of bacterially expressed SRF and YY1 on a wild-type SRE oligonucleotide probe are shown. The amounts of SRF in the titration were 50, 25, and 10 ng. +, indicates addition of 20 ng of YY1. The Y35/36 competitor (comp.) oligonucleotide contains sequences that correspond to positions −10 to +10 of the adeno-associated virus P5 promoter and binds YY1 but not SRF. Oligonucleotide pm12 is a mutant version of SRE that binds neither SRF nor YY1 (9, 41).

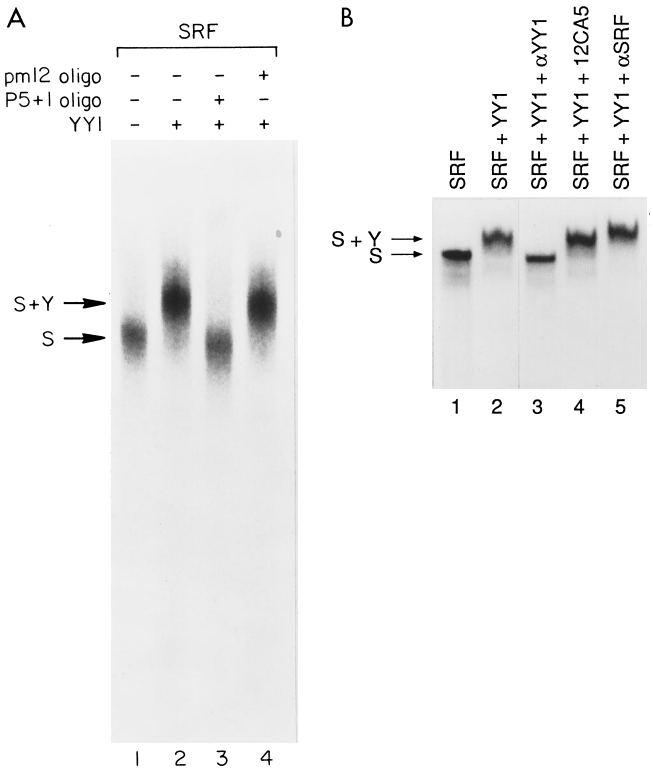


FIG. 5. YY1 and SRF can cooccupy the SRE. (A) Mobility shift assays of bacterially expressed YY1 and SRF on an SRE oligonucleotide probe were analyzed by electrophoresis in Tris-glycine buffer containing 5 mM $MgCl_2$. The presence (+) or absence (−) of YY1 and SRF is indicated above the lanes. The complexes containing SRF (S) and YY1 (Y) are indicated. YY1 by itself does not form a stable complex with the SRE under these electrophoretic conditions. The Y35/36 and pm12 competitor oligonucleotides (oligo) are described in the legend to Fig. 4. They were added at 100-fold molar excesses at the same time as YY1 and SRF. The electrophoresis was carried out for approximately 5 h at 200 V. The free probe cannot be seen, as it ran out of the gel during the extended electrophoresis. (B) Results of mobility shift assays of bacterially expressed GST-SRF and histidine-tagged YY1 in the presence of monoclonal antibody to YY1 (lane 3), irrelevant monoclonal antibody (lane 4), and polyclonal antiserum to SRF (lane 5).

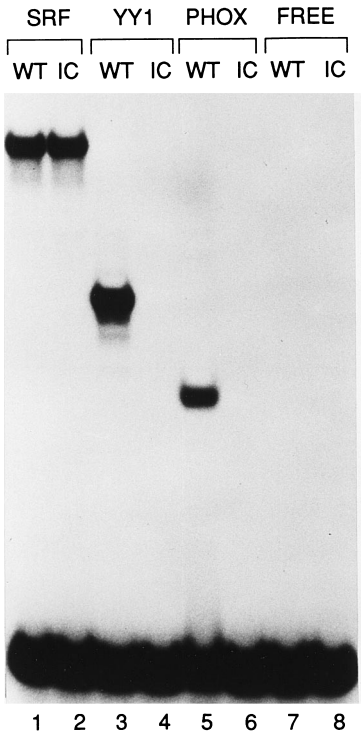
this putative isomerization can be accelerated both by modifications of SRF and by agents such as spermidine that affect DNA structure (31), we believe that this isomerization most likely involves a concerted change in the structure of both SRF and the SRE.

Proteins such as Phox1 and YY1 could enhance the rate of SRF binding through an effect on either the conformation of SRF or the structure of DNA. Phox1 appears to enhance SRF binding primarily through an effect on SRF conformation. Under in vitro conditions in which this enhancement is readily observed, the DNA binding activity of Phox1 is not required and Phox1 enhances the binding of SRF at sites to which Phox1 itself does not detectably bind. Furthermore, circular permutation analysis of the interaction of Phox1 with DNA indicates that Phox1 does not detectably influence DNA structure (32). In contrast, YY1 induces a sharp bend upon binding to SRE DNA, and its ability to bind to the SRE is absolutely required for enhancement of SRF binding. This suggests that the primary mode of enhancement of SRF binding by YY1 is through an effect on DNA structure: YY1 induces a DNA conformation that is kinetically favorable for binding of SRF.

We cannot, however, rule out additional contributions from direct protein-protein interactions between SRF and YY1. Indeed, the requirement for YY1 DNA binding could also reflect a role for the DNA in positioning YY1 and SRF so that they can interact directly. These interactions would presumably be too weak to permit complex formation in the absence of DNA, since we do not observe such complexes in vitro. In this case, the YY1-SRF interaction would resemble the interaction of HMGI/Y with NF-κB and ATF-2 at the beta-interferon promoter (5, 49, 50), in which HMGI/Y has the dual functions of organizing the DNA and interacting with the other proteins.

Given that genomic footprinting studies indicate that the SRE is constitutively occupied by a protein with the same footprint as SRF, what is the functional relevance of regulated SRF DNA binding activity? We have been unable to assess the effect of YY1 activity on the ability of SRF to mediate a serum response in mammalian cells, because most cell lines contain functionally saturating amounts of SRF and YY1. Cointroduction of either protein with an SRE-containing reporter gene results in only nonspecific effects on gene expression (data not shown, but see reference 21). Consequently, we resorted to the use of *Drosophila* cells to show that YY1 can interact with SRF within living cells with functional consequences for a reporter gene.

One possible scenario in which the effect of YY1 may be relevant is if the activation of c-*fos* transcription through the SRE requires the exchange of a new SRF molecule onto the



WT GGATGTCCATATTAGGACATCT

IC GGATGTCCICICCIGGACATCT

FIG. 6. Changes in the major groove of the SRE prevent binding of YY1 but not SRF. The nucleotide sequences of wild-type (WT) and inosine · cytosine-substituted (IC) SRE oligonucleotides are shown at the bottom. Mobility shift assays of recombinant SRF, YY1, and Phox1 were performed with a wild-type SRE probe (lanes 1, 3, and 5) and inosine · cytosine-substituted SRE probes (lanes 2, 4, and 6). Lanes 7 and 8, free probes.

site. In this case, the rate at which the exchange occurs will determine the kinetics of gene activation. Moreover, since the signals that activate c-*fos* transcription are short-lived, SRF exchange must occur within a prescribed time window. Slow exchange could preclude activation entirely. By enhancing the rate of exchange of SRF with the SRE, YY1 could promote serum-responsive transcription.

Because SRF half-sites and the YY1 binding consensus are nearly identical, most SRF binding sites should also bind YY1. Therefore, the requirement for YY1 cofactor activity may be widespread among SRF-regulated genes. We speculate that the state of rapid exchange maintained at such sites by YY1 and its direct influence on the three-dimensional structure of the DNA are critical for the timely association of a multicomponent protein-DNA complex capable of responding to very transient signals. Because YY1 is a ubiquitously expressed protein, SRF binding sites that contain a YY1 recognition sequence should be signal responsive in all cells. In contrast, certain potential SRF binding sites lack a CCAT motif and do not bind YY1. Such sites presumably require an alternative mechanism for loading SRF. One such mechanism may be interaction with proteins of the homeodomain class (14, 15). We propose that whereas YY1 is a ubiquitous cofactor required for activation of c-*fos* and other non-cell-specific genes, homeodomain proteins are cell-type-specific cofactors for SRF that fulfill the same function at genes that are activated by signals in a cell-type-specific pattern.

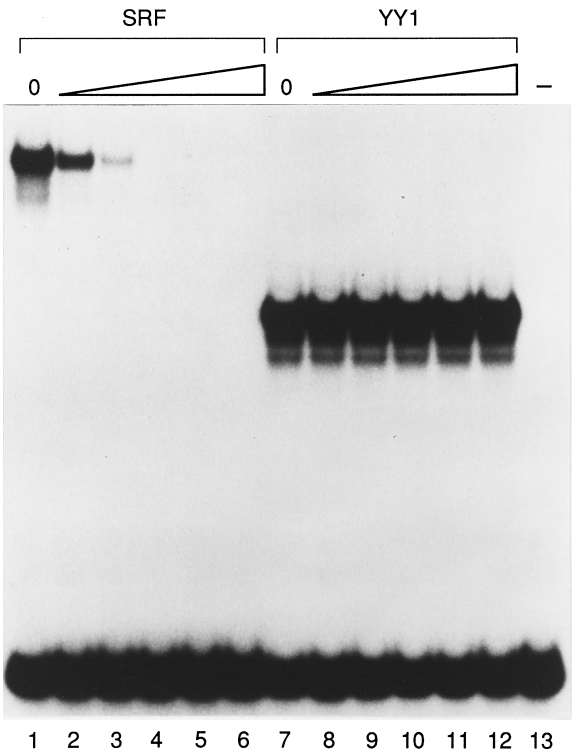


FIG. 7. Distamycin inhibits binding of SRF but not YY1 to the SRE. Mobility shift assays were performed with recombinant SRF (lanes 1 to 6) and YY1 (lanes 7 to 12) in the presence of distamycin at 0.1, 0.2, 0.4, 0.8, and 1.6 μM. All reaction mixtures were adjusted to the same final concentration of dimethyl sulfoxide vehicle. Lanes 1 and 7, dimethyl sulfoxide alone; lane 13, free probe.

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