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Transcriptional Repression of the c-*fos* Gene by YY1 Is Mediated by a Direct Interaction with ATF/CREB

QINGJUN ZHOU, RICHARD W. GEDRICH, AND DANIEL A. ENGEL*

Department of Microbiology and Cancer Center, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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Transcriptional activation of the mouse c-*fos* gene by the adenovirus 243-amino-acid E1A protein requires a binding site for transcription factor YY1 located at −54 of the c-*fos* promoter. YY1 normally represses transcription of c-*fos*, and this repression depends on the presence of a cyclic AMP (cAMP) response element located immediately upstream of the −54 YY1 DNA-binding site. This finding suggested that the mechanism of transcriptional repression by YY1 might involve a direct interaction with members of the ATF/CREB family of transcription factors. In vitro and in vivo binding assays were used to demonstrate that YY1 can interact with ATF/CREB proteins, including CREB, ATF-2, ATFa1, ATFa2, and ATFa3. Structure-function analyses of YY1 and ATFa2 revealed that the C-terminal zinc finger domain of YY1 is necessary and sufficient for binding to ATFa2 and that the basic-leucine zipper region of ATFa2 is necessary and sufficient for binding to YY1. Overexpression of YY1 in HeLa cells resulted in repression of a mutant c-*fos* chloramphenicol acetyltransferase reporter that lacked binding sites for YY1, suggesting that repression can be triggered through protein-protein interactions with ATF/CREB family members. Consistent with this finding, repression was relieved upon removal of the upstream cAMP response element. These data support a model in which YY1 binds simultaneously to its own DNA-binding site in the c-*fos* promoter and also to adjacent DNA-bound ATF/CREB proteins in order to effect repression. They further suggest that the ATF/CREB-YY1 complex serves as a target for the adenovirus 243-amino-acid E1A protein.

Transcription factor YY1 (13, 19, 32, 36) is a multifunctional DNA-binding protein that can serve as a target of the adenovirus E1A proteins (16, 29, 36). At the adeno-associated virus P5 promoter, YY1 can function as an initiator protein (35, 40). In this capacity, it stimulates recruitment of RNA polymerase II to the site of transcription initiation (40). In addition to its initiator function, YY1 has been shown to repress transcription of the c-*fos* proto-oncogene (16, 17, 31) and activate transcription of the c-*myc* proto-oncogene (33), suggesting that it plays an important role in cell cycle control. Several other cellular and viral genes are either activated or repressed by YY1 (3, 4, 13, 19, 28, 32), apparently depending on the sequence context in which DNA-binding sites for YY1 are located.

Two general mechanisms for the action of YY1 can be envisioned. They can be applied to both positive and negative regulation by YY1 and are not mutually exclusive. YY1 has been demonstrated to bend DNA, leading to the suggestion that it acts structurally to organize protein components of the transcription complex (31, 39). A second possibility is that YY1 acts as a functioning component of the transcription complex, with intrinsic activity separate from simple DNA binding. This leads to the experimental prediction that YY1 may be able to act even in the absence of direct binding to DNA, through protein-protein interactions.

Previously we have shown that the c-*fos* proto-oncogene can be transcriptionally activated by the 243-amino-acid residue E1A protein [$E1A_{(243)}$] (12, 15, 16). Recently we identified an E1A response element in the c-*fos* promoter, which is composed of a cyclic AMP response element (CRE) located at −67 and a DNA-binding site for YY1 located at −54 (16). The CRE and YY1 binding sites appear to interact functionally in the response to $E1A_{(243)}$, since maximal responsiveness requires both sites, and the YY1 site alone is not sufficient to confer a response in the absence of the −67 CRE (16). Other studies with the c-*fos* promoter have also indicated a functional interaction between the −67 CRE and the −54 YY1 DNA-binding site. In HeLa cells transfected with c-*fos* reporter constructs, the −54 YY1 site was shown to mediate transcriptional repression, and an intact −67 CRE was absolutely required for this function (31).

The close proximity of the CRE and YY1 sites in the c-*fos* promoter and the apparent functional interaction between these sites suggested to us that YY1 might physically interact with specific CRE-binding proteins. Since both the CRE and YY1 sites are required for maximal responsiveness to $E1A_{(243)}$, the identification of such a physical interaction would be important to our understanding of the mechanism of transcriptional activation of the c-*fos* gene by $E1A_{(243)}$. YY1 has been shown to bind transcription factors Sp1 (26, 34), c-Myc (37), and $TAF_{II}55$ (10) and the nucleolar phosphoprotein B23 (21), suggesting that its general mechanism of action involves specific protein-protein contacts. Here we demonstrate that YY1 can specifically interact with several members of the activating transcription factor/CRE-binding protein (ATF/CREB) family of transcription factors, which are known to bind to the CRE. This interaction is mediated by the C-terminal zinc finger domain of YY1 and the basic-leucine zipper (bZIP) region of ATF/CREB. We also provide evidence that the ATF/CREB-YY1 interaction can occur at the c-*fos* promoter in living cells and that it requires both the CRE and YY1 sites for maximum efficiency. Furthermore, we find that the ATF/CREB-YY1 interaction can result in transcriptional repression of the c-*fos* promoter, even in the absence of DNA-binding sites for YY1.

* Corresponding author. Phone: (804) 924-2817. Fax: (804) 982-1071. Electronic mail address: dae2s@virginia.edu.

MATERIALS AND METHODS

Cell culture. Monolayer HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, penicillin, and streptomycin at 37°C in a 10% CO_2 incubator. All media, sera, and antibiotics were from GIBCO/BRL.

Plasmids. pKATFa1, pKATFa2, and pKATFa3 were constructed by PCR synthesis with primers 5′-CTAGGATCCACCATGGGAGACGACAGACCG (containing an artificial *Bam*HI site and Kozak consensus sequence CCACC) and 5′-CTAGTCGACTCATCTGCCCGCAGACTG (containing an artificial *Sal*I site), using plasmids pATFa1, pATFa2, and pATFa3 (9), respectively, as templates. The PCR products were digested with *Bam*HI and *Sal*I and cloned into pGEM3. Plasmids pGSTbZIP1, pGSTbZIP2, and pGSTbZIP3 were constructed by PCR synthesis using pATFa2 as the template DNA and the following primers: ZQ1 (5′-AGTCGAATTCAGGGCAGTCTTTATGAGC), ZQ2 (5′-AGTCGAATTCTCTGCCCGCAGACTGGGA), ZQ3 (5′-GATCGGATCCAC TGGGGGGCGACGGCGG), and ZQ4 (5′-GATCGGATCCCGACGGCAGC GCTTTCTG). Primer pairs were as follows: for pGSTbZIP1, ZQ3 and ZQ2; for pGSTbZIP2, ZQ3 and ZQ1; and for pGSTbZIP3, ZQ4 and ZQ2. Each PCR product was digested with *Bam*HI and *Eco*RI (artificial sites near the ends of the PCR primers) and cloned into plasmid pGEX-2TK (23). Plasmid GST-YY1(282-330) was constructed by PCR amplification using primers ZQ13 (ATGCGG ATCCATGAAGCCAAGAAAAATT) and ZQ14 (GATCGAATTCACATTCT GCACAGACGTG), with YY1 cDNA as the template, the product of which was cloned into pGEX-2TK. Plasmid pKATFa2*dl*2-95 was constructed by digestion of pKATFa2 with *Eco*RI and self-ligation of the larger of the two resultant fragments. Plasmid pKATFa2*dl*28-284 was constructed by digestion of pKATFa2 with *Nco*I and religation of the two largest of three resultant fragments. The structure of the plasmid was confirmed by restriction analysis. To construct plasmid pYY1(C-term)-VP16, a PCR product was synthesized by using YY1 cDNA as the template and primers 5′-CTAGGATCCTCACTGGTTGT TTTTGGC and 5′-CTAGAATTCCCTCCTGGAGGAATACCT. The ends of the PCR product were digested with *Eco*RI and *Bam*HI, and the products were cloned into pCMV5 to make plasmid pYY1(C-term). This plasmid was then digested with *Eco*RI and *Hin*dIII, and the smaller of two fragments was isolated and ligated with the larger of two fragments generated from plasmid pCMVYY1/VP16 that had also been digested with *Eco*RI and *Hin*dIII. The wild-type −76/+10 fosCAT plasmid was made by PCR synthesis with primers 5′-ACTGAA GCTTTCCGCCCAGTGACGTA (with an artificial *Hin*dIII site) and primer RG3 (5′-CATGTCTAGACAGTCGCGGTTGGAGT [with an artificial *Xba*I site]), using −356/+109 DNA (7) as the template. The PCR products were digested with *Hin*dIII and *Xba*I and ligated with the largest fragment produced by digestion of −56/+109 DNA (7) with *Hin*dIII and *Xba*I. To make plasmid *pm*27, plasmid −76/+10 fosCAT was digested with *Hin*dIII and *Xba*I, and the smaller of two fragments was ligated into plasmid pAlter (Promega) that had been digested with *Hin*dIII and *Xba*I. This plasmid was subjected to mutagenesis by using the Altered Sites system (Promega) and oligonucleotide GAAGCGCT GTGATTTTTTTTACTTCCTACGTCACTG, containing nucleotides (underlined) that mutate both YY1 site CCAT core sequences. Resultant mutants were screened by DNA sequencing. Plasmid *pm*28 was constructed by PCR synthesis with primer BQ5 (5′-TATCAAGCTTTCCGCCCAGTGAGGTAGGAAG [with an artificial *Hin*dIII site and underlined CRE mutation at position −64]) and primer RG3 (see above), using −76/+10 fosCAT DNA as the template. The PCR products were digested with *Hin*dIII and *Xba*I and ligated to the large fragment produced by digestion of −76/+10 fosCAT with *Hin*dIII and *Xba*I. The plasmid was confirmed by sequencing. Plasmid *pm*27.28 was constructed by PCR synthesis with primers BQ5 and RG3, using *pm*27 DNA as the template. This resulted in a construct with mutant CRE and YY1 sites. The PCR products were digested with *Hin*dIII and *Xba*I and ligated with the large fragment produced by digestion of −76/+10 fosCAT with *Hin*dIII and *Xba*I. The plasmid was confirmed by sequencing. Plasmid CMV-YY1 was constructed by digestion of Gal4-YY1 (36) with *Eco*RI and ligation of the smaller fragment into *Eco*RI-digested pCMV5. The correct orientation was established by restriction analysis. Plasmid Gal4-VP16 was constructed by digestion of plasmid PadhGV16 (6) with *Hin*dIII and *Xba*I and ligation of the smallest of the three resulting fragments with pCMV5 that had been digested with *Hin*dIII and *Xba*I. CREBZR (a gift from Terry Meyer, Harvard University) contains the human CREB327 cDNA (38) in pGEM7zf(+). pGEM3-ATF-1, pGEM3-ATF-2, and pGEM3-ATF-3 (gifts from Michael Green, University of Massachusetts) contain human cDNAs for ATF-1, ATF-2, and ATF-3 (18), respectively, in pGEM3. Constructs described previously include pGST-YY1 plasmids, containing wild-type or deletion mutants of human YY1 cDNA, and pCMVYY1/VP16 (26), pCMV5 (1), plasmids GST-SH2 and GST-SH3 (14), and plasmid Gal4-YY1 (36).

In vitro transcription and translation. One microgram of linearized DNA was used as the template for in vitro synthesis of RNA, using an RNA transcription kit from Stratagene as instructed by the manufacturer. The plasmids and, in parentheses, restriction enzymes and RNA polymerases used were as follows: CREBZR (*Xba*I, SP6), pGEM3-ATF-1 (*Ssp*I, T7), pGEM3-ATF-2 (*Hin*cII, T7), pGEM3-ATF-3 (*Sac*I, T7), pKATFa1 (*Sal*I, T7), pKATFa2 (*Sal*I, T7), and pKATFa3 (*Sal*I, T7). For synthesis of C-terminal deletions of ATFa2, pKATFa2 was digested with *Pvu*II (for *dl*374-483) or with ScaI (for *dl*319-483) and transcribed with T7 RNA polymerase. One-tenth of the products of each reaction (or chloramphenicol acetyltransferase [CAT] RNA supplied by GIBCO/BRL) was added to in vitro translation reaction mixtures, using a rabbit reticulocyte translation system from GIBCO/BRL and [^{35}S]methionine from NEN. Successful in vitro translation was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography.

Production of GST fusion proteins and in vitro binding reactions. *Escherichia coli* DH5 cells transformed with glutathione *S*-transferase (GST) expression vectors were grown overnight at 37°C with shaking in 500 ml of LB medium containing ampicillin. A 1:10 dilution of the overnight culture was added to 500 ml of LB medium containing ampicillin and incubated at 37°C for 1 h with shaking. Isopropylthiogalactopyranoside (IPTG) was then added to 0.5 mM, and the cells were incubated for an additional 3 h. The cells were then pelleted and resuspended in 5 ml of NETN buffer (100 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5% Nonidet P-40) and sonicated for 1 min two to three times with a cell disruptor (model W-375; Heat Systems-Ultrasonics, Inc.) set at output = 5 and duty cycle = 50%. The sonic extract was centrifuged at 12,000 rpm in an SS-34 rotor at 4°C for 10 min, and the supernatant was supplemented with glycerol to 10% (vol/vol). To this, 25 μl of a 1:1 slurry of glutathione-agarose beads (Sigma) in NETN–0.5% nonfat dry milk was added, and then the mixture was rocked at 4°C for 30 min. The beads were pelleted in a microcentrifuge, washed twice with 1 ml of NETN, incubated with 1 ml of NETN–0.5% nonfat dry milk at 4°C for 1 h, and again pelleted. The amount of GST protein recovered was determined by SDS-PAGE followed by staining with Coomassie blue and comparison with protein standards.

Binding reactions were performed essentially as described previously (34). Pelleted beads corresponding to 0.5 to 1.0 μg of GST fusion protein were incubated with 300 μl of incubation buffer (50 mM KCl, 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 5 mM 2-mercaptoethanol, 0.1% Tween 20, 0.5% nonfat dry milk) and 1/10 of the products from the appropriate in vitro translation reaction for 1 h at 4°C. The beads were then pelleted and washed twice in 1 ml of incubation buffer except that the KCl concentration was 100 mM instead of 50 mM. Bound proteins were analyzed by addition of sample buffer followed by boiling, SDS-PAGE, and fluorography.

Western immunoblot analysis. Western blot analysis was performed essentially as described previously (20). Products of GST-binding reactions were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with either a monoclonal antibody against human YY1 (a gift of Tom Shenk) or monoclonal antibody 8C10 against p190 (8) (a gift of Sarah Parsons). The blots were incubated with affinity-purified goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Boehringer Mannheim), and specific immune complexes were visualized using an enhanced chemiluminescence kit from Amersham.

Transfection and CAT assay. HeLa cell monolayers in 10-cm-diameter dishes were transfected by the calcium phosphate method exactly as described previously (2). The amounts (in micrograms) of DNA for each experiment are indicated in the figure legends. For all transfections, salmon sperm DNA was used to supplement the DNA up to a total of 25 μg per plate. The control expression plasmid for experiments involving YY1-VP16, YY1(C-term)-VP16, Gal4-VP16, and YY1 was pCMV5. For all experiments, transfections were performed in duplicate to confirm reproducibility within the experiment. Entire experiments presented in the figures and tables were repeated up to five times. Medium was exchanged 16 h after transfection, and the cells were harvested 48 h after transfection. CAT assays were performed as described previously (2), with modifications. Cells were resuspended in 50 μl of 250 mM Tris-Cl (pH 7.8) and then lysed by freezing (in liquid N_2) and thawing (at 37°C) three times. Lysates were incubated at 65°C for 10 min prior to pelleting of debris by centrifugation. All 50 μl of the lysate was assayed for CAT activity. Reactions were done in a volume of 100 μl containing 50 μl of lysate plus 50 μl of a premix containing 200 mM Tris (pH 7.8), 0.5 mg of *n*-butyryl coenzyme A (Sigma) per ml, and 0.5 μCi of [^{14}C]chloramphenicol (Amersham). Reaction mixtures were incubated at 37°C for 1 h and then extracted with 300 μl of mixed xylenes and back extracted twice with 100 μl of 250 mM Tris (pH 7.8). CAT activity was measured by liquid scintillation counting of 200 μl of the xylene extracts in 5 ml of Econofluor (NEN) scintillation fluid.

RESULTS

YY1 physically interacts with ATF/CREB proteins in vitro. Figure 1 illustrates the −76 to −23 region of the mouse c-*fos* promoter, containing binding sites for transcription factors ATF/CREB, YY1, and the TATA box-binding protein. There are two closely spaced binding sites for YY1, whose core CCAT sequences are located at −54 and −50 (31). The −54 YY1 site (i.e., the CRE-proximal site) mediates repression by YY1 in a manner that depends on the −67 CRE, whereas the −50 YY1 site has no apparent influence on transcription (16, 31).

A GST-YY1 fusion protein was produced in *E. coli* and

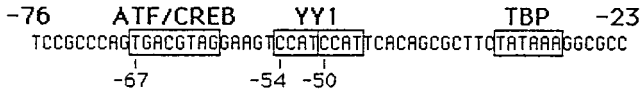

FIG. 1. The −76 to −23 region of the mouse c-*fos* promoter. Shown are binding sites for ATF/CREB, YY1, and TATA box-binding protein (TBP). The nucleotide positions of the 5′ ends of the ATF/CREB and YY1 sites are indicated. For YY1, only the core CCAT sequences are shown, of which there are two. Methylation interference analysis of these two YY1 sites demonstrated that YY1 can bind to either of the sites but suggested that simultaneous binding to both sites does not occur (31).

purified by using glutathione-agarose beads. Beads containing immobilized GST-YY1 were incubated with ^{35}S-labeled, in vitro-translated CREB protein and washed to remove nonspecifically bound protein. The products of the binding reaction were then subjected to SDS-PAGE. As a control, ^{35}S-CREB was also incubated with beads coupled to GST alone or to GST fused to the SH2 or SH3 protein interaction domain of the v-Src oncoprotein (14). The results are shown in Fig. 2A. ^{35}S-CREB was clearly retained by the GST-YY1 beads but not the control reagents, indicating a specific and direct physical interaction between CREB and YY1. In addition, ^{35}S-labeled, in vitro-translated CAT protein was not bound by GST or GST-YY1, again demonstrating the specificity of the interaction.

Members of the ATF/CREB family of transcription factors are structurally related. Therefore, several ATF proteins were also tested for the ability to interact with YY1. The results (Fig. 2B and C) demonstrate that ATF-2, ATFa1, ATFa2, and ATFa3 were all able to form complexes with YY1 in vitro. The interactions between YY1 and members of the ATF/CREB family were found to be completely resistant to high concentrations of ethidium bromide (not shown), demonstrating that they were not due to nonspecific binding of ATF/CREB and YY1 to trace amounts of contaminating DNA in the binding reactions (25).

These data demonstrate that several known CRE-binding proteins (CREB, ATF-2, ATFa1, ATFa2, and ATFa3) are capable of direct, specific interaction with YY1. Since transcriptional repression of the c-*fos* gene by YY1 depends on an intact CRE located just upstream of the −54 YY1 binding site (31), these results suggested that the interaction between YY1 and ATF/CREB proteins is mechanistically involved in transcriptional repression by YY1.

Interaction with ATFa2 is mediated by the C-terminal zinc finger domain of YY1. A series of GST-YY1 deletion mutants (a kind gift of Yang Shi) was used to identify the domains of YY1 that are responsible for the interaction with one member of the ATF/CREB family, ATFa2. The structures of the deletion mutants are shown in Fig. 3, along with the results of the in vitro interaction assays. Removal of the C-terminal 84 amino acid residues (in GST-YY1 *dl*331-414) had no effect on binding to ^{35}S-ATFa2, but removal of an additional 107 residues (in GST-YY1 *dl*224-414) completely abolished binding. Therefore, residues 224 to 330 constitute a domain of YY1 that is required for the interaction with ATFa2. All of the remaining C-terminal deletion mutants failed to bind ATFa2, consistent with the requirement for the 224 to 330 region. All N-terminal deletion mutants of YY1 were found to be equally capable of interacting with ATFa2, including mutant GST-YY1 *dl*1-331, which encoded only the C-terminal 83 residues of the protein.

Despite the fact that the C-terminal 83 residues of YY1 were sufficient for binding to ATFa2 (as revealed by mutant *dl*1-331), mutant *dl*331-414, which lacked the C-terminal 84 residues, also bound ATFa2 efficiently. These data demonstrate that there are two distinct domains of YY1 that can mediate an interaction with ATFa2. One domain requires the 224 to 330 region, as is evident from a comparison of the activities of *dl*331-414 and *dl*224-414. The other is the neighboring 332 to 414 region, present in *dl*1-331, which is sufficient for interacting with ATFa2.

YY1 contains four zinc fingers located between amino acids 290 and 414. The 332 to 414 region in *dl*1-331 is composed entirely of two intact zinc fingers and one interrupted zinc finger, which strongly suggests that the zinc finger structures function to bind ATFa2. An additional intact zinc finger is located in the 224 to 330 region of *dl*331-414, suggesting it could be responsible for the ability of this mutant to bind ATFa2. To test this, the protein GST-YY1(282-330) was produced. It contained the lone zinc finger found in mutant *dl*331-414, located between amino acids 282 and 330. As shown in Fig. 3B, this protein bound efficiently to ^{35}S-ATFa2. These data demonstrate that there are two distinct zinc finger-containing domains capable of binding to ATFa2.

The bZIP domain of ATFa2 is necessary and sufficient for interacting with YY1. Several deletion mutants of ATFa2 were produced in order to identify the domain of this protein responsible for interacting with YY1. Each mutant protein was

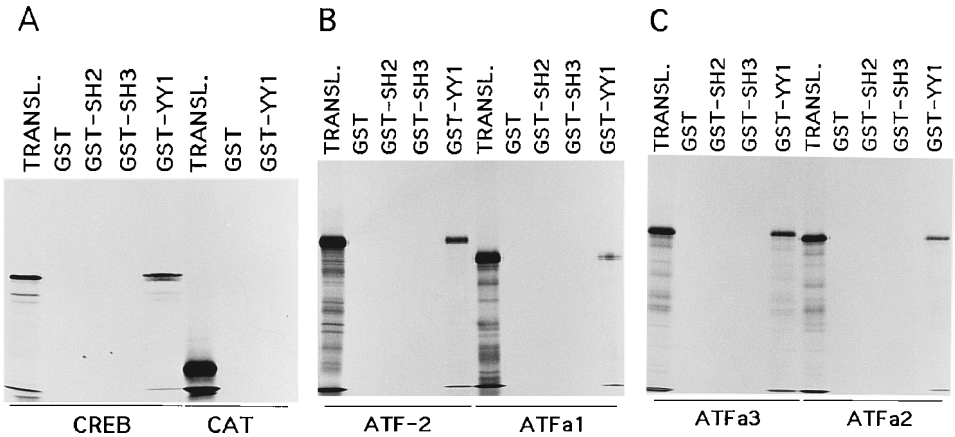

FIG. 2. In vitro assay of the ATF/CREB-YY1 interaction. Glutathione-agarose beads coupled to the indicated GST fusion proteins were incubated with ^{35}S-labeled, in vitro-translated CREB or CAT (A), ATF-2 or ATFa1 (B), and ATFa2 or ATFa3 (C). The reaction products were washed and then analyzed by SDS-PAGE. TRANSL., in vitro translation products of the indicated proteins, in half the amount added to the binding assays.

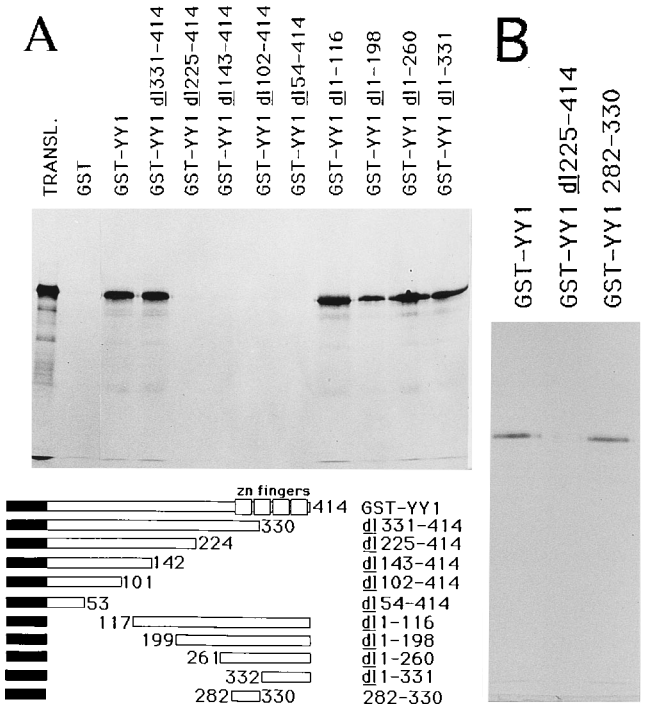

FIG. 3. Deletion analysis of YY1. GST-YY1 and the indicated GST-YY1 deletion mutants were tested for binding in vitro to ^{35}S-labeled ATFa2 as described in Materials and Methods. Aliquots of the purified GST-YY1 derivatives were analyzed by SDS-PAGE and Coomassie blue staining (not shown). Equivalent amounts of GST and each GST-YY1 protein were added to the binding reaction mixtures. The products of the binding reactions were analyzed by SDS-PAGE. The structures of the GST-YY1 derivatives are shown. Each black box indicates the position of the GST moiety, which is not drawn to scale. The locations of the four YY1 zinc finger domains are indicated for the wild-type YY1 protein. Numbers represent amino acid residue positions. TRANSL., products of ATFa2 translation in vitro, in half the amount added to the binding assays.

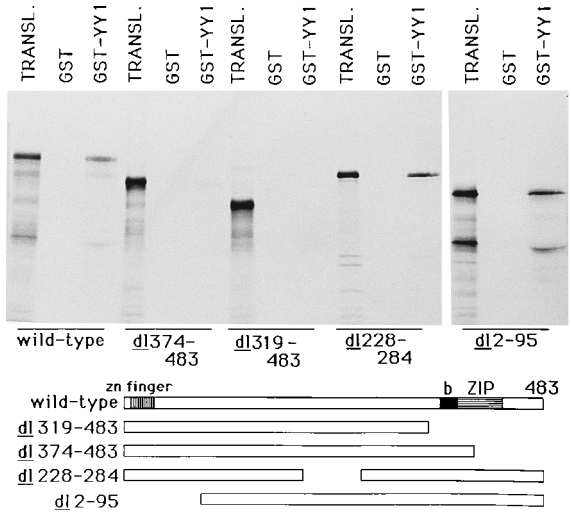

FIG. 4. Deletion analysis of ATFa2. The indicated ATFa2 deletion mutants were translated in vitro in the presence of [^{35}S]methionine and tested for binding to wild-type GST-YY1 as described in Materials and Methods. The products of the binding reactions were analyzed by SDS-PAGE. The structures of the ATFa2 derivatives are shown, along with the locations of the zinc finger and bZIP domains in the wild-type ATFa2 protein. Numbers represent amino acid residue positions. TRANSL., products of ATFa2 translation in vitro, in half the amount added to the binding assays.

translated in vitro in the presence of [^{35}S]methionine and used in in vitro interaction assays as described above. The results are shown in Fig. 4, as are the structures of the mutants. Of the mutants tested, only those with deletions in the bZIP region of the protein were altered in the ability to bind YY1. Specifically, the YY1-binding activities of two mutants, *dl*374-483 and *dl*319-483, were each drastically reduced. Mutant *dl*374-483, which retained part of the leucine zipper region and all of the basic region, had some residual YY1-binding activity, as judged from a longer exposure of the autoradiogram (not shown). Mutant *dl*319-483 however, which contained none of the bZIP domain, was completely unable to interact with YY1. In contrast *dl*2-95, which lacked an N-terminal zinc finger structure, and *dl*228-284 were as active as the wild-type protein in binding to YY1. These data demonstrate that the bZIP domain of ATFa2 was necessary for interacting with YY1.

The bZIP region functions as a DNA-binding and protein dimerization domain for a large class of transcription factors. Dimerization among particular members of this class is mediated by the leucine zipper structure, and DNA binding is carried out by a neighboring region containing a high proportion of basic amino acid residues (27). The requirement of the ATFa2 bZIP region for binding to YY1 suggested that this domain was directly involved in the interaction. To test this, three GST-bZIP clones were constructed, and the resulting proteins were assayed for the ability to bind YY1 in vitro. For these experiments, the GST-bZIP proteins were produced and purified from *E. coli* and then coupled to glutathione-agarose beads. The beads were then incubated with purified histidine-tagged YY1 (a kind gift of Michael Atchison), washed, and subjected to SDS-PAGE. Again, glutathione-agarose beads coupled to GST alone and to GST-SH3 were used as negative controls for nonspecific binding. Following electrophoresis, the separated proteins were transferred to nitrocellulose and probed with an anti-YY1 monoclonal antibody (a kind gift of Tom Shenk). The structures of the three GST-bZIP proteins are shown in Fig. 5A, and the results are shown in Fig. 5B. Clone GST-bZIP1 contains ATFa2 amino acid residues 319 to 483, which encompass a region starting 16 residues N terminal to the start of the basic region and ending at the C terminus of the protein (residue 483). GST-bZIP2 is a C-terminal truncation of GST-bZIP1 that ends 11 residues past the final leucine of the leucine zipper. GST-bZIP3 is an N-terminal truncation of GST-bZIP1 which starts just at the beginning of the basic region (at residue 334). Strong binding to each of the GST-bZIP proteins but not to the negative controls, was observed, indicating that regions N terminal and C terminal to the bZIP region are dispensable for binding.

An unrelated histidine-tagged protein, His-p190-GTPase (a kind gift of Sarah Parsons), was also tested for binding to YY1 to determine if the histidine residues at the N terminus of His-YY1 might be responsible for the interaction with the GST-bZIP proteins. No such binding was detected (Fig. 5C). Also arguing against this possibility is the fact that YY1 itself contains a stretch of 11 consecutive histidine residues at positions 70 to 80 (36), and this region of the protein was found to be dispensable for binding to ATFa2 in the experiments shown in Fig. 3. In sum, these data demonstrate that the bZIP domain of ATFa2, which also serves as the dimerization and DNA-binding domain of the protein, is necessary and sufficient for interacting with transcription factor YY1.

YY1 interacts with ATF/CREB at the c-*fos* promoter. The interaction between YY1 and ATF/CREB proteins was further explored with an in vivo "tethering" assay. HeLa cells were

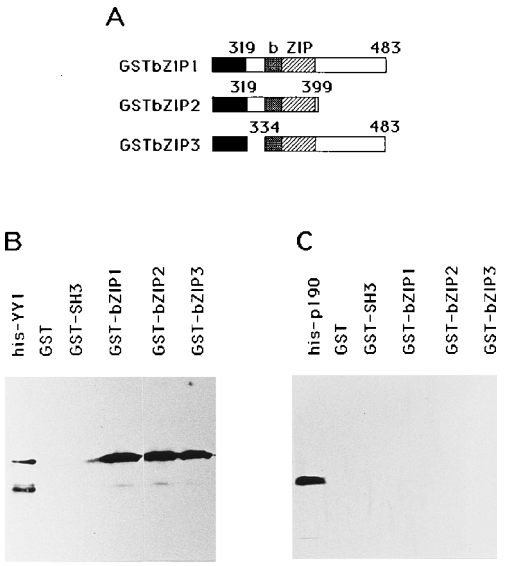

FIG. 5. Assay of the bZIP-YY1 interaction. (A) Structures of GST-bZIP proteins. The locations of the basic region (b) and leucine zipper domain (ZIP) are shown, and the amino acid endpoints of the ATFa2 portion of the fusion proteins are indicated. Each black box represents GST, which is not drawn to scale. (B and C) The indicated immobilized GST fusion proteins were incubated with bacterially produced his-YY1 (B) or His-p190-GTPase (C), washed, subjected to SDS-PAGE, and transferred to nitrocellulose. The filters were probed with an anti-YY1 (A) or anti-p190 (B) monoclonal antibody, and then the resultant immune complexes were visualized by enhanced chemiluminescence as described in Materials and Methods. The first lanes in panels B and C contain an aliquot of the indicated His-tagged protein loaded directly for SDS-PAGE, without GST affinity purification.

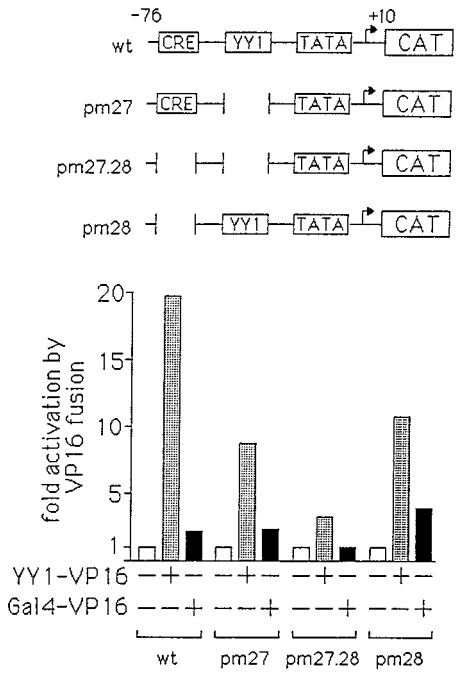

FIG. 6. In vivo assay of the ATF/CREB-YY1 interaction. HeLa cells were cotransfected with 5 μg of the indicated reporter plasmids and 2.5 μg of a plasmid encoding either YY1-VP16 or Gal4-VP16, or a control expression plasmid lacking gene sequences, as described in Materials and Methods. After 48 h, cells were harvested and assayed by the liquid scintillation CAT assay. The transfection protocol and CAT assay were performed as described in Materials and Methods. Fold activation was calculated as CAT activity (in counts per minute) from cells expressing the VP16 fusion protein divided by CAT activity from cells transfected with empty expression vector. wt, wild type.

transiently transfected with two plasmids. One (fosCAT) contained the −76 to +10 region of the murine c-*fos* gene, carrying the closely spaced ATF/CREB and YY1 DNA-binding sites, the TATA element, and the transcription initiation site linked to the CAT reporter gene. The other was an expression plasmid encoding full-length YY1 protein fused to the transcriptional activation domain of the herpes simplex virus VP16 protein. In this system, transcriptional activation mediated by the VP16 activation domain occurs if the YY1-VP16 fusion protein is tethered to the promoter either by a direct protein-DNA interaction or by an interaction with proteins that are specifically bound to the DNA. In the absence of such tethering, significant activation by the VP16 activation domain does not occur. In these experiments, therefore, YY1 was converted from a repressor to a transcriptional activator for the purpose of determining if it can be tethered to the promoter through protein-protein contacts. When the fosCAT construct was transfected along with the YY1-VP16 expression plasmid, an 18- to 70-fold activation was observed (Fig. 6 and Table 1). No such activation occurred when the parental expression plasmid lacking gene sequences was used instead of the YY1-VP16 plasmid. Furthermore, this activation required the VP16 activation domain, since expression of YY1 without the VP16 activation domain did not result in activation (not shown; see also results of repression experiments in Table 3). An additional control cotransfection with the fosCAT construct and a plasmid encoding a yeast Gal4-VP16 fusion protein resulted in only about a twofold activation. This result demonstrated that YY1-VP16 was specifically tethered to the fosCAT promoter construct through the YY1 portion of the fusion protein, resulting in a large activation of the promoter. These results were anticipated, because the fosCAT construct contains YY1 DNA-binding sites and no Gal4 DNA-binding sites (the slight activation by Gal4-VP16 could be due to a direct effect of the VP16 activation domain on the general transcription factors). We reasoned, however, that the observed tethering of YY1-VP16 was also due to the interaction between YY1-VP16 and endogenous ATF/CREB factors bound at the nearby −67 CRE.

Next, we tested the hypothesis that YY1-VP16 could be tethered to the promoter even in the absence of a YY1 site, since the neighboring −67 CRE would be bound by ATF/CREB proteins that could be recognized by YY1-VP16. Figure 6 shows that mutant *pm*27, which lacked both the −54 and −50 YY1 sites, was nonetheless still activated by YY1-VP16, although to a lesser degree than the wild-type construct (see also Table 1). The effect of YY1-VP16 on this construct varied from 6.8- to 14-fold. Again, the Gal4-VP16 protein was unable

TABLE 1. Assay of the ATF/CREB-YY1 interaction in vivo[a]

Reporter	Relative basal transcription[b]	Fold activation by YY1-VP16			
		Expt 1	Expt 2	Expt 3	Expt 4
Wild type	1.0	19.6	48	18	70
*pm*27	2.5	8.7	14	10	6.8
*pm*27.28	0.32	3.2	1.8	2.2	2.3
*pm*28	0.20	10.7	6.7	3.5	17.6

[a] Data are from four independent experiments, performed as described in the legend to Fig. 6 and in Materials and Methods. Experiment 1 is the same as that shown in Fig. 6, with the Gal4-VP16 data omitted.

[b] Average relative basal transcription for each of the indicated promoters in the absence of YY1-VP16.

TABLE 2. The zinc finger domain of YY1 can interact with ATF/CREB in vivo[a]

Reporter	Fold activation by YY1(C-term)-VP16	
	Expt 1	Expt 2
Wild type	42	27
*pm*27	9	6.6
*pm*27.28	2.5	1.8
*pm*28	5	6.1

[a] Data are from two independent experiments, performed as described in the legend to Fig. 6 and in Materials and Methods. See Fig. 6 for structures of the reporter constructs.

to significantly alter the transcription level of this construct, indicating that the transcriptional activation by YY1-VP16 depended on the presence of the YY1 moiety. These data demonstrate that YY1-VP16 could be tethered to the promoter through an interaction that did not involve direct binding to YY1 DNA-binding sites. To test directly the idea that ATF/CREB factors bound to the CRE were responsible for this tethering, we made a double mutant fosCAT construct, *pm*27.28, that contained mutant CRE and YY1 sites. Figure 6 and Table 1 show that this construct was activated only 1.8- to 3.2-fold by YY1-VP16, similar to the effect of Gal4-VP16 on any of the mutants tested. This result clearly demonstrates that tethering of YY1-VP16 to the *pm*27 mutant depended nearly entirely on CRE-bound ATF/CREB. Finally, the effect of YY1-VP16 was tested on a fosCAT mutant, *pm*28, that retained its YY1 binding sites but lacked a functional CRE (Fig. 6 and Table 1). Again, there was a clear activation of this construct by YY1-VP16 but not Gal4-VP16. Interestingly, despite the presence of intact YY1 binding sites in the *pm*28 mutant, activation of this construct by YY1-VP16 was reproducibly lower than activation of the wild-type construct that contained the YY1 sites as well as the CRE.

These data demonstrate that YY1 can interact with the c-*fos* promoter in vivo, even in the absence of a YY1 binding site in the DNA. The interaction that took place in the absence of the YY1 DNA-binding site depended entirely on the CRE, which binds endogenous ATF/CREB factors. The data are completely consistent with the in vitro experiments shown above that demonstrate a direct physical interaction between YY1 and ATF/CREB. In addition, they show that a maximally efficient interaction of YY1 with the promoter requires both an intact YY1 site and an intact CRE, since maximal activation by YY1-VP16 occurred only with the wild-type fosCAT construct.

To rule out the possibility that YY1-VP16 was being tethered by direct binding to the DNA at the CRE, a DNA band shift experiment was performed in which *E. coli*-produced GST-YY1 was incubated with wild-type or mutant ^{32}P-labeled c-*fos* probes spanning the −76 to +10 region and containing the c-*fos* CRE and/or −54 YY1 site. As expected, GST-YY1 bound efficiently to the wild-type probe but not at all to a mutant probe lacking the binding site for YY1 (not shown). Since the mutant probe contained an intact CRE, it is clear that YY1 did not bind directly to the CRE or any other part of the DNA. In agreement with this finding, Natesan and Gilman have also demonstrated that binding of recombinant YY1 protein to the identical region of the c-*fos* promoter absolutely requires the −54 and −50 YY1 binding sites, even in the presence of the −67 CRE (31). We therefore conclude that the role of the CRE in tethering YY1-VP16 did not involve a direct interaction between YY1-VP16 and the CRE. It follows that the observed CRE-dependent tethering of YY1-VP16 involved the interaction of YY1-VP16 with CRE-bound ATF/CREB.

The C-terminal zinc finger domain of YY1 is sufficient for interaction with ATF/CREB in vivo. In vivo tethering experiments similar to those presented above were also performed with a plasmid encoding a YY1-VP16 fusion protein that contained only the 282 to 414 zinc finger domain of YY1. As demonstrated by the data presented in Fig. 3, this region of YY1 contains domains necessary and sufficient for binding to ATFa2 in vitro. The results of the tethering assay are shown in Table 2. Like full-length YY1-VP16, the YY1(C-term)-VP16 protein activated transcription of the fosCAT reporters in a manner that depended on both the CRE and YY1 binding sites. Again, maximal activation was observed only with the wild-type fosCAT reporter that contained intact CRE and YY1 sites. These data demonstrate that the C-terminal zinc finger domain of YY1 is sufficient to interact with endogenous HeLa cell ATF/CREB proteins at the c-*fos* promoter. They correlate nicely with the results of the in vitro interaction experiments presented above.

The c-*fos* −67 CRE mediates transcriptional repression by YY1 in the absence of DNA-binding sites for YY1. YY1 represses transcription of the c-*fos* promoter through the −54 YY1 site, and this repression depends on the presence of an intact CRE at −67 (31). If the interaction between YY1 and ATF/CREB proteins is functionally important in transcriptional repression, then overexpression of YY1 might be expected to lead to repression of transcription from the c-*fos* promoter even in the absence of binding sites for YY1. Under this hypothesis, repression would be mediated by the direct interaction of YY1 with DNA-bound ATF/CREB proteins. To test this idea, HeLa cells were cotransfected with the mutant fosCAT reporter construct *pm*27 and an expression plasmid encoding native YY1 (or a control parental expression plasmid lacking gene sequences). Mutant *pm*27 lacks YY1 DNA-binding sites but contains an intact −67 CRE. As shown in Table 3, overexpression of YY1 consistently resulted in repression of

TABLE 3. CRE-dependent repression of the c-*fos* promoter by YY1[a]

Condition	CAT activity (cpm)							
	Expt 1		Expt 2		Expt 3		Expt 4	
	*pm*27	*pm*27.28	*pm*27	*pm*27.28	*pm*27	*pm*27.28	*pm*27	*pm*27.28
−YY1	25,508	1,093	17,520	995	41,164	2,563	17,497	353
+YY1	7,052	573	5,070	396	12,108	1,578	4,982	160
Repression	3.6	1.9	3.5	2.5	3.4	1.6	3.5	2.2

[a] HeLa cells were transfected with 5 μg of the indicated reporter plasmids and 10 μg of either pCMVYY1 (+YY1) or the control plasmid pCMV5 (−YY1) as described in Materials and Methods. Cells were harvested 48 h later and assayed for CAT activity. Fold repression is calculated as CAT activity (in counts per minute) from cells cotransfected with the reporter and pCMVYY1 divided by CAT activity from cells cotransfected with the same reporter and the parental expression vector pCMV5.

the *pm*27 fosCAT reporter (an average of 3.5 ± 0.1-fold), demonstrating that intact binding sites for YY1 were not required for this effect. However, the effect of YY1 expression on the double mutant *pm*27.28, which lacked both the CRE and YY1 binding sites, was reproducibly diminished (2.1 ± 0.3-fold). Consistent with this finding, expression of a mutant YY1 protein lacking the C-terminal ATF/CREB binding region failed to repress transcription (data not shown). This finding clearly shows that repression by YY1 can take place in the absence of the −54 and −50 YY1 DNA-binding sites and that such repression depends at least in part on an intact CRE.

It is important to point out that YY1 binding-site mutations in the c-*fos* promoter that lead to loss of YY1 function result in a two- to fourfold increase in both basal and inducible transcription of the promoter (16, 31). We observed that overexpression of YY1 resulted in a 3.5-fold repression of a promoter lacking binding sites for YY1 and that nearly half of this effect depended on the presence of the −67 CRE. Given the in vitro and in vivo interaction data concerning YY1 and ATF/CREB, our results support a model in which the interaction of YY1 with DNA-bound ATF/CREB proteins contributes significantly to transcriptional repression by YY1.

DISCUSSION

The experiments presented here demonstrate that YY1 can interact directly with members of the ATF/CREB family of transcription factors both in vitro and at the c-*fos* promoter in living cells and that this interaction correlates with the ability of YY1 to repress transcription. That the ATF/CREB-YY1 complex is truly involved in regulation of c-*fos* transcription is supported by the following evidence. First, binding sites for ATF/CREB and YY1 are located next to each other in the c-*fos* promoter, making the possibility of contact between the two factors exceedingly high. Second, the ability of YY1 to repress transcription from the c-*fos* promoter depends on an intact ATF/CREB binding site (16, 31), indicating that the presence of ATF/CREB at the promoter is required for YY1 function. Third, the in vivo interaction experiments using YY1-VP16 took advantage of endogenous ATF/CREB factors that are positioned naturally on the c-*fos* promoter. The fact that YY1-VP16 could interact with these factors in the context of the transfected fosCAT reporter plasmid argues strongly that the ATF/CREB-YY1 interaction normally takes place at this promoter. In addition to these findings, we have recently shown that a short region of the c-*fos* promoter that encompasses only the ATF/CREB and YY1 DNA-binding sites constitutes a novel adenovirus E1A response element and that the function of this element depends on both the ATF/CREB binding site and the −54 YY1 binding site (16). Thus, there is a convergence of physical and functional data that support the existence of the ATF/CREB-YY1 complex as a functional unit at the c-*fos* promoter.

In addition, we have shown that transcriptional repression of the c-*fos* promoter by YY1 can take place in the absence of DNA-binding sites for YY1 that are known to be targets for YY1-induced DNA bending (31). We therefore conclude that YY1-induced repression can take place in the absence of YY1-induced DNA bending. Coupled with the in vitro and in vivo interaction data, this finding also leads to the conclusion that YY1 behaves as a functional component of the transcription complex. In this regard, YY1 could act by directly altering the activity of ATF/CREB in the transcription complex. Such effects might involve changes in the ability of ATF/CREB to bind DNA or to interact with other proteins of the transcription complex. Natesan and Gilman showed that CREB and YY1 can bind simultaneously in vitro to a DNA probe containing the −67 CRE and adjacent YY1 sites, suggesting that YY1 does not act by reducing ATF/CREB DNA binding (31). Our in vivo interaction experiments support this finding, since YY1-VP16 could be tethered to the promoter solely through DNA-bound ATF-CREB. If YY1 was preventing the binding of ATF/CREB to DNA, efficient tethering would not have been observed. In addition, maximal activation of our fosCAT reporter by YY1-VP16 occurred only when both the CRE and YY1 sites were present. This finding is also consistent with simultaneous and efficient binding of both ATF/CREB and YY1 to the DNA. Therefore, we favor the possibility that YY1 alters the activity of the transcription complex without drastic effects on the ability of ATF/CREB to bind DNA.

In addition to altering the transcriptional activation function of ATF/CREB, YY1 might contain a transcriptional repression domain that acts by specifically contacting one or more of the general transcription factors. Under this hypothesis, the role of the interaction with ATF/CREB would be to increase the efficiency of YY1 binding to the promoter or to position it properly so that it could repress transcription through the general factors. This hypothesis is consistent with our results. We found that YY1 repressed transcription in the absence of DNA-binding sites for YY1 but that only about half of this repression was relieved when the −67 CRE was mutated (Table 3). One possibility to explain this result is that overexpressed YY1 is able to repress transcription by acting directly on the general factors, even in the absence of promoter-bound ATF/CREB. This component of the repression would not be expected to depend on the CRE. Consistent with this possibility, YY1 has recently been shown to bind to $TAF_{II}55$, a component of TFIID (10).

Our results do not exclude the role of DNA bending in the mechanism of transcriptional repression by YY1. Such bending may indeed contribute to repression by altering the physical relationship of ATF/CREB to other components of the transcription complex, as proposed previously (31). It is also possible that YY1 binding to ATF/CREB factors can alter the ability of ATF/CREB to bend DNA (24), thus changing the physical state of the transcription complex. The data presented here do establish, however, that DNA bending induced directly by YY1 is not the sole mechanism by which YY1-induced repression is effected.

The bZIP region functions as a DNA-binding domain and also mediates homo- and heterodimerization among bZIP-containing proteins, as inferred from the interaction between leucine-containing alpha helices. In addition, the bZIP region can participate in important interactions with non-bZIP proteins. Transcriptional activation by $E1A_{(289)}$ involves a direct interaction between E1A and the bZIP region of ATF-2 (30). The bZIP region of ATF-2 also has been shown to interact specifically with the high-mobility-group protein HMG I(Y) in the regulation of the human beta interferon gene (11). Also, the leucine zipper domain of c-*jun* has been demonstrated to be involved in a direct interaction with the MyoD protein (5). Our results provide an additional example of the physical and functional versatility of the bZIP region. We speculate that the interaction with YY1 leads to an additional level of regulation of the activity of ATF/CREB. For instance, the ability of YY1 to interact with ATF/CREB may be controlled by cellular or extracellular events that are distinct from those that directly influence the activity of ATF/CREB, such as protein kinase A.

Adenovirus $E1A_{(243)}$ and $E1A_{(289)}$ have recently been shown to interact physically with YY1, and it is interesting that residues 15 to 35 of $E1A_{(243)}$, which constitute a binding site for YY1, overlap a region of $E1A_{(243)}$ required for activation

of c-*fos* transcription (15, 16) and induction of cellular transformation (22, 41). Given the involvement of YY1 in the activation of the c-*fos* promoter by $E1A_{(243)}$ (16), it is likely that an interaction between $E1A_{(243)}$ and YY1 is important in this process, and it will be important to determine how such an interaction might alter control of the c-*fos* promoter by the ATF/CREB-YY1 complex. An interesting possibility is that $E1A_{(243)}$ binds to YY1 and acts to relieve transcriptional repression mediated by the ATF/CREB-YY1 interaction. This and other possibilities are currently being examined.

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