Intramolecular inhibition of activating transcription factor-2 function by its DNA-binding domain

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ATF-2 is a cellular basic region–leucine zipper (bZIP) transcription factor that can mediate diverse transcriptional responses, including activation by the adenovirus E1a protein. ATF-2 contains an activation domain, required for transcriptional activity, but in the absence of an appropriate inducer, full-length ATF-2 is transcriptionally inactive. Here we have investigated the mechanism underlying this regulated inhibition of ATF-2 transcriptional activity. We show that the region of ATF-2 that suppresses the activation region is the bZIP DNA-binding domain and that maximal inhibition requires both the basic region and leucine zipper subdomains. Inhibition is activation domain specific: The ATF-2 bZIP suppresses the ATF-2 and the related E1a activation domains but not acidic- and glutamine-rich activation domains. In vitro protein interaction assays demonstrate that the ATF-2 activation domain and bZIP specifically bind to one another. Finally, we show that bZIP-mediated inhibition can be modulated in a cell-type-specific manner by another sequence element within ATF-2. On the basis of these and other data, we propose that the ATF-2 bZIP and activation domain are engaged in an inhibitory intramolecular interaction and that inducers of ATF-2 disrupt this interaction to activate transcription.

[Key Words: ATF-2; transcription activator; bZIP DNA-binding domain; activation domain; cell-type specificity]

Received August 21, 1995; revised version accepted January 24, 1996.

Eukaryotic gene expression is largely controlled at the transcriptional level through regulation of promoter-specific transcription factors. For example, the activity of various transcription factors can be affected by small molecule ligands (e.g., steroid hormone receptors), by phosphorylation, and by interactions with regulatory proteins (for review, see Evans 1988; Martin and Green 1992; Karin and Hunter 1995). An important class of regulatory proteins are viral transcription activators such as adenovirus E1a (for review, see Flint and Shenk 1989; Martin and Green 1992) and herpes simplex virus VP16 (for review, see Thompson and McKnight 1992; O'Hare 1993). These viral proteins do not bind directly to DNA but instead function through interactions with promoter-specific transcription factors that have sequence-specific DNA-binding activity.

A promoter-specific transcription factor that has received considerable attention is activating transcription factor-2 (ATF-2), a member of the ATF/CREB (cAMP responsive element binding) protein family of basic region–leucine zipper (bZIP) DNA-binding proteins (Hai et al. 1989; Maekawa et al. 1989). ATF-2 is of particular interest because it can be regulated by several, appar-

ently diverse inducers. In particular, ATF-2 can mediate transcriptional activation by the 289-amino-acid adenovirus E1a protein (Liu and Green 1990; Maekawa et al. 1991; Chatton et al. 1993; Liu and Green 1994; Livingstone et al. 1995). In addition, ATF-2 has been implicated as the target for the hepatitis B virus protein pX (Maguire et al. 1991), the human T-cell leukemia virus type I (HTLV-I) activator Tax (Franklin et al. 1993; Wagner and Green 1993), as well as the retinoblastoma gene product (pRB) (Kim et al. 1992). ATF-2 is also required for viral RNA-induced activation of the human interferon β gene, a mechanism that involves interaction with the high mobility group protein [HMGI(Y)] (Du et al. 1993). Finally, in response to extracellular signals ATF-2 has been shown to be regulated by phosphorylation (Abdel-Hafiz et al. 1992b; Gupta et al. 1995; Livingstone et al. 1995; van Dam et al. 1995).

An important aspect of transcription factor activation, is the mechanism that renders the factor inactive in the absence of an inducer. Full-length ATF-2, either in its native form (Georgopoulos et al. 1992; Ivashkiv et al. 1992) or as a GAL4 fusion protein (Liu and Green 1990), is transcriptionally inactive. However, ATF-2 contains an activation domain and when this minimal activation domain is directed to a promoter it is a potent activator

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of transcription (Liu and Green 1994; Livingstone et al. 1995; see below). Thus, the intrinsic activity of the minimal ATF-2 activation domain is suppressed in the context of the full-length protein.

Here, we have performed experiments to understand why in the absence of an inducer full-length ATF-2 is transcriptionally inactive. We delineate the element in ATF-2 required for inhibition and find, surprisingly, that it is the bZIP DNA-binding domain. We show further that inhibition involves interaction between the bZIP and ATF-2 activation domain. Our results suggest a model in which the ATF-2 bZIP DNA-binding and activation domains are coordinately regulated.

Results

The ATF-2 activation domain is inhibited by its bZIP DNA-binding domain

The transcriptional activity of ATF-2 and derivatives was tested in a transient expression assay following fusion to the GAL4(1–147) DNA-binding domain. In this experiment and those presented below, comparable expression of each GAL4 fusion protein was verified by immunoblotting (see below; data not shown). Consistent with previous results (Liu and Green 1990; Georgopou-

los et al. 1992; Ivashkiv et al. 1992), Figure 1 shows that full-length ATF-2 [GAL4–ATF-2(1–505)] failed to activate transcription, whereas a minimal ATF-2 activation domain [GAL4–ATF-2(N)] was highly active. These results suggested that a sequence within ATF-2 inhibits the intrinsic activity of the activation domain. To identify this inhibitory sequence, we analyzed a series of ATF-2 carboxy-terminal deletion mutants. Figure 1 shows that GAL4–ATF-2(1–415), which lacks the sequence carboxy-terminal to the bZIP DNA-binding domain, was a poor transcriptional activator. Significantly, however, GAL4–ATF-2(1–349), which differs from GAL4–ATF-2(1–415) only by the absence of the bZIP DNA-binding domain, was 200-fold more active than GAL4–ATF-2(1–415). Further deletion of residues 110–349 resulted in a modest (about fourfold) increase in activity. These results indicate that inhibition of the ATF-2 activation domain depends on the bZIP motif. To verify this idea, we fused the bZIP domain directly to the activation domain and found that the resulting protein [GAL4–ATF-2(N,bZIP)] was inactive. Thus, the ATF-2 bZIP is sufficient for inhibition.

The activities and expression levels of the GAL4–ATF-2 fusion proteins were analyzed in COS-1 cells. Figure 2A shows that as in HeLa cells, the bZIP strongly

Figure 1. Transcriptional activities of GAL4–ATF-2 deletion derivatives in HeLa cells. HeLa cells were cotransfected with plasmids expressing the GAL4–ATF-2 fusion proteins, indicated at the top of each lane, the CAT reporter pG5E1bCAT (Lillie and Green 1989), and the internal control plasmid RSV- β -gal by the calcium-phosphate transfection procedure. Cell extracts were prepared 36 hr post-transfection and assayed for CAT and β -galactosidase activity. A representative autoradiograph is shown. The CAT activities normalized to that of GAL4–ATF-2(N) are shown at the bottom; variations in CAT activity were <20%. Diagrams of the GAL4–ATF-2 fusion proteins are also indicated.

inhibited the ATF-2 activation domain. Immunoblotting analysis (Fig. 2B) shows that the differences in transcriptional activities of the GAL4-ATF-2 fusion proteins cannot be explained by variations in their levels of expression.

Efficient inhibition of ATF-2 activation domain requires both the basic region and the leucine zipper subdomains

The bZIP domain consists of two functionally separable subdomains: the basic region and the leucine zipper (LZ) (Johnson and McKnight 1989). To analyze the role of these subdomains in transcriptional inhibition we tested the activities of carboxy-terminal deletion mutants lacking either the LZ region [GAL4-ATF-2(1-380)] or the entire bZIP domain [GAL4-ATF-2(1-349)]. Figure 3A shows that both fusion proteins were comparably active, indicating that the basic region is not sufficient for inhibition.

To analyze the role of the LZ, we fused it directly to the carboxyl terminus of the activation domain (N) and found that the resulting protein [GAL4-ATF-2(N,LZ)] was significantly more active than that containing the intact bZIP domain [GAL4-ATF-2(N,bZIP)] (Fig. 3B). From these results, we conclude that efficient inhibition requires both the basic region and the LZ.

Ability of heterologous bZIPs to inhibit the ATF-2 activation domain

Next, we asked whether inhibition was specific to the

ATF-2 bZIP by testing whether the bZIP of CREB, an ATF/CREB family member, the bZIPs of c-Jun and c-Fos, Jun/Fos family members, and the bZIP of C/EBP (CCAAT/enhancer binding protein) a C/EBP family member, could inhibit the ATF-2 activation domain. Each bZIP was fused to the carboxyl terminus of GAL4-ATF-2(1-349). Figure 4 shows that the CREB bZIP inhibited as well as the ATF-2 bZIP domain, and that the c-Jun and C/EBP bZIPs also inhibited but with a slightly lower efficiency. Significantly, the c-Fos bZIP, which cannot homodimerize (see, e.g., O'Shea et al. 1989), was not inhibitory. This result, in conjunction with those of Figure 3A, indicates that inhibition requires a bZIP that can dimerize.

Inhibition does not involve a bZIP-DNA interaction

One possible, but unlikely, explanation for the observed inhibition was that the bZIP domain sequesters the GAL4 fusion protein to irrelevant bZIP DNA-binding sites on genomic DNA. To rule out this possibility, we analyzed a fusion protein containing a CREB bZIP domain mutant, K304E, which is defective in DNA binding (Dwarki et al. 1990). Figure 5 shows that this CREB bZIP mutant inhibited the ATF-2 activation domain as efficiently as the wild-type CREB bZIP. Thus, inhibition of the ATF-2 activation domain does not involve bZIP DNA binding.

Figure 2. Analysis of GAL4-ATF-2 deletion derivatives in COS-1 cells. COS-1 cells were cotransfected with the indicated amount of expression plasmid and 2 μg of the CAT reporter pG5E1bCAT (Lillie and Green 1989). [A] CAT assay; [B] Immunoblot analysis.

Figure 3. Efficient inhibition of the ATF-2 activation domain requires both the basic region and leucine zipper (see Fig. 1 legend). (A) Analysis of the basic region (B); (B) analysis of the leucine zipper (LZ).

Activation domain specificity of bZIP-mediated inhibition

To determine whether bZIP-mediated inhibition was activation domain specific, we analyzed fusion proteins in which the ATF-2 bZIP was attached to the carboxyl terminus of the acidic VP16 activation domain (Triezenberg et al. 1988) [GAL4-VP16-ATF-2(bZIP)], the glutamine-rich activation region A of Sp1 (Courey and Tjian 1988)

[GAL4-SP1(Q)-ATF-2(bZIP)], or the zinc-binding activation domain of the adenovirus E1a protein (Lillie and Green 1989) [GAL4-E1a(CR3)-ATF-2(bZIP)]. The activity of each fusion protein was compared with that of its respective counterpart, which lacked the ATF2 bZIP. Figure 6 shows that neither the VP16 activation domain nor the SP1 glutamine-rich activation domain was inhibited by the ATF-2 bZIP, whereas the E1a activation domain was rendered inactive. Immunoblotting analysis (Fig. 6A) shows that the differences in transcriptional activities of the GAL4-ATF-2 fusion proteins cannot be explained by variations in their levels of expression. As discussed below, the ATF-2 and E1a activation domains have common structural and functional properties. Therefore, the bZIP domain can inhibit only certain classes of activation domains.

Direct interaction between the ATF-2 activation domain and bZIP

One possible mechanism for the observed inhibition is a direct, intramolecular interaction between the bZIP and ATF-2 activation domains. To test this idea, we performed an *in vitro* protein interaction experiment. ATF-2 fragments 1-349 or 1-109, which contain the activation domain, were expressed as glutathione *S*-transferase (GST) fusion proteins, immobilized on glutathione-agarose beads (see Fig. 7A) and incubated with bacterially expressed ATF-2 fragment (350-505) containing

Figure 4. Inhibition of the ATF-2 activation domain by different bZIPs (see Fig. 1 legend).

Figure 5. A mutant bZIP defective in DNA binding can inhibit the ATF-2 activation domain [see Fig. 1 for legend]. As indicated in the diagram, CREB bZIPm contains a single amino acid substitution (Lys → Glu) at position 304 in the bZIP (Dwarki et al. 1990).

the bZIP domain. After extensive washing, retention of ATF-2(350–505) was analyzed by immunoblotting. Figure 7B shows that both GST–ATF-2(1–349) and GST–ATF-2(1–109) efficiently bound the bZIP-containing fragment, ATF-2(350–505). In contrast, ATF-2(350–505) did not bind to the control GST protein, or to GST–

VP16, which can interact with a variety of other proteins (for review, see Triezenberg 1995). We conclude that the ATF-2 activation domain and the ATF-2 bZIP DNA-binding domain interact specifically with one another.

We also tested the ability of the CREB and c-Fos bZIPs to interact with the ATF-2 activation domain. Figure 7C shows that the CREB bZIP bound to immobilized GST–ATF-2(1–109), whereas the c-Fos bZIP did not. Therefore, there is a direct correlation between the ability of bZIPs to inhibit and interact with the ATF-2 activation domain.

Cell-type specificity of bZIP-mediated inhibition

Some promoter-specific transcription factors are active only in certain cell types (see, e.g., Baichwal and Tjian 1990). To determine whether bZIP-mediated inhibition was cell-type specific, we analyzed the GAL4–ATF-2 fusion proteins in several other cell lines. The results of Figure 8 show that the relative activities of the various GAL4–ATF-2 fusion proteins in monkey kidney (CV-1), Chinese hamster ovary (CHO), and HeLa cells were very similar. In contrast, in mink lung CCL64 cells, often used in signal transduction studies, the activities of GAL4–ATF-2 or GAL4–ATF-2(1–415), which both contain the bZIP, were comparable to that of GAL4–ATF-2(1–349), which lacks the bZIP. Thus, inhibition of the ATF-2 activation domain by the bZIP is cell-type specific.

A specific ATF-2 sequence is responsible for cell-type-specific regulation

The data of Figure 9 show that in CCL64 cells, GAL4–

Figure 6. Activation domain specificity of bZIP-mediated inhibition. (A) As in the Fig. 1 legend, except that for the different types of activation domains, the amount of cell extract analyzed was adjusted so that roughly comparable autoradiographic signals were obtained. The activity of the GAL4 fusion protein containing the activation domain alone was arbitrarily assigned as 100. (B) Immunoblot analysis. COS-1 cells were transfected with 0.5 µg of each expression plasmid.

Figure 7. Direct interaction between the ATF-2 activation domain and bZIP. Glutathione-agarose beads containing immobilized GST, GST-VP16, GST-ATF-2(1-349), or GST-ATF-2(1-109) were incubated with bacterially expressed 6His-ATF-2(350-505), 6His-CREB(bZIP), or 6His-c-Fos(bZIP). After washing, bound His-tagged proteins were detected by immunoblotting with monoclonal T7-Tag antibody (Novagen). (A) Coomassie-blue stained SDS-polyacrylamide gel demonstrating that equal amounts of immobilized fusion proteins were used for the protein-affinity chromatography assays. (B) Immunoblot of 6His-ATF-2(350-505). (C) Immunoblot of 6His-ATF-2(350-505), 6His-CREB(bZIP), and 6His-c-Fos(bZIP).

ATF-2 is constitutively active, whereas GAL4-ATF-2(N,bZIP), in which the bZIP is fused directly to its carboxyl terminus, is not. These data suggest that a sequence between the activation domain and the bZIP is important for counteracting inhibition. Such a sequence could function merely to increase spacing between these two domains or have a more active role. To distinguish between these possibilities, we tested whether the relative position of the bZIP and activation domain affected inhibition. In the first set of experiments, we analyzed a fusion protein, GAL4-ATF-2(bZIP,N), in which the

ATF-2 activation domain was transferred to the carboxyl terminus. Figure 9A shows that GAL4-ATF-2(bZIP,N) was inactive in both CCL64 and HeLa cells, indicating that inhibition by the bZIP is independent of its position relative to the activation domain. Next, we compared the activities of GAL4-ATF-2(bZIP,1-349) and GAL4-ATF-2(1-415), which contain the same sequences but differ by the relative positions of the bZIP and activation domain. In GAL4-ATF-2(bZIP,1-349) the two domains are located close to each other, whereas in GAL4-ATF-2(1-415) they are separated by residues 110-349. The

Figure 8. Cell-type specificity of bZIP-mediated inhibition. Relative transcriptional activities in different cells normalized to the activity of GAL4-ATF-2(N) are indicated together with diagrams for the GAL4 fusion proteins. (NT) Not tested.

Figure 9. A specific ATF-2 sequence is responsible for cell-type-specific regulation (see Fig. 1 legend). (A) The role of ATF-2 sequences 110–349 in cell-type-specific activity. The cell type transfected is indicated below the autoradiograph. (B) Delineation of the ATF-2 sequence responsible for activity in CCL64 cells. (C) Immunoblot analysis (as in Fig. 6).

results of Figure 9A show that both proteins were active in CCL64 but not HeLa cells, indicating that ATF-2 sequences 110–349 can modulate bZIP-mediated inhibition in a position-independent manner.

To delineate further the sequence element responsible for counteracting inhibition, we constructed a series of internal deletion mutants and tested their activity in CCL64 cells. Figure 9B shows that deletion of residues 110–143, or residues 150–247, did not significantly affect activity, whereas deletion of residues 110–195 strongly reduced transcription. Figure 9C shows that the GAL4 fusion proteins were expressed at comparable levels. Therefore, the transcriptional activity of ATF-2 in CCL64 cells can be attributed to a short sequence (residues 110–195) that counteracts bZIP-mediated inhibition in a cell-type-specific manner.

Discussion

A central question in the eukaryotic transcription field is how are promoter-specific transcription factors regu-

lated? Several mechanisms by which activators are silenced in the absence of their inducers have been described and include, for example, sequestration in the cytoplasm (Picard and Yamamoto 1987; Baeuerle and Baltimore 1988; Beg and Baldwin 1993; Whiteside and Goodbourn 1993); interaction with inhibitory proteins (Moman et al. 1992; Helin and Harlow 1993); conformational inactivity (Morimoto 1993); and underphosphorylation (Karin and Hunter 1995). The collective results of this study reveal a new mechanism for rendering a transcription factor inactive, which involves a direct, intramolecular interaction between the activation domain and the DNA-binding domain. The data supporting this model, and the implications for activation, are discussed below.

Communication between the ATF-2 activation domain and bZIP

A direct interaction between the ATF-2 activation domain and bZIP is suggested by the results of both in vitro

and in vivo experiments. In an in vitro protein affinity–chromatography assay, the ATF-2 activation domain specifically bound to its own bZIP. The functional significance of this in vitro interaction is supported by transfection experiments. The ATF-2 and E1a activation domains were strongly inhibited by the ATF-2 bZIP in vivo, whereas the VP16 activation domain and the glutamine-rich activation domain A of Sp1 were not. Accordingly, the E1a (Liu and Green 1994; Livingstone et al. 1995) and the ATF-2 activation domain (this study), but not the VP16 activation domain, interact with bZIP in vitro. The CREB bZIP both inhibited and interacted with the ATF-2 activation domain [also see Abdel-Hafiz et al. 1992a]. In contrast, the c-Fos bZIP neither inhibited nor interacted with the ATF2 activation domain.

Complementary to our results is the previous demonstration that the activation domain of ATF-2 strongly inhibits its ability to bind DNA in vitro (Abdel-Hafiz et al. 1992b). Taken together, these observations suggest that the ATF-2 activation domain and bZIP antagonize each other's function.

We note that both the ATF-2 and E1a activation domains, which interact with the ATF-2 bZIP, contain metal binding sites (Culp et al. 1988; Zu et al. 1991; Chatton et al. 1993). It is intriguing that several other protein–protein interactions appear to also involve a bZIP domain–metal binding site contact (Diamond et al. 1990; Jonat et al. 1990; Semmes and Jeang 1992; Wagner and Green 1993).

Activation of ATF-2

ATF-2 can be activated by both regulatory proteins and through phosphorylation. Our results may be relevant to both of these activation mechanisms. Protein activators of ATF-2 include adenovirus E1a, HTLV-I Tax, and HMG I(Y). Significantly, each of these protein activators has been shown to interact with the ATF-2 bZIP (Du et al. 1993; Franklin et al. 1993; Wagner and Green 1993; Liu and Green 1994). These observations suggest a model in which binding of these regulatory proteins to the ATF-2 bZIP disrupts the intramolecular activation domain–bZIP interaction leading to activation.

A second ATF-2 activation mechanism involves phosphorylation. Several studies have shown that the activation domain of ATF-2 can be phosphorylated by stress-activated protein kinases (SAPKs) (Gupta et al. 1995; Livingstone et al. 1995; van Dam et al. 1995), including JNK, the c-Jun amino (N)-terminal protein kinase. An attractive idea is that phosphorylation disrupts the activation domain–bZIP interaction. Consistent with this possibility, phosphorylation of ATF-2 by MAP kinase dramatically increases its in vitro DNA-binding activity (Abdel-Hafiz et al. 1992b).

Coordinate regulation of DNA-binding and activation domains

An important implication of our model is that the ATF-2 activation domain and DNA-binding domain are coordi-

nately regulated. We suggest that such coordinate regulation may be critical for activator function and proper transcriptional regulation. First, the ability of the DNA-binding domain to suppress the activation domain when not bound to DNA would help prevent the “squenching” of general transcription factors (or coactivators) (Ptashne 1988; Johansen and Prywes 1993). Second, coordinate regulation may also be important for function of a transcription factor family whose members have similar DNA-binding specificity. For example, the ATF/CREB factor family comprises more than a dozen proteins with virtually identical DNA-binding specificities (Hoeffler et al. 1988; Gonzalez et al. 1989; Hai et al. 1989; Maekawa et al. 1989; Gaire et al. 1990; Yoshimura et al. 1990; Foulkes et al. 1991) but whose responses to various inducing agents differ significantly (Gonzalez and Montminy 1989; Liu and Green 1990; Abdel-Hafiz et al. 1992). ATF/CREB proteins not involved in a particular transcriptional response may need to be prevented from binding to DNA to avoid blocking binding of the relevant ATF/CREB protein. Coordinate regulation of the DNA-binding and activation domain would help ensure that a factor binds to DNA only in the presence of its inducer.

Our results with ATF-2 may be applicable to other activators. For example, for several other promoter-specific transcription factors, the DNA-binding domain, or the act of DNA binding, appears to modulate the activation domain (Fujita et al. 1992; Johansen and Prywes 1993; Hill et al. 1994; Lefstin et al. 1994). Whether this is attributable to direct interaction between the DNA binding and activation domains, as with ATF-2, remains to be determined.

Materials and methods

Plasmids

pSGATF-2 (Liu and Green 1990) and pSGATF-2(N) (segment N corresponding to residues 1–109) (Liu and Green 1994) have been previously described. pSGATF-2(1–243), pSGATF-2(1–349), pSGATF-2(1–380), pSGATF-2(1–415) contain the appropriate DNA fragments encoding residues 1–243, 1–349, 1–380, or 1–415 of ATF-2 inserted in-frame to the GAL4(1–147) sequence in the pSG424 vector (Sadowski and Ptashne 1989).

To generate pSGATF-2(1–349)–bZIPs [pSGATF-2(1–349)–Jun(bZIP), etc.], the DNA fragments encoding the bZIPs of CREB (residues 280–314) from rat, c-Jun (residues 248–340), c-Fos (residues 134–200) from mouse, and c/EBP (residues 278–357) from rat were amplified by PCR; the resulting PCR products were then inserted in-frame to the GAL4(1–147)–ATF-2(1–349) sequence in pSGATF-2(1–349). To create pSGATF-2(N)–CREB(bZIP), pSGATF-2(N)–CREB(bZIPm), pSGATF-2(N,bZIP), and pSGATF-2(N,LZ), the PCR-amplified DNA fragments encoding the CREB bZIP (residues 280–314), the CREB bZIP mutant containing a point mutation at position 304 (K304E) (Dwarki et al. 1990), the ATF-2 bZIP (residues 350–415), and the LZ of ATF-2 (375–415) were, respectively, inserted in-frame to the GAL4(1–147)–ATF-2(N) sequence in pSGATF-2(N). pSGATF-2(bZIP,N) and pSGATF-2(bZIP,1–349) were created by inserting the DNA fragment encoding ATF-2 bZIP into the junction between the GAL4(1–147) sequence and the ATF-2 activation domain se-

quence in pSGATF-2(N) or residue 1–349 of ATF-2 in pSGATF-2(1–349).

pSGVP16 contains the DNA fragment encompassing the activation domain (residues 414–490) of the herpes simplex virion protein VP16 (Triezenberg et al. 1988) inserted in-frame to the GAL4(1–147) sequence in the pSG424 vector. pSGVP16–ATF-2(bZIP) was derived by ligating the DNA fragment encoding ATF-2 bZIP to the GAL4(1–147)–VP16 sequence in pSGVP16. pCGSp1(Q) has been described (Southgate and Green 1995). pCGSp1(Q)–ATF-2(bZIP) was constructed by inserting the DNA fragment of the ATF-2 bZIP to the GAL4(1–95)–Sp1(Q) sequence in pCGSp1(Q). To construct pGAL4–E1a(CR3)–ATF-2(bZIP), the DNA fragment of ATF-2 bZIP was inserted in-frame downstream of the GAL4(1–147)–E1a(121–223) sequence in pSG147–E1a (Lillie and Green 1989).

To construct pSGATF-2(Δ110–143), the *AccI*–*Bam*HI fragment of ATF-2 (144–505) was inserted into the *SacI* site of pSG424 by blunt-end ligation; the *EcoRI*–*Bam*HI fragment from the resulting plasmid was inserted into the *XbaI* site of pSGATF-2(N) by blunt-end ligation. To construct pSGATF-2(Δ110–195), the *ScaI*–*Bam*HI fragment of ATF-2 (196–505) was inserted into the *Bam*HI site of the pSG424 plasmid by blunt-end ligation; the *EcoRI* fragment from the resulting plasmid was then inserted into the *XbaI* site of the pSGATF-2(N) by blunt-end ligation. pSGATF-2(Δ150–247) is a GAL4 fusion of CRE–BP2, an isoform of ATF-2 that lacks residues 150–247 of full-length ATF-2 as a result of alternative pre-mRNA splicing (Georgopoulos et al. 1992).

The reporter plasmid used in all of the transient transfection assays is pG5E1bCAT (Lillie and Green 1989), which contains five copies of the GAL4 DNA-binding site upstream of adenovirus E1b TATA element and the chloramphenicol acetyltransferase (CAT) gene.

The plasmids used for protein expression in *Escherichia coli*, including pGEX2T (Smith and Johnson 1988), pGVP (Lin and Green 1991), pGEX–ATF-2(1–349) (Liu and Green 1994), pGEX–ATF-2(1–109) (Liu and Green 1994), and pRsetB–ATF-2(350–505) (Wagner and Green 1993) have been described previously. pRsetC–CREB(bZIP) and pRsetC–cFos(bZIP) were constructed by inserting the DNA fragments encoding the respective bZIP sequences to the 6His–T7 tag sequence in pRsetC vector (Invitrogen).

Cell culture and transfection

HeLa, monkey kidney CV-1, mink lung CCL64, and COS-1 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS). The cells were split 1:8 (for HeLa and CV-1 cells), 1:16 (for CCL64 cells), or 1:7 (for COS-1 cells) ~20 hr prior to transfection by the calcium-phosphate transfection technique (Wigler et al. 1978). Each transfection mixture for HeLa, CV-1, and CCL64 cells contained 2 μg of reporter plasmid, pG5E1bCAT, 2 μg of plasmid encoding one of the GAL4 fusion proteins, as well as 3 μg of RSV–β-gal plasmid as internal control. In all cases, the total amount of DNA for each transfection was adjusted to 20 μg with pGEM3 (Promega). The cells were exposed to the precipitate for ~20 hr. After washing, the cells were grown further for 36 hr before being harvested.

CHO cells were grown in α minimal essential medium with nucleotides supplemented with 10% FBS. The cells were split 1:8 24 hr before transfection. DNA was introduced into the cells by the DEAE–dextran procedure (Wigler et al. 1978). Fourteen hours post-transfection cells were treated with dimethylsulfoxide (DMSO), followed by a treatment with chloroquine for 2.5 hr. The cells were harvested 48 hr after the DMSO shock.

Cell extracts from the transfected cells were assayed for β-galactosidase activity and CAT activity as described (Sambrook et al. 1989). For CAT assays, the amount of cell extract used for each reaction was normalized by the β-galactosidase activity. All of the transfection experiments were repeated at least twice, the autoradiographs from the CAT assays presented are representative of each type of experiment, and the CAT activities shown are the average values from each type of experiment.

To analyze the expression of GAL4 fusion proteins, cell extracts prepared from transiently transfected COS-1 cells were fractionated by SDS-PAGE. GAL4 fusion proteins were detected by immunoblotting with a polyclonal GAL4(DBD) antibody (Santa Cruz Biotech., Inc.) using ECL (Amersham).

Protein-affinity chromatography

His–ATF-2(350–505), 6His–CREB(bZIP), and 6His–c-Fos(bZIP) were expressed and purified from *E. coli* using plasmids pRsetB–ATF-2(350–505), pRsetC–CREB(bZIP), and pRsetC–c-Fos(bZIP) as described previously (Wagner and Green 1993). GST, GST–VP16, GST–ATF-2(1–349), and GST–ATF-2(1–109) were expressed and purified from *E. coli* as described previously (Lin and Green 1991), with the following modifications. The *E. coli* lysate was prepared with buffer A [20 mM Tris–HCl at pH 8.0, 1.0 M NaCl, 20 μM ZnCl₂, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] containing 1 μg/ml of pepstatin A, and 1 μg/ml of leupeptin. After incubation of pre-swollen glutathione–agarose beads with precleared *E. coli* lysate, the beads were washed extensively, first with buffer A and then with buffer B [20 mM HEPES (pH 8.0), 0.1 M KCl, 20 μM ZnCl₂, 10% glycerol, 5 mM β-mercaptoethanol, 0.5 mM PMSF]. The proteins on the beads were eluted with 40 mM glutathione in buffer B that contained 150 mM HEPES (pH 8.0) and were dialyzed extensively against buffer B.

For protein-affinity chromatography, the purified GST or GST fusion proteins were recoupled to glutathione–agarose beads. Twenty microliters of the resulting beads containing equal amounts (5 μg) of GST or one of the GST fusion proteins was incubated with 1.0 μg of 6His–ATF-2(350–505), 6His–CREB(bZIP), or 6His–c-Fos(bZIP) in 0.6 ml of the protein-binding buffer (10 mM HEPES at pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, 50 μM ZnCl₂, 0.025% NP-40, 0.5 mM DTT, 0.5 mM PMSF) containing 60 μg of bovine serum albumin. After incubating at 4°C for 2 hr with continuous mixing, the beads were washed four times with 1 ml of the protein-binding buffer over a period of 1 hr. 6His–ATF-2(350–505), 6His–CREB(bZIP), and 6His–c-Fos(bZIP) bound to the beads were eluted by SDS sample buffer, fractionated by SDS-PAGE, and detected by immunoblotting with a monoclonal T7–Tag antibody (Novagen).

Acknowledgments

We thank F. Liu and W. Du for plasmids; and F. Liu, J. Valcarcel, S. Roberts, S. Wagner, J. Reese, G. Perini, and other members of the Green laboratory for critical reading of the manuscript and helpful discussions. M.R.G. is an investigator for the Howard Hughes Medical Institute. This work was supported by a grant from the National Institutes of Health to M.R.G.

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Intramolecular inhibition of activating transcription factor-2 function by its DNA-binding domain.

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Genes Dev. 1996, **10**:

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