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Interaction of a Common Factor with ATF, Sp1, or TATAA Promoter Elements Is Required for These Sequences To Mediate Transactivation by the Adenoviral Oncogene E1a

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The adenovirus protein E1a stimulates transcription of both viral and cellular genes. Unlike most other transcription factors, it induces transactivation through several different promoter elements. The mechanism by which elements of diverse sequence mediate the effect of E1a is the focus of this study. Three E1a-responsive elements (an ATF site, an Sp1 site, and a TATA box containing the sequence TATAA) were studied to determine whether their interaction with a common factor is necessary for transactivation. In transfection assays, each element was used as a competitor against promoter constructs containing the other elements. The elements as competitors had no effect on basal transcription, but each competitor completely inhibited transactivation by E1a. Competitors that were not E1a responsive failed to inhibit transactivation. Therefore, either E1a itself or an E1a-inducible factor interacts with each of the elements to cause transactivation, most likely though an association with each element's specific binding protein.

The 289-amino-acid form of E1a is an adenovirus early-gene product that is widely studied because it is both oncogenic and a strong transcriptional activator (10, 24). These are independent activities, since mutations that inhibit one of these activities without affecting the other can be made in E1a.

Three separate regions in E1a are important for transformation (14, 16, 20, 23, 25, 30, 32, 33, 37, 38). These are two regions (CRI and CRII) whose sequences are conserved in different strains of adenovirus and the amino-terminal region of the protein. Each of these regions binds to specific cellular proteins. CRI and CRII are both required for binding the retinoblastoma susceptibility gene product and cyclin A (p60), which are important in cell cycle control, whereas CRII alone binds a 107-kDa protein and the amino-terminal region binds a 300-kDa protein. These interactions appear to be integral to E1a's oncogenic activity, since mutations that disrupt binding of any of these proteins inhibit the ability of E1a to transform cells (8, 33, 36, 38).

A third region of E1a (CRIII), whose sequence is also conserved in different adenovirus strains, causes transactivation by E1a (10). Unlike CRI, CRII, and the amino-terminal region, this region is not required for transformation. However, it is essential for productive infection, in which it serves to transactivate adenovirus early genes as well as certain cellular genes that may facilitate infection, such as heat shock genes (10).

Efforts to identify promoter elements that mediate transactivation by CRIII of E1a led to the surprising finding that apparently unrelated elements, such as ATF sites and TATA boxes containing the sequence TATAA (TATAA element), are responsive (29, 31, 39), and in this report we demonstrate that an Sp1 site is also responsive. E1a is not targeted directly to these elements, since it does not bind DNA in a sequence-specific fashion (3, 9). Several studies, however, propose that it stimulates transcription by causing phosphorylation of the transcription factors that do bind these elements (2, 11), whereas others suggest that it participates directly in transcription complexes (19, 21).

A recent report indicates that E1a can be targeted to ATF sites in the promoter through its interaction with an ATF site-binding protein, ATF-2 (CRE-BP1), and that this interaction is required for transactivation through these sites (21). We hypothesized that analogous interactions occur at other responsive elements. To test this idea, a series of competition assays was devised to determine whether the interaction of E1a with the Sp1 site and TATAA element is also required for transactivation of these responsive sequences.

MATERIALS AND METHODS

Cell culture and DNA transfection. The human fibrosarcoma cell line HT-1080 was grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum at 37°C in 5% CO_2. Transfection assays were done by the Ca_2PO_4 method, and chloramphenicol acetyltransferase (CAT) activity was determined as described previously (5). The medium with the Ca_2PO_4 precipitate was replaced with fresh medium after 4 to 8 h, and the cells were harvested 24 to 36 h later.

Plasmid construction. pE1a12S and pE1a13S encode the 243- and 289-amino-acid forms of E1a, respectively (29). The E1a promoter, which contains 498 bp of 5′ flanking sequence (the 5′ end is cloned into the *Eco*RI site in the parent vector, Bluescript SK [Stratagene Inc., La Jolla, Calif.]), drives expression in each of these plasmids. The 3′ ends of the E1a cDNAs are cloned into the *Bam*HI site. Plasmids pTATAA-CAT, pTA-CAT, pSp1-TA-CAT, and pATF-TA-CAT were constructed as follows. Oligonucleotides (see below) were synthesized such that after hybridization of complementary strands, a *Hin*dIII site was present on the 5′ end and a *Pst*I site was present on the 3′ end. Hybridized oligonucleotides were ligated into p65CAT (7), containing the CAT structural gene, which had been digested with *Hin*dIII and *Pst*I. pTATAA-CAT contains the fibronectin gene sequence from

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+8 to −36 (6). The TATAA sequence is the only known element present in this region. pTA-CAT contains the same region except that the sequence TATAA which extends from −20 to −24 is replaced by the simian virus 40 (SV40) early-gene TATA box equivalent TATTTAT, which is unresponsive to E1a (31). pSp1-TA-CAT was constructed by adding the Sp1 sequence, 5′-GGGGCGGG-3′, 5′ to position −36 in pTA-CAT, and pATF-TA-CAT was constructed by adding the sequence 5′-CCCGTGACGTCACCCG-3′, which corresponds to the fibronectin gene ATF site between positions −161 to −176 (5), 5′ to position −36 in pTA-CAT. Competitor plasmids were constructed by cloning synthetic double-stranded oligonucleotides containing these ATF and Sp1 sites into the vector. The nuclear factor 1 (NF-1) competitor plasmid was constructed by cloning synthetic double-stranded oligonucleotides containing the adenovirus NF-1 site (5′-AGCTTGGTCTGGCTTTGGGCCAAGAGC CGA-3′) (28) into the vector. pE1aCAT was constructed by first using the polymerase chain reaction (PCR) to amplify the E1a promoter in pE1a13S and then cloning the amplified promoter upstream of the CAT gene in pSKCAT, in which the CAT structural gene is cloned into the *Pst*I (5′ end) and *Bam*HI (3′ end) sites of Bluescript SK. For PCR, pE1a13S was linearized with *Bam*HI and the E1a promoter was amplified by using the T7 primer from SK, which is located 5′ of the 5′ end of the E1a promoter (position −498 [*Eco*RI site of SK]). The 3′ primer for PCR was the oligonucleotide 5′-AGTCACTGCAGTTTCAGTCCCGGTGTCGGAGC-3′, which extends from position +41 to position +61 of the E1a gene. This oligonucleotide contains a *Pst*I site (underlined) near its 5′ end for cloning purposes. After PCR, the promoter fragment was digested with *Eco*RI and *Pst*I and cloned into the corresponding sites of pSKCAT.

RESULTS AND DISCUSSION

ATF, Sp1, and TATAA elements are responsive to E1a in transient transfection assays. E1a appears to be targeted to ATF sites in promoters through its interaction with the ATF site-binding protein ATF-2 (21). Therefore, it was conceivable that analogous interactions involving E1a might occur at other E1a-responsive elements. We reasoned that it might be possible to detect such interactions by using a series of competition assays.

Three different E1a-responsive elements were examined in our studies: the ATF site, the Sp1 site, and a TATAA element. First, we demonstrated that constructs containing these elements were responsive to E1a in transfection assays. pTATAA-CAT expression was stimulated when the E1a expression vector pE1a13S was cotransfected (Fig. 1A) but not when pE1a13S was replaced with the control plasmid pE1aG (data not shown), which is identical to pE1a13S except that the sequence encoding human β-globin was substituted for the sequence encoding E1a (29). When the SV40 early-gene TATA box equivalent TATTTAT, which is known to be unresponsive to E1a (31), was substituted for TATAA (pTA-CAT), E1a responsiveness was lost (Fig. 1B). This result indicates that in pTATAA-CAT, the sequence TATAA is required for transactivation by E1a. Insertion of an Sp1 or an ATF site upstream of the TATTTAT element in pTA-CAT conferred responsiveness, indicating that each of these elements is also capable of independently mediating transactivation by E1a when coupled with the unresponsive TATTTAT element (Fig. 1C and D). The constructs were not significantly transactivated when pE1a12S (encodes the 243-amino-acid form of E1a, which lacks CRIII) was substituted for pE1a13S (Fig. 1E), indicating that transactivation is CRIII dependent.

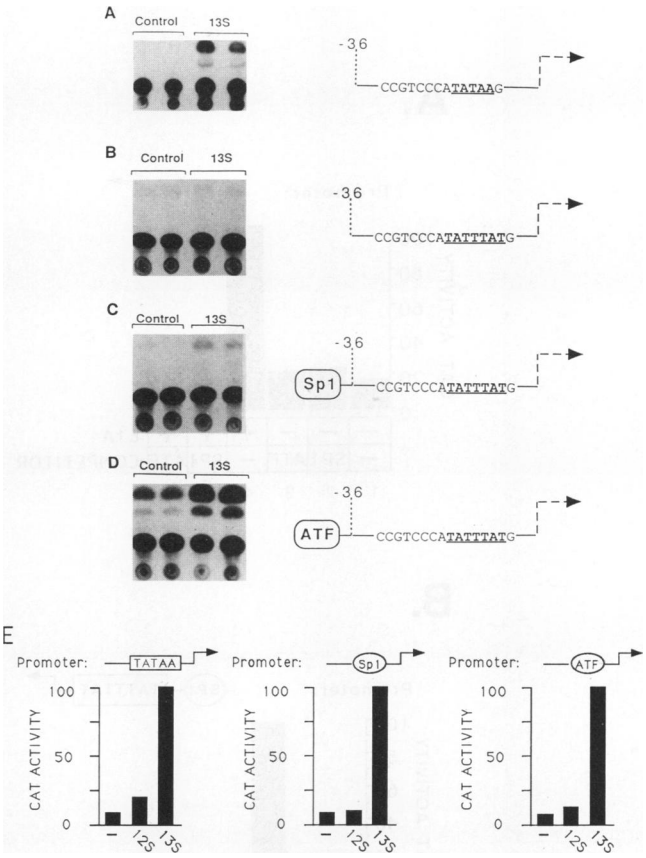

FIG. 1. Independent responsiveness of three different promoter elements to E1a. (A) The TATAA box is E1a responsive. Three micrograms of pTATAA-CAT was cotransfected into the HT-1080 cell line along with 1 μg of pE1a13S (13S). (B) Mutation of the TATAA box to resemble the SV40 early-gene TATA box equivalent TATTTAT eliminated E1A responsiveness. pTA-CAT was transfected along with pE1a13S as described above. (C) An Sp1 site is E1a responsive when coupled to the unresponsive TATTTAT box. pSp1-TA-CAT was cotransfected with pE1a13S. (D) An ATF site is also E1a responsive when coupled to the TATTTAT box. pATF-TA-CAT was cotransfected with pE1a13S. Duplicate assays are shown in each panel. (E) Activation by E1a is dependent on CRIII. Assays were done as in panels A to D except that where indicated, 1 μg of pE1a12S (12S) was cotransfected. In this panel, relative CAT activity is expressed graphically as a percentage. Each experiment was repeated at least three different times, and the results shown are representative of each assay. Schematic diagrams of reporter plasmids are shown.

TATAA, Sp1, and ATF elements each interact with an E1a-inducible factor that is required for transactivation. Next, the effect of competition with the Sp1 or ATF site on transactivation through the TATAA box was examined (Fig. 2A). A plasmid containing either an Sp1 or ATF site was used as a competitor in transfection assays with pTATAA-CAT. Neither competitor appeared to alter the basal expression of this construct (compare lanes 2 and 3 with lane 1 in Fig. 2; also see below)(however, since basal expression of pTATAA-CAT is low [only two- to threefold above background], it is impossible to demonstrate this unequivocally), which was not surprising because ATF and Sp1 sites bind different proteins than the TATAA box (i.e., ATF and Sp1

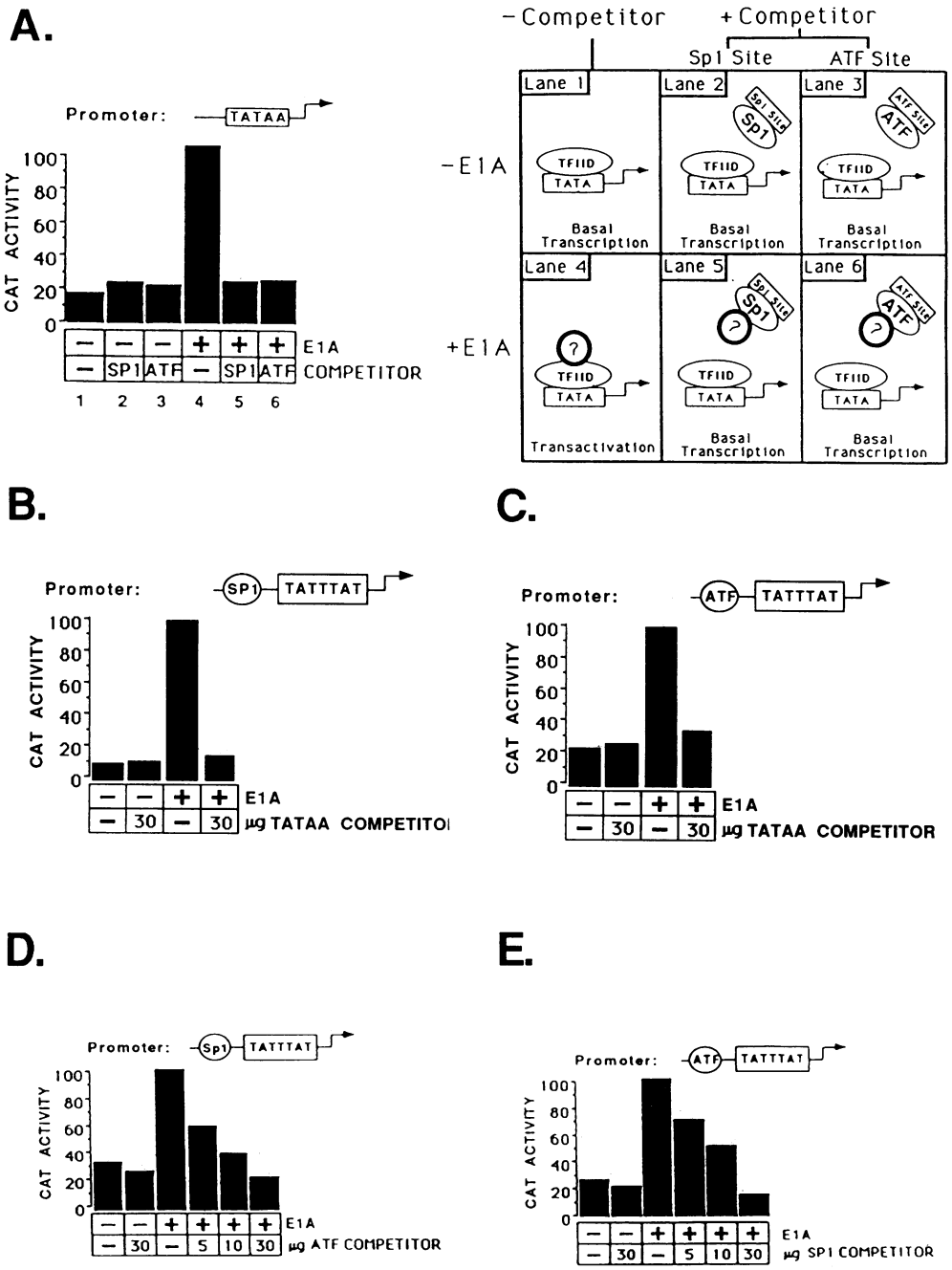

FIG. 2. Requirement of a common factor for transactivation by E1a through three different responsive elements. (A) Competition with either the ATF or Sp1 site had no effect on basal transcription, but each eliminated responsiveness of the TATAA box. Three micrograms of pTATAA-CAT and 1 µg of pE1a13S were transfected as described for Fig. 1. Thirty micrograms of competitor plasmid containing either no element (−), an Sp1 site, or an ATF site was cotransfected. The right portion of panel A is a schematic illustration of each assay. The circled question mark represents a factor required for transactivation. (B) Competition with the TATAA box eliminated E1a responsiveness of the Sp1 site. Experiments were done as described for panel A except that the reporter construct pSp1-TA-CAT was transfected and a TATAA box competitor was cotransfected. (C) Competition with the TATAA box eliminated responsiveness of the ATF site. Experiments were done as described for panel B except that the promoter construct pATF-TA-CAT was transfected. (D) Competition with the ATF site eliminated responsiveness of the Sp1 site. Experiments were done as described for panel B except that an ATF site competitor was substituted for the TATAA competitor. (E) Competition with an Sp1 site competitor eliminated responsiveness of the ATF site. Experiments were done as described for panel C except that an Sp1 site competitor was substituted for the TATAA box competitor. Transfections were done as described for Fig. 1, and relative CAT activity is expressed graphically as a percentage in each panel.

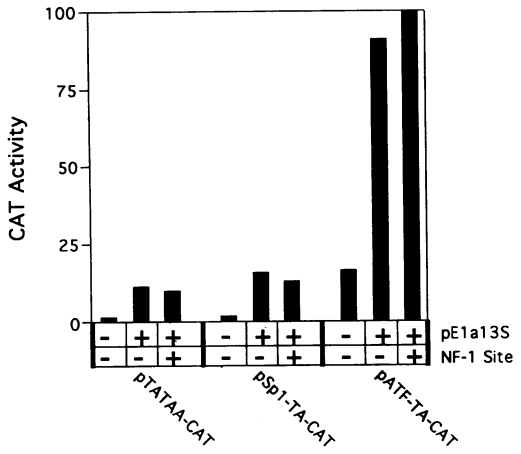

FIG. 3. Evidence that a competitor element that is not E1a responsive does inhibit transactivation of the TATAA, Sp1, or ATF site. Assays were done as described for Fig. 2; however, where indicated, 30 μg of a competitor plasmid containing the adenovirus NF-1 site was included as a competitor. The NF-1 site is not E1a responsive (15), and it was unable to inhibit transactivation of any of the three responsive elements.

versus TFIID). Each of the competitors, however, completely inhibited transactivation of the TATAA element by E1a (Fig. 2A; compare lanes 5 and 6 with the E1a-induced level of transcription in lane 4 and the uninduced level in lane 1), whereas a control plasmid without a competitor element or with an element that is not E1a responsive (NF-1 site from adenovirus) (15) did not (Fig. 2A, lane 4; Fig. 3). These findings suggest that a factor that interacts with the TATAA element and is required for transactivation but not for basal transcription is also bound specifically by the ATF and Sp1 competitors; presumably, by binding this factor the competitors sequester it, preventing it from interacting with the TATAA element in the reporter plasmid (see the schematic in Fig. 2A).

Because ATF and Sp1 site competitors were able to bind a factor required for transactivation of the TATAA element, it appeared likely that this factor would also be important for transactivation of ATF and Sp1 sites in a promoter. To determine whether this was the case, we first performed the converse of the previous experiment: the TATAA box was used as a competitor in assays with promoters containing either an ATF or Sp1 site. This competitor had no measurable affect on pSp1-TA-CAT expression (Fig. 2B; compare lanes 1 and 2), but as with pTATAA-CAT, basal expression of pSp1-TA-CAT was low, making an unequivocal statement concerning an effect of the competitor on basal expression impossible; however, this competitor clearly had no effect on basal expression of pATF-TA-CAT, which is greater than 20-fold above background (Fig. 2C; compare lanes 1 and 2). Nevertheless, E1a induction of the Sp1 and ATF reporter constructs was inhibited completely by the TATAA competitor (Fig. 2B and C; compare lane 4 with the E1a-induced level in lane 3 and the uninduced level in lane 1). When the vector alone or a plasmid containing an NF-1 site was used as a competitor, E1a induction was not affected (Fig. 2B and C; Fig. 3).

It should be noted that both the ATF and Sp1 reporter plasmids in this experiment contain an SV40 early-gene TATA equivalent TATTTAT that, like the TATAA site, binds TFIID (the two sites appear to bind TFIID with similar affinity, as judged by competition assays using gel retardation assays; data not shown). The inability of the TATAA competitor to affect basal expression of these constructs suggests that even though its concentration is sufficient to sequester a factor involved in activation by E1a, it is not high enough to sequester enough TFIID to decrease basal transcription levels.

Next, we examined the effect of competition with the ATF site on transactivation through the Sp1 site in pSp1-TA-CAT (Fig. 2D). The results of this experiment were similar to those outlined above for the TATAA element: the competitor ATF site had no measurable effect on basal transcription of the Sp1 promoter construct (Fig. 2D; compare lanes 1 and 2), but it completely disrupted transactivation (Fig. 2D; compare lanes 4 to 6 with the E1a-induced level in lane 3 and the uninduced level in lane 1). Control plasmids lacking an E1a-responsive element or containing an NF-1 site had no effect (Fig. 2D, lane 3; Fig. 3). These results suggest that a factor that would normally effect transactivation through the Sp1 site is bound by the ATF competitor. When the Sp1 site was used as a competitor against pATF-TA-CAT, similar results were observed (Fig. 2E); the competitor had no effect on basal transcription but completely disrupted transactivation, suggesting that the Sp1 site competitor binds a factor that would normally bring about transactivation through the ATF site.

We also examined the effect of the ATF competitor on a reporter construct with a promoter that consists of three Sp1 sites upstream of a TATAA box (this construct, −122FNCAT, corresponds to the first 122 bp of the fibronectin gene) (5). The basal activity of this construct is well above background. An ATF competitor eliminated E1a induction of the construct but did not inhibit basal expression (data not shown), providing further evidence that an ATF competitor does not inhibit basal activity of the TATAA box or Sp1 site.

It was conceivable that the competitors did not inhibit transactivation by E1a but simply blocked expression of E1a by inhibiting activity of the E1a promoter in pE1a13S. To rule out this possibility, the effects of ATF and Sp1 site competitors on E1a promoter activity were examined by using pE1aCAT, in which the identical E1a promoter fragment found in pE1a13S drives the CAT gene as a reporter plasmid. The competitors had no effect on CAT activity (Fig. 4), indicating that they do not inhibit E1a promoter activity. Therefore, the results from the competition assays with the three E1a-responsive elements suggest that each element interacts with a common factor required for transactivation by E1a.

It should be noted that the E1a promoter is activated by E1a (data not shown); however, this activation is not CRIII dependent, since pE1a12S efficiently transactivates pE1aCAT (data not shown). It is known that E2F sites mediate activation by E1a through a mechanism that involves regions outside of CRIII (1). Since the E1a promoter contains E2F sites, it is possible that they are responsible, at least in part, for mediating activation by E1a, and it is likely that the competitors we have used compete only for activation by CRIII.

Our results do not allow us to determine whether the common factor that we have identified is E1a itself or whether it is an E1a-inducible factor; however, the simplest explanation is that the factor is E1a. A previous study indicated that interaction of E1a with the ATF site (through ATF-2) is required for transactivation of this element (21), and we show here that the interaction of a common E1a-inducible factor is required for transactivation of ATF, Sp1,

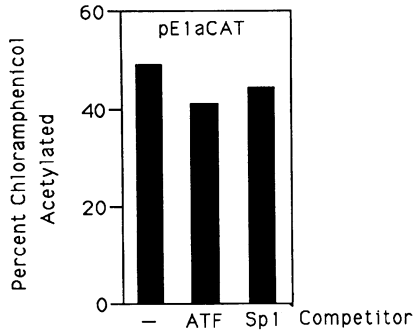

FIG. 4. Evidence that cotransfection of competitor plasmids containing either an Sp1 or an ATF site does not affect the activity of the E1a promoter. One microgram of pE1a13S and 3 μg of pE1aCAT were transfected along with either 30 μg of competitor plasmid or control vector (−) as described for Fig. 1.

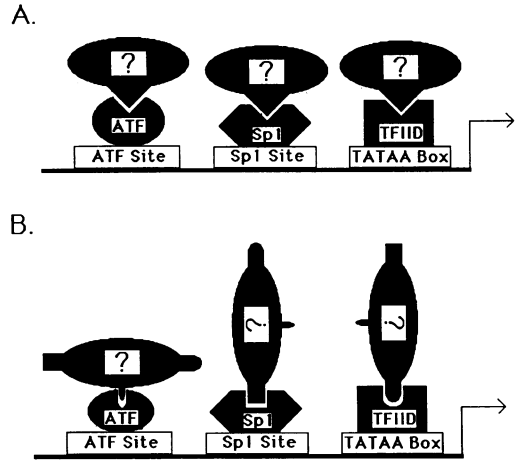

FIG. 5. Potential interactions between an E1a-inducible factor and responsive-element-binding proteins. (A) A common motif in each DNA-binding protein is recognized by a single site in the E1a-inducible factor (?). (B) A separate domain in the factor recognizes a unique motif in each DNA-binding protein. The simplest model of such interactions is shown; intermediates may lie between the factor and the DNA-binding proteins.

and TATAA elements. Taken together, these results suggest that the common factor is E1a itself.

Potential models for the interaction of an E1a-inducible factor with the ATF, Sp1, and TATAA elements. How might a common factor recognize a group of sequences as dissimilar as those of the ATF, Sp1, and TATAA promoter elements? If the factor is indeed E1a, it is unlikely that it is targeted to these elements through direct interactions with DNA, because E1a is not a sequence-specific DNA-binding protein (3, 9). Moreover, it is improbable that any protein could selectively recognize a group of elements with sequences as dissimilar as those of the ATF, Sp1, and TATAA elements. Therefore, the only apparent way that this factor could be targeted to these elements is through an interaction with their respective binding proteins.

We present two possible mechanisms for such interactions. First, the DNA-binding proteins could share a common protein binding motif, such as a leucine zipper (17) or helix-loop-helix (26), that recognizes a domain in the factor (Fig. 5A). Second, separate domains in this factor may interact with different motifs on the DNA-binding protein (Fig. 5B). E1a itself is an example of a protein that acts in this fashion: separate domains in E1a bind different sets of cellular proteins (8, 21, 33, 36, 38).

In support of the protein-protein interactions proposed in Fig. 5, ATF-2, Sp1, and TFIID each are known to interact with other nuclear proteins at the promoter, as in the following examples. (i) In addition to interacting with E1a, ATF-2 interacts with protein X of hepatitis B virus to form a DNA-binding complex (22). (ii) The bovine papillomavirus enhancer protein E2 can be tethered to the Sp1 site by Sp1, and when bound in this fashion E2 increases promoter activity; if Sp1 is not available to act as a tether, E2 has no effect (18). Additionally, Sp1 molecules can associate with other Sp1 molecules (35), and wild-type Sp1 can tether a mutant form of Sp1 that lacks a DNA-binding domain to a promoter (27). (iii) TFIID can interact with the potent transactivating factor VP16 (34), and there is also evidence that is associated with ATF and GAL4 (12, 13). Furthermore, TFIID interacts either directly or indirectly with at least five other factors that are required for general transcription: TFIIA, TFIIB, TFIIE, TFIIF, and RNA polymerase.

Finally, in Fig. 5, an E1a-inducible factor is shown interacting with DNA-binding proteins that are bound to their respective promoter elements. Our results are consistent with a model in which this interaction occurs only with DNA-binding proteins that are bound to DNA. This is suggested by the fact that even though responsive-element-binding proteins are normally present in the cell, they are apparently able to sequester the E1a-inducible factor only when competitor plasmids are present (as evidenced by the requirement for competitor plasmids for inhibition of transactivation; Fig. 2).

How might E1a selectively interact with these proteins only after they are DNA bound? One possible explanation is that the conformation of the proteins is altered upon DNA binding, thus revealing an E1a-binding site. More complex mechanisms are also possible. For example, CRIII of E1a contains a zinc finger, a common motif in the DNA-binding domains of several different transcription factors (4). It is conceivable that this domain may bind surrounding DNA sequences when E1a is tethered to transcription factors, thus facilitating the interaction. Further studies are required to discriminate between these and several other possible mechanisms.

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