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Transcriptional Activation by the Adenovirus Larger E1a Product Is Mediated by Members of the Cellular Transcription Factor ATF Family Which Can Directly Associate with E1a

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We recently isolated three cDNA clones encoding closely related proteins (ATFa1, ATFa2, and ATFa3) that belong to the activating transcription factor–cyclic AMP-responsive element family of cellular transcription factors. Using cotransfection experiments, we showed that these proteins mediate the transcriptional activation induced by the adenovirus E1a 13S mRNA gene product and that the zinc-binding domains present in both E1a conserved region 3 and the most N-terminal portion of the ATFa proteins play crucial roles in this activity. Reciprocal coimmunoprecipitation experiments demonstrated direct interactions between these proteins. Neither the conserved region 3 domain of E1a nor the N-terminal metal-binding element of ATFa is essential for these interactions. The simultaneous alteration of both the N-terminal and the C-terminal domains of ATFa abolished E1a binding, while either mutation alone failed to impair these interactions.

E1a, one of the adenovirus oncogenes, codes for nuclear proteins that exert pleiotropic effects on the host cell physiology, including transcriptional activation or inhibition of viral and cellular genes, alteration of the cell cycle, cell immortalization or, in cooperation with other oncogenes (e.g., E1b and *ras*), complete cell transformation (7, 15, 46). Some of these effects involve modifications of the patterns of binding of cellular transcription factors to particular promoter elements.

Early in infection with adenovirus 2 or adenovirus 5 (Ad2 or Ad5, respectively), expression of the E1a gene yields two polypeptides, of 243 and 289 amino acids (243R and 289R, respectively), which differ only by a 46-amino-acid element with zinc-binding activity. Although the smaller of these gene products has been reported to stimulate promoter activities in some instances (33, 37, 57), the larger has a more universal transactivating capability (5, 32).

The molecular mechanisms of the transcriptional activation induced by E1a have been extensively studied, and most attention has been paid to the 289R protein. The general picture that has emerged is that this E1a protein can act on a wide range of promoters, of viral or cellular origin, that do not necessarily share common sequence elements. Thus, extensive mutational analyses of adenovirus E1a-inducible promoters revealed that most, if not all, of the host cell promoter-binding factors involved in the constitutive function of these promoters (e.g., TFIID, activating transcription factor [ATF], E4F, E2F, AP1, and TFIIIC) also contribute to their E1a-induced activity (5, 32). Furthermore, the E1a protein weakly binds to double-stranded DNA, but with no particular sequence specificity (8, 13). Finally, studies with fusion proteins clearly indicated that targeting the E1a protein to a promoter by providing a DNA binding domain to portions of the 289R protein comprising conserved region 3 (CR3) (46) is essential for transcriptional activation (38, 44). It has been concluded that the E1a protein may associate indirectly with promoter DNA by interacting with cellular transcription factors already bound.

The E1a protein has been shown to interact with a number of distinct polypeptides, including, among others, the retinoblastoma susceptibility protein and cyclin A (18, 23, 56, 59). Although these proteins do not by themselves bind to DNA, recent evidence has demonstrated their contribution to promoter modulation through interactions with the cellular E2F transcription factor (1, 9, 10, 47, 49) or a related factor, DRTF1, which is down-regulated during embryonal carcinoma stem cell differentiation (2, 3). A direct protein-protein interaction between E1a and the mammalian TATA box factor, TFIID, has been reported (27, 36). This observation reinforces the conclusion that TFIID may mediate the E1a-induced promoter activation of certain genes (51, 58).

Multiple, independent cDNA clones encoding ATF or related cyclic AMP-responsive element (CRE) binding proteins have been isolated (6, 17, 19, 22, 24, 28, 39, 42). Cotransfection experiments with vectors directing the expression of human ATF fusion proteins (GAL4–ATF-2 and cMyb-CRE-BP1) and E1a have shown that the ATF derivatives mediate the transcriptional activation induced by E1a (40, 41). The results indirectly suggest that ATFs may act by recruiting the E1a gene product to the promoter, thereby activating the initiation complex.

We have performed similar analyses with related, but distinct, ATF clones (the ATFa series), which we isolated from HeLa cell cDNA libraries (17). Our results show that these proteins can mediate E1a-induced transactivation and that domains comprising the potential zinc-binding elements of the 289R E1a and the ATFa proteins are essential for this effect. In addition, we show that the ATFa zinc-binding domain is not sufficient by itself to support this activation. Finally, we demonstrate that the E1a proteins directly contact the ATFa proteins and that regions distinct from the zinc-binding domains of the ATFa and the 289R E1a proteins are required in addition for this interaction.

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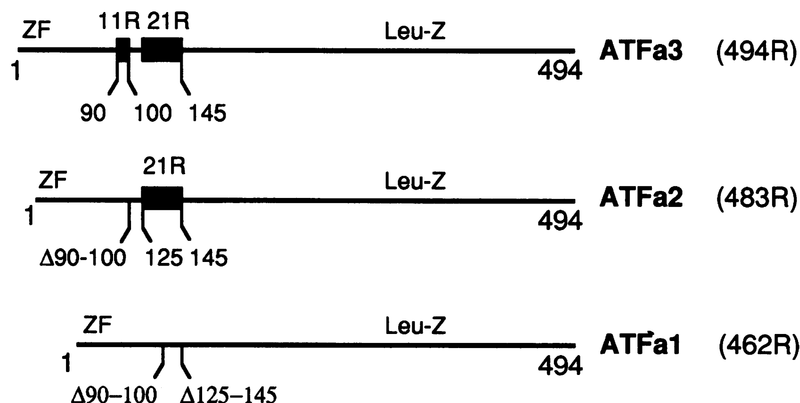

FIG. 1. Structures of the ATFa clones used in this study. The peptides corresponding to the open reading frames of the three related clones that we isolated from a HeLa cell cDNA library (17; unpublished results) are schematically represented, with coordinates corresponding to the numbering of the amino acid residues (R) of the largest protein (ATFa3). The closed boxes, with corresponding lengths given above, represent peptide elements retained or deleted (Δ) in each protein, as indicated. The approximate locations of a putative zinc-binding motif (ZF) and a leucine zipper element (Leu-Z) are shown. The overall sizes of the proteins are indicated on the right.

MATERIALS AND METHODS

ATFa cDNA clones. We initially isolated two related HeLa cell cDNA clones (formerly called ATF-a and ATF-aΔ; EMBL accession number X52943) with strong ATF-CRE binding activity (17). More recently, secondary screenings of a lambda-ZAP library led to the identification of an additional cDNA clone belonging to the same family (EMBL accession number X57197; unpublished results). While the two first clones only differ by a 63-bp coding element (retained in ATF-a and absent in ATF-aΔ), the third one has an additional 33-bp coding element with respect to ATF-a. These cDNA clones were renamed according to their increasing complexity (ATFa1, ATFa2, and ATFa3, respectively), as depicted in Fig. 1.

Recombinant plasmid constructions. Plasmid pE1a comprises the entire Ad2 E1a transcription unit (0 to 4.5 map units) with a simian virus 40 (SV40)-derived polyadenylation site; p13S and p12S are intron-defective recombinants derived from pE1a and producing only the 13S and 12S E1a mRNAs; and pE1a$^-$ is an E1a-defective recombinant derived from pE1a by deletion of the E1a-transcribed sequences located downstream of position +129. All the above-mentioned plasmids have been described previously (37). Plasmids pT178 and pS185 were derived from p13S by oligonucleotide-directed mutagenesis (34) to generate in the 289R protein substitutions of Tyr-178 and Ser-185 with Pro and Arg, respectively. Plasmid pC157 was a gift from L. C. Webster and R. P. Ricciardi and corresponds to mutant C157S, in which Cys-157 of the 289R protein has been replaced by Ser (53).

Plasmids p(E2-97)CAT and p(E2-70)CAT contain fragments from positions −97 and −70 to +46 of the Ad2 E2a early promoter region, respectively, linked to the bacterial chloramphenicol acetyltransferase (CAT) gene (29).

The pATFa1, pATFa2, and pATFa3 recombinants contain the entire coding regions of the corresponding ATFa cDNAs inserted into the polylinker of the pTL1 expression vector, a derivative of pSG5 (21). Recombinants G4, G4-ATFa1, and A, B, C, D, E, and F deletion derivatives of G4-ATFa1 contain the sequences encoding the DNA binding domain of the yeast GAL4 protein alone or fused in-frame to sequences encoding the ATFa1 protein (see Fig. 3A). The junctions of these fusion proteins were verified by DNA sequencing. The pm22 and pm27 mutations correspond, respectively, to substitutions of Asp-22 and His-27 of ATFa1 with Lys and Asn (see Fig. 4). The ΔN325 and ΔC263 mutations correspond to ATFa1 deletions removing the 324 N-terminal and the 262 C-terminal residues, respectively. The ΔC263-pm27 mutation corresponds to pm27 with a deletion of the 262 C-terminal residues.

The G4-TK-CAT reporter (54) contains the CAT gene driven by the herpes simplex virus thymidine kinase (TK) promoter (−105 to +51) and bears a single GAL4 binding site inserted 5′ to the TK promoter (see Fig. 3C).

For bacterial expression and in vitro RNA transcription-translation, appropriate E1a, ATFa, and CRE-binding protein (CREB) (19) cDNAs were inserted into the pET3-1 expression vector (17).

Transfections and CAT assay. HeLa or COS-7 cells were grown in Dulbecco medium supplemented with 5% calf serum. The cells were transfected by calcium phosphate coprecipitation (4), 16 h after plating, with the amounts of recombinant DNA indicated in the figure legends, adjusted to 14 μg per 9-cm petri dish with double-stranded carrier DNA (Bluescript). The medium was changed after 15 to 20 h. After an additional 20 to 24 h, cells were harvested and cellular extracts were prepared and assayed for CAT activities as previously described (20, 55). To compare the CAT activities in the absence and presence of E1a, we normalized the amounts of cell extracts on the basis of protein concentrations. Each transfection experiment was repeated at least four times with different plasmid preparations. The percent acetylation of chloramphenicol was determined by thin-layer chromatography and then scintillation counting. The values always agreed by within 20% from one experiment to another.

Cell-free transcription and translation of cloned cDNAs. Plasmids carrying cDNAs under the control of the T7 phage promoter (pET3 series) were cut at positions 3′ to the termination codons by appropriate restriction enzyme digestion. The linearized DNA was transcribed in vitro by T7 RNA polymerase (Promega). Transcription yields were determined by measuring labelled nucleotide incorporation in a microreaction run in parallel under the same conditions as the cold macroreaction. After transcription, the template DNA was digested with RNase-free DNase, and RNA was

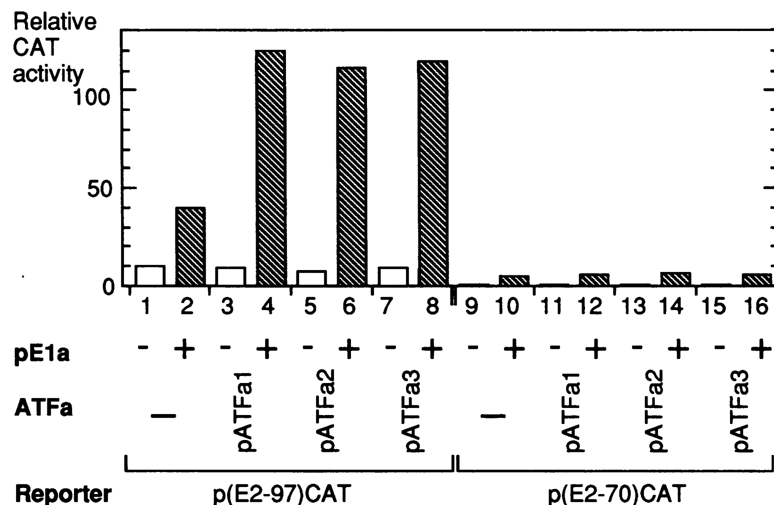

FIG. 2. ATFa proteins mediate the induction of the E2a promoter by E1a. HeLa cells were cotransfected with plasmids directing the synthesis of the ATFa proteins (0.5 μg), pE1a (1 μg), and either the p(E2-97)CAT or the p(E2-70)CAT reporter plasmid (5 μg), as indicated. When the ATFa or E1a proteins were not to be expressed (−), a vector without an insert (pTL1) or containing only the E1a 5′ region (pE1a⁻) was transfected instead, respectively. After 36 h, cells were harvested and assayed for CAT activities. The mean relative values for three independent experiments are shown.

extracted and ethanol precipitated. One-microgram samples of the individual purified RNAs were translated in the presence of a micrococcal nuclease-treated rabbit reticulocyte lysate, under the conditions recommended by the manufacturer (Promega), in the presence of 15 μCi of [^{35}S]methionine (1,060 Ci/mmol) per 35-μl reaction mixture (labelled synthesis) or in the presence of 0.85 mM cold methionine (unlabelled synthesis).

Antibodies. ATFa proteins were overexpressed in *Escherichia coli*. The resulting inclusion bodies were recovered from the bacterial lysate by centrifugation and separated by semipreparative sodium dodecyl sulfate (SDS) gel electrophoresis. The band corresponding to the ATFa3 protein was excised from the Coomassie-stained gel and pulverized in liquid nitrogen, and the protein was used to immunize rabbits. M73, a monoclonal antibody specific for E1a proteins, was obtained from E. Harlow (23). E1a-specific polyclonal antibodies were raised in rabbits by serial subcutaneous injections of partially purified inclusion bodies produced in *E. coli* upon overexpression of the 289R E1a protein.

Immunoprecipitation analysis. In vitro-translated proteins (see above) were incubated with 40 μl of a 50% protein A-Sepharose suspension (Pharmacia) for 15 min at 4°C in 300 μl of phosphate-buffered saline–0.1% Nonidet P-40 to remove immunoglobulins present in the reticulocyte lysate. Immunoprecipitation was performed essentially as previously described (30). Specific antisera (5 μl) were added to the supernatant. After 90 min at 4°C, 30 μl of protein A-Sepharose was added, and the mixture was incubated for an additional hour. The Sepharose beads were washed once in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.5)–1 M NaCl and three times in 20 mM HEPES (pH 7.5)–250 mM NaCl–0.5% Nonidet P-40. Adsorbed proteins were dissociated by being boiled for 5 min in 20 μl of Laemmli sample buffer and were resolved by SDS–12.5% polyacrylamide gel electrophoresis (35).

Zinc blot analysis. Bacteria induced to overexpress recombinant proteins were lysed by ultrasonication. The inclusion bodies were recovered by centrifugation, dissolved in 6 M guanidinium hydrochloride, and reprecipitated with ethanol as previously described (48). The proteins were pelleted, resuspended in Laemmli sample buffer, and resolved on SDS–12.5% polyacrylamide gels (35). After electrophoresis, the gels were treated as previously described (45). In brief, the proteins were reduced in the presence of 5% 2-mercaptoethanol before electrotransfer to nitrocellulose membranes. The blot was incubated in the presence of $^{65}ZnCl_2$ (0.2 μCi/ml), washed, and exposed for autoradiography.

RESULTS

ATFa proteins can mediate transcriptional activation by the adenovirus E1a proteins. We recently isolated three HeLa cell cDNA clones, ATFa1 (formerly called ATF-aΔ), ATFa2 (formerly called ATF-a), and ATFa3 (see Materials and Methods and Fig. 1). A comparative blot analysis of HeLa cell RNAs revealed that the relative amounts of mRNAs coding for related ATFa (17), ATF-1 (22), CREB (19), and CRE-BP1 (42) are about 1:20:20:5 (18a). This observation suggests, if translation efficiencies and protein stabilities are comparable, that the ATFa proteins represent minor species among the members of the ATF-CRE family in HeLa cells.

For assay of the transcriptional activity of the proteins encoded by these ATF cDNA clones, the clones were inserted into an expression vector (pTL1; see Materials and Methods). The resulting recombinants were transfected into HeLa cells, together with CAT reporter plasmids driven by Ad2 E2a promoter fragments comprising (E2-97) or not comprising (E2-70) the ATF binding site. Under the transfection conditions used (Fig. 2), the basal activity of the E2a promoter [p(E2-97)CAT] was not stimulated by coexpression of any of the ATFa proteins (compare lane 1 with lanes 3, 5, and 7), suggesting that these proteins lack an intrinsic activation domain. In this respect, the three ATFa proteins resemble the ATF-2 protein, another member of the ATF-CRE family (40).

We also tested the ability of the E1a 289R protein to *trans*-activate the viral E2a promoter in the absence and presence of the ATFa proteins. As expected (60), the E1a gene product stimulated E2a promoter activity (Fig. 2,

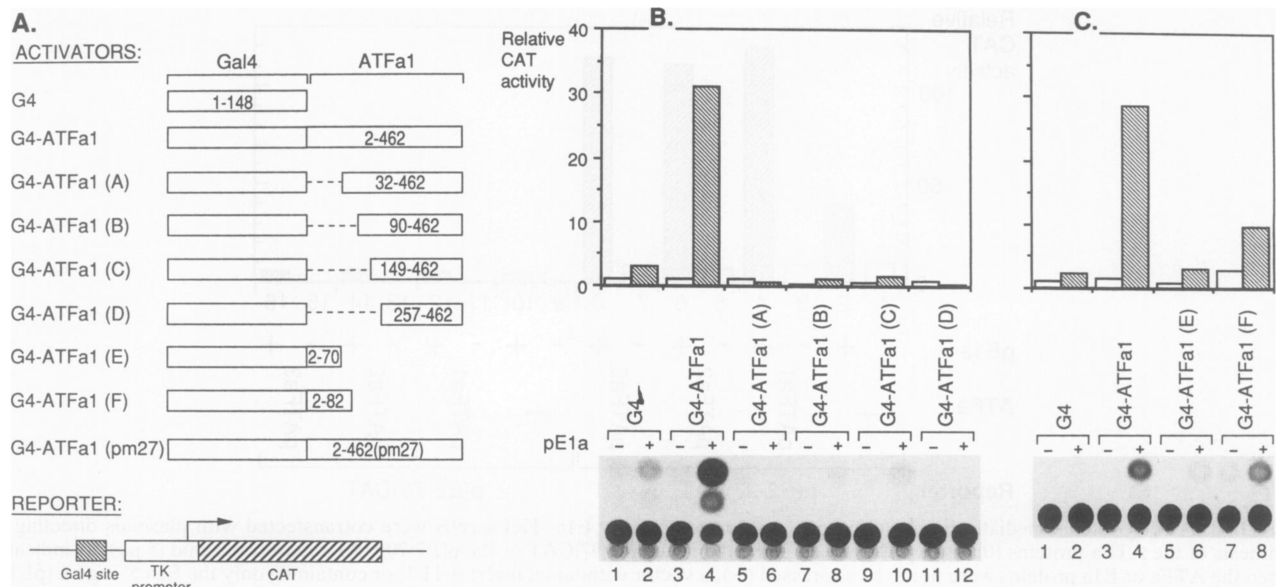

FIG. 3. Deletion analysis of ATFa activity. (A) The structures of the GAL4 derivatives are schematically represented; the amino acid coordinates of the GAL4 and ATFa1 sequences retained in the various hybrids are shown. The structure of the G4-TK-CAT reporter gene is shown below. (B and C) COS-7 cells were cotransfected with plasmids directing the expression of the chimeric protein (0.5 μg), pE1a (1 μg), and the reporter plasmid (5 μg). When the E1a proteins were not to be expressed (−), plasmid pE1a⁻ was used instead of plasmid pE1a. CAT expression was analyzed as described in the legend to Fig. 2. The results of a typical experiment are shown (bottom) along with the mean relative values for three or four independent experiments (top).

compare lanes 1 and 2). Surprisingly, the level of this stimulation was significantly increased upon coexpression of the ATFa proteins (lanes 4, 6, and 8). This additional stimulation was clearly dependent on the ATF binding site, since no such activation was observed with the truncated E2a promoter [p(E2-70)CAT] (compare lane 10 with lanes 12, 14, and 16), strongly supporting the conclusion that the E1a effect is, at least in part, mediated by the ATFa proteins. Furthermore, these experiments showed that the three ATFa species have similar stimulating effects in the presence of E1a.

To circumvent the potential interference of the endogenous ATF proteins and gain further insight into the mechanism of this ATFa-mediated activation, we used the GAL4 protein fusion system (40) for the study of E1a action. We linked the ATFa1 protein to the yeast GAL4 DNA binding domain (GAL4, 1 to 148) and assayed the activity of the chimeric protein (G4-ATFa1; Fig. 3A) in cotransfection experiments with a G4-TK-CAT reporter plasmid that carries a single GAL4 binding site upstream of the TK promoter linked to the CAT coding sequences. As shown in Fig. 3B, this minimal promoter was only weakly responsive to E1a in the absence of G4-ATFa1 expression (compare lanes 1 and 2). In contrast, a dramatic enhancement of reporter E1a responsiveness occurred upon coexpression of the chimeric protein (Fig. 3B, lanes 3 and 4), confirming the contribution of an ATFa domain to E1a action. By itself, G4-ATFa1 failed to stimulate transcription from the G4-TK-CAT reporter (Fig. 3B, compare lanes 1 and 3), further supporting the conclusion that the ATFa proteins lack a proper activation domain (see above).

The E1a activation-mediating activity of the ATFa proteins depends on the most N-terminal portion of the ATFa proteins, which includes a potential zinc finger motif. To delineate the ATFa protein domain responsible for the mediation of E1a activation, we created a series of deletions from the 5′ end of the ATFa1 cDNA and cloned the truncated fragments in-frame with the GAL4 DNA binding domain (Fig. 3A). The resulting recombinants were assayed in cotransfection experiments with the G4-TK-CAT reporter. As shown in Fig. 3B, deletion of the first 31 residues of ATFa1 abolished E1a responsiveness, although nearly identical amounts of the various GAL4 derivatives accumulated, as revealed by immunoblot analysis of the transfected cell extracts with antibodies directed against the GAL4 moiety of the ATFa1 derivatives (data not shown). These results suggest that the domain spanning residues 1 to 31 (R1-31) of ATFa1 comprises an element that is essential for supporting E1a responsiveness.

To examine whether this ATFa domain was by itself able to mediate the E1a effect, we tested GAL4 recombinants carrying the R2-70 and R2-82 N-terminal portions of ATFa1 [G4-ATFa1 (E) and G4-ATFa1 (F), respectively; Fig. 3A] by cotransfection with the G4-TK-CAT reporter. As shown in Fig. 3C, only the recombinant retaining R2-82 of ATFa1 significantly mediated E1a-dependent transactivation of the reporter, although at a reduced level compared with the recombinant bearing the entire ATFa1 coding region. This result clearly suggests that sequences between R31 and R82 (and possibly extending beyond R82) also contribute to the E1a activation-mediating activity of ATFa.

A potential metal-binding site could be identified between R9 and R27 by inspection of the amino acid sequence of this ATFa region (Fig. 4). To examine the role of this structural motif, we introduced a single mutation into the G4-ATFa1 chimera, changing His-27 of the ATFa1 moiety to Asn. As shown in Fig. 5, this point mutation (pm27), like the deletion of the entire element [G4-ATFa1 (A)], abrogated the ability of the recombinant ATFa1 protein to mediate E1a-induced transactivation. Altogether, our results suggest that the E1a

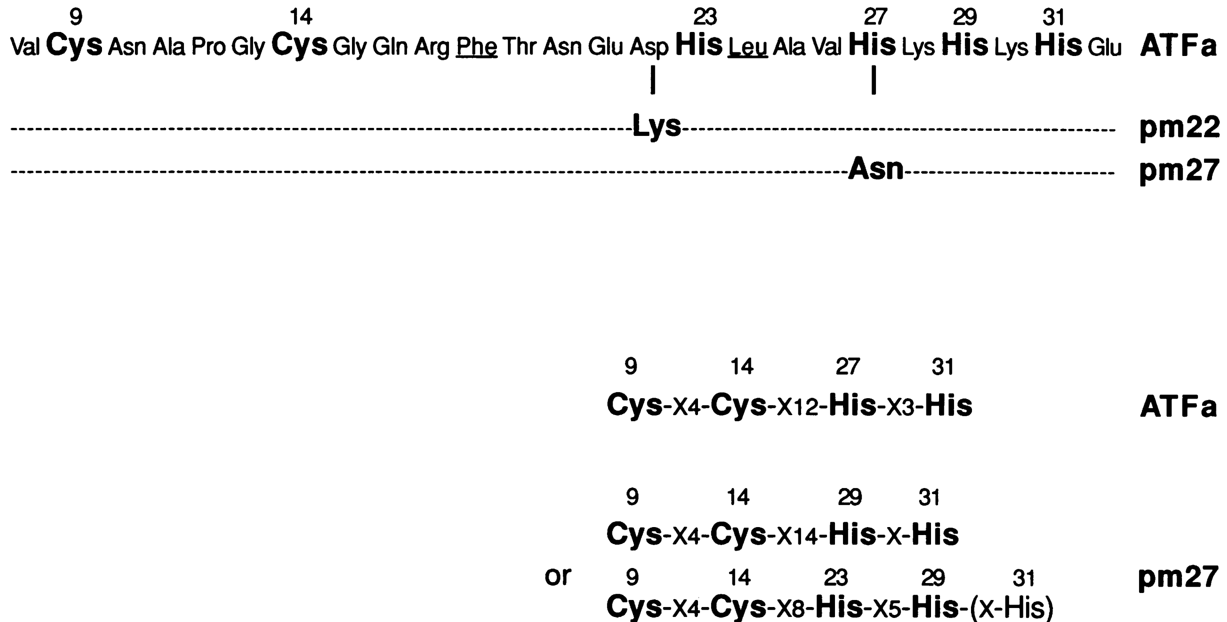

FIG. 4. Structure of the potential ATFa metal-binding domain. The peptide sequence (R8-32) of ATFa is shown at the top; Cys and His residues are in boldface type, and the invariant aromatic (Phe) and Leu residues in C2H2-type zinc fingers are underlined (12). The Asp→Lys and His→Asn alterations in the pm22 and pm27 mutations, respectively, are shown. The best fit with the prototype C2H2 motif is given for the ATFa protein. Alternative, remote structures can be obtained with the pm27 mutation.

effect is mediated by an N-terminal structural domain of ATFa extending at least to R82 and comprising a potential metal-binding site in which His-27 plays a central role.

A single mutation of the putative zinc finger of E1a abolishes its ATFa-mediated activity. To identify the domain of the E1a proteins implicated in their ATFa-mediated activity, we first compared the effects of plasmids expressing both the 13S (289R) and the 12S (243R) mRNA products (pE1a) or either one of the two products separately (p13S and p12S). Figure 5 shows that pE1a and p13S exhibited strong transactivating properties (lanes 7 and 9), while at the plasmid concentrations used in this assay, p12S had no more effect than a control plasmid expressing only the first 43 N-terminal residues of the E1a protein (pE1a$^-$). These results indicate that CR3, which is unique to the E1a 13S mRNA product, is essential for ATFa-mediated E1a activity in agreement with the conclusion previously reached about a distinct, but related, ATF clone, ATF-2 (40).

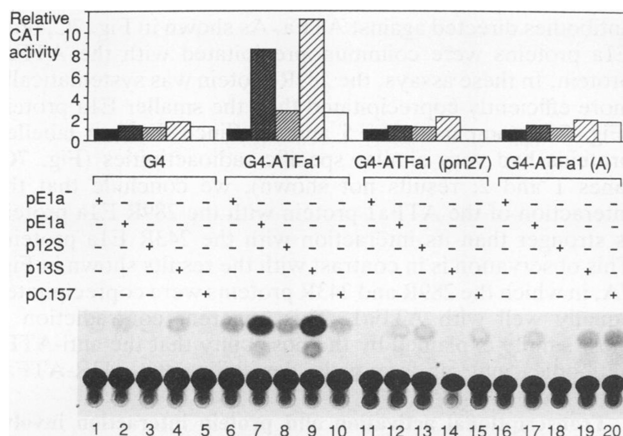

FIG. 5. Metal-binding motifs within the ATFa and E1a 289R proteins are involved in ATFa-mediated E1a induced activation. COS-7 cells were cotransfected with the indicated plasmids (G4 recombinants, 0.5 μg; E1a recombinants, 1 μg) and the G4-TK-CAT reporter (5 μg) and analyzed as described in the legend to Fig. 3.

Within this CR3 domain of E1a, a consensus metal-binding motif has been identified between R154 and R174 (11). In addition, this motif effectively binds zinc, and the presence of all four Cys residues is essential for transactivation of the viral E3 promoter by E1a. We examined whether the integrity of this zinc-binding structure was also critical for ATFa-mediated activation by E1a. As shown in Fig. 5, a single mutation within the potential E1a zinc finger, changing Cys-157 to Ser (as in pC157), abolished the ATFa-supported E1a responsiveness of the reporter promoter (lane 10). Immunoblot analysis of the transfected cell extracts with antibodies against E1a revealed (data not shown) that nearly identical amounts of E1a protein were expressed from both the p13S and the pC157 recombinants. These results indicate that the E1a zinc-binding element is essential for the ATFa-mediated response.

The N-terminal potential metal-binding motif of ATFa binds zinc. To determine whether the ATFa proteins were able to bind zinc, we used a protein blotting technique (45) by which zinc-binding proteins could be detected by their ability to bind radioactive zinc. The validity of this procedure was confirmed by the analysis of bacterially synthesized E1a proteins. It was previously demonstrated by atomic absorption spectrophotometry that the 289R but not the 243R E1a protein contains zinc (11). As expected from these results, only the larger of these proteins produced a radioactive signal after a protein blot was probed with a zinc radioisotope (Fig. 6, compare lanes 3 and 4). The control immunoblot (lanes 7 and 8) confirmed that roughly equal amounts of both the 289 and the 243R E1a compounds were present on the blot.

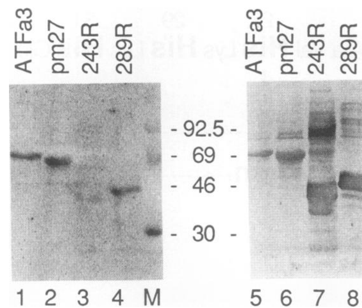

FIG. 6. Zinc-binding activity of bacterially synthesized proteins. About 1 μg of protein from bacteria expressing the ATFa and E1a proteins (as indicated) was fractionated by SDS gel electrophoresis and transferred to nitrocellulose. The membranes were incubated with radioactive zinc (lanes 1 to 4) or a mixture of antisera specific for the ATFa and E1a proteins (lanes 5 to 8) and processed as described in Materials and Methods. M, prestained molecular mass markers (in kilodaltons).

When analyzed under the same experimental conditions, the bacterially produced ATFa3 protein (for some reason, ATFa1 could not be overproduced) and pm27 protein, the ATFa1 derivative altered at the level of the putative N-terminal metal-binding motif (see Fig. 4), generated radioactive signals of roughly equal intensities and at the expected positions (Fig. 6, lanes 1 and 2). Immunoblot (Western) analysis confirmed that similar amounts of the two proteins were present (Fig. 6, lanes 5 and 6). These results strongly suggest that the ATFa proteins do indeed bind zinc but that His-27 of these proteins, although essential for the mediation of E1a activity is dispensable for metal binding. This latter observation is most likely explained by the particular His richness of the N-terminal portion of ATFa (see Fig. 4). Although the wild-type sequence shows the best fit with the consensus C2H2-type zinc finger sequence (52), alternative arrangements may substitute for the prototype motif and account for the residual zinc-binding activity of the pm27 derivative. This result indicates that the zinc-binding capacity of ATFa is not by itself responsible for the mediation of E1a activity. A distinct derivative (pm22), altered within the finger loop of this structure, also retained zinc-binding activity but had lost its transcriptional activity (8a), indicating that essential elements are located between the potential zinc-chelating residues.

The E1a and ATFa proteins interact directly. In contrast to ATFa, E1a is not a sequence-specific DNA binding protein (13) and may be anchored to the promoter through factors, like the ATF proteins, already bound to the DNA (40). We therefore examined whether the E1a and ATFa proteins could directly associate with each other.

Preliminary protein blot and band shift experiments (results not shown) suggested that such interactions may indeed occur. The ATFa protein could reproducibly be detected on electrophoretic blots of extracts from bacteria programmed with ATFa expression vectors after probing with labelled E1a proteins. Conversely, when protein blots of extracts from bacteria producing E1a proteins were probed with labelled extracts containing the ATFa1 protein, bands at the positions of the 289R and 243R E1a proteins could be observed in some of our experiments. Similarly, electrophoretic band shift assays with an ATF target sequence as a probe in the presence of in vitro-cosynthesized ATFa1 and E1a proteins revealed the expected retarded ATF complex and, in several experiments, an even more slowly migrating complex the formation of which was prevented by preincubation of the protein mixture with E1a antibodies. These experiments were the first indications of direct interactions between the ATFa and E1a proteins. Their lack of systematic reproducibility may be related to the experimental conditions, involving denaturing SDS gel electrophoresis on the one hand and multiprotein complex electrophoretic analysis on the other.

To assay for complex formation between the E1a proteins and the ATFa1 protein under other conditions, we performed indirect immunoprecipitations with specific antibodies directed against either the E1a or the ATFa proteins. To this end, we synthesized the wild-type E1a and ATFa proteins and appropriate mutants thereof by successive in vitro transcription and translation. Under the conditions used (see Materials and Methods), the ATFa transcripts gave rise to a major immunoprecipitable translation product (Fig. 7A, lanes 1 and 2) with an apparent molecular mass of about 60 kDa, and the 13S and 12S E1a transcripts were translated as major polypeptides with apparent molecular masses of about 46 and 40 kDa, respectively (Fig. 7C, lanes 1 and 2). The amounts of proteins synthesized and their specific activities (when labelled) were in each case carefully monitored by Western analysis and radioautography (data not shown). Identical amounts of unlabelled, in vitro-synthesized 289R or 243R E1a protein were mixed with labelled, in vitro-synthesized ATFa1 protein and incubated for 15 min at 25°C before immunoprecipitation with antibodies directed against E1a. As shown in Fig. 7A, the ATFa1 protein was clearly detected in the immunoprecipitates, in the presence of either the 289R or the 243R protein (lanes 3 and 5). The fact that in the absence of E1a, virtually no ATFa1 protein was precipitated by the anti-E1a serum (Fig. 7A, lanes 7 and 8) strongly suggests that the ATFa1 protein was coprecipitated along with either E1a protein because these proteins stably interacted during the assay. Since, under these conditions, the coprecipitation of ATFa1 or its point-mutated derivative (pm27; Fig. 7A, lanes 4 and 6) was about equally efficient in the presence of either the 289R or the 243R E1a protein, we conclude that the zinc-binding domains of ATFa and E1a are not essential for the E1a-ATFa interaction.

To confirm these results, we performed the reverse experiment. Unlabelled ATFa1 protein was incubated with labelled E1a proteins, and the mixtures were treated with antibodies directed against ATFa. As shown in Fig. 7C, both E1a proteins were coimmunoprecipitated with the ATFa1 protein. In these assays, the 289R protein was systematically more efficiently coprecipitated than the smaller E1a protein (Fig. 7C, compare lanes 5 and 6). Since the two labelled proteins had very similar specific radioactivities (Fig. 7C, lanes 1 and 2; results not shown), we conclude that the interaction of the ATFa1 protein with the 289R E1a protein is stronger than its interaction with the 243R E1a protein. This observation is in contrast with the results shown in Fig. 7A, in which the 289R and 243R proteins were coprecipitated equally well with ATFa1. This apparent contradiction is most simply explained by the possibility that the anti-ATFa antibodies may preferentially destabilize the 243R-ATFa1 complex.

Transcriptional activation and protein interaction involve both common and separate domains of ATFa and E1a. To roughly circumscribe the ATFa domains involved in the E1a-ATFa interactions, we first compared the ability of various ATFa1 mutants to be coprecipitated with the labelled E1a proteins. Point mutations altering the zinc-bind-

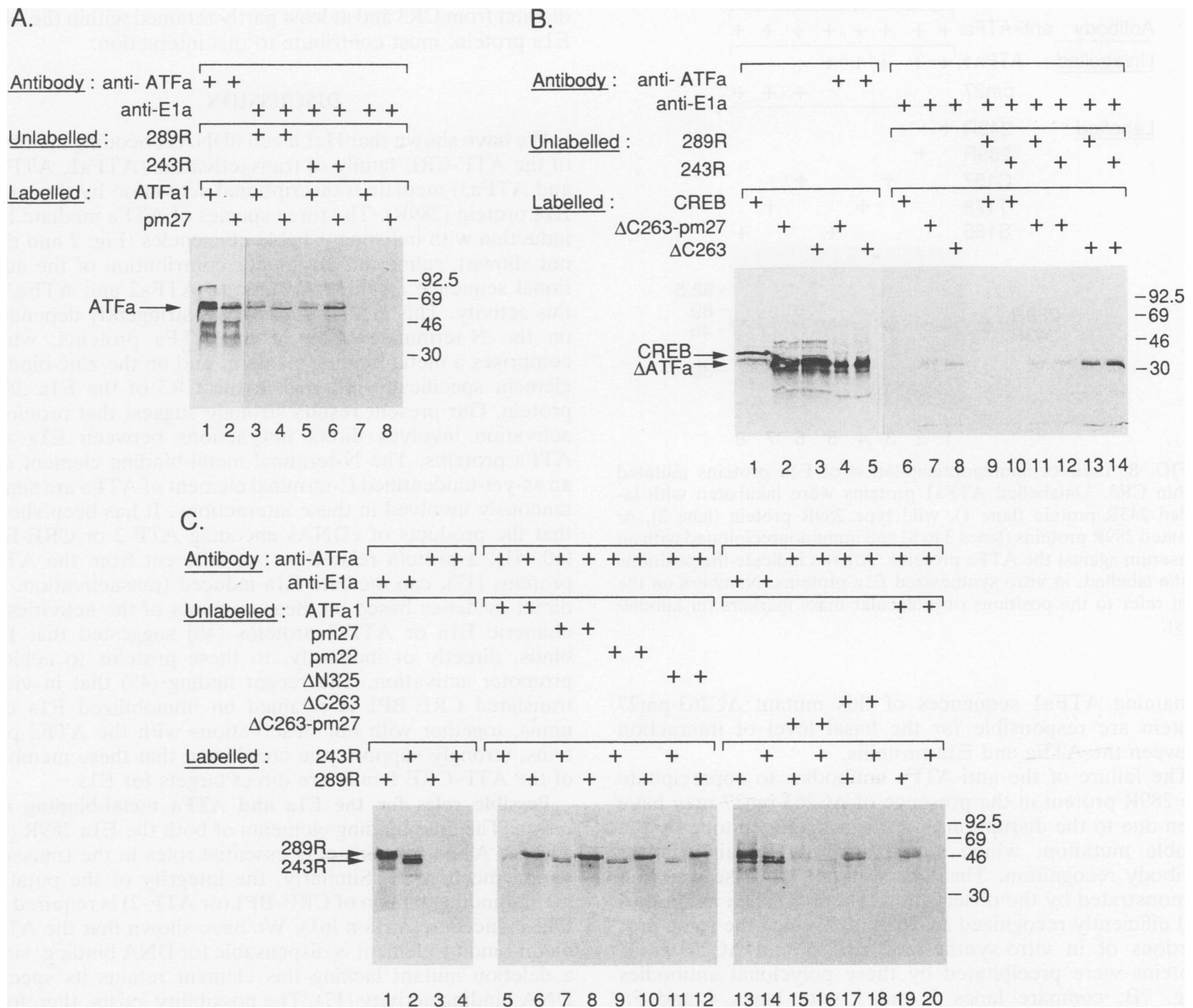

FIG. 7. Indirect immunoprecipitation of labelled ATFa (A and B) or E1a (C) proteins. When indicated (+), 5 μl each of the corresponding in vitro-synthesized proteins (labelled or unlabelled synthesis, adjusted for equivalent amounts of specific protein, as judged by Western analysis; data not shown) were mixed, incubated for 90 min at 4°C, immunoprecipitated with specific antibodies, and analyzed as described in Materials and Methods. (A) Labelled ATFa proteins were incubated with unlabelled E1a proteins and immunoprecipitated with monoclonal antibody M73, directed against the E1a proteins (lanes 4 to 8), or an antiserum against the ATFa proteins (lanes 1 and 2). The arrow indicates the positions of the labelled, in vitro-synthesized ATFa major proteins. Lanes 1 and 2 were exposed for 7 h, and the remaining lanes were exposed for about 24 h. (B) Labelled ATFa1 mutant and CREB proteins were loaded directly on the gel (lanes 1 to 3), after direct precipitation with anti-ATFa (lanes 4 and 5) or anti-E1a (lanes 6 to 8) antibodies, or after preincubation with the unlabelled E1a proteins and immunoprecipitation with anti-E1a antibodies (lanes 9 to 14). Arrows indicate the positions of the labelled, in vitro-synthesized CREB and ATFa major proteins. (C) Labelled E1a proteins (289R or 243R) were incubated with unlabelled wild-type (lanes 5 and 6), mutated (lanes 7 to 10), or truncated (lanes 11, 12, and 15 to 18) ATFa proteins and immunoprecipitated with an antiserum against the ATFa proteins or with monoclonal antibody M73 (lanes 1, 2, 13, and 14). Arrows indicate the positions of the labelled, in vitro-synthesized E1a proteins. Numbers on the right refer to the positions of molecular mass markers (in kilodaltons).

ing element (pm27 and pm22) had no effect on the E1a binding activity of ATFa (Fig. 7C, lanes 5 to 10), confirming that other portions of the ATFa molecule are critical for E1a binding (see above). Our observation that a mutant with a deletion of the N-terminal two-thirds of the molecule (ΔN325) retained essentially wild-type E1a binding activity (lanes 11 and 12) indicates that an essential binding domain is located within the 170 C-terminal residues of ATFa1. Since deletion of this C-terminal portion of ATFa1 (ΔC263) did not abolish E1a coprecipitation (lanes 17 to 20), we conclude that another essential binding domain is present within the N-terminal half of ATFa1 and that either the C- or the N-terminal element is required for E1a interaction, as measured under these conditions. In agreement with this conclusion, the simultaneous alteration of the zinc-binding element and deletion of the C-terminal portion of ATFa1 (ΔC263-pm27) reduced the 289R E1a coprecipitation efficiency to the level of that of 243R E1a (compare lanes 15 to 20). The fact that the 243R signal was itself not markedly diminished (compare lanes 16 and 18) suggests that the

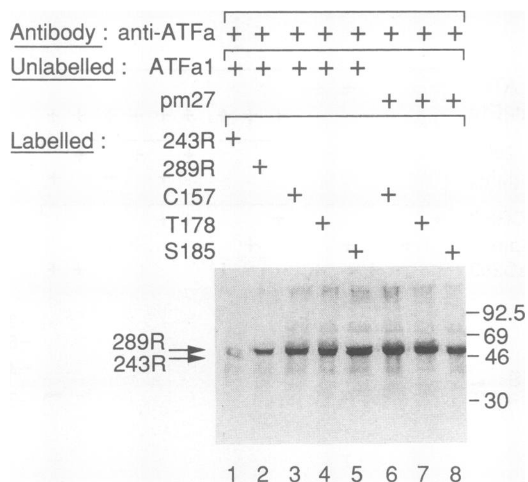

FIG. 8. Indirect immunoprecipitation of E1a proteins mutated within CR3. Unlabelled ATFa1 proteins were incubated with labelled 243R protein (lane 1), wild type 289R protein (lane 2), or mutated 289R proteins (lanes 3 to 8) and immunoprecipitated with an antiserum against the ATFa proteins. Arrows indicate the positions of the labelled, in vitro-synthesized E1a proteins. Numbers on the right refer to the positions of molecular mass markers (in kilodaltons).

remaining ATFa1 sequences of this mutant ΔC263-pm27 protein are responsible for the basal level of interaction between the ATFa and E1a proteins.

The failure of the anti-ATFa antibodies to coprecipitate the 289R protein in the presence of ΔC263-pm27 may have been due to the disruption of a major ATFa epitope by the double mutation, which would severely impair efficient antibody recognition. That this was not the case was first demonstrated by the observation that anti-ATFa antibodies still efficiently recognized ΔC263-pm27, since the same proportions of in vitro-synthesized ΔC263 and ΔC263-pm27 proteins were precipitated by these polyclonal antibodies (Fig. 7B, compare lanes 2 and 3 with lanes 4 and 5). Furthermore, when the same amounts of labelled ΔC263 and ΔC263-pm27 proteins were incubated in the presence of unlabelled E1a proteins and treated with anti-E1a antibodies, only the ΔC263 protein could be detected in the coprecipitate, at levels significantly above the nonspecific immunoprecipitate back-ground (compare lanes 13 and 14 with lanes 7, 8, 11, and 12). Finally, as a negative control for these coprecipitation experiments, we also used the rat brain CREB factor, which has been shown to lack E1a-activation-mediating activity (14). As expected (Fig. 7B), this factor was not coimmunoprecipitated with E1a.

We also tested the effect of point modifications within the CR3 domain of E1a; we chose mutations (C157, altering the zinc finger of CR3, and S185, altering the C-terminal portion of CR3) that abolished ATFa-mediated transcriptional activation (Fig. 5) (8a, 44, 53) and a mutation (T178, altering the linker region between the zinc finger and the carboxyl portion of CR3) that had no transcriptional effect (53). As shown in Fig. 8, none of these mutations significantly affected the efficiency of the coprecipitation of the 289R E1a protein with either wild-type ATFa1 or the pm27 derivative. These results confirm that the zinc-binding elements of E1a and ATFa, which are both essential for transcriptional activation, are dispensible for the interaction between these proteins. In addition, they suggest that an E1a element(s), distinct from CR3 and at least partly retained within the 243R E1a protein, must contribute to this interaction.

DISCUSSION

We have shown that HeLa cell cDNAs encoding members of the ATF-CRE family of transactivators (ATFa1, ATFa2, and ATFa3) mediate transcriptional activation by the larger E1a protein (289R). The three species of ATFa mediate E1a induction with indistinguishable efficiencies (Fig. 2 and data not shown), ruling out any major contribution of the additional sequence elements specific to ATFa2 and ATFa3 in this activity. This mediator activity is stringently dependent on the N-terminal region of the ATFa proteins, which comprises a metal-binding element, and on the zinc-binding element specifically retained in the CR3 of the E1a 289R protein. Our present results strongly suggest that promoter activation involves direct interactions between E1a and ATFa proteins. The N-terminal metal-binding element and an as-yet-unidentified C-terminal element of ATFa are simultaneously involved in these interactions. It has been shown that the products of cDNAs encoding ATF-2 or CRE-BP1 (40, 41), a protein related to but different from the ATFa proteins (17), can mediate E1a-induced transactivation. Indirect evidence based on measurements of the activities of chimeric E1a or ATF-2 proteins (40) suggested that E1a binds, directly or indirectly, to these proteins to achieve promoter activation. The recent finding (43) that in vitro-translated CRE-BP1 is retained on immobilized E1a columns, together with our observations with the ATFa proteins, strongly supports the conclusion that these members of the ATF-CRE family are direct targets for E1a.

Possible roles for the E1a and ATFa metal-binding elements. The zinc-binding elements of both the E1a 289R (44) and the ATFa proteins play essential roles in the transactivation mechanism. Similarly, the integrity of the putative metal-binding domain of CRE-BP1 (or ATF-2) is required for E1a-induced activation (61). We have shown that the ATFa metal-binding element is dispensable for DNA binding, since a deletion mutant lacking this element retains its specific DNA binding activity (17). The possibility exists, therefore, that the ATFa and E1a zinc-binding elements are involved in protein-protein interactions. Precedents for such interactions via metal-binding domains have in fact been reported (16, 25).

Whereas point mutations altering the ATFa (pm27 and pm22) or E1a (C157) metal-binding elements essentially abolish ATFa-mediated E1a responsiveness, none of these mutations significantly affects the coimmunoprecipitation of these proteins. This result indicates that the particular configuration of the zinc-binding elements is critical for transcriptional activation. Protein interaction is likely to be maintained through additional domains, distinct from the metal-binding elements. For the ATFa proteins, we have shown that elements within the C-terminal region contribute to this interaction. Further studies will be necessary to map the elements involved. On the other hand, the results of an extensive mutational analysis of the *trans*-activating domain of the 289R E1a protein (53) revealed that the C-terminal region of the CR3 domain, half of which is common to both E1a proteins, may be involved in interactions with upstream transcription factors, such as ATFs.

E1a interacts with other cellular transcription factors. The TATA binding factor TFIID has been shown to specifically interact with the larger E1a protein (27, 36). Interestingly, the N-terminal portion of the CR3 region of E1a appears to

be involved in this binding (36). Nuclease protection experiments have shown that a partially purified ATF activity cooperates with TFIID in the assembly of functional preinitiation complexes on the Ad5 E4 promoter by transiently interacting with TFIID (26). It is tempting to speculate that the E1a protein further stimulates this complex formation by simultaneously binding to the ATF and TFIID factors. In fact, cotransfection experiments (our unpublished data) have shown that the cloned ATFa proteins used in the present study are able to mediate the E1a-induced stimulation of the Ad5 E4 promoter, which contains several ATF binding sites critical for E1a responsiveness (31).

Examples of E1a interactions with other transcription factors have also been documented. Thus, Sp1, c-Jun, and c-Fos can individually bind to E1a (43), suggesting a role for these cellular upstream transcription activators in E1a-mediated gene control. Oct-4, a transcription factor that recognizes an octamer motif found in various cellular promoters, including the immunoglobulin heavy-chain enhancer, has been shown to interact with the 289R E1a protein (50).

E1a interacts directly with the transcription factors mentioned above, no additional intermediary protein apparently being required for the association. Furthermore, no particular posttranslational modification of either of the partners seems to be needed, although in some cases (CRE-BP1 and c-Fos), the binding is markedly increased by preincubation of the in vitro-synthesized protein with a nuclear extract (43). For the ATFa proteins, we have observed no significative change in the overall efficiency of interaction with E1a upon preincubation with nuclear or whole-cell extracts. In addition, the interaction occurs in the absence of DNA, and its efficiency is not modified in the presence of the ATFa recognition sequences (data not shown).

The elucidation of the precise molecular mechanisms underlying promoter activation will largely depend on the availability of in vitro transcription systems faithfully mimicking in vivo transcriptional controls.

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