Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3957–3961, May 1991
Biochemistry

Induction of c-fos mRNA and AP-1 DNA-binding activity by cAMP in cooperation with either the adenovirus 243- or the adenovirus 289-amino acid E1A protein

(transcription/transcription factor/transformation/cAMP-dependent protein kinase)

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Communicated by Oscar L. Miller, January 31, 1991 (received for review November 11, 1990)

ABSTRACT **Products of the adenovirus *E1A* gene can act synergistically with cAMP to activate transcription of several viral early genes and the cellular genes *c-fos* and *jun-B*. Transcription factor AP-1-binding activity is also induced by the combined action of E1A and cAMP. Mouse S49 cells were infected with adenovirus variants expressing either the 243- or 289-amino acid E1A protein and treated with the cAMP analog dibutyryl-cAMP. Significant E1A-dependent induction of c-fos mRNA and AP-1-binding activity was observed in cells expressing either E1A protein. These effects absolutely required the presence of cAMP. In contrast, the 243-amino acid protein was a poor activator of the viral early genes *E2* and *E4* compared with the 289-amino acid protein. These data suggest that the 243- and 289-amino acid E1A proteins both interact functionally with the cAMP signaling system to activate transcription of a cellular gene and AP-1-binding activity. The mechanism involved in this process is probably different from the mechanism of transcriptional activation of viral genes.**

The E1A proteins of adenovirus are able to regulate transcription of a number of viral and cellular genes (for review, see ref. 1). In cooperation with an activated Ha-*ras* oncogene or the viral *E1B* gene, the E1A proteins can transform primary rodent cells in culture (2, 3). There are two major E1A mRNA species, designated 12S and 13S, that arise from alternative splicing of a common precursor transcript (4, 5). The resulting proteins of 243 and 289 amino acids (aa) differ only by an internal 46-aa region that is unique to the 289-aa protein. The E1A-coding regions have been subjected to extensive genetic analysis to determine the protein domains responsible for the transcriptional activation and transformation functions (for review, see ref. 6). Results of these studies indicate that transcriptional activation requires the unique 46-aa region of the 289-aa protein. Mutations within this region severely affect transcriptional activation of viral promoters, and a synthetic peptide containing the 46-aa region is sufficient for transcriptional activation when added to cell-free extracts or microinjected into cells (7, 8). The 12S mRNA-encoded protein (243-aa), which lacks the 46-aa region, is a poor activator of viral transcription, although results differ as to the extent of its transcriptional activation capability (9–11). Interestingly, the 243-aa protein can cooperate with either viral E1B proteins or with activated Ha-ras protein to transform primary cells, arguing that the transcriptional activation associated with the unique 46-aa region of the 289-aa protein is not essential for transformation (10–13). It is conceivable that transformation by the 243-aa species requires the modest transcriptional activation potential of this protein, or that the 243-aa species has a distinct transcriptional activation function that operates on certain cellular but not viral genes.

Previously we and others (14–22) identified a link between the action of the E1A proteins and the cAMP-dependent protein kinase pathway. Sequences with homology to the consensus cAMP response element (CRE) 5′-TGACGTCA-3′ are located upstream of the E1A-inducible adenovirus early genes *E1A*, *E2*, *E3*, and *E4*. Cellular factors that recognize these sequences have been identified, including the CREB/ATF and AP-1 families (14–16, 18, 20–25). In adenovirus-infected mouse S49 cells, cAMP activates transcription of the early viral genes. Moreover, the combination of E1A protein and cAMP activates the *E1A* and *E4* genes synergistically, indicating that the cAMP-mediated and E1A-mediated responses are somehow linked or interactive (19). E1A also acts together with cAMP to regulate the level of transcription factor AP-1 (22). AP-1 activity has been implicated in both positive and negative transcriptional regulation by adenovirus E1A proteins (26, 27). Treatment of adenovirus-infected S49 cells with cAMP induces significantly greater AP-1 activity than treatment of uninfected cells. Expression of the *E1A* gene is required for this effect. The induced AP-1 activity contains proteins immunologically related to the fos and jun families of proteins. There is a corresponding induction of mRNAs encoded by the cellular *c-fos* and *jun-B* genes in adenovirus-infected cells treated with cAMP (22).

Here we report that both the 243-aa and 289-aa E1A proteins can act with cAMP to induce AP-1 DNA-binding activity and c-fos mRNA. These results raise interesting questions about the role of the cAMP signaling system in the transcriptional activation and transformation functions of the E1A proteins.

MATERIALS AND METHODS

Cells, Viruses, and Infections. S49 cells were obtained from the University of California at San Francisco Cell Culture Facility. They were grown in suspension in tissue culture dishes, in Dulbecco's modified Eagle medium/10% heat-inactivated horse serum (GIBCO). Viruses *dl*309, *dl*347, *dl*348, and *dl*343 were propagated in 293 cells to produce virus stocks. Infection of S49 cells and treatment with $N^6,O^{2'}$-dibutyryl-cAMP (Bt_2cAMP) were done as described (19).

Cytoplasmic RNA Isolation and Analysis. Cells were harvested by centrifugation, and washed in 1 ml of ice-cold phosphate-buffered saline. Cytoplasmic RNA was isolated and analyzed by ribonuclease protection as described (19). The *c-fos*-specific probe was synthesized from a plasmid

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Abbreviations: aa, amino acid; Bt_2cAMP, $N^6,O^{2'}$-dibutyryl-cAMP; pfu, plaque-forming units.
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containing a *Bgl* II fragment derived from Finkel–Biskis–Jinkins murine sarcoma virus spanning nucleotides 890–1500 containing a portion of the v-*fos* gene (from M. Cole, Princeton University). The plasmid was linearized with *Rsa* I and transcribed with T7 RNA polymerase in the presence of [^{32}P]UTP. The resulting RNA hybridized with a 303-base region of the c-fos mRNA. The *E4* and *E2*-specific probes were synthesized as described (19, 28). RNase-resistant products were analyzed by electrophoresis through a 5% polyacrylamide gel containing 7 M urea, and visualized by autoradiography.

Preparation of Nuclear Extracts and DNA Band-Shift Analysis. To prepare nuclear extracts, cells (0.4–1 × 10^8, depending on the experiment) were harvested and washed with ice-cold phosphate-buffered saline. Lysis and nuclear extract preparation were done as described (22). Extracts were standardized for protein concentration by using the Bio-Rad protein assay. DNA band-shift analysis was done essentially as described (16), except that the polyacrylamide gels were electrophoresed in 0.25× Tris/boric acid/EDTA (TBE) buffer (1× TBE is 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3), and no recirculation was done. The oligonucleotide 5′-GGATGTTATAAAGCATGAGTCAGA-CACCTCTGGCT-3′ containing the human collagenase AP-1 binding site (complementary strand not shown) was used in the DNA band-shift assays.

RESULTS

The 243-aa and 289-aa Proteins Can Each Act with cAMP to Induce AP-1 DNA-Binding Activity and c-fos mRNA. Previously we reported the induction of AP-1 DNA-binding activity and c-fos mRNA accumulation by the combined action of E1A and cAMP in mouse S49 cells (22). We next wanted to know whether the observed responses to the combination of E1A and cAMP correlate with the activity of only the 289-aa protein or whether both E1A proteins can effect this response. This question is of interest because the 243-aa and 289-aa E1A proteins are each able, in cooperation with an activated *ras* oncogene, to induce transformation of primary cells, whereas only the 289-aa protein is an efficient activator of viral early gene transcription. Rodent cells are known to be targets of the transforming properties of the E1A proteins. Because S49 cells are a rodent cell line we sought to explore the possible relationship between the observed cAMP response and the transformation properties of E1A.

To test the ability of the 243-aa and 289-aa E1A proteins individually to cooperate with cAMP, S49 cells were infected with viruses that express either the 12S or 13S E1A mRNAs. These viruses, designated *dl*347 and *dl*348, contain cDNA copies of the 12S (*dl*347) or 13S (*dl*348) E1A mRNAs in place of the wild-type gene. The normal *E1A* promoter and upstream sequences are intact (10).

Exponentially growing S49 cells were mock-infected or infected with *dl*347, *dl*348, *dl*309 (phenotypically wild type), or *dl*343 at a multiplicity of 20 plaque-forming units (pfu) per cell and plated at a density of 5 × 10^5 cells per ml. Mutant *dl*343 was used to control for effects specific to E1A proteins; this mutant contains an out-of-frame deletion in the 12S and 13S 5′ exons of the *E1A* gene. E1A-dependent transcriptional activation is not seen in *dl*343-infected cells, and the virus is defective for growth (29). At 5, 3, and 1 hr before harvesting, cells were treated with 1 mM Bt_2cAMP. The cells were harvested at 24 hr after infection, lysed in buffer containing Triton X-100, and processed for isolation of nuclear proteins and cytoplasmic RNA.

Fig. 1 shows the results of a DNA band-shift assay that used nuclear extracts prepared from adenovirus-infected, Bt_2cAMP-treated S49 cells. The extracts were incubated with a ^{32}P-labeled 35-base-pair (bp) oligonucleotide containing the

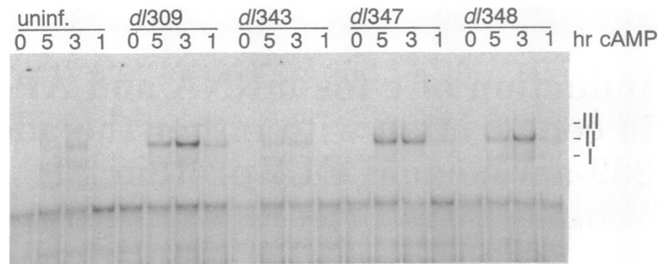

FIG. 1. Induction of AP-1 activity by Bt_2cAMP in adenovirus-infected S49 cells. Cells were infected with the indicated viruses at a multiplicity of infection of 20 pfu per cell, and nuclear extracts were prepared at 24 hr after infection. Treatment with Bt_2cAMP was for the times indicated before harvesting. Five micrograms of extract was analyzed by the DNA band-shift assay with a ^{32}P-labeled double-stranded oligonucleotide containing the collagenase AP-1 binding site. Specific DNA–protein complexes (I, II, and III) were resolved on a native 4% polyacrylamide gel; the gel was dried and autoradiographed (the signal representing excess free DNA probe was cut off the bottom of the autoradiogram). hr cAMP, hours of Bt_2cAMP treatment; uninf., uninfected.

human collagenase AP-1-binding site. Three bands representing specific complexes between AP-1 and the labeled oligonucleotide were evident. These complexes have been previously characterized and termed complexes I, II, and III (22). Consistent with our previous report (22), the induction by Bt_2cAMP of complex II was dramatically increased in cells infected by wild-type adenovirus *dl*309, compared with mock-infected cells or cells infected with mutant *dl*343 that fails to produce functional E1A protein. The effect of adenovirus infection on the response to cAMP has been shown previously to be due to E1A action. The possibility that other early viral genes are required for this effect has been excluded by showing that viruses expressing E1A proteins but carrying large deletions in the *E1B*, *E2*, *E3*, and *E4* genes are fully capable of triggering an increased response to cAMP (22). Interestingly, complex II was also substantially induced in cells infected with *dl*347, which produces only the 243-aa E1A protein. An equal induction was also seen in cells infected with *dl*348, which produces only the 289-aa E1A protein. The magnitude of the effect produced by the individually expressed 12S and 13S gene products was reproducibly somewhat less than that seen in wild-type infected cells. In addition, in some experiments *dl*347 was slightly less efficient at triggering this response than *dl*348 (data not shown). Immunoblot analysis was used to assess the relative amounts of E1A proteins produced in cells infected with *dl*309 and *dl*347. There was 2- to 3-fold less E1A in cells infected with *dl*347 than with *dl*309 (data not shown). This difference is due to the fact that the 243-aa protein produced in *dl*347-infected cells is unable to positively regulate transcription from the *E1A* gene. We conclude that the 243-aa and 289-aa E1A proteins are each able to increase the induction of AP-1 DNA-binding activity by cAMP.

The binding specificity of the factors generating the complexes induced in mock-infected, *dl*309-infected, and *dl*347-infected cells was tested (data not shown). Formation of the complexes was shown to be decreased by addition to the binding mixture of excess unlabeled homologous DNA, demonstrating the specificity of the interaction. Competition experiments were also done with a double-stranded oligonucleotide containing a point mutation within the AP-1 binding site (22). The mutated DNA fragment competed poorly with the wild-type sequence for formation of the complexes (data not shown). These data indicate that the DNA-binding proteins induced upon Bt_2cAMP treatment of mock-infected, *dl*309-infected, and *dl*347-infected all are specific for the AP-1-binding site.

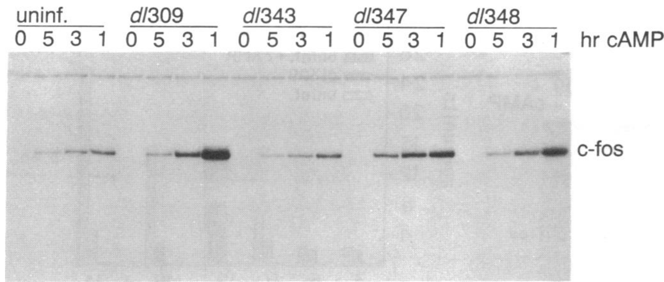

FIG. 2. Induction of c-fos mRNA by Bt_2cAMP in adenovirus-infected S49 cells. Cells were infected with the indicated viruses at a multiplicity of infection of 20 pfu per cell, and cytoplasmic RNA was prepared at 24 hr after infection. Treatment with Bt_2cAMP was for the times indicated before harvesting. RNase protection analysis was done with 10 μg of RNA per sample and a ^{32}P-labeled v-*fos* probe specific for a 303-base region of the mouse c-fos mRNA. RNase-resistant products were separated on a denaturing 5% polyacrylamide gel followed by autoradiography. Data were quantified by using a Bio-Rad model 620 Video densitometer. hr cAMP, hours of Bt_2cAMP treatment; uninf., uninfected.

Transcription factor AP-1 is a complex formed by products of the *fos* and *jun* gene families (for review, see ref. 30). The factor(s) induced in adenovirus-infected cells by cAMP are immunologically related to the fos and jun proteins (22). Previously we have shown that E1A acts in synergy with cAMP to elevate cytoplasmic levels of the c-fos and jun-B mRNAs (22). The induction of c-fos mRNA in virus-infected Bt_2cAMP-treated cells is due to an increase in the transcription rate of the c-*fos* gene as determined by run-on analysis with isolated nuclei (D.A.E. and T.S., unpublished work). Presumably the increase in fos and jun-B mRNA levels leads to the observed induction of AP-1 DNA-binding activity (22). After the observation that AP-1-binding activity was increased in *dl*347- and *dl*348-infected Bt_2cAMP-treated cells, cytoplasmic RNA was analyzed for a corresponding induction of c-fos mRNA.

Total cytoplasmic RNA was isolated from virus-infected cells by using the same conditions of infection and Bt_2cAMP treatment used to observe the increase in AP-1 DNA-binding activity. The RNA was analyzed by the RNase protection procedure with a ^{32}P-labeled probe homologous to a 303-base region of the cellular c-fos mRNA. The results of this analysis are shown in Fig. 2. As has been previously described, uninfected S49 cells exhibited an increase in c-fos mRNA after treatment with Bt_2cAMP (22). Identical results were obtained with cells infected with the E1A mutant *dl*343. Maximal induction was seen ≈1 hr after Bt_2cAMP treatment (Fig. 2, ref. 22, and data not shown). The induction was increased by a factor of 8 in cells infected with wild-type virus (*dl*309) and by a factor of 3 in cells infected with either *dl*347 or *dl*348. Again, maximal induction occurred ≈1 hr after Bt_2cAMP treatment. Similar experiments were also done with virus *dl*520 (from Stanley Bayley, McMaster University) which, like *dl*347, produces only the 243-aa E1A (12). Results with *dl*520 were identical to those with *dl*347 (data not shown). These data correlate well with the AP-1 DNA-binding data: the 243-aa and 289-aa E1A proteins are both able to act with cAMP to induce AP-1-binding activity and c-fos mRNA accumulation.

The 243-aa E1A Protein Is a Poor Activator of Viral Transcription in S49 Cells. The c-*fos* gene is known to be regulated at the level of transcription by cAMP (ref. 31; D.A.E. and T.S., unpublished work). The enhanced accumulation of c-fos mRNA in response to cAMP in cells expressing the 243-aa protein represents a heretofore-uncharacterized action of the 243-aa protein. Whereas there are few reports of transcriptional activation of cellular genes by the 243-aa protein (32–34), the response of the adenovirus early gene promoters to the 243-aa and 289-aa proteins has been well studied. The available data indicate that the 243-aa protein can activate early viral promoters, but the induction is very poor compared with the 289-aa protein (9, 10).

Does the ability of the 243-aa E1A protein to cooperate with cAMP correlate with its ability to transcriptionally activate viral genes in S49 cells? To answer this question, S49 cells were infected with viruses *dl*309, *dl*347, and *dl*348 and harvested at 12, 24, and 36 hr after infection for preparation of cytoplasmic RNA. The RNA was analyzed by RNase protection for the early viral mRNAs encoded by the *E2* and *E4* genes. The results are shown in Fig. 3 *A* and *B*. Clearly the ability of the 243-aa E1A protein encoded by *dl*347 to activate transcription of the viral *E2* and *E4* genes was poor in S49 cells compared with wild-type *dl*309 and *dl*348. These data are in line with most published reports analyzing the relative strengths of the 243-aa and 289-aa proteins to activate transcription. Therefore, despite the fact that the 243-aa protein is a poor activator of viral transcription in S49 cells, this protein can efficiently act with cAMP to trigger a transcriptional response of the c-*fos* gene and to induce AP-1 DNA-binding activity.

Regulation of c-fos Is an Early Effect of E1A. To test when the effects of E1A and cAMP are first observable during infection, cells were mock-infected or infected with *dl*309 and harvested 3, 6, 9, 12, 18, or 24 hr later. For each time point, cells were left untreated or were treated for 1 hr with Bt_2cAMP before harvesting. Cytoplasmic RNA was isolated and analyzed for c-fos mRNA (Fig. 4 *A* and *B*). A mild cooperative effect of E1A and cAMP was evident as early as 9–12 hr after infection, and this effect increased throughout the infection time course. It is important to emphasize that in S49 cells, this time frame represents the early phase of viral infection. It is well before the onset of viral DNA replication

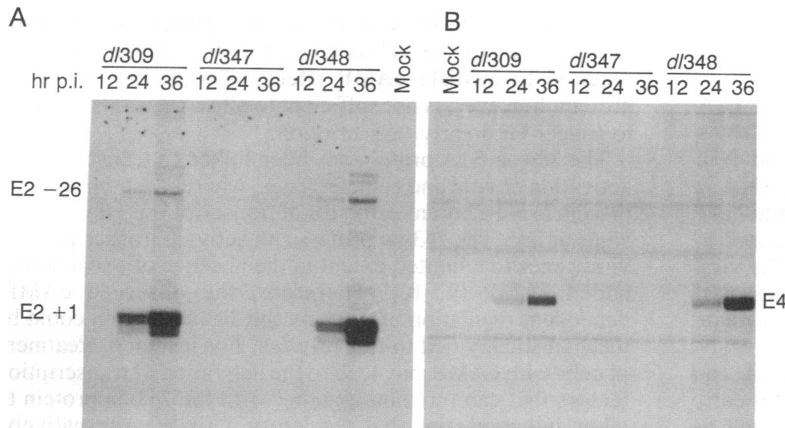

FIG. 3. Expression of adenovirus early genes *E2* and *E4* in S49 cells. Cells (5 × 10^6 cells per ml) were infected at a multiplicity of infection of 20 pfu per cell and plated at a density of 5 × 10^5 cells per ml 1 hr after infection. Cells were harvested at the indicated times after infection for cytoplasmic RNA isolation and analysis. RNase protection analysis was performed with 10 μg of RNA per sample and a ^{32}P-labeled E2 early (*A*) or E4 (*B*) probe. RNase-resistant products were separated on a denaturing 5% polyacrylamide gel followed by autoradiography. In *A*, positions of E2 mRNAs transcribed from the major (1) and minor (−26) transcription start sites are indicated. hr p.i., hours after infection.

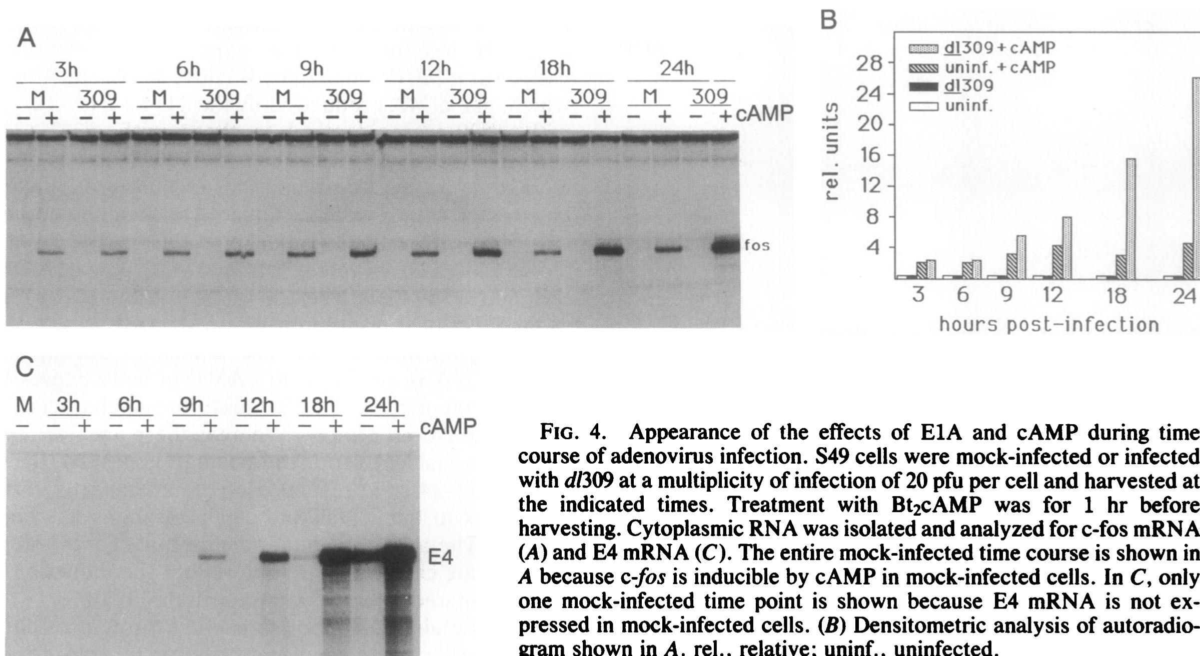

FIG. 4. Appearance of the effects of E1A and cAMP during time course of adenovirus infection. S49 cells were mock-infected or infected with *dl*309 at a multiplicity of infection of 20 pfu per cell and harvested at the indicated times. Treatment with Bt_2cAMP was for 1 hr before harvesting. Cytoplasmic RNA was isolated and analyzed for c-fos mRNA (*A*) and E4 mRNA (*C*). The entire mock-infected time course is shown in *A* because c-*fos* is inducible by cAMP in mock-infected cells. In *C*, only one mock-infected time point is shown because E4 mRNA is not expressed in mock-infected cells. (*B*) Densitometric analysis of autoradiogram shown in *A*. rel., relative; uninf., uninfected.

at ≈30 hr after infection (19). Interestingly, there was no observable induction of c-fos mRNA by E1A alone over the entire 24-hour time course of infection (Fig. 4*A*; compare untreated lanes for uninfected and *dl*309 infected; also see Fig. 2, compare untreated lanes for uninfected, *dl*309, *dl*347, and *dl*348). The effect of E1A was observed only in the presence of Bt_2cAMP. In another experiment, cells were infected and harvested after even shorter time points (0.5, 1, and 3 hr). Again, no induction of c-fos mRNA was detected in the absence of Bt_2cAMP (data not shown).

The response of the c-*fos* gene was then compared with that of the viral early gene *E4* (Fig. 4*C*). In contrast to c-fos mRNA, E4 mRNA began to appear in the cytoplasm between 12 and 18 hr after infection, even in the absence of Bt_2cAMP. This is roughly the same point at which the cooperation between E1A and cAMP to induce c-fos mRNA was first observed. As reported earlier (19) E4 mRNA levels could be further increased by Bt_2cAMP treatment.

We conclude from these data that the E1A proteins can alter the response of the c-*fos* gene to cAMP during the early phase of infection and that cAMP is required to be present to see the effect of E1A on c-*fos* expression. This finding is clearly different from results with the viral early gene *E4*, where E1A alone is sufficient to activate transcription, and cAMP increases the magnitude of this effect (see Fig. 4*C*). These data suggest that the mechanisms by which E1A regulates transcription of the c-*fos* and *E4* genes are somehow different.

DISCUSSION

Data presented here show that the 243-aa and 289-aa E1A proteins can each act with cAMP to induce AP-1 DNA-binding activity (Fig. 1) and c-fos mRNA (Figs. 2 and 4) in adenovirus-infected S49 cells. Induction of c-fos mRNA is not effected by E1A proteins alone; E1A is only seen to have an effect on c-*fos* expression when the cells are exposed to cAMP. In contrast, E1A activates transcription of the viral *E4* gene in the absence of cAMP. These data suggest that there are different mechanisms by which E1A can regulate transcription of these two genes.

There may be several mechanisms by which E1A can activate transcription of the *E4* gene (and other viral early genes). These might include cAMP-dependent as well as cAMP-independent mechanisms. If this is the case, in the absence of cAMP, the cAMP-dependent mechanism would be inactive, whereas the other mechanisms would remain active. Therefore, in the absence of cAMP, the *E4* gene would still be able to respond to E1A through a cAMP-independent mechanism. Perhaps in the case of c-*fos*, only the cAMP-dependent mechanism is a potential target for E1A-mediated transcriptional activation, so in the absence of cAMP the c-*fos* gene does not respond to E1A.

Why is the 243-aa protein unable to activate viral early gene transcription but is able to cooperate with cAMP to induce c-fos mRNA accumulation and AP-1 DNA-binding activity? Activation of viral early genes is known to require the unique 46-aa region found only in the 289-aa E1A protein. Because the 243-aa protein lacks this region, it does not activate viral genes efficiently. Also, cis-acting elements that control the viral early genes may contain specific sequences that respond to the presence of this 46-aa region. In the context of the system under study, it makes sense to speculate that the c-*fos* gene lacks sequences that allow it to respond to the 46-aa region, because the 289-aa protein alone does not trigger induction of c-fos mRNA. Despite this, c-*fos* does respond to either the 243-aa or the 289-aa E1A protein when cAMP is present. This result suggests that regions in common between the 243-aa and 289-aa proteins are important for this effect. There is growing evidence that these regions of E1A act by binding to, and affecting the function of, specific cellular proteins. A mechanism that explains the actions of the 243-aa protein in this system is that a cellular protein (or proteins) exists that binds the 243-aa E1A protein, and that protein is required to mediate cAMP effects. The action of such a protein, however, is not sufficient to allow the 243-aa protein to trigger viral early transcription.

The 289-aa E1A protein has been linked to transcriptional activation of viral and cellular genes, whereas the 243-aa E1A protein is not a potent activator of transcription. However, in some assays, the 243-aa protein can activate transcription of viral genes to a limited extent in the absence of exogenously added cAMP (9, 10). Therefore, the observed cAMP-dependent regulation of c-*fos* by the 243-aa protein could be mechanistically tied to this process. For instance, treatment of cells with cAMP may lead to the activation of transcription factors that can function together with the 243-aa protein to allow full transcriptional regulatory activity. Alternatively,

the mechanism underlying the induction of c-*fos* could be, at least in some aspects, different from the mechanism of viral transcriptional activation.

The E1A proteins have a number of activities that may relate to their ability to transform rodent cells. These include (*i*) cooperation with an activated Ha-*ras* oncogene to fully transform primary fibroblasts (3); (*ii*) immortalization of primary cells in culture (2); (*iii*) stimulation of cellular DNA synthesis and proliferation (35); (*iv*) transcriptional repression (36, 37); (*v*) induction of an epithelial growth factor (38); and (*vi*), physical association with several cellular proteins including the retinoblastoma gene product Rb (39–41). Our results indicate a functional interaction between both the 243-aa and 289-aa proteins and the cAMP-signaling system. Perhaps the observed cAMP-dependent regulation of c-*fos* transcription by these proteins is involved in the mechanism of cellular transformation by E1A. Consistent with this notion is the fact that the c-*fos* gene is known to be involved in cell growth regulation.

Recently the Rb protein has been reported to function to repress c-*fos* expression and AP-1 transcriptional activity (42). Interaction of the Rb protein with the E1A proteins is thought to inhibit Rb function and allow transformation. Our data are consistent with the possibility that the induction of c-fos mRNA and AP-1 activity in cAMP-treated adenovirus-infected cells is from an inhibition of Rb protein function.

If c-*fos* only responds to E1A proteins in the presence of cAMP, then what is the physiological relevance of this observation with regard to the E1A action? Cells generally express cAMP-dependent protein kinase, and the level of kinase activity in the cell depends on the level of expression of the enzyme and the intracellular level of cAMP. The steady-state level of cAMP may vary with cell type, thus resulting in different levels of kinase activity between cells. In addition, intracellular levels of cAMP are known to change in response to hormone or growth factor stimulation. Therefore, E1A probably can regulate c-*fos* transcription in some cells (depending upon cell type or condition) in the absence of externally added cAMP. These cells may be the *in vivo* targets of the growth-regulatory effects of E1A proteins.

We thank Matt Marton for the E2 RNase protection probe and for helpful discussions. This work was supported by a grant from the U.S. Public Health Service (CA-38965) to T.S. and by the National Cancer Institute Cancer Center Support Grant 2P30CA44579-04 and the American Cancer Society Institutional Research Grant IRG-149G to D.A.E. D.A.E. was a postdoctoral fellow of the National Institutes of Health (CA-08210) during the initial phase of this work. U.M. received fellowship support from the Fritz Thyssen Stiftung and the Deutsche Akademische Austausch Dienst, and T.S. is an American Cancer Society Professor.

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