JOURNAL OF VIROLOGY, Oct. 1992, p. 5867–5878
0022-538X/92/105867-12$02.00/0

Vol. 66, No. 10

Adenovirus E4orf4 Protein Reduces Phosphorylation of c-Fos and E1A Proteins While Simultaneously Reducing the Level of AP-1

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Received 27 May 1992/Accepted 14 July 1992

Adenovirus E1A protein and cyclic AMP cooperate to induce transcription factor AP-1 and viral gene expression in mouse S49 cells. We report that a protein encoded within the viral E4 gene region acts to counterbalance the induction of AP-1 DNA-binding activity by E1A and cyclic AMP. Studies with mutant adenoviruses demonstrated that in the absence of E4orf4 protein, AP-1 DNA-binding activity is induced to substantially higher levels than in wild-type virus-infected cells. The induction is the result of increased production of JunB and c-Fos proteins. Hyperphosphorylated forms of c-Fos and E1A proteins accumulate in the absence of functional E4orf4 protein. We propose that the E4orf4 protein acts to inhibit the activity of a cellular kinase that phosphorylates both the E1A and c-Fos proteins. Phosphorylation-dependent alterations in the activity of c-Fos, E1A, or some unidentified protein might, then, lead to decreased synthesis of AP-1 components. This E4 function likely plays an important role in natural infections, since a mutant virus unable to express the E4orf4 protein is considerably more cytotoxic than the wild-type virus.

Adenovirus E1A proteins appear to activate transcription through multiple mechanisms, including modification of cellular transcription factors, binding to transcription factors, and causing an increase in the levels of transcription factors. Modification can be achieved by altering the association of transcription factors with other cellular proteins. For example, the E1A proteins bind to the retinoblastoma protein, removing it from an association with E2F (4, 11, 12) and presumably freeing it to activate transcription. Modification might also be achieved by protein phosphorylation through E1A-associated kinases (24, 41, 82, 86). Indeed, the phosphorylation state of transcription factors TFIIIC, E2F, and E4F has been reported to be altered in infected cells (3, 36, 67). Physical interaction of E1A with a variety of cellular transcription factors (TFIID, AP-1, CRE-BP1/ATF2, and Oct-4) has been reported (37, 45, 53, 74), indicating that E1A proteins can function within a transcription complex. Finally, the amount of some cellular transcription factors (e.g., AP-1) is increased in the presence of E1A proteins (reviewed in reference 77).

AP-1, composed of polypeptides encoded by members of the *fos* and *jun* gene families (reviewed in references 16 and 85), has been linked to transcriptional regulation by E1A in both mouse S49 and human HeLa cells. Originally, it was noticed that many E1A-inducible viral promoters contain the cyclic AMP (cAMP)-responsive element CRE, which serves as a binding site for both the AP-1 and ATF/CREB families of transcription factors (10, 27, 28, 30, 35, 39, 44, 46, 48, 56, 59, 62, 71). Subsequently it was shown that cAMP can activate the expression of E1A-inducible viral genes and that E1A can activate transcription through CRE. Members of both the AP-1 and ATF/CREB families have been implicated in mediating the transcriptional activation effect of E1A and cAMP (10, 19, 30, 39, 46–49, 57, 59, 60, 65, 72). A link between transcriptional activation by E1A, cAMP, and transcription factor AP-1 was established in mouse S49 cells, in which E1A protein and cAMP cooperate to induce adenovirus gene expression and AP-1 DNA-binding activity with similar kinetics, suggesting that AP-1 activates viral gene expression (21, 59). Both the 243- and 289-amino-acid E1A proteins cooperate with cAMP to induce AP-1 activity, albeit with different efficiencies (22). More recently, de Groot et al. (19) reported E1A-dependent activation of AP-1 DNA-binding activity in infected HeLa cells. AP-1 DNA-binding activity is elevated by increased synthesis of AP-1 components in both S49 and HeLa cells. Transient transfection assays confirm that AP-1 DNA-binding sites can confer enhanced E1A responsiveness to heterologous promoters (10, 19, 60, 79). Interestingly, E1A proteins can cooperate with c-Jun, a component of AP-1, to activate transcription via AP-1 sites (19). Depending on the promoter construct tested and the experimental conditions employed, E1A is also able to repress transcription through AP-1 sites, suggesting that E1A regulates AP-1 activity in a complex manner (19, 63).

Our previous findings with S49 cells established a role for cAMP-dependent protein kinase A (PKA) in the induction of AP-1 activity by E1A and cAMP (59). Similarly, induction of viral gene expression by these stimuli was dependent on PKA (21). Both E1A proteins and AP-1 components are phosphorylated at multiple sites (5, 15, 17, 58, 69, 78, 90). Alterations in the phosphorylation state by cellular kinases such as PKA may alter the activity of these proteins. In the case of E1A protein, mutational analysis has failed to establish a role for the phosphorylation sites in the transcriptional activation properties of the protein (20, 81). In contrast, the phosphorylation state of AP-1 components can modulate their transcriptional activation potential in both positive and negative manners. Phosphorylation in a domain adjacent to the DNA-binding domain prevents DNA binding and transcriptional activation by c-Jun (8). Increased phos-

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phorylation in the transcriptional activation domain of c-Jun increases the ability of c-Jun to activate transcription without an effect on its DNA-binding activity (6, 66). Mutational analysis suggests that phosphorylation sites within the C terminus of the c-Fos protein are essential for the ability of c-Fos to negatively regulate its own promoter (64).

The effects of protein phosphorylation on the activity of AP-1 components led us to search for PKA-dependent alterations in the phosphorylation of AP-1 constituents and E1A proteins. In cAMP-treated adenovirus-infected S49 cells, the 289-amino-acid E1A protein and c-Fos protein accumulated in hypophosphorylated forms. Other phosphoproteins, such as JunB, the E1B 55-kDa protein, and the 24-kDa mRNA cap-binding protein (38), were not affected. Analysis of mutant adenoviruses revealed that hypophosphorylation was dependent on a 14-kDa viral protein encoded by open reading frame (orf) 4 of the viral E4 transcription unit. The results of pulse-chase experiments are consistent with the interpretation that the E4orf4 protein inhibits the activity of a cellular kinase which phosphorylates both the c-Fos and E1A proteins. In addition to phosphorylation changes, AP-1 DNA-binding activity was more strongly induced by cAMP treatment in cells infected with an E4orf4 mutant virus than in cells infected with the wild-type virus. Increased AP-1 DNA-binding activity resulted from increased accumulation of AP-1 components JunB and c-Fos. The concomitant alterations in phosphorylation and AP-1 DNA-binding activity raise the possibility that the two events are related, i.e., altered phosphorylation might directly or indirectly influence AP-1 activity.

Regardless of the mechanism, we can conclude that the E4orf4 protein functions to antagonize the induction of AP-1 DNA-binding activity mediated by E1A plus cAMP. This E4 function likely plays an important role in natural infections, since an adenovirus variant with a mutation in E4orf4 is more cytotoxic than the wild-type virus.

MATERIALS AND METHODS

Cells, viruses, and infection protocol. S49 cells (obtained from the University of California at San Francisco Cell Culture Facility) and B1R cells (a gift from Vincent Groppi, The Upjohn Company) were grown in suspension culture in medium containing 10% heat-inactivated horse serum. HeLa cells and W162 cells (which express adenovirus type 2 E4 proteins; 87) were grown as monolayers in medium complemented with 10% calf serum. Wild-type and mutant viruses were grown and titrated as described previously (40, 54). Each experiment was carried out with at least two independently grown stocks of virus to ensure reproducibility. The following mutant viruses used in this study have been described elsewhere: *dl*343 (32); *dl*347 and *dl*348 (89); *dl*312 and *dl*313 (40); *dl*802 (68); *dl*327 (80); *dl*339 (50); *dl*355, *dl*356, *dl*358, and *dl*366 (29); and *dl*1003, *dl*1006, and *dl*1014 (9).

In general, infections of exponentially growing S49 and B1R cells were carried out as described previously (59) at a multiplicity of 20 PFU per cell, as determined by plaque assay on HeLa cells. However, the titers of some of the E4 deletion viruses had to be determined on E4-complementing cell line W162. When this was necessary, the wild-type virus titer was determined in parallel. The plaque yield for wild-type virus was about 100-fold less on W162 than on HeLa cells. Therefore, for viruses whose titers had been determined on W162 cells a multiplicity of 0.2 PFU was employed. Control experiments were performed to ensure that viral genes such as E1A and E4 were efficiently expressed.

RNA analyses and DNA band shift assays. The preparation of nuclear extracts and the analysis of DNA-protein complexes by DNA band shift assay, as well as the sequences of double-stranded oligonucleotides used for the analysis, were as described elsewhere (59).

The preparation of cytoplasmic RNA and RNase protection protocols have been described by Engel et al. (21). The c-*fos* probe was a gift from M. Cole, and the *junB* probe was provided by D. Nathans (The Johns Hopkins School of Medicine).

Nuclear run-on assays were carried out as described by Greenberg and Ziff (25), with some modifications. S49 nuclei were prepared (59) and resuspended at a density of 10^7/100 μl in solution I (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.5], 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). A total of 10^7 nuclei in solution I were added to 100 μl of solution II (50 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 300 mM NaCl, 0.5 mM ATP-CTP-GTP, 1.25 mM dithiothreitol). A total of 150 μCi of [α^{32}P]UTP (800 Ci/mmol) was added, and elongation reactions were carried out for 30 min at 30°C. A 1-μl volume of DNase I (10 mg/ml; RNase free) was added, and incubation was continued for 5 min at 37°C. Proteins were digested with sodium dodecyl sulfate [SDS]-proteinase K and phenol extracted, and RNA was precipitated with 5% trichloroacetic acid in a total volume of 6 ml for 30 min at 0°C. Nuclear RNA was collected by filtration onto Millipore GFC filters (0.45-μm pore size) and was washed three times with 5% trichloroacetic acid and once with ethanol. Filters were incubated for 30 min at 37°C with 4 μg of DNase I in 1.5 ml of solution III (20 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 1 mM $CaCl_2$), 45 μl of 0.5 M EDTA and 140 μl of 10% SDS were added, and the RNA was eluted by heating the solution for 10 min at 65°C. RNA was precipitated with ethanol, suspended in 500 μl of solution IV (10 mM HEPES [pH 7.5], 10 mM EDTA, 0.2% SDS, 0.1 M NaCl), fragmented by adding 100 μl of 1 M NaOH and incubation for 10 min at 0°C, and neutralized with 200 μl of 1 M HEPES (pH 5.6). Linearized and heat-denatured plasmid DNA was spotted onto Gene Screen Plus membranes by using a slot blot apparatus. Filters were prehybidized for 15 min at 70°C in solution V (1% SDS, 300 mM NaCl, 10% dextran sulfate) and heat denatured, and radiolabeled nuclear RNA was added. Hybridization was carried out for 4 days at 70°C. Filters were washed once for 15 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS, twice for 30 min each time at 70°C with 2× SSC–1% SDS and a grain of proteinase K, once for 30 min at 37°C in 1× SSC–10 mg of RNase A per ml, and once for 1 h at 65°C in 2× SSC–1% SDS. Filters were air dried prior to autoradiography.

Phosphopeptide analysis. Infected S49 cells were labeled with $^{32}P_i$ (9,000 Ci/mmol) in phosphate-free medium beginning at 5 h prior to harvest and treated with cAMP beginning at 3 h prior to harvest. Labeled 13S E1A proteins were isolated by excision from SDS-containing polyacrylamide gels and subjected to tryptic phosphopeptide analysis (90).

Immunoprecipitation and Western blotting (immunoblotting). S49 cells were harvested, washed once with Dulbecco modified Eagle medium lacking methionine or phosphate, and resuspended at a density of 10^7/ml in the same medium containing 10% serum and 1 mCi of [^{35}S]Translabel (1,000 to 1,350 Ci/mmol) or 1 mCi of $^{32}P_i$ (9,000 Ci/mmol). Cells were harvested after the appropriate incubation time (see figure legends), and total cell extracts were prepared in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl [pH 8.0]). To obtain

denatured cell lysates, cells were suspended in 0.5% SDS–0.1% β-mercaptoethanol–50 mM Tris-HCl (pH 8.0), heated for 10 min to 95°C, and diluted to RIPA conditions. Extracts were precleared with normal rabbit serum and incubated with monoclonal antibodies or affinity-purified polyclonal antiserum. Extracts were incubated with antibodies for 60 min at 4°C, protein A-Sepharose CL4B was added to a final concentration of 0.1 mg/ml, and antigen-antibody complexes were harvested by centrifugation after an additional incubation for 60 min at 4°C. The complexes were washed five times in RIPA buffer and analyzed by electrophoresis on SDS–7.5 or 10% polyacrylamide gels as previously described (43). Potato acid phosphatase treatment was carried out after immunoprecipitation. Precipitates were washed five times in RIPA buffer and once in 10 mM PIPES-HCl (pH 6.0) and incubated in a total volume of 30 μl of 10 mM PIPES-HCl (pH 6.0) in the presence of 5 U of potato acid phosphatase (Sigma Chemical Co., St. Louis, Mo.) for 15 min at 37°C.

For Western blots, 20 μg of nuclear protein was resolved on SDS-polyacrylamide gels and transferred for 60 min at 4°C and 0.5 A to nitrocellulose filters (0.45-μm pore size; BA85; Schleicher & Schuell) by using an electroblotting transfer system. All of the following steps were carried out at room temperature. Filters were incubated for 60 min in phosphate-buffered saline (PBS) containing 1% nonfat dry milk and then for 60 min in the same buffer containing antibody. Filters were washed twice in PBS and incubated for 60 min in PBS containing 1% nonfat dry milk and a secondary antibody linked to alkaline phosphatase. Filters were washed three times for 15 min each time in PBS and once with AP buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) and incubated in AP buffer supplemented with 5-bromo-4-chloro-3-indolylphosphate toluidinium (15 mg/100 ml) and nitroblue tetrazolium (30 mg/100 ml). The color reaction was stopped by rinsing the filters extensively in H_2O.

Monoclonal E1A antibody M73 and c-Fos antiserum, gifts from E. Harlow (Massachusetts General Hospital) and T. Curran (Roche Institute of Molecular Biology), have been described elsewhere (18, 31). JunB antiserum was provided by R. Bravo (Bristol-Myers Squibb). It is a polyclonal rabbit antiserum and specific for the JunB protein.

RESULTS

Altered phosphorylation of E1A proteins after cAMP treatment. We previously reported that E1A protein and cAMP cooperate to induce transcription factor AP-1 in adenovirus-infected S49 cells (59). S49 cells were employed for these experiments, since unlike HeLa cells, the more common adenovirus laboratory host, they are highly responsive to cAMP. Furthermore, S49 sublines with deficiencies in the cAMP-triggered signal transduction pathway are available. One of these cell lines, B1R (7, 14), does not express cAMP-activated PKA (reviewed in reference 61). Employing B1R cells, we have previously demonstrated that PKA is required for induction of AP-1 by E1A plus cAMP. This led us to determine whether cAMP treatment of adenovirus-infected cells would lead to changes in the phosphorylation of proteins such as E1A that are involved in the induction process. Nuclear extracts were prepared from infected cells after various periods of cAMP treatment, and E1A proteins were detected by Western blot analysis. In cells infected with a virus expressing only the 243-amino-acid E1A protein (*dl*347) or only the 289-amino-acid E1A protein (*dl*348), two major E1A-specific bands were observed (*dl*347, 243-1 and 243-2; *dl*348, 289-1 and 289-2 [Fig. 1A]). The bands represent different phosphoforms of the E1A proteins, with the more highly phosphorylated form migrating slower in the gel system employed (20, 31, 69, 81). All four bands were evident in extracts from wild-type (*wt*300) virus-infected cells (Fig. 1A, 0-h cAMP). Changes in the mobility of E1A proteins, indicative of changes in their phosphorylation, were observed when extracts from cAMP-treated cells were analyzed. Alterations in the mobility of E1A proteins were first evident after cAMP treatment for 1 h (data not shown) and became pronounced by 3 h (Fig. 1A). The intensity of the bands designated 289-1 and 289-2 decreased after extended periods of cAMP treatment (see Fig. 2B), while bands of faster mobility, 289-3 and 289-4 (identified as 289 specific in *dl*348-infected cells; data not shown), accumulated (Fig. 1A). There also appeared to be a slight increase in the proportion of less-phosphorylated relative to highly phosphorylated 243-amino-acid E1A protein (243-1 versus 243-2, Fig. 1). However, the predominant effect of treating

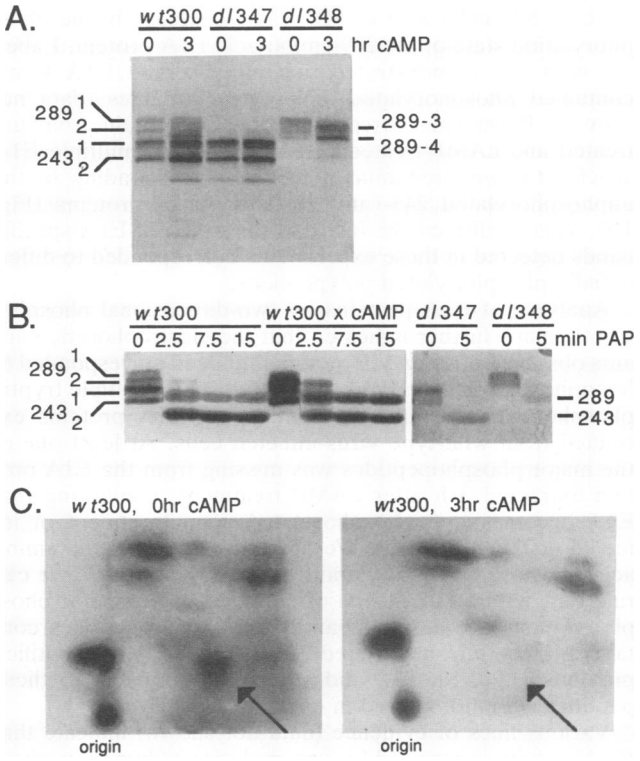

FIG. 1. Alterations in the phosphorylation of the E1A protein. (A) S49 cells were infected with *wt*300, *dl*347, and *dl*348 at a multiplicity of 20 PFU per cell, and cAMP was added for the indicated periods before harvesting at 24 h after infection. Nuclear proteins (20 μg) were resolved on an 8% denaturing polyacrylamide gel, transferred to nitrocellulose filters, and probed with M73, an E1A-specific monoclonal antibody. E1A-specific bands are designated (243-1, 243-2, and 289-1 to 289-4). (B) Nuclear extracts were prepared at 48 h after infection of S49 cells with *wt*300, *dl*347, or *dl*348 and treated with potato acid phosphatase (PAP) for the indicated times. (C) For phosphopeptide analysis, ^{32}P-labeled 13S E1A proteins were electrophoretically purified from total cell extracts of untreated or cAMP-treated S49 cells at 24 h after infection with *wt*300 and subjected to tryptic digestion. Electrophoresis was from left (negative) to right (positive), and chromatography was from bottom to top. The arrows mark the major phosphopeptide that was not present in E1A protein from cAMP-treated cells.

infected S49 cells with cAMP was a decrease in the phosphorylation state of the 289-amino-acid E1A protein. Labeling with $^{32}P_i$ demonstrated that all observed E1A forms contained phosphorylated amino acid residues (data not shown). Phosphatase treatment of E1A protein from untreated and cAMP-treated cells reduced the multiple E1A species to two predominant bands, corresponding to the unphosphorylated 243- and 289-amino-acid proteins (Fig. 1B). This confirmed the fact that the different E1A-specific bands detected in these experiments corresponded to differentially phosphorylated polypeptides.

Analyses of E1A proteins by two-dimensional phosphotryptic maps further indicated that the electrophoretic variants observed after cAMP treatment indeed corresponded to hypophosphorylated E1A protein (Fig. 1C). Multiple tryptic phosphopeptides could be identified in E1A proteins extracted from wild-type virus-infected cells. At least one of the major phosphopeptides was missing from the E1A protein extracted 3 h after cAMP treatment, confirming that E1A becomes hypophosphorylated upon treatment of infected cells with cAMP. We have not identified the amino acid(s) whose phosphorylation is altered. However, we can rule out, with a fair degree of confidence, the major phosphorylation sites at Ser-89 and -219. Tryptic peptides containing these phosphorylated residues have been identified previously (20, 81, 83), and spots corresponding to these peptides were not altered in our experiment.

Various lines of evidence (data not shown) indicate that PKA activity is required for accumulation of hypophosphorylated E1A protein. (i) Accumulation of hypophosphorylated E1A protein was abolished in the presence of H8, a chemical kinase blocker with marked preference for PKA (34). (ii) Treatment with TPA, an inducer of PKC but not PKA, did not induce phosphorylation changes in E1A protein. (iii) Hypophosphorylated E1A proteins did not accumulate upon cAMP treatment of infected B1R cells, a mutant subline of S49 cells deficient in PKA activity (7, 14). B1R cells contained the same four E1A species that were present in S49 cells prior to cAMP treatment. Thus, while PKA activity was required for accumulation of hypophosphorylated E1A protein, the kinase did not seem to phosphorylate E1A protein directly to generate the electrophoretic variants observed in one-dimensional gels.

Altered phosphorylation of E1A requires the adenovirus E4orf4 product. cAMP might cooperate with E1A proteins to mediate the efficient expression of another viral gene, which in turn could influence the phosphorylation state of E1A proteins. Accordingly, viral mutants were analyzed for the ability to cooperate with cAMP in the induction of hypophosphorylated forms of E1A protein. In cells infected with mutant viruses lacking E1B (*dl*313 and *dl*339), E2 (*dl*802), and E3 (*dl*327) functions, hypophosphorylated forms of E1A accumulated with kinetics similar to those in wild-type virus-infected cells (Table 1). In contrast, accumulation of hypophosphorylated E1A protein was abolished when infections were carried out with mutant viruses lacking the E4 gene region (Fig. 2). At least six different E4 proteins can be translated from seven open reading frames within the E4 gene region; they are termed orfs 1, 2, 3, 3/4, 4, 6, and 6/7 (23, 33, 55, 84; Fig. 2A). To identify which of the orfs encodes the viral function required for the phosphorylation change, we evaluated a series of mutant viruses carrying deletions within the E4 gene region (Fig. 2A). The results for some viruses are shown in Fig. 2B and summarized in Table 1. All mutants that carried deletions affecting orf4 (*dl*366, *dl*1003, and *dl*358) were unable to cooperate with cAMP in the induction of hypophosphorylated E1A proteins. One of these mutant viruses (*dl*358) carried a 20-bp deletion within orf4 which did not affect any of the other E4 orfs. Furthermore, orf4 encoded the only E4 product required, since hypophosphorylated E1A proteins accumulated upon cAMP treatment of S49 cells infected with *dl*1014 which expressed orf4 as the only E4 product (Table 1).

TABLE 1. Phenotypes of mutant adenoviruses

Virus	Mutated gene	Hypophosphorylation		Increased AP-1
		E1A	c-Fos	
*wt*300	None	+	+	−
*dl*347	E1A-289aa	+	+	−
*dl*348	E1A-243aa	+	+	−
*dl*313	E1A/E1B	+	+	−
*dl*339	E1B	+	+	ND[a]
*dl*802	E2	+	+	ND
*dl*327	E3	+	+	ND
*dl*366	E4	−	−	+
*dl*1003	E4orf4, 6, 6/7	−	−	+
*dl*1006	E4orf1, 2, 3, 3/4	+	+	−
*dl*355	E4orf6	+	+	−
*dl*356	E4orf6/7	+	+	−
*dl*358	E4orf4	−	−	+
*dl*1014	E4orf1, 2, 3, 3/4, 6, 6/7	+	+	−

[a] ND, not determined.

We also analyzed the phosphorylation changes of E1A protein by pulse-chase experiments (Fig. 2C). Two bands representing different phosphoforms of the 289-amino-acid protein were evident in *dl*366- and *dl*358-infected, cAMP-treated cells after 10 min of pulse labeling, and the hypophosphorylated band was readily chased into the more highly phosphorylated form within 1 h. In contrast, only the hypophosphorylated, faster-migrating form accumulated to substantial levels in *wt*300-infected cells, and it was not chased into a more highly phosphorylated form, even after 2 h. Phosphorylation changes in the 243-amino-acid E1A protein were not evident. Our data suggest that the accumulation of hypophosporylated 289-amino-acid E1A protein in infected cells resulted from inhibition of a kinase by the E4orf4 product. However, we cannot exclude the possibility that a phosphatase which removed phosphate from newly synthesized E1A protein was activated. If a phosphatase was involved, it must have been activated slowly over at least 1 h, since this was the first time after cAMP treatment when hypophosphorylated E1A protein was evident.

To determine whether E4orf4 proteins would influence the phosphorylation of E1A proteins in HeLa cells, the cells were infected with *wt*300, *dl*366, or *dl*358 and E1A proteins were analyzed by immunoprecipitation of ^{35}S-labeled extracts prepared at various times during the infection cycle (Fig. 2D). Two different phosphoforms of the 289-amino-acid E1A protein were present in equimolar amounts in *dl*366- and *dl*358-infected cells throughout the time course. In contrast, the less-phosphorylated form predominated in *wt*300-infected cells. Thus, we conclude that the regulation of E1A protein phosphorylation by E4orf4 is not restricted to S49 cells and may have a function during the viral life cycle in HeLa cells. Exogenously added cAMP was not required to induce hypophosphorylation in HeLa cells, presumably because they contain constitutively high levels of cAMP (54a).

Altered phosphorylation of c-Fos protein. We next determined whether the phosphorylation of other proteins was

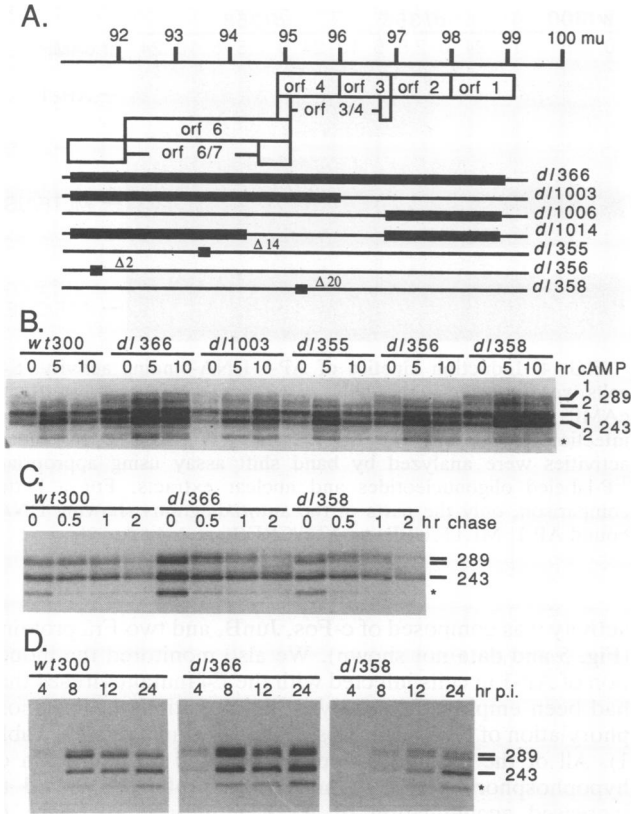

FIG. 2. E4orf4 is required for hypophosphorylation of E1A protein. (A) Map of the E4 gene region. The thick black line on top represents the right-end 91 to 100 map units (mu) of the viral genome containing the E4 gene region. The direction of transcription is right to left. The open rectangles represent different orfs within the E4 gene region. Deletions (black boxes) in various E4 mutant viruses and virus nomenclature are indicated at the bottom. (B) Alterations in E1A phosphorylation within E4 mutant virus-infected cells. S49 cells were infected with various E4 mutant viruses and treated with cAMP for the indicated times immediately before harvesting at 24 h postinfection. Nuclear extracts were prepared, and E1A proteins were analyzed in a Western blot employing M73 antibody. The dot marks a protein band of unknown origin. It was observed only in some experiments and most likely reflects an E1A degradation product. (C) Pulse-chase analysis. S49 cells were infected with *wt*300, *dl*366, or *dl*358, and cAMP was added 20 h later. Cells were labeled for 10 min with [^{35}S]methionine 2.5 h after addition of cAMP and chased with an excess of unlabeled methionine for 0- to 2-h intervals as indicated. Total cell extracts were analyzed by immunoprecipitation with M73 antibody. The dot marks a band of unknown origin (see Fig. 3B). (D) HeLa cells were infected with *wt*300, *dl*366, or *dl*358 and labeled with [^{35}S]methionine for the final 1 h before harvesting at the indicated times. E1A proteins were immunoprecipitated from total cell extracts with M73 antibody, resolved on SDS–10% polyacrylamide gels, and visualized by fluorography. The sizes (amino acids) of marker proteins are indicated, and the dot marks a band of unknown origin.

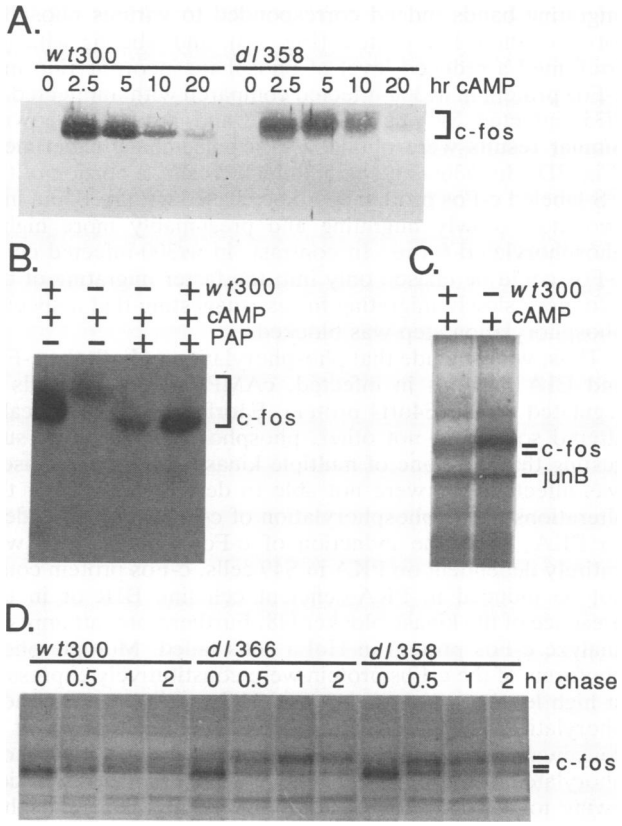

FIG. 3. Alterations in c-Fos phosphorylation. (A) Nuclear extracts were prepared from *wt*300- or *dl*358-infected cells after various periods of cAMP treatment and analyzed for c-Fos protein in a Western blot employing a polyclonal c-Fos antiserum. (B) Potato acid phosphatase (PAP) treatment of c-Fos protein in nuclear extracts of uninfected or *wt*300-infected S49 cells that were treated with cAMP as indicated. Bands corresponding to phosphorylated and dephosphorylated Fos proteins are labeled. (C) Immunoprecipitation of c-Fos protein from extracts of ^{32}P-labeled uninfected or *wt*300-infected cells prepared in RIPA buffer. Bands corresponding to c-Fos protein and coprecipitating JunB proteins are labeled. (D) Pulse-chase experiment. Total cell extracts were prepared from S49 cells infected with *wt*300, *dl*366, or *dl*358; treated for 3 h with cAMP; labeled with [^{35}S]methionine; and chased in the presence of excess unlabeled methionine for the times indicated. c-Fos proteins were then immunoprecipitated by using a polyclonal antiserum.

altered upon cAMP treatment of infected S49 cells. We observed no alterations in the overall phosphorylation pattern of cellular proteins as analyzed by one-dimensional gel electrophoresis of ^{32}P-labeled total cell extracts, excluding the possibility that infection and cAMP treatment led to a general reduction of protein phosphorylation (data not shown). The 24-kDa mRNA cap-binding protein was monitored, since its phosphorylation state is known to be altered in adenovirus-infected cells (38), but phosphorylation of the protein was not influenced by cAMP treatment (data not shown). Additionally, we observed no changes in the phosphorylation of the viral E1B 55-kDa protein (data not shown) or the JunB protein (see Fig. 3C and 5B) when we analyzed them by immunoprecipitation from ^{35}S- or ^{32}P-labeled extracts. In contrast, the phosphorylation of the c-Fos protein, a component of transcription factor AP-1, was altered in infected and cAMP-treated cells in an E4orf4-dependent manner. Figure 3A shows a Western blot analysis of c-Fos protein induced by cAMP treatment in *wt*300- and *dl*358-infected cells. In both cases, c-Fos protein was induced within 2.5 h of cAMP treatment. With time, the mobility of the c-Fos protein decreased in *dl*358- but not *wt*300-infected cells, suggesting that more highly phosphorylated c-Fos protein accumulated in mutant virus-infected cells. Treatment with phosphatase demonstrated that the differentially

migrating bands indeed corresponded to various phosphoforms of the c-Fos protein (Fig. 3B), and labeling with ^{32}P confirmed a reduced level of phosphate incorporation into c-Fos protein in *wt*300-infected compared with uninfected or *dl*358-infected S49 cells (Fig. 3C and data not shown). Similar results were obtained in a pulse-chase experiment (Fig. 3D). In *dl*366- and *dl*358-infected cells, a portion of the ^{35}S-labeled c-Fos protein could be chased within 30 min into two more slowly migrating and presumably more highly phosphorylated forms. In contrast, in *wt*300-infected cells, c-Fos could be chased only into the faster migrating of the two more slowly migrating forms, suggesting that a specific phosphorylation step was blocked.

Thus, we conclude that phosphorylation of both the c-Fos and E1A proteins in infected, cAMP-treated S49 cells is regulated by the E4orf4 protein. E4orf4 protein specifically altered some, but not other, phosphorylation events, suggesting that only one of multiple kinases (or phosphatases) was affected. We were not able to determine whether the alterations in the phosphorylation of c-Fos were dependent on PKA, since the induction of c-Fos protein itself was entirely dependent on PKA in S49 cells. c-Fos protein could not be induced in PKA-deficient cell line B1R or in the presence of the kinase blocker H8. Furthermore, attempts to analyze c-Fos protein in HeLa cells failed. Multiple phosphoforms of the c-Fos protein were constitutively expressed at high levels even in uninfected HeLa cells, and no phosphorylation changes were observed upon infection. It is conceivable that the accumulation of a specific hypophosphorylated form of the c-Fos protein escaped detection owing to the presence of additional comigrating phosphoforms. Alternatively, the c-Fos amino acid residue(s) dephosphorylated in S49 cells may not be phosphorylated in HeLa cells.

E4orf4 regulates the level of AP-1. Since c-Fos is a component of AP-1, we determined whether the change in c-Fos phosphorylation would alter the physical properties of AP-1 or its level of DNA-binding activity. We searched for changes in the bound half-life of AP-1–DNA complexes or in binding specificity for a variety of different recognition sites, but no alterations were observed (data not shown), suggesting that the DNA-binding properties of AP-1 were not influenced by the phosphorylation events under study. However, induction of AP-1 DNA-binding activity by cAMP was altered in E4 mutant-infected versus wild-type virus-infected cells (Fig. 4). S49 cells were infected with the *wt*300 or E4 mutant virus, nuclear extracts were prepared after various times of cAMP treatment, and AP-1 levels were analyzed by DNA band shift assay. As reported earlier for *wt*300-infected cells (59), treatment with cAMP led to a transient increase in AP-1 DNA-binding activity, which reached maximal levels around 2.5 to 5 h after addition of cAMP and decreased thereafter. In contrast, the induced AP-1 activity reached higher levels and remained elevated for a longer time in cells infected with viruses lacking the entire E4 gene (*dl*366) or E4orf4 (*dl*358). As in *wt*300 infections, maximal levels of AP-1 were induced after 2.5 to 5 h of cAMP treatment but levels stayed high for at least 24 h, the last time point tested. The effect was reproducibly somewhat more pronounced for *dl*366 than *dl*358 infections, and it was specific for AP-1 activity since no effect was observed for two other transcription factors, MLTF/USF and ATF/CREB. Immunoprecipitation experiments with c-Fos and JunB antisera confirmed that the induced AP-1 activity was identical in wild-type virus-infected and mutant virus-infected cells and did not change during the course of cAMP treatment. The AP-1

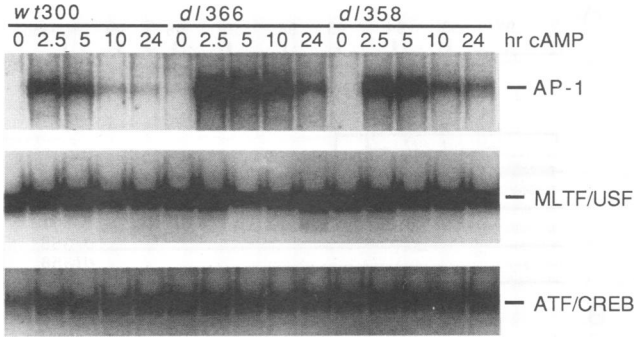

FIG. 4. Induction kinetics of AP-1 DNA-binding activity. S49 cells were infected with *wt*300, *dl*366, and *dl*358 and treated with cAMP for the indicated periods before harvesting at 24 h after infection. Levels of AP-1, MLTF, and ATF/CREB DNA-binding activities were analyzed by band shift assay using appropriate ^{32}P-labeled oligonucleotides and nuclear extracts. For a better comparison, only the parts of the autoradiograms showing DNA-bound AP-1, MLTF/USF, or ATF/CREB are reproduced.

activity was composed of c-Fos, JunB, and two Fra proteins (Fig. 5 and data not shown). We also monitored the induction of AP-1 in cells infected with the E4 mutant viruses that had been employed to analyze the alterations in the phosphorylation of E1A and c-Fos proteins (summarized in Table 1). All of the mutations which prevented accumulation of hypophosphorylated E1A and c-Fos proteins also led to increased accumulation of AP-1 activity in response to cAMP.

E4orf4 regulates both the accumulation of AP-1 DNA-binding activity and the phosphorylation of two proteins involved in its induction: c-Fos and E1A.

Phosphorylation state of c-Fos does not influence binding to JunB. We have previously shown that the AP-1 activity induced by cAMP in adenovirus-infected S49 cells contains c-Fos and JunB proteins (59). Experiments were carried out to determine whether the phosphorylation state of c-Fos influences its ability to associate with JunB. ^{35}S-labeled extracts were prepared, and immunoprecipitations were performed by using antibodies to JunB- or c-Fos (Fig. 5). More labeled extract from wild-type virus-infected than mutant virus-infected cells was employed to generate equivalent amounts of immunoprecipitated proteins (*dl*358-infected cells contain more c-Fos and JunB; see Fig. 7). Immunoprecipitation of c-Fos from extracts of *wt*300 and *dl*358-infected cells confirmed that the c-Fos protein in *dl*358-infected cells included species with slower mobility on denaturing polyacrylamide gels than the c-Fos protein from *wt*300-infected cells, indicative of a difference in its phosphorylation state (Fig. 5A). Three JunB-specific polypeptides were coimmunoprecipitated with c-Fos. The identity of the JunB polypeptides was revealed by immunoprecipitation with a JunB-specific antibody, and all three bands were shifted into one band by phosphatase treatment, indicating that they represented different phosphoforms of JunB (Fig. 5B). As expected, the three phosphoforms of JunB coimmunoprecipitated with c-Fos antibodies from native, but not denatured, extracts. All three phosphoforms of JunB were evident in extracts from both *wt*300- and *dl*358-infected cells, and they coimmunoprecipitated in equal amounts with the different c-Fos phosphoforms (Fig. 5A). Thus, the interaction of c-Fos with the JunB protein was not detectably altered by the phosphorylation state of the c-Fos protein.

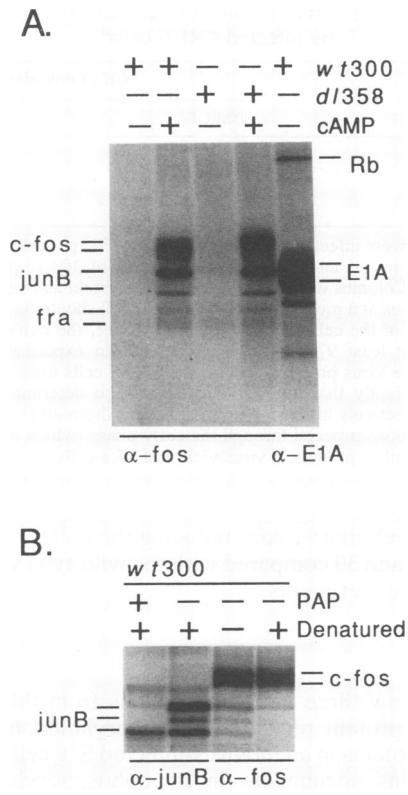

FIG. 5. Heterodimerization of c-Fos and JunB. (A) Immunoprecipitation were performed with a c-Fos antibody using ^{35}S-labeled total cell extracts from *wt*300- and *dl*358-infected cells. Where indicated, the cells had been treated for 3 h with cAMP prior to extract preparation. c-Fos, JunB, and Fra-specific bands are labeled. As a control, E1A and associated proteins were immunoprecipitated from the *wt*300-infected cell extract by using antibody M73, and bands corresponding to the E1A (289 [upper band] and 243 [lower band] amino acids) and retinoblastoma proteins (Rb) are labeled. (B) Denatured or native ^{35}S-labeled total cell lysates were prepared from cAMP-treated *wt*300-infected cells, and immunoprecipitations were carried out with a JunB- or c-Fos-specific antibody. Where indicated, immunoprecipitates were treated with potato-acid phosphatase (PAP) prior to electrophoresis. Bands corresponding to c-Fos and JunB are labeled.

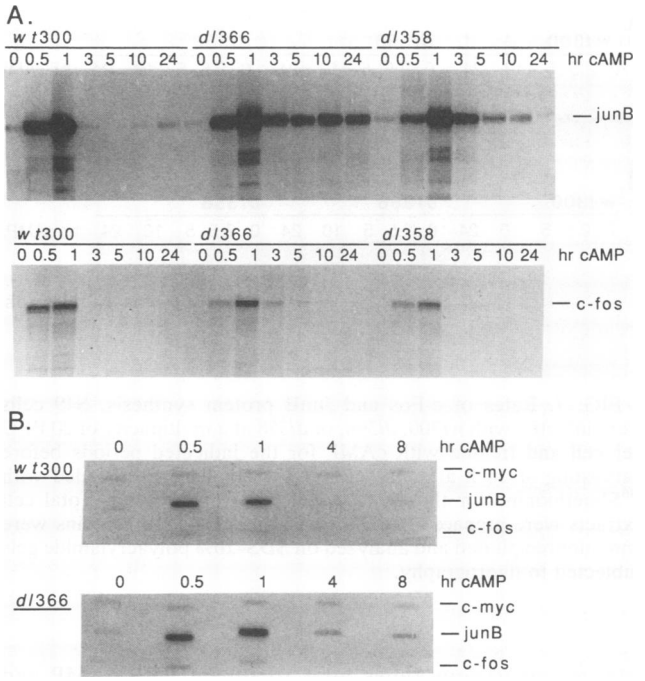

FIG. 6. mRNA levels and transcription rates of *junB* and c-*fos*. (A) Steady-state mRNA levels were measured in an RNase protection experiment. S49 cells were infected with *wt*300, *dl*366, or *dl*358 at a multiplicity of 20 PFU per cell and treated with cAMP for the indicated periods before harvesting at 24 h after infection. Cytoplasmic RNA was analyzed by using c-*fos*- and *junB*-specific probe RNAs. (B) The transcription rates of the c-*fos*, *junB*, and c-*myc* genes were measured in a nuclear run-on experiment. Infection conditions and cAMP treatment were as in panel A.

The data in Fig. 5 also confirmed that phosphorylation of the JunB protein, at least as analyzed by one-dimensional gel electrophoresis, was not detectably affected by the E4orf4 mutation.

Mechanism of AP-1 regulation by E4orf4. The increased accumulation of AP-1 DNA-binding activity in E4 mutant virus-infected cells could result from alterations in the rate of transcription or translation of AP-1 components. Alternatively, alterations in the phosphorylation of c-Fos might change the half-life of c-Fos protein and AP-1 complexes. Experiments were performed to distinguish among these possibilities.

RNase protection experiments revealed that c-*fos* and *junB* mRNAs increased to peak levels within 1 h of cAMP treatment of wild-type or mutant virus-infected cells (Fig. 6A). Both mRNAs returned nearly to basal levels by 3 h after cAMP treatment of wild-type virus-infected cells. In contrast, levels of *junB* mRNA remained elevated for extended periods after cAMP treatment in E4 mutant-infected cells: at least 10 h for *dl*358 and 24 h for *dl*366. The difference between the two mutants was reproduced in multiple independent experiments and is consistent with the difference observed for AP-1 DNA-binding activity (Fig. 4). Less c-*fos* than *junB* mRNA was present at all times tested, and the level of c-*fos* mRNA also appeared to remain somewhat elevated for a longer time in *dl*366-infected cells than in cells infected with *wt*300 (compare 3- and 5-h time points, Fig. 6A). However, the effect was much less dramatic than that observed for *junB* mRNA.

We confirmed by nuclear run-on experiments (Fig. 6B) that the increased accumulation of *junB* mRNA was the result of an increased transcription rate of the *junB* gene. In *wt*300-infected cells, the transcription rates of both the c-*fos* and *junB* genes were induced to peak levels within 30 min of cAMP treatment and decreased after 1 h. In *dl*366-infected cells, *junB* and c-*fos* transcription was also induced within 30 min. However, even though the *junB* transcription rate was reduced after 1 h, it remained elevated above basal levels for at least 8 h, the last time point tested. Transcription of the c-*fos* gene remained only slightly above basal levels (1.5-fold) at 4 h after cAMP treatment was initiated. This marginal enhancement is consistent with the slight increase observed for c-*fos* mRNA levels. Treatment with cAMP had no effect on c-*myc* expression, which served as a control.

We next examined the half-life and synthesis rate of the JunB and c-Fos proteins. The stability of the two proteins was the same in *dl*366-, *dl*358-, and *wt*300-infected cells (Fig. 3B and data not shown). The rate of protein synthesis was monitored by labeling cells with [^{35}S]methionine for 10-min

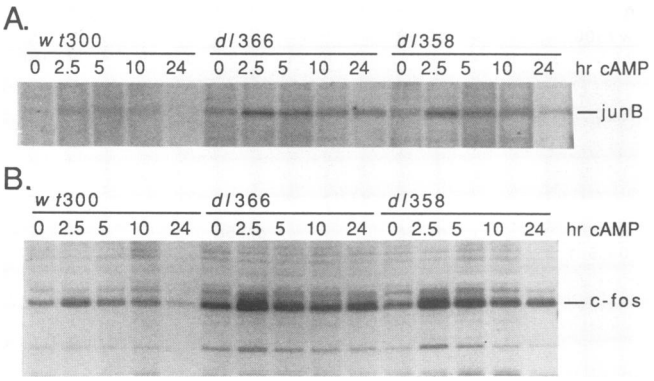

FIG. 7. Rates of c-Fos and JunB protein synthesis. S49 cells were infected with *wt*300, *dl*366, or *dl*358 at a multiplicity of 20 PFU per cell and treated with cAMP for the indicated periods before harvesting at 24 h after infection. All samples were labeled with [^{35}S]methionine for the final 10 min before harvesting. Total cell extracts were prepared and JunB (A) and c-Fos (B) proteins were immunoprecipitated and analyzed on SDS–10% polyacrylamide gels subjected to fluorography.

intervals at various times after treatment with cAMP and then performing immunoprecipitations by using conditions determined to capture all c-Fos or JunB protein. The total protein synthesis rate was induced only very mildly by cAMP and to a similar extent in wild-type and mutant virus-infected cells (data not shown). The rate of JunB synthesis was substantially enhanced and maintained at elevated levels for a much longer time after cAMP treatment in mutant-infected than in wild-type virus-infected cells (Fig. 7A). Substantially more JunB protein was synthesized at 5 and 10 h after the start of cAMP treatment in mutant-infected than in wild-type virus-infected cells. The enhanced rate of JunB synthesis (Fig. 7A) appears to be a direct consequence of its enhanced mRNA levels (Fig. 6). After 2.5 h of cAMP treatment, the rate of c-Fos protein synthesis was enhanced to a greater extent in mutant-infected than in wild-type virus-infected cells (*wt*300, 1.5-fold; *dl*366, 5-fold; *dl*358, 5-fold [Fig. 7B]). Further, an enhanced level of c-Fos synthesis was maintained for a longer time after cAMP treatment in mutant-infected than in wild-type virus-infected cells (e.g., compare the 24-h assays for *wt*300 and *dl*366, Fig. 7B). Overall, then, more c-Fos protein is synthesized after cAMP treatment in mutant virus-infected cells than in cells infected with wild-type virus.

It is difficult to be certain whether the modulation of c-Fos by the E4orf4 gene product is due entirely to its modest effect on transcription. A translational component might also be involved. It is, nevertheless, clear that the E4 protein affected JunB accumulation primarily at the level of transcription.

E4orf4 reduces virus-mediated cytotoxicity. It seemed likely that a persistently high level of a regulatory molecule such as AP-1 could adversely affect the cell, so we determined whether the E4orf4 mutant was more toxic than the wild-type virus. Rat fibroblasts (CREF cells) were employed for the assay, since they are nonpermissive for adenovirus type 5, permitting expression of early but not late viral genes. Cells were infected with *dl*358 or *wt*300 and then assayed for colony formation to assess the number of viable cells (Table 2). Infection with the wild-type virus reduced the number of colonies by about 40%. However, the mutant virus was much more toxic, reducing the colony number by factors of 10 and 30 compared with the wild-type virus in two independent experiments.

TABLE 2. E4orf4 protein enhances colony formation by infected CREF cells[a]

Virus	No. of colonies	
	Expt 1	Expt 2
None	66	79
*wt*300	40	46
*dl*358	2	8

[a] CREF cells were infected at a multiplicity of 1 PFU per cell, and 20 h later they were trypsinized and replated at a density of 100 cells per 60-mm-diameter plate. Colonies were stained with Giemsa stain and counted 1 week later. Mutant virus at a multiplicity of 1 PFU per cell should, in theory, infect only about 30% of the cells in the culture. However, the mutant virus must have infected at least 97 and 90% of the cells in experiments 1 and 2, respectively. The virus probably infects the CREF cells used in this experiment more efficiently than the HeLa cells used to determine viral titers, producing an effectively higher input multiplicity. Alternatively, it is possible that many infections proceed through the early phase (which would score in this assay) but fail to produce a virus yield in HeLa cells.

DISCUSSION

We can draw three main conclusions from this work. (i) The E4orf4 protein regulates phosphorylation of the E1A and c-Fos proteins in adenovirus-infected S49 cells. E1A and c-Fos proteins accumulate in hypophosphorylated forms upon cAMP treatment of wild-type virus-infected but not E4orf4 mutant virus-infected cells (Fig. 1 to 3). Accumulation of hypophosphorylated proteins is prevented in the presence of a chemical PKA blocker or in B1R cells (data not shown), indicating that PKA is required for the E4orf4-mediated hypophosphorylation event. The simplest explanation for this requirement is that PKA is needed to induce transcription of the E4 unit in cooperation with E1A protein (21) to generate sufficient quantities of the E4orf4 protein which, in turn, induce hypophosphorylation. However, we cannot rule out an additional, more direct role for PKA in the process. E4orf4 protein also regulates the phosphorylation of E1A in HeLa cells, even in the absence of exogenously applied cAMP (Fig. 2D). PKA is constitutively activated in HeLa cells, owing to high endogenous levels of cAMP (54a). (ii) The E4orf4 protein plays a central role in the regulated induction of AP-1 DNA-binding activity by E1A and cAMP. AP-1 DNA-binding levels are higher, and stay elevated for longer times (24 versus 5 h), in E4orf4 mutant-infected cells than in wild-type virus-infected cells (Fig. 4). The increase in AP-1 DNA-binding activity is due to enhanced accumulation of the JunB and c-Fos proteins. Expression of *junB* is modulated at the level of transcription, and c-*fos* might be influenced at both the transcriptional and the translational levels (Fig. 6 and 7). (iii) The E4orf4 protein reduces the cytotoxic effect of early viral gene expression (Table 2). This might result from the altered E1A and c-Fos phosphorylation or the reduction in AP-1 activity, but we have no evidence for cause and effect beyond the correlation. Such a protective role could be important in natural infections, in which adenoviruses maintain long-term associations with their infected hosts.

Induction of AP-1 DNA-binding activity by E1A and cAMP is a transient process. The degree and duration of the induction is apparently regulated by the E4orf4 protein, since AP-1 DNA-binding activity reaches higher levels and

remains elevated for an extended time after cAMP treatment in the absence of the E4 product. We observed repeatedly that AP-1 was induced to even higher levels when the total E4 transcription unit, rather than just orf4, was deleted. Conceivably, a second E4 protein contributes to the regulation of AP-1 accumulation. Nevertheless, the process was clearly dependent on the E4orf4 protein, since increased accumulation of AP-1 activity was not observed upon infection with *dl*1014, which contains only orf4 in its E4 region (Table 1).

Hypophosphorylation and down regulation of AP-1 DNA-binding activity could not be uncoupled by the use of mutants (Table 1), and the two events occur simultaneously, beginning after 1 h of cAMP treatment. Thus, it seems likely that the hypophosphorylation of some target molecule is responsible for down regulation of AP-1 by the E4orf4 protein. No E4orf4-dependent change in the overall migration or phosphate content of total cellular phosphoproteins was observed, ruling out a global effect on phosphorylation (data not shown). Further, the phosphorylation states of the JunB protein (Fig. 5), the adenovirus E1B 55-kDa protein, and the 24-kDa cap-binding protein (data not shown) were not detectably altered by the E4orf4 protein. Hypophosphorylation has been observed only for the E1A and c-Fos proteins. Although we cannot rule out a key role for an unidentified protein that is hypophosphorylated, this correlation raises the possibility that E4orf4 protein-mediated accumulation of hypophosphorylated E1A and/or c-Fos proteins is directly linked to a reduction in AP-1 DNA-binding activity. Either E1A, which is responsible for the original induction of AP-1 activity, or c-Fos, which is a component of AP-1 activity, might reasonably be expected to play a direct role in the E4orf4-mediated regulatory process.

The ability of E1A proteins to induce AP-1 DNA-binding activity could be influenced by its phosphorylation state. Although it has previously been demonstrated that point mutations within the major phosphorylation sites of E1A protein do not affect its ability to activate transcription (20, 81), it is possible that a minor site or combination of sites not tested is responsible for the effect. Further, as discussed in the introduction, E1A can activate through multiple mechanisms. Loss of an effect on AP-1 might have been masked by activation through other pathways. Thus, it would be premature to rule out a direct role for E1A phosphorylation in the process.

We favor the hypothesis that the change in c-Fos phosphorylation is key to the regulation of AP-1 DNA-binding levels. In fact, c-Fos may contribute to the differential regulation of *junB* and c-*fos* promoter activities we have observed (Fig. 6B). It has previously been demonstrated that c-Fos, as part of AP-1, can activate the expression of a variety of target genes, including the c-*jun* gene (13, 52, 75). c-*fos* can also negatively regulate its own promoter (73, 75, 88), independently of its ability to bind as part of AP-1 to AP-1 sites within the c-*fos* transcriptional control region. Repression is mediated by the serum response element within the c-Fos regulatory region and requires C-terminal amino acids of the c-Fos protein, a domain that is not required to form active AP-1 complexes (26, 42, 51, 70, 88). In vitro transcription studies also indicate that the c-Fos protein contains independent repression and activation domains (1, 2). The c-Fos protein is phosphorylated at multiple sites, some of which are located in the C terminus of the protein (5, 17, 58). It is conceivable that the phosphorylation state of c-Fos differentially influences its repression or activation function. It has recently been demonstrated that point mutations which prevent phosporylation in the C terminus of c-Fos abolish its ability to negatively regulate the c-*fos* promoter (72). Perhaps the E4orf4 protein regulates the phosphorylation status of sites within the transactivation domain of c-Fos but not in the C-terminal domain involved in repression. This could lead to an increased transactivation potential of c-Fos in the absence of E4orf4 protein without an effect on negative autoregulation. As a consequence, in E4orf4 mutant virus infections, c-*fos* transcription would be transiently induced, while expression of *junB* could be enhanced on a continuing basis. In agreement with this hypothesis, a sequence element resembling the AP-1 motif has been identified within the promoter of the human *junB* gene (76).

This model for regulation of c-Fos activity by phosphorylation shows an interesting parallel to the regulation of c-Jun activity. Two different phosphorylation domains have been identified within the c-Jun protein. One of the domains is located adjacent to the DNA-binding domain and, if phosphorylated, prevents DNA binding and transcriptional activation by c-Jun (8). The second domain is contained within the transcriptional activation domain of c-Jun; when phosphorylated, it increases the transactivation potential of c-Jun without an influence on its DNA-binding activity (6). Phosphorylation can be induced by *ras* and may be mediated by MAP-1 kinase (66).

Although we cannot definitively rule out the action of a phosphatase, it seems likely that the accumulation of hypophosphorylated E1A and c-Fos proteins resulted from inhibition of a kinase. This assertion follows from two findings. (i) Hypophosphorylated E1A proteins accumulated rather slowly; fully phosphorylated forms of E1A were still detectable after 1 h of cAMP treatment, although in reduced amounts. This suggests that fully phosphorylated E1A proteins were not actively dephosphorylated but rather turned over within 1 to 2 h owing to their relatively short half-life. (ii) Pulse-chase experiments show that in *wt*300-infected cells both the c-Fos protein and the E1A 289-amino-acid E1A protein were not phosphorylated to completion during the chase period (Fig. 2C and 3D). Partially phosphorylated c-Fos and E1A proteins accumulated during the chase period, suggesting that they may be phosphorylated by several different kinases, one of which is regulated in virus-infected cells. A cellular kinase could be inhibited by direct binding of the E4orf4 protein, which would then serve as a negatively acting regulatory subunit; however, it is equally possible that the viral protein initiates a cascade of events, leading to inhibition by a more indirect mechanism. Alternatively, PKA may play a more direct role in the process than simply participation in induction of the E4orf4 protein. PKA could phosphorylate a kinase to regulate its activity or phosphorylate another protein, which in turn could regulate kinase activity. The adenovirus type 5 E4orf4 protein itself contains a PKA consensus phosphorylation site, suggesting that it is a regulatory molecule activated by PKA. It should be noted, however, that the consensus phosphorylation site is not conserved in the E4orf4 proteins of all adenovirus serotypes.

ACKNOWLEDGMENTS

We thank T. Curran for c-Fos antiserum, R. Bravo for JunB antiserum, N. Sonenberg for antiserum against the 24-kDa mRNA cap-binding protein, E. Harlow for the hybridoma cell line that produces E1A antibodies (M73), K. Ryder and D. Nathans for *junB* plasmid DNA, M. Cole for c-*fos* and c-*myc* plasmid DNAs, and G. Ketner for *dl*1003, *dl*1006, and *dl*1014. We are also grateful to M. Resh for help with two-dimensional gel analysis of phosphotryptic

fragments and R. HydeRuyscher for help with nuclear run-on assays. We thank M. Roberts, R. Javier, and N. Kralli for many discussions and suggestions during the course of this work and R. Weinmann for critical reading of the manuscript.

This work was supported by a grant from the National Cancer Institute (CA38965). U.M. was a graduate student during the course of this work at the Universität zu Köln and received fellowship support from the Fritz Thyssen Stiftung and the Deutscher Akademischer Austauschdienst while performing this work at Princeton University. T.K. received fellowship support from EMBO, and T.S. is an American Cancer Society Professor and an Investigator of the Howard Hughes Medical Institute.

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