Phosphorylation within the Transactivation Domain of Adenovirus E1A Protein by Mitogen-Activated Protein Kinase Regulates Expression of Early Region 4

STEVE G. WHALEN,¹ RICHARD C. MARCELLUS,¹ ANNE WHALEN,² NATALIE G. AHN,²
ROBERT P. RICCIARDI,³ AND PHILIP E. BRANTON^{1,4*}

*Departments of Biochemistry¹ and Oncology,⁴ McGill University, Montreal, Quebec H3G 1Y6, Canada;
Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, University of Colorado,
Boulder, Colorado 80309²; and Department of Microbiology, School of Dental Medicine,
University of Pennsylvania, Philadelphia, Pennsylvania 19104³*

Received 14 August 1996/Accepted 14 February 1997

A critical role of the 289-residue (289R) E1A protein of human adenovirus type 5 during productive infection is to transactivate expression of all early viral transcription. Sequences within and proximal to conserved region 3 (CR3) promote expression of these viral genes through interactions with a variety of transcription factors requiring the zinc binding motif in CR3 and in some cases a region at the carboxy-terminal end of CR3, including residues 183 to 188. It is known that 3',5' cyclic AMP (cAMP) reduces the level of phosphorylation of the 289R E1A protein through the activation of protein phosphatase 2A by the E4orf4 protein. This study was designed to identify the E1A phosphorylation sites affected by E4orf4 expression and to determine their importance in regulation of E1A activity. We report here that two previously unidentified sites at Ser-185 and Ser-188 are the targets for decreased phosphorylation in response to cAMP. At least one of these sites, presumably Ser-185, is phosphorylated *in vitro* by purified mitogen-activated protein kinase (MAPK), and both are hyperphosphorylated in cells which express a constitutively active form of MAPK kinase. Analysis of E1A-mediated transactivation activity indicated that elevated phosphorylation at these sites increased expression of the E4 promoter but not that of E3. We have recently shown that one or more E4 products induce cell death due to p53-independent apoptosis, and thus it seems likely that one role of the E4orf4 protein is to limit production of toxic E4 products by limiting expression of the E4 promoter.

The 289-residue (289R) product of the 13S mRNA from early region 1A (E1A) of human adenovirus type 5 (Ad5) has been shown to transactivate both viral and cellular genes (for reviews, see references 8, 20, and 57). At least two independent, but not mutually exclusive, mechanisms exist by which E1A products enhance gene expression. One mechanism involves activation of E2F transcription factors through complex formation with the retinoblastoma tumor suppressor family of proteins (reviewed in reference 52). Such interactions, which involve conserved regions 1 and 2 (CR1 and CR2) of the E1A protein (see Fig. 1), are important both in the activation of viral early region 2 (E2) and in the induction of cellular S-phase genes, which results in unscheduled DNA synthesis and cell transformation (reviewed in references 5 and 52). The other mechanism is involved in transactivation of E3 and E4, and to some extent E2, and requires residues 140 to 185, which comprise CR3, as well as residues 186 to 188 (1, 8, 20, 36, 57, 63). CR3 is absent in the otherwise identical 243R product of the 12S E1A mRNA (see Fig. 1), and thus while the 243R protein can enhance E2 expression, it cannot transactivate the E3 and E4 promoters. Residues 147 to 177 contain a zinc finger motif which interacts with TATA box binding protein (TBP) of the basic transcription initiation complex (23, 35), as well as with the TBP-associated factor TAF_{II}110 (46). A region including residues 183 to 188 binds to several unrelated tran-

scription factors, including ATF-2, thus tethering E1A proteins to the promoter and enhancing interactions with TBP (36, 38, 39, 49, 54, 63). Genetic analysis of the E1A transactivation domain identified the four Cys residues within the zinc finger as essential but also showed that none of the residues between amino acids 183 and 188 could be altered even by highly conservative substitutions (24, 63).

Previous studies with mouse S49 lymphoma cells, which are unable to synthesize 3',5' cyclic AMP (cAMP), showed that expression of E1A in the presence of exogenous dibutyryl cAMP (Bt₂cAMP) causes an increase in expression of *c-fos*, E1A, and other early viral genes and a rise in AP-1 transcription factor activity (17, 51). The increase in *c-fos* expression has been linked to regions at the amino termini and in CR1 of both the 289R and 243R E1A proteins, which are required for complex formation with p300 and/or related proteins (18, 23). It is currently believed that such interactions in the presence of cAMP cause the disruption of an ATF/CREB-YY1 repressor complex and the increased expression of the *c-fos* promoter (21, 22, 69, 70). Somewhat later in these cells a decrease in the phosphorylation of both c-Fos and the 289R E1A protein has been observed. Such hypophosphorylation was shown to be caused by E1A transactivation of E4 and specifically by expression of the E4orf4 protein (50). In the presence of E4orf4, the 289R E1A protein is labeled by [³²P]orthophosphate at lower levels and exhibits increased gel mobility in keeping with decreased phosphorylation (50). Mutants defective in E4orf4 exhibit both increased AP-1 activity and heightened cytotoxicity (50). E4orf4 was shown to bind to and activate protein phosphatase 2A (PP2A); however, the kinetics of hypophosphorylation of c-Fos and the 289R E1A protein suggested to Klein-

* Corresponding author. Mailing address: Departments of Biochemistry and Oncology, McGill University, McIntyre Medical Sciences Building, 3655 Drummond St., Montreal, Quebec H3G 1Y6, Canada. Phone: (514) 398-7263. Fax: (514) 398-7384. E-mail: branton@medcor.mcgill.ca.

berger and Shenk (34) and Müller et al. (50) that their hypophosphorylation occurred through the PP2A-induced inactivation of a protein kinase, rather than by PP2A directly. It was suggested that mitogen-activated protein kinase (MAPK) is a likely candidate, an idea that seems reasonable as the MAPK signaling pathway is known to be inhibited by cAMP in some cell lines (11, 41, 67). Thus, either directly or indirectly, MAPK may regulate phosphorylation of E1A proteins. The sites of E1A hypophosphorylation in response to E4orf4 remained unidentified.

Previous work by our group and others has identified several sites of phosphorylation on E1A proteins. Major sites were mapped to Ser-219 (60–62) and Ser-89 (60, 61) and shown to be substrates for p34^{cdc2} or a related protein kinase (13), possibly Cdk2, which is present in E1A complexes containing the pRB-related proteins p107 and p130 and cyclin A or E (4, 42). Another phosphorylation site, which is a substrate for casein kinase II, has recently been identified at Ser-132 (66). That study also suggested that an additional site may be present in the tryptic phosphopeptide from the 243R E1A protein, which contains Ser-132, probably at Ser-188. Ser-96 was believed to be a minor phosphorylation site (14, 60, 61), and there was circumstantial evidence that multiple serines between residues 227 and 237 may also be phosphorylated (61). However, recent biochemical and genetic analyses have failed to yield any direct evidence that either of these possibilities is highly likely (65).

The functional importance of E1A protein phosphorylation remains uncertain. No biological effect was noted with a mutant containing a substitution at Ser-219 (62). Phosphorylation of Ser-89 is largely responsible for the major shift in gel mobilities of both the 289R and the 243R proteins (15, 55, 58, 68) and somewhat increases the efficiency of E1A-mediated cell transformation (14) and interactions with pRB and related proteins (3, 42). Phosphorylation of Ser-132 appears to enhance complex formation somewhat with pRB and related proteins, and in the case of the 289R E1A protein only, seems to reduce cytotoxicity, which leads to a substantial increase in cell transformation efficiency (66).

In this report we show that Ser-185 and Ser-188 within the E1A transactivation domain are phosphorylated, apparently via MAPK, and that they are the targets of hypophosphorylation in response to cAMP and E4orf4. Furthermore, we show that phosphorylation of these sites regulates transactivation of the E4 promoter but not that of E3. The fact that the E4 region encodes one or more products that induce p53-independent apoptosis (45, 59) suggests that by regulating phosphorylation at these sites, E4orf4 may autoregulate expression of these toxic products.

MATERIALS AND METHODS

Cells. HeLa and CHO cells were cultured on 60-mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in alpha minimal essential medium (alpha-MEM) supplemented with 10% fetal calf serum and in Dulbecco's MEM (DMEM) supplemented with 10% heat-inactivated horse serum, respectively. S49 cells were grown in suspension with DMEM and 10% heat-inactivated horse serum. Ad5-transformed human 293 cells that express E1A and E1B proteins (28) were cultured under conditions similar to those for HeLa cells.

Viruses. Cells were infected with wild-type (wt) or mutant Ad5 at 35 PFU, as described previously (56). Mutant and wt viruses were plaque purified three times, grown, and titrated on 293 cells. Viruses used in this study included Ad5 wt dl309 (33); *hr5* (32), which contains a Ser-185-to-Asn (S185N) substitution in the 289R E1A protein and a G139D substitution in the 243R E1A protein (24); *pm975*, which produces 13S but not 12S E1A mRNA and thus the 289R but not the 243R protein (49); and *dl520*, which expresses 12S but not 13S E1A mRNA and thus the 243R but not the 289R protein (30).

Construction of additional viral mutants. DNA from plasmid pSK(-)E1A, which contains a cDNA corresponding to the Ad5 E1A 13S mRNA and carries either wt sequences or sequences harboring S185G, S185T, or S188T substitutions (63), was digested with *Xma*I and *Xba*I and cloned into the pXC38 vector,

which expresses the entire E1A and E1B regions of Ad5 (48). The resulting plasmids express only the 289R E1A protein and have been designated pXC13 (wt), pXC13/S185G, pXC13/S185T, and pXC13/S188T. pXC13/S185G was used as a template for PCR mutagenesis to change the codon for Ser-188 to Asp, producing the double-point mutant pXC13/S185G/S188D. The entire coding region for the transactivation domain was sequenced in all plasmids. Mutations were rescued into virus which expresses only 13S E1A mRNA by the method of McGrory et al. (47).

Radioactive labeling. Ad5-infected S49 cells were labeled from 20 to 24 h postinfection (p.i.) with [³²P]orthophosphate (3,000 Ci/mmol; New England Nuclear) in 2.5 ml of phosphate-free medium. In some cases Bt₂cAMP (final concentration, 1 mM) was added at 22 h p.i., and all cells were harvested at 24 h p.i. In experiments in which 293 cells were electroporated with the mutant plasmid constructs, cells were labeled from 14 to 18 h following treatment.

Analysis of tryptic peptides by TLC. ³²P-labeled E1A proteins were purified from cell extracts by immunoprecipitation with M73 mouse anti-E1A monoclonal antibodies (31). E1A products were purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and they were eluted, oxidized, and digested with 20 mg of trypsin (Worthington) for 3 h, followed by addition of a further 20 mg of trypsin and incubation for an additional 3 h, as previously described (61). Tryptic phosphopeptides were analyzed by thin-layer chromatography (TLC) with Polygram CEL 300 plates, as previously described (15).

In vitro phosphorylation of E1A proteins by purified MAPK. The 289R E1A protein (5 µg) produced in and purified from *Escherichia coli* (16, 19) was resuspended in kinase buffer (12.5 mM MOPS [morpholinopropanesulfonic acid, pH 7.2] containing 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM MgCl₂, 0.05 mM sodium phosphate, and 2 mM [final concentration] dithiothreitol) containing 50 mCi of [γ-³²P]ATP (10.0 mCi/ml, specific activity, 3,000 Ci/mmol; New England Nuclear) and 0.2 mg of purified MAPK (a kind gift from Steve Pellech) at 30°C for 5 min, and the reaction was terminated by the addition of an equal volume of double-strength SDS-PAGE sample buffer. Tryptic peptides were prepared as described above.

Analysis of E1A proteins by Western blotting. Nuclear extracts were prepared from cells which were harvested, washed three times in ice-cold phosphate-buffered saline, and lysed in 2.5 cell volumes of lysis buffer (10 mM Tris-HCl [pH 7.5] containing 150 mM NaCl, 1 mM MgCl₂, and 0.08% Triton X-100 [vol/vol]). Nuclei were separated by centrifugation at 0°C for 5 min, washed once, and then lysed in 2 volumes of extraction buffer C (20 mM HEPES [pH 7.9] containing 25% glycerol [vol/vol], 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and incubated for 90 min on ice. Debris was removed by centrifugation, and the supernatant containing the nuclear extract was aliquoted and stored at -80°C until use. Equal amounts of extract were separated by SDS-PAGE, and the material was transferred to nitrocellulose membranes with transfer buffer (48 mM Tris-HCl [pH 7.2] containing 39 mM glycine, 0.03% SDS, and 20% [vol/vol] methanol) at 4°C. Enhanced chemiluminescence immunoblotting with M73 anti-E1A antibodies was performed under the conditions recommended by the manufacturer (Amersham).

MAPKK expression. Plasmid DNAs containing cDNAs encoding hemagglutinin antigen (HA)-tagged wt MAPK kinase (MAPKK) or mutant forms ΔN3-S218E-S222D and K97M were introduced into 293 cells by electroporation, as previously described (43). Expression of MAPKK protein was confirmed by Western blot analysis with the anti-HA-tag antibody 12CA5 (data not shown). The ΔN3-S218E-S222D mutant form of MAPKK was shown to increase MAPKK activity about 400-fold and to enhance MAPK activity by at least 60-fold, whereas the K97M form was found to be catalytically inactive (43). MAPKK-expressing cells were either used to prepare nuclear extracts for Western blot analysis or labeled with [³²P]orthophosphate, and E1A proteins from equal amounts of total cell extract were purified by immunoprecipitation and SDS-PAGE and subjected to tryptic phosphopeptide analysis by TLC.

Measurement of E1A-mediated transactivation of the Ad5 E4 promoter. Transactivation assays were performed with CHO cells plated at a density of 2 × 10⁵ cells on 60-mm-diameter dishes. The E4 reporter plasmid was E4-CAT, which contains the E4 promoter upstream of the chloramphenicol acetyltransferase (CAT) gene (64). Transient cotransfections were performed by the calcium phosphate precipitation method (29) with 2.5 µg of reporter plasmid DNA and 2.5 µg of DNA from plasmids expressing wt or mutant E1A products. The plasmid pSV2CAT (28) was used as a positive control. In addition, 3 µg of plasmid RSVβ-Gal (53) was included to allow normalization of transfection efficiency by measuring β-galactosidase activity. Cells were glycerol shocked after 12 h and then harvested 36 h later. CAT assays were performed with cell extracts exhibiting equal levels of β-galactosidase activity, essentially as described previously (27). The levels of activity were quantified from TLC plates with a Fujix Bas 2000 phosphorimager.

RESULTS

E1A protein phosphorylation in mouse S49 cells in the presence and absence of cAMP. Previous studies carried out for the most part by our group have identified three major phosphor-

FIG. 1. Phosphorylation sites in Ad5 E1A proteins. The Ad5 289R and 243R E1A proteins are illustrated, and the positions of CR1, CR2, CR3, ar1, and ar2 (see the text) and the sites of phosphorylation (P) have been noted. N and C termini are marked. Presented below is the amino acid sequence of tryptic peptide T8 (residues 178 to 205), which is found only in the 289R protein. Ser-188 in the 243R protein is located in peptide T4B (residues 106 to 139 fused to the residues corresponding to 188 to 205). Ser-185 and Ser-188 sites and the residues within T8 that were shown to be essential for transactivation (63) have been indicated.

ylation sites at serine residues 89, 132, and 219 in E1A proteins synthesized in Ad5-infected KB or HeLa cells (Fig. 1). In our original study (61), the predicted E1A tryptic peptides were numbered in order from the amino terminus. Ser-89 lies in the tryptic peptide designated T2 (residues 3 to 97), and Ser-219 lies in peptide T11 (residues 217 to 223). T2 migrates as multiple species by TLC, due almost certainly to inefficient trypsin cleavage caused by the proximity of several proline residues (15, 60, 65). T11 is the most prominent phosphopeptide and is the only species that migrates towards the cathode on TLC plates. Ser-132 lies within a tryptic peptide T4A in the 289R E1A protein (residues 106 to 155). Due to differential splicing of the 12S mRNA, Ser-132 from the 243R E1A protein is present in a peptide termed T4B, which is comprised of residues 106 to 139 fused to a sequence corresponding to amino acids 186 to 205 of the 289R protein. Recent studies indicated that whereas T4A migrates as a single spot by TLC, T4B is present as multiple species (66). Although partial proteolysis again may account for some of this heterogeneity, genetic analysis implied the presence of one or more additional sites within T4B, probably including Ser-188 (66).

To analyze the basis of E1A hypophosphorylation in response to cAMP, mouse S49 lymphoma cells were infected with wt Ad5 in the presence or absence of Bt₂cAMP. E1A proteins were either examined directly by SDS-PAGE and Western blotting or labeled in vivo with [³²P]orthophosphate, and tryptic peptides were prepared and analyzed by TLC. Figure 2A shows that addition of cAMP caused a clear shift in the mobility of the 289R E1A protein from slower- to faster-migrating species. Such was not the case in a similar analysis with *dl520*, which produces only the 243R E1A protein (Fig. 2A). These results were identical to those of a previous study and reflected an increased gel mobility caused by hypophosphorylation in response to cAMP (50). Figure 2B shows an analysis of E1A phosphopeptides from wt-infected S49 cells in the absence of Bt₂cAMP and indicated the presence of tryptic phosphopeptides observed previously, including T11 (Ser-219), multiple T2 species (Ser-89), T4A from the 289R protein (Ser-132), and T4B from the 243R protein (Ser-132 and probably Ser-188). However, additional species, labeled a, b, and c in Fig. 2B, which had never been prominent in our analyses of E1A proteins from infected KB cells were also apparent. Fig-

FIG. 2. Analysis of E1A proteins in S49 cells in the presence and absence of cAMP. Mouse S49 cells were infected with wt *dl309*, *dl520*, or mutant *hr5*, and some cultures were treated with Bt₂cAMP prior to either labeling or cell lysis. (A) Equal amounts of nuclear extract from cultures either treated (+) or untreated (-) with Bt₂cAMP (cAMP) were separated by SDS-PAGE and analyzed by Western blotting with M73 anti-E1A antibody. Contents of the lanes are as indicated in the figure. (B to E) Extracts from infected cells labeled with [³²P]orthophosphate were precipitated with M73 antibody, E1A proteins were separated by SDS-PAGE and digested with trypsin, and tryptic peptides derived from equal amounts of material were separated by TLC, as described in Materials and Methods. Peptides T11, T2, and T4A (see the text) have been indicated as have three novel T8 phosphopeptides (a, b, and c [marked with arrowheads]). Electrophoresis was from right to left, and ascending chromatography was from bottom to top. Panels contain wt *dl309* (B), wt *dl309* plus cAMP (C), *hr5* (D), and *hr5* plus cAMP (E).

ure 2C shows that addition of Bt₂cAMP had little effect on the levels of any of the E1A tryptic phosphopeptides apart from the novel species. (The apparent increase in peptide T4A in Fig. 2C was not observed in repeated analysis.) Thus, peptides a, b, and c appeared to be the targets of cAMP-induced E4orf4-mediated hypophosphorylation in these cells.

In attempting to interpret these results and those of Müller et al. (50), it was apparent that E1A hypophosphorylation must involve novel sites phosphorylated in S49 cells but much less so in human KB or HeLa cells. Such sites might be located within CR3 because the 289R E1A protein but not the 243R E1A protein appeared to be affected. As suggested previously, it seemed possible that such sites are substrates of MAPK (34, 50). CR3 contains potential sites at Ser-156, Thr-164, Ser-172, Thr-178, and Ser-185; however, only Ser-185 is located within a sequence characteristic of a MAPK site (11) (Fig. 1). Mutant *hr5* is one of a series of host-range mutants isolated almost 20 years ago by Harrison et al. (32) and shown to contain an

FIG. 3. Analysis of Ser-185 and -188 mutant E1A proteins in S49 cells in the presence and absence of cAMP. The method used was similar to that described in the legend to Fig. 2 and employed *pm975* or a mutant affecting Ser-185 (*pm975/S185G*) or both Ser-185 and Ser-188 (*pm975/S185G/S188D*). (A) Western blot analysis with M73 anti-E1A antibodies of equal amounts of extracts from cells treated (+) or not treated (–) with Bt_2cAMP (cAMP); (B to F) analysis of ^{32}P -labeled E1A tryptic phosphopeptides from equal amounts of Bt_2cAMP -treated and untreated infected cells. (B) Mock-infected cells (material in the positions of E1A proteins was analyzed); (C) *pm975*; (D) *pm975* plus cAMP; (E) *pm975/S185G*; (F) *pm975/S185G/S188D*. Phosphopeptides a, b, and c are marked with arrowheads.

S185N substitution (24). Analysis of E1A products from *hr5*-infected S49 cells indicated no change in migration by SDS-PAGE following addition of Bt_2cAMP (Fig. 2A) and the complete absence of the novel tryptic phosphopeptides observed with the wt (compare Fig. 2D and E). These results strongly suggested that Ser-185 is phosphorylated in S49 cells and is the target of hypophosphorylation in response to cAMP.

Phosphorylation of both Ser-185 and Ser-188 is regulated by cAMP. To assess the role of Ser-185 in the E1A 289R protein further, mutations were introduced into *pm975*, which expresses the 289R but not the 243R protein (49). Mutations included those affecting Ser-185 and nearby Ser-188, the latter of which had been suggested as a potential site in other studies (66). Ser-185 is present on tryptic phosphopeptide T8 from the 289R protein (Fig. 1), whereas Ser-188 exists on peptide T8 from the 289R protein and on peptide T4B from the 243R protein. E1A proteins produced by these mutants in infected S49 cells were studied in the presence and absence of exogenous Bt_2cAMP . Figure 3A shows that in the absence of cAMP, wt *pm975* yielded a major form of the 289R E1A protein, which appeared as two closely migrating species. Addition of Bt_2cAMP induced the loss of the upper “supershifted” species. These results were similar to those shown in Fig. 2A with wt Ad5, except that here the effect on the 289R protein was clearer as no 243R protein species were present. Figure 3A shows that with mutants *pm975/S185G* and *pm975/S185G/S188D*, only a single E1A species was present with no super-

shifted form and that addition of Bt_2cAMP had little effect, thus confirming at least Ser-185 as a phosphorylation site. It was noted that substitution of a Gly residue at Ser-185 caused a change in gel migration of the 289R protein that appeared to be unrelated to phosphorylation. It is possible that this shift resulted from a direct effect on the E1A protein conformation which altered gel mobility in SDS-PAGE analysis (see below and Discussion). To examine the phosphorylation pattern directly, tryptic phosphopeptides derived from mutant and wt E1A proteins were examined by TLC. A problem which made such analyses of E1A proteins from S49 cells difficult was the presence of phosphopeptides from a contaminating cellular protein which was present nonspecifically and to various degrees in all immunoprecipitates prepared with E1A-specific M73 monoclonal antibodies. Figure 3B shows the pattern obtained by analyzing material from precipitates of mock-infected cells which migrated in the region of the gel normally occupied by E1A products. We observed a complex set of phosphopeptides which were also present at various levels in all other samples (Fig. 3C to F), including those prepared from mock-infected cells treated with Bt_2cAMP (data not shown). We made serious efforts to eliminate this material but were largely unsuccessful in repeatedly duplicating the low background shown in Fig. 2D to E. Nevertheless, as seen previously in Fig. 2B, Fig. 3C does show that the wt 289R protein yielded the three novel a, b, and c phosphopeptides in addition to the well-studied T11, T2, and T4A tryptic phosphopeptides and

the background material. It was noted that the c species was somewhat variable from experiment to experiment. Figure 3D again shows that these novel peptides, but not T11, T2, and T4A, were eliminated by treatment with Bt_2cAMP . Analysis of E1A products from mutant *pm975/S185G* indicated that the a peptide was absent, again suggesting Ser-185 as a site of phosphorylation. However, analysis of E1A peptides from mutant *pm975/S185G/S188D* (Fig. 3F) indicated the complete absence of all three novel peptides, as was the case with E1A products from Bt_2cAMP -treated mutant-infected cells (data not shown). These results suggested that both Ser-185 and Ser-188 from tryptic peptide T8 were phosphorylated. The presence of three species originating from the T8 peptide may reflect incomplete proteolysis, differential phosphorylation, or both (see below).

An additional point should be made equating phosphorylation and the formation of the supershifted form of the 289R E1A protein. Phosphorylation of the a, b, and c peptides shown in Fig. 3C was relatively minor compared to that of peptide T11 harboring the major Ser-219 site. However, the supershifted form of the 289R protein was present in about equimolar amounts with the faster-migrating unshifted species (Fig. 3A, first lane). These results suggested that transient phosphorylation may be sufficient to induce a change in gel mobility (see Discussion).

The 289R E1A protein is phosphorylated in vitro by MAPK.

As discussed above, Ser-185 lies within a classic MAPK substrate consensus sequence; however, Ser-188 does not (10). To determine directly if either of these sites is phosphorylated by MAPK, the 289R E1A protein synthesized in and purified from *E. coli* was incubated in vitro with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and purified MAPK. Tryptic peptides were prepared, and the pattern obtained by TLC was compared with that of the 289R protein labeled in vivo with $[\text{}^{32}\text{P}]\text{orthophosphate}$. Figure 4B shows that MAPK phosphorylated several peptides in vitro. Both T11 and T2 were labeled, and this result was perhaps expected as these sites are phosphorylated by a Cdk-type kinase which has a substrate specificity very similar to that of MAPK. Previous studies using purified $\text{p34}^{\text{cdc}2}$ or Cdk2 present in E1A-p107-cyclin A complexes showed that only T11 and T2 were phosphorylated in vitro by this class of enzyme (4, 13). However, with MAPK at least two additional peptides were phosphorylated. One migrated in the position of peptide b, which originates from T8, as confirmed in a mixing experiment (Fig. 4C) in which a sample containing E1A phosphopeptides labeled in vivo (Fig. 4A) was combined with those labeled by MAPK in vitro. All three T8 peptides (a, b, and c) were evident in both samples; however, the radioactivity present in peptide b in the mixture was clearly increased (Fig. 4C). The origin of the second major species labeled by MAPK (Fig. 4B) is unclear. The mixing experiment (Fig. 4C) clearly indicated that it does not correspond to either peptide a or c. We believe that it represents a larger form of the T8 peptide which was produced by partial trypsin digestion, as samples prepared from cell extracts contain only very small quantities of E1A protein whereas large amounts of the bacterially produced material were used. Nevertheless these results confirmed that MAPK is able to phosphorylate the T8 peptide, apparently at only one site, presumably Ser-185.

Phosphorylation of the 289R E1A protein in 293 cells expressing a constitutively active MAPKK. While activation of PP2A by E4orf4 may play a direct role in the inactivation of MAPK activity and thus the decreased phosphorylation of Ser-185, it is likely that the synergy provided by cAMP rests with its negative regulatory role upstream in the MAPK signaling pathway. We therefore carried out experiments in which an attempt was made to enhance the phosphorylation of E1A proteins by

FIG. 4. Analysis by TLC of tryptic phosphopeptides from 289R E1A products labeled in vitro with purified MAPK. The 289R E1A protein (5 mg) synthesized in and purified from *E. coli* was incubated in vitro with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and purified MAPK, and the tryptic phosphopeptide TLC pattern was compared with that of E1A products of *pm975*, which had been labeled in vivo in S49 cells as described for Fig. 3C. (A) 289R protein labeled in vivo; (B) 289R protein labeled in vitro by MAPK; (C) mixture of in vivo- and in vitro-labeled samples. Phosphopeptides a, b, and c, are marked with arrowheads.

expressing a constitutively active form of MAPKK, which should result in increased MAPK activity. Such transfection experiments were not practical in lytically infected HeLa cells, and thus Ad5-transformed human 293 embryonic kidney cells, which express both the 289R and the 243R E1A proteins constitutively, were employed (28). 293 cells were transfected with DNA from a plasmid expressing a constitutively active HA-tagged form of MAPKK, termed $\Delta\text{N3-S218E-S222D}$, which has been shown to increase MAPK activity by about 60-fold (43). As a control, an inactive HA-tagged MAPKK mutant, termed K97M, was also introduced into some cells (43). Expression of both MAPKK proteins at high levels was confirmed by Western blot analysis with the anti-HA-tag antibody 12CA5 (data not shown). Figure 5A shows the results of Western blot analysis with anti-E1A M73 antibodies to characterize the pattern of E1A proteins. Expression of the active $\Delta\text{N3-S218E-S222D}$ form of MAPKK caused a shift in migration of the 289R E1A protein compared to the pattern observed in cells expressing the inactive K97M form. We also carried out an experiment in which E1A proteins were labeled in these cells in vivo with $[\text{}^{32}\text{P}]\text{orthophosphate}$ and then immunoprecipitated with M73 antibody and analyzed by SDS-PAGE. Figure 5B shows that in cells expressing the active $\Delta\text{N3-S218E-S222D}$ form of MAPKK, the level of labeling was

FIG. 5. Analysis of E1A proteins in 293 cells following ectopic expression of MAPKK. (A) 293 cells were transfected with plasmid DNA encoding HA-tagged catalytically inactive (K97M) or constitutively active (Δ N3-S218E-S222D) MAPKK, and equal amounts of cell extracts were analyzed by Western blotting with M73 anti-E1A antibodies. (B) An experiment similar to that described for panel A was performed, except that cells were labeled with [32 P]orthophosphate and E1A products were immunoprecipitated with M73 antibodies and analyzed by SDS-PAGE.

enhanced and the pattern of E1A products was compatible with that shown by Western blot analysis, although individual E1A protein species were less well resolved.

To determine what phosphorylation site(s) was affected by expression of MAPKK, 32 P-labeled 289R E1A protein was excised from gels such as that shown in Fig. 5B and digested with trypsin and the resulting phosphopeptides were separated by TLC. Figure 6 shows the patterns obtained. In the case of 293 cells transfected with plasmid DNA expressing the inactive K97M form of MAPKK (Fig. 6A), the pattern was very similar to those presented earlier. In particular, the a, b, and c species corresponding to tryptic peptide T8 were labeled only weakly if at all. However, in the case of cells expressing the active Δ N3-S218E-S222D form of MAPKK (Fig. 6B), the a and b phosphopeptides were preferentially labeled with 32 P. It is unclear why the c peptide was unlabeled, although as indicated above, it was present to various degrees from experiment to experiment with S49 cells. These results clearly indicated that increasing MAPKK leads to an increase in phosphorylation of the Ser-185 and -188 sites *in vivo*.

Increased phosphorylation of Ser-185 and -188 correlates with increased transactivation of the E4 but not the E3 promoter. If phosphorylation at Ser-185 and -188 is of any functional importance, it appears most likely that it functions in the regulation of E1A-mediated transactivation, as the sites lie within the region known to interact with certain transcription factors. Prior genetic analysis had shown that point mutations

or deletions affecting either Ser-185 or Ser-188 abolished E1A transactivation activity entirely (24, 25, 36, 37, 39, 40, 63). Even conversion of these Ser sites to Thr residues, which we have found are still phosphorylated at least to some degree (data not shown), resulted in the complete loss of transactivation activity (63). In addition, site-specific mutations of residues surrounding this region (Gly-180, Val-183, Tyr-184, Pro-186, and Val-187) also abolished activity (63). These results indicated that this region of the E1A molecule is particularly sensitive to changes in primary and thus possibly tertiary structures. Thus, it was not possible to employ a genetic approach in assessing the role of Ser-185 and -188 phosphorylation. In order to determine if phosphorylation affects E1A transactivation activity, we took advantage of the fact that phosphorylation of Ser-185 and Ser-188 could be increased by expression of MAPKK. Studies of 293 cells could not be conducted because it was not possible to assess gene expression in the absence of E1A products. Thus, CHO cells were transfected with constructs expressing either wt MAPKK, the constitutively active Δ N3-S218E-S222D form, or the inactive K97K form, as well as plasmid DNA expressing the 289R E1A protein along with reporter plasmids containing CAT linked to either the Ad5 E3 or E4 promoter. Figure 7 shows that with both E3-CAT and E4-CAT, CAT activity was relatively unaffected by expression of any of the MAPKK constructs in the absence of E1A. These results indicated that phosphorylation of transcription factors or transcription machinery as a result of the introduction of MAPKK had little direct effect on transcriptional activity. E3 and E4 expression was differentially affected by coexpression of E1A. In the case of E3-CAT, CAT activity was increased by expression of the 289R E1A protein, but addition of wt MAPKK or the constitutively active or inactive MAPKK mutant had no further effect. These results indicated that E3 expression was unaffected by increased phosphorylation of the 289R E1A protein at Ser-185 or -188. E1A also increased expression of E4, but MAPKK augmented the levels further. Expression of wt MAPKK increased CAT activity by an average of about 50%, and expression of the constitutively active Δ N3-S218E-S222D (R4F in Fig. 7) form increased CAT activity on the average by about 80%. Expression of the catalytically inactive K97M (8e in Fig. 7) form had no stimulatory effect. While these reproducible increases may be seen as somewhat modest, it should be remembered that the level of increase in Ser-185 and -188 phosphorylation may not have been as great as that seen in 293 cells in Fig. 6. In addition, the degree of phosphorylation of the a and b peptides observed in the labeling experiment in Fig. 6 may not accurately reflect the overall level of phosphorylation of these sites in the total E1A protein. Thus, these results suggested that phosphorylation at Ser-185 and -188 regulates E4 expression but not that of E3.

FIG. 6. Analysis of E1A tryptic phosphopeptides in 293 cells expressing MAPKK. 293 cells were transfected with plasmid DNA encoding HA-tagged catalytically inactive (K97M) or constitutively active (Δ N3-S218E-S222D) MAPKK and then labeled with [32 P]orthophosphate as described for Fig. 5B. E1A tryptic peptides were prepared and analyzed by TLC. (A) K97M-expressing cells; (B) Δ N3-S218E-S222D-expressing cells (arrowheads indicate the a and b phosphopeptides).

DISCUSSION

In this report we have identified two new sites of E1A protein phosphorylation in tryptic peptide T8 located at Ser-185 and Ser-188 at the carboxy-terminal end of the E1A transactivation domain. Both of these sites have been conserved in other adenovirus serotypes, including Ad3, Ad12, Ad7, Ad40, and AD4, and thus may be of general importance in adenovirus replication. Two or three forms of the T8 peptide, termed peptides a, b, and c, were observed. The a and b peptides migrated in an ascending diagonal towards the cathode in a pattern characteristic for phosphorylation at two sites on the same peptide (7). Thus, peptide b contains one and peptide a contains two phosphates. However, a third peptide, c, which migrated directly below peptide b, was also frequently ob-

FIG. 7. Analysis of E3 and E4 promoter activities in cells expressing E1A proteins and MAPKK. CHO cells were transfected with plasmid DNA encoding the 289R E1A protein, CAT under either the Ad5 E3 or the Ad5 E4 promoter, and either the inactive (K97M [8e]), constitutively active (Δ N3-S218E-S222D [R4F]), or wt form of HA-tagged MAPKK. Cell extracts were assayed for CAT activity, and the activity was plotted as a percentage of that obtained with the wt. Seven independent assays were performed for each mutant, and the standard error for each has been indicated with a T bar. A map of factor binding sites has been included for each promoter.

served. Although this peptide may arise as a phosphoisomer resulting from phosphorylation at another site within T8, possibly Thr-204, it is much more likely the result of partial proteolysis caused by the close proximity of several Pro residues. Previous studies showed that phosphorylation at Ser-89 is responsible for a major shift in gel migration, thus generating two major forms of the 289R E1A protein, termed 52K and 48.5K, and two 243R E1A protein forms, termed 50K and 45K (15, 55, 58, 68). The slower-migrating species generally predominate (as in Fig. 2A and 3A) but can be converted entirely to the faster-migrating forms by treatment with potato acid phosphatase. We show here that phosphorylation at Ser-185 and -188 causes a smaller supershift in gel migration of the 289R protein 52K species. As pointed out above, the level of 32 P labeling of the T8 peptide containing these sites was considerably lower than that of the major site at Ser-219 in tryptic peptide T11. It is possible that the Ser-185 and -188 sites are particularly susceptible to dephosphorylation by protein phosphatases during cell lysis or preparation of cell extracts. It is also possible

that phosphorylation induces a conformational change in the 289R E1A protein that is responsible for the supershift in SDS-PAGE analysis and that persists even following dephosphorylation. Further studies will be required to distinguish between these possibilities.

A previous report by Müller et al. (50) showed that phosphorylation of the 289R E1A protein is reduced when Ad5-infected S49 cells are treated with cAMP. We show here that Ser-185 and Ser-188 are the E1A phosphorylation sites regulated by cAMP. This regulation has been shown to require the E4orf4 protein, which binds to and activates PP2A. It had been proposed that hypophosphorylation of the 289R E1A protein does not result directly from the action of PP2A but rather appears to result indirectly from the inhibition of a protein kinase by PP2A (34). Both MAPK and its regulator MAPKK can be regulated by PP2A (2, 26). Our data showing that purified MAPK can phosphorylate an appropriate site in vitro and that overexpression of MAPKK increases Ser-185 and -188 phosphorylation convincingly links MAPK to E1A phosphor-

ylation. These results differ from those in a recent report which showed that E1A proteins were not labeled in vitro by MAPK (42). However, that study was conducted with E1A products incubated with immunoprecipitates containing MAPK rather than with purified MAPK. Ser-185 lies in a classic MAPK consensus sequence (10). Although Ser-188 does not have an immediately adjacent Pro residue, as is usually the case, other atypical MAPK sites have been reported to be phosphorylated at low efficiency by MAPK, at least in vitro (10). In addition, it is possible that the unusual (Glu-Pro)₆ repeat just downstream of Ser-188 may enhance recognition of the site by MAPK. It should be noted, however, that only one residue appeared to be phosphorylated in vitro by purified MAPK. Thus, it appears more likely that Ser-188 is phosphorylated by another kinase which may be regulated by MAPK.

The major question which emerged in this study was does phosphorylation at Ser-185 and -188 have any functional significance? This question was difficult to answer because mutations affecting either Ser-185 or Ser-188 had previously been shown to decrease dramatically the ability of E1A proteins to transactivate all promoters. However, mutants with defects at other nearby residues, which presumably did not all affect phosphorylation, were also defective (63). This region in E1A molecules therefore appears to be particularly sensitive to changes in primary structure. Thus, it was not possible to test the role of phosphorylation by a genetic approach by taking advantage of mutants in which the Ser-185 and Ser-188 sites had been converted to another residue. In this study we were able to test the role of Ser-185 and -188 phosphorylation by increasing phosphorylation of these sites specifically through overexpression of MAPKK. Such treatment had no effect on either E3 or E4 expression in the absence of E1A products. In the presence of E1A, no change in expression of the E3 promoter occurred in response to MAPKK; however, expression of E4 was significantly increased. Thus, phosphorylation of Ser-185 and -188 in response to MAPK appears to activate E4 expression specifically.

A recent report suggested that the effects induced by cAMP and E4orf4 were entirely due to the dephosphorylation by PP2A of transcription factors, including E4F, which regulates E4 expression, and that all E1A-induced transactivation was reduced (6). Thus, at least one consequence of activation of PP2A by E4orf4 appears to be a generalized suppression of E1A-transactivating activity. However, our results on E3 and E4 expression were obtained in the absence of activated PP2A (and E4orf4) and the effects of MAPKK expression were to increase expression from the E4 promoter but not that of E3. In other experiments involving fragments of E1A products linked to Gal4 DNA binding domains, Bondesson et al. (6) argued further that hypophosphorylation of E1A proteins is of no functional importance in the regulation of E1A transactivation activity. Our results argue strongly that with full-length E1A proteins and natural promoters regulated by E1A products, such is not the case.

Our data suggested a subtle level of regulation of E1A activity by the E4orf4 product. Initially, E1A proteins transactivate all early promoters, and in response to phosphorylation of Ser-185 and -188 by MAPK, E1A efficiently activates expression of E4 transcripts. Production of E4orf4 results in activation of PP2A and, as suggested by Bondesson et al. (6), a generalized decrease in E1A transactivation. However, PP2A also inactivates MAPK and MAPKK, leading to a decrease in phosphorylation of Ser-185 and -188 in newly synthesized E1A molecules. Hypophosphorylation reduces production of E4 products but does not affect expression of other early regions, thus allowing viral replication to continue. Why would such

specificity in regulation of E4 expression be of value in virus replication? In addition to the activation of PP2A by E4orf4, E4 products are involved in a variety of functions, including host cell shutoff, transport and accumulation of late mRNAs, viral DNA synthesis, and enhancement of E2 promoter activity (references 9 and 12 and references therein). Of particular interest in the context of this study are our recent findings that one or more E4 products induce p53-independent apoptosis (44, 45, 59). We are currently attempting to identify these E4-death-inducing proteins. Although the 19-kDa E1B protein provides protection against such cell death, expression of sufficiently high levels of E4 may nevertheless be toxic (44), as is elimination of E4orf4 synthesis (50). We therefore suggest that one major role for the E4orf4-induced suppression of E4 expression is to limit the expression of the E4-death-inducing protein(s), thus maintaining cell viability to allow sufficient virus production. We attempt to test this model further in continuing studies.

ACKNOWLEDGMENTS

We thank many of our colleagues for generous gifts, including Tom Shenk for S49 cells and *dl309*, Arnold Berk for *pm975*, Nick Jones for *dl520*, Frank Graham for *hr5* and 293 cells, Steve Pellech for purified MAPK, and Ed Harlow for M73 hybridoma cells. Special thanks go to Anne-Claude Gingras and Nahum Sonenberg for helpful discussions and to undergraduate students Juanita Chan and Nathalie Campbell for making the plasmid mutant pXC13/S185G/S188D.

This work was supported through grants from the National Cancer Institute of Canada, the Medical Research Council of Canada (PEB), and the National Institutes of Health (grant NIH CA29797 to R.P.R.).

REFERENCES

1. Akusjärvi, G. 1993. Proteins with transcription regulatory properties encoded by human adenoviruses. *Trends Microbiol.* **1**:163–170.
2. Anderson, N. G., J. L. Maller, N. K. Tonks, and T. W. Sturgill. 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature (London)* **343**:651–653.
3. Barbeau, D. 1994. M.Sc. thesis. McMaster University, Hamilton, Ontario, Canada.
4. Barbeau, D., R. Charbonneau, S. G. Whalen, S. T. Bayley, and P. E. Branton. 1994. Functional interactions within adenovirus E1A protein complexes. *Oncogene* **9**:359–373.
5. Bayley, S. T., and J. S. Mymryk. 1994. Adenovirus E1A proteins and transformation: a review. *Int. J. Oncol.* **5**:425–444.
6. Bondesson, M., K. Öhman, M. Mannervik, S. Fan, and G. Akusjärvi. 1996. Adenovirus E4 open reading frame 4 protein autoregulates E4 transcription by inhibiting E1A transactivation of the E4 promoter. *J. Virol.* **70**:3844–3851.
7. Boyle, W. J., P. van der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* **201**:110–149.
8. Braithwaite, A., C. Nelson, A. Skulimowski, J. McGovern, D. Pigott, and J. Jenkins. 1990. Transactivation of the p53 oncogene by E1a gene products. *Virology* **177**:595–605.
9. Bridge, E., S. Medghalchi, S. Ubol, M. Leesong, and G. Ketner. 1993. Adenovirus early region 4 and viral DNA synthesis. *Virology* **193**:794–801.
10. Clark-Lewis, I., J. S. Sanghera, and S. L. Pellech. 1991. Definition of a consensus sequence for peptide substrate recognition by p44^{mapk}, the meiosis-activated myelin basic protein kinase. *J. Biol. Chem.* **266**:15180–15184.
11. Cook, S. J., and F. McCormick. 1993. Inhibition by cAMP of ras-dependent activation of raf. *Science* **262**:1069–1072.
12. Cutt, J. R., T. Shenk, and P. Hearing. 1987. Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. *J. Virol.* **61**:543–552.
13. Dumont, D. J., and P. E. Branton. 1992. Phosphorylation of adenovirus E1A proteins by the p34cdc2 protein kinase. *Virology* **189**:111–120.
14. Dumont, D. J., R. C. Marcellus, S. T. Bayley, and P. E. Branton. 1993. Role of phosphorylation near the amino terminus of adenovirus type 5 early region 1A proteins. *J. Gen. Virol.* **74**:583–595.
15. Dumont, D. J., M. L. Tremblay, and P. E. Branton. 1989. Phosphorylation at serine 89 induces a shift in gel mobility but has little effect on the function of adenovirus type 5 E1A proteins. *J. Virol.* **63**:987–991.
16. Egan, C., S.-P. Yee, B. Ferguson, M. Rosenberg, and P. E. Branton. 1987. Binding of cellular polypeptides to human adenovirus type 5 E1A proteins produced in *Escherichia coli*. *Virology* **60**:292–296.
17. Engel, D. A., S. Hardy, and T. Shenk. 1988. cAMP acts in synergy with E1A

- protein to activate transcription of the adenovirus genes E4 and E1A. *Genes Dev.* 2:1517-1528.
18. Engel, D. A., U. Müller, R. W. Gedrich, J. S. Eubanks, and T. Shenk. 1991. 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. *Proc. Natl. Acad. Sci. USA* 88:3957-3961.
 19. Ferguson, B., N. Jones, J. Richter, and M. Rosenberg. 1984. Adenovirus E1A gene product expressed at high levels in *Escherichia coli* is functional. *Science* 224:1343-1346.
 20. Flint, J., and T. Shenk. 1989. Adenovirus E1A protein paradigm viral transactivator. *Annu. Rev. Genet.* 23:141-161.
 21. Gedrich, R. W., S. T. Bayley, and D. A. Engel. 1992. Induction of AP-1 DNA-binding activity and *c-fos* mRNA by the adenovirus 243R E1A protein and cyclic AMP requires domains necessary for transformation. *J. Virol.* 66:5849-5859.
 22. Gedrich, R. W., and D. A. Engel. 1995. Identification of a novel E1A response element in the mouse *c-fos* promoter. *J. Virol.* 69:2333-2340.
 23. Geisberg, J. V., W. S. Lee, A. J. Berk, and R. P. Ricciardi. 1994. The zinc finger region of the adenovirus E1A transactivating domain complexes with the TATA box binding protein. *Proc. Natl. Acad. Sci. USA* 91:2488-2492.
 24. Glenn, G. M., and R. P. Ricciardi. 1985. Adenovirus 5 early region 1A host range mutants *hr3*, *hr4*, and *hr5* contain point mutations which generate single amino acid substitutions. *J. Virol.* 56:66-74.
 25. Glenn, G. M., and R. P. Ricciardi. 1987. An adenovirus type 5 E1A protein with a single amino acid substitution blocks wild-type E1A transactivation. *Mol. Cell. Biol.* 7:1004-1011.
 26. Gomez, N., and P. Cohen. 1991. Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. *Nature (London)* 353:170-173.
 27. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
 28. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59-72.
 29. Graham, F. L., and A. J. van der Eb. 1972. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
 30. Haley, K. P., J. Overhauser, L. E. Babiss, H. S. Ginsberg, and N. C. Jones. 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. *Proc. Natl. Acad. Sci. USA* 81:5734-5738.
 31. Harlow, E., B. R. Franza, Jr., and C. Schley. 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region E1A products. *J. Virol.* 55:533-546.
 32. Harrison, T., F. Graham, and J. Williams. 1977. Host range mutants of adenovirus type 5 defective for growth in HeLa cells. *Virology* 77:319-329.
 33. Jones, N., and T. Shenk. 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. *Proc. Natl. Acad. Sci. USA* 76:3665-3669.
 34. Kleinberger, T., and T. Shenk. 1993. Adenovirus E4orf4 protein binds to protein phosphatase 2A, and the complex down regulates E1A-enhanced *junB* transcription. *J. Virol.* 67:7556-7560.
 35. Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk. 1991. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. *Cell* 87:365-376.
 36. Lillie, J. W., and M. R. Green. 1989. Transcription activation by the adenovirus E1a proteins. *Nature (London)* 338:39-44.
 37. Lillie, J. W., M. Green, and M. R. Green. 1986. An adenovirus E1A protein region required for transformation and transcriptional repression. *Cell* 46:1043-1051.
 38. Lillie, J. W., P. M. Loewenstein, M. R. Green, and M. Green. 1987. Functional domains of adenovirus type 5 E1a proteins. *Cell* 50:1091-1100.
 39. Liu, F., and M. R. Green. 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1a protein. *Cell* 61:1217-1224.
 40. Liu, F., and M. R. Green. 1994. Promoter targeting by adenovirus E1a through interaction with different cellular DNA-binding domains. *Nature (London)* 368:520-525.
 41. Ludlow, J. W. 1992. Selective ability of S-phase cell extracts to phosphorylate SV40 large T antigen *in vitro*. *Oncogene* 7:1011-1014.
 42. Mal, A., A. Piotrkowski, and M. L. Harter. 1996. Cyclin-dependent kinases phosphorylate the adenovirus E1A protein, enhancing its ability to bind pRb and disrupt pRb-E2F complexes. *J. Virol.* 70:2911-2921.
 43. Mansour, S., W. T. Matten, A. S. Hermann, J. M. Candia, S. Rong, K. Fukasawa, G. F. Vande Woude, and N. G. Ahn. 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* 265:966-970.
 44. Marcellus, R. C., and P. E. Branton. Unpublished results.
 45. Marcellus, R. C., J. G. Teodoro, T. Wu, D. E. Brough, G. Ketner, G. C. Shore, and P. E. Branton. 1996. Adenovirus type 5 early region 4 is responsible for E1A-induced p53-independent apoptosis. *J. Virol.* 70:6207-6215.
 46. Mazzarelli, J. M., G. B. Atkins, J. V. Geisberg, and R. P. Ricciardi. 1995. The viral oncoproteins Ad5 E1A, HPV16 E7 and SV40 TAg bind a common region of the TBP-associated factor-110. *Oncogene* 11:1859-1864.
 47. McGrory, W. J., D. D. Bautista, and F. L. Graham. 1988. A simple technique for the rescue of early region 1 mutations into infectious human adenovirus type 5. *Virology* 163:614-617.
 48. McKinnon, R. D., S. Bacchetti, and F. L. Graham. 1982. Tn5 mutagenesis of the transforming genes of human adenovirus type 5. *Gene* 19:33-42.
 49. Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk. 1982. Resolving the functions of overlapping viral genes by site specific mutagenesis at a mRNA splice site. *Nature (London)* 295:380-384.
 50. Müller, U., T. Kleinberger, and T. Shenk. 1992. Adenovirus E4orf4 protein reduces phosphorylation of c-Fos and E1A proteins while simultaneously reducing the level of AP-1. *J. Virol.* 66:5867-5878.
 51. Müller, U., M. P. Roberts, D. A. Engel, W. Doerfler, and T. Shenk. 1989. Induction of transcription factor AP-1 by adenovirus E1A protein and cAMP. *Genes Dev.* 3:1991-2002.
 52. Nevins, J. R. 1991. Transcriptional activation by viral regulatory proteins. *Trends Biochem. Sci.* 16:435-439.
 53. Pöpperl, H., and M. S. Featherstone. 1992. An autoregulatory element of the murine *Hox-4.4* gene. *EMBO J.* 11:3673-3680.
 54. Ricciardi, R. P., R. L. Jones, C. L. Cepko, P. A. Sharp, and B. E. Roberts. 1981. Expression of early adenovirus genes requires a viral encoded acidic polypeptide. *Proc. Natl. Acad. Sci. USA* 78:6121-6125.
 55. Richter, J. D., J. M. Slavicek, J. F. Schneider, and N. C. Jones. 1988. Heterogeneity of adenovirus type 5 E1A proteins: multiple serine phosphorylations induce slow-migrating electrophoretic variants but do not effect E1A-induced transcriptional activation or transformation. *J. Virol.* 62:1948-1955.
 56. Rowe, D. T., F. L. Graham, and P. E. Branton. 1983. Intracellular localization of adenovirus type 5 tumor antigens in productively infected cells. *Virology* 129:456-468.
 57. Shenk, T., and J. Flint. 1991. Transcriptional and transforming activities of the adenovirus E1A proteins. *Adv. Cancer Res.* 57:47-85.
 58. Smith, C. L., C. Debouck, M. Rosenberg, and J. S. Culp. 1989. Phosphorylation of serine residue 89 of human adenovirus E1A proteins is responsible for their characteristic electrophoretic mobility shifts, and its mutation affects biological function. *J. Virol.* 63:1569-1577.
 59. Teodoro, J. G., G. C. Shore, and P. E. Branton. 1995. Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53-independent mechanisms. *Oncogene* 11:467-474.
 60. Tremblay, M. L., D. J. Dumont, and P. E. Branton. 1989. Analysis of phosphorylation sites in exon 1 region of E1A proteins of human adenovirus type 5. *Virology* 169:397-407.
 61. Tremblay, M. L., C. J. McGlade, G. E. Gerber, and P. E. Branton. 1988. Identification of the phosphorylation sites in early region 1A proteins of adenovirus type 5 by amino acid sequencing of peptide fragments. *J. Biol. Chem.* 263:6375-6383.
 62. Tsukamoto, A. S., A. Ponticelli, A. J. Berk, and R. B. Gaynor. 1986. Genetic mapping of a major site of phosphorylation in adenovirus type 2 E1A proteins. *J. Virol.* 59:14-22.
 63. Webster, L. C., and R. P. Ricciardi. 1991. *trans*-dominant mutants of E1A provide genetic evidence that the zinc finger of the *trans*-activating domain binds a transcription factor. *Mol. Cell. Biol.* 11:4287-4296.
 64. Weeks, D. L., and N. C. Jones. 1983. E1A control of gene expression is mediated by sequences 5' to the transcriptional starts of the early viral genes. *Mol. Cell. Biol.* 3:1222-1234.
 65. Whalen, S. G., and P. E. Branton. Unpublished results.
 66. Whalen, S. G., R. C. Marcellus, D. Barbeau, and P. E. Branton. 1996. Importance of the Ser-132 phosphorylation site in cell transformation and apoptosis induced by the adenovirus type 5 E1A protein. *J. Virol.* 70:5373-5383.
 67. Wu, J., P. Dent, T. Jelinek, A. Woltman, M. J. Weber, and T. W. Sturgill. 1993. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. *Science* 262:1065-1069.
 68. Yee, S.-P., D. T. Rowe, M. L. Tremblay, M. McDermott, and P. E. Branton. 1983. Identification of human adenovirus early region 1 products using antisera against synthetic peptides corresponding to the predicted carboxy terminus. *Virology* 129:1003-1013.
 69. Zhou, Q., and D. A. Engel. 1995. Adenovirus E1A₂₄₃ disrupts the ATF/CREB-YY1 complex at the mouse *c-fos* promoter. *J. Virol.* 69:7402-7409.
 70. Zhou, Q., R. W. Gedrich, and D. A. Engel. 1995. Transcriptional repression of the *c-fos* gene by YY1 is mediated by a direct interaction with ATF/CREB. *J. Virol.* 69:4323-4330.