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Regulation of p53-Dependent Apoptosis, Transcriptional Repression, and Cell Transformation by Phosphorylation of the 55-Kilodalton E1B Protein of Human Adenovirus Type 5

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The adenovirus type 5 55-kDa E1B protein (E1B-55kDa) cooperates with E1A gene products to induce cell transformation. E1A proteins stimulate DNA synthesis and cell proliferation; however, they also cause rapid cell death by p53-dependent and p53-independent apoptosis. It is believed that the role of the E1B-55kDa protein in transformation is to protect against p53-dependent apoptosis by binding to and inactivating p53. It has been shown previously that the 55-kDa polypeptide abrogates p53-mediated transactivation and that mutants defective in p53 binding are unable to cooperate with E1A in transformation. We have previously mapped phosphorylation sites near the carboxy terminus of the E1B-55kDa protein at Ser-490 and Ser-491, which lie within casein kinase II consensus sequences. Conversion of these sites to alanine residues greatly reduced transforming activity, and although the mutant 55-kDa protein was found to interact with p53 at normal levels, it was somewhat defective for suppression of p53 transactivation activity. We now report that a nearby residue, Thr-495, also appears to be phosphorylated. We demonstrate directly that the wild-type 55-kDa protein is able to block E1A-induced p53-dependent apoptosis, whereas cells infected by mutant *pm*490/1/5A, which contains alanine residues at all three phosphorylation sites, exhibited extensive DNA fragmentation and classic apoptotic cell death. The E1B-55kDa product has been shown to exhibit intrinsic transcriptional repression activity when localized to promoters, such as by fusion with the GAL4 DNA-binding domain, even in the absence of p53. Such repression activity was totally absent with mutant *pm*490/1/5A. These data suggested that inhibition of p53-dependent apoptosis may depend on the transcriptional repression function of the 55-kDa protein, which appears to be regulated be phosphorylation at the carboxy terminus.

Early region 1B (E1B) of human adenoviruses encodes polypeptides which are essential for both viral replication and cell transformation. The E1B gene of adenovirus serotype 5 (Ad5) expresses two major proteins, a 19-kDa polypeptide of 176 residues (176R) and a 496-residue protein (496R) of 55 kDa which is produced from an internal initiation site in an alternative reading frame (9, 22, 53). Later in infection, other E1B species of 84, 93, and 156 residues (84R, 93R, and 156R, respectively) are produced from alternatively spliced mRNAs, but their roles are unclear (2, 28, 36, 39, 66, 71). All of the latter species share a 79-residue amino terminus with the 55-kDa protein, and whereas the remaining sequences of 84R and 93R are unique, 156R is completed by the 77-residue carboxy terminus of the 55-kDa polypeptide. The E1B products of the closely related Ad2 are virtually identical in sequence but are one or two residues shorter than those of Ad5 and exist as 495R, 82R, 92R, and 155R species (2).

The 55-kDa E1B protein (E1B-55kDa) is essential for several processes, including viral replication, accumulation of late viral mRNAs, late viral protein synthesis, and shutoff of host protein synthesis (3–5, 8, 11, 29, 35, 37, 46, 54, 58, 76). Synthesis occurs early after infection, and the 55-kDa protein is found largely in the nucleus and the perinuclear region of the cytoplasm (56, 60, 80). Products of early region 1A (E1A) induce DNA synthesis and cell transformation, but stable transformants are produced only in presence of E1B products (reviewed in reference 6). Both the 55- and 19-kDa proteins are independently able to transform rodent cells in cooperation with E1A, but transforming activity is increased when both are present (5, 8, 46, 72, 76, 81). In the absence of E1B, E1A proteins induce rapid apoptosis characterized by extensive DNA degradation, chromatin condensation, and cell death (15, 38, 68). The E1B-19kDa product probably cooperates in E1A-mediated cell transformation by blocking apoptosis via a mechanism which is functionally analogous to that of the Bcl-2 suppressor (10, 13, 15, 30, 49, 55, 73).

The function of the E1B-55kDa protein is at least partly linked to formation of complexes with the cellular p53 tumor suppressor protein (59), as p53 binding appears to be required for cell transformation mediated by this E1B product (75). p53 is a transcription factor which can both activate and repress gene expression (19, 24, 64, 79). The amino terminus contains an acidic activation domain which, when fused to the GAL4 DNA-binding region, is capable of transcriptional activation in both mammalian and yeast cells (20). Changes in gene expression induced by p53 are believed to play key roles in both growth arrest and induction of apoptosis (16, 21, 23, 34, 78), including cell death induced by E1A products (15, 38, 41, 68). The E1B-55kDa protein binds to the activation domain of p53 and inhibits p53-dependent transactivation activity (75). The 55-kDa polypeptide possesses intrinsic transcriptional repression activity which suppresses basal transcription when localized to a promoter, for example, by fusion with the GAL4 DNA-binding domain (77). Thus, complex formation with p53 targets repression activity of the 55-kDa product to promoters containing p53-specific binding sites, presumably accounting for inhibition of p53 transactivation activity (67, 75, 77). It has

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been suggested that the 55-kDa species may function in transformation by blocking p53-dependent apoptosis induced by E1A products.

The E1B-55kDa protein is known to be highly phosphorylated on both serine and threonine residues (40, 60, 74). Recently we mapped two phosphorylation sites to the extreme carboxy terminus of the Ad5 55-kDa protein at Ser-490 and Ser-491 (67). Both of these sites are conserved in the E1B products of other adenovirus serotypes. In addition to decreasing its level of phosphorylation, conversion of these sites to alanine residues greatly reduced transforming activity and partially blocked repression of p53-dependent transcription by the 55-kDa protein. Interestingly, a mutant of the 55-kDa product containing Ala in place of Ser-490 and Ser-491 was able to bind to p53 at normal levels. Thus, complex formation with p53 is not sufficient for either transcriptional repression or cell transformation mediated by the 55-kDa polypeptide and appeared to require a function which is regulated by phosphorylation at these carboxy-terminal sites.

In the present studies we demonstrate that phosphorylation of the E1B-55kDa protein appears to be exclusively at the carboxy terminus at serine residues 490 and 491, as well as at a third conserved residue, Thr-495. Mutation of all of these sites to Ala residues resulted in elimination of all detectable phosphorylation and complete abrogation of both transforming and transcriptional repression activities. We also show directly for the first time that the E1B-55kDa protein blocks E1A-induced p53-dependent apoptosis and that this activity is prevented by alteration of the carboxy-terminal phosphorylation sites. These data suggest that the mechanism by which the E1B-55kDa product cooperates with E1A in cell transformation is by blocking E1A-induced p53-dependent apoptosis.

MATERIALS AND METHODS

Cells and virus infection. Human KB and 293 cells were cultured on 150-mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in α-minimal essential medium supplemented with 10% fetal calf serum and were normally infected with mutant or wild-type (wt) Ad5 at a multiplicity of 35 PFU per cell, as described previously (56). Chinese hamster ovary (CHO), primary baby mouse kidney (BMK), and primary baby rat kidney (BRK) cells were cultured under similar conditions in 60-mm-diameter dishes.

Virus mutants. The virus used as the wt in these studies is that described by Harrison et al. (31). Mutant *pm*490A/491A (or *pm*490/1A), which contains Ala residues in place of Ser-490 and Ser-491 of the E1B-55kDa protein, has been described previously (67). Additional mutants prepared for these studies included *pm*490/1/5A, which contains Ala at all three phosphorylation sites at Ser-490, Ser-491, and Thr-495 of the 55-kDa protein, and *pm*490/1D, which contains codons for Asp in place of Ser-490 (CAT) and Ser-491 (GAC). Mutants were generated by site-directed mutagenesis exactly as described previously (67). The altered Ad5 DNA fragments were subcloned into plasmid pXC38 (45), which contains the complete E1A and E1B sequences (bases 22 to 5788), to produce E1 plasmids p490/1/5A and p490/1D. These mutations were also subcloned into a previously described plasmid, p1716/2072 (46), a derivative of pXC38 which contains mutations at the codons for Met-1 and Met-120 of the E1B-19kDa protein and thus fails to express this E1B polypeptide. The mutations within the context of the p1716/2072 background have been designated p490/1/5A(19K−) and p490/1D(19K−). In addition, these mutations were introduced into the background of E1A mutant *dl*520, which produces 12S but not 13S E1A mRNA (29a), to form mutants *dl*520/490/1A(19K−) and *dl*520/490/1/5A(19K−). These constructs were created by subcloning the 2,041-bp *Hpa*I-*Aat*II fragment from the E1A plasmid pLE2 (33), which expresses *dl*520, into p490A/491A(19K−) and p490/1/5A(19K−). Some experiments were also carried out with mutant *dl*520/E1B−, which expresses 12S E1A mRNA and no E1B products (63). Ad5 mutations were rescued into virus by the method of homologous recombination described previously (44). Virus mutants were screened, and mutations were confirmed by DNA sequencing, restriction enzyme digestion, or Southern blot analysis, as described previously (17, 46, 69). Viral plaque assays were performed with Ad5-transformed 293 cells (26).

Antisera and immunoprecipitation. Immunoprecipitations were carried out with antipeptide serum 58-N2, which is directed against the amino terminus of the E1B-55kDa protein and thus also recognizes the related 84R, 93R, and 156R products (46, 66). Mouse monoclonal antibody 2A6 (60) was also used to identify or immunoprecipitate the 55-kDa protein in some experiments. Infected-cell extracts were immunoprecipitated exactly as described previously (43) by using 30 μl of 58-N2 or 2 μl of 2A6 ascites fluid.

Radioactive labeling. Ad5- or mock-infected human KB cells were normally labeled from 16 to 18 h postinfection (p.i.), and BRK cells were labeled from 20 to 24 h p.i. Labeling was performed with 100 μCi of [^{35}S]methionine (specific activity, 1,300 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml or 0.5 mCi of [^{32}P]orthophosphate per ml in methionine- or phosphate-free medium (Gibco BRL), respectively. In vitro phosphorylation experiments were carried out with 50 ng of the Ad2 E1B-55kDa protein expressed in and purified from insect cells infected by a recombinant baculovirus (a kind gift from Arnie Berk) or with α-casein (Boehringer Mannheim) as a control. These proteins were incubated in vitro with 200 U of purified casein kinase I (CKI) or casein kinase II (CKII) (New England Biolabs). Reactions with CKI were carried out in 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$ and 5 mM dithiothreitol, and those with CKII were carried out in 20 mM Tris-HCl (pH 7.5) containing 50 mM KCl_2 and 10 mM $MgCl_2$. Such reactions were carried out for various periods at 30°C in a volume of 50 μl in the presence of 100 μM [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham Corp.).

SDS-PAGE. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10 or 15% polyacrylamide separating and 5% stacking gels, as described previously (56).

Analysis of phosphopeptides by HPLC. The ^{32}P-labeled 55-kDa protein was immunoprecipitated from infected cells and purified by SDS-PAGE. Regions of gels containing this species were excised, and the eluted proteins were digested with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington), as described previously (70). Tryptic peptides were separated by using an Ultrasphere octyldecyl silane (C_{18}) reverse-phase column (4.6 by 250 mm) operating on a Waters dual-pump high-performance liquid chromatography (HPLC) system with a 600E controller. The column was preequilibrated with solution A (5% formic acid in water), and peptides were eluted from the column with a linear gradient of solution B (5% formic acid in ethanol) for 95 min, as described previously (70). The flow rate was maintained at 1 ml/min, and labeled phosphopeptides were detected with an on-line LB507A isotope detector (Bertold).

Transformation of BRK cells. Cells were prepared from 4- or 5-day-old Wistar rats, as described previously (46). BRK cells were transfected at approximately 80% confluence by calcium phosphate coprecipitation with 5 μg of plasmid DNA containing E1A and mutant or wt E1B (27). Transformed foci were identified by Giemsa staining after 2 weeks in culture in Joklik's medium (S-minimal essential medium; Gibco BRL) supplemented with 10% horse serum.

Transcriptional repression assay. The ability of wt and mutant E1B-55kDa proteins to repress transcription was determined in assays involving a construct described previously (77) which expresses a fusion protein containing Ad2 E1B-495R-55kDa protein linked to the GAL4 DNA-binding domain (GAL4-55K). The GAL4 fusion proteins of wt and mutant Ad5 E1B-496R-55kDa proteins were created by replacing the *Kpn*I-*Afl*II fragment of GAL4-55K with the equivalent fragment from pXC38, p490A/491A, p490D/491/D, or p490/1/5A. Transcriptional repression activity was determined by measuring the ability of the GAL4-55K fusion proteins to suppress transcription of the thymidine kinase (TK) promoter. The reporter construct used in this assay was the previously described construct G_5tkCAT (77), which contains five GAL4 DNA-binding sites linked to the herpes simplex virus (HSV) TK promoter linked to the chloramphenicol acetyltransferase (CAT)-coding sequence. Assays were performed by transfecting CHO cells, which had been plated at a density of 2×10^5 cells per 60-mm-diameter dish, with 1 μg of G_5tkCAT reporter DNA and 1, 5, or 10 μg of wt or mutant GAL4-55K plasmid DNA. As a negative control, cells were transfected with DNA of plasmid pSG424, which expresses the DNA-binding region of GAL4 alone. All transfections were performed by calcium phosphate coprecipitation (27), and transfection efficiency was standardized by including 1 μg of HSVβ-Gal in all transfections and measuring β-galactosidase activity. Sonicated salmon sperm DNA (Pharmacia) was added to make a total of 15 μg of DNA per plate. Cells were harvested at 40 h posttransfection, and CAT assays were performed as previously described (25) with volumes of cell extracts containing equal amounts of β-galactosidase activity. CAT activity was quantified from thin-layer chromatography plates with a Fuji BAS2000 phosphorimager.

DNA fragmentation assay. The presence of nucleosome-sized DNA fragments was determined by isolating low-molecular-weight DNA by using a modified Hirt extraction procedure, exactly as described previously (68). Sixty-millimeter-diameter plates of BMK cells were harvested at 40 h p.i. and lysed in pronase lysis buffer (10 mM Tris-HCl [pH 8.5] containing 5 mM EDTA, 100 mM NaCl, and 1 mg of pronase per ml, to which SDS was added to 0.5% [wt/vol]). Cell lysates were incubated at 37°C for 2 h, and NaCl was added to a final concentration of 1 M. Samples were incubated overnight at 4°C and centrifuged at 15,000 × *g* for 30 min. DNA was ethanol precipitated, treated with RNase A, and analyzed on 1% agarose gels stained with ethidium bromide.

RESULTS

Mapping of an additional phosphorylation site at the carboxy terminus of the E1B-55kDa protein. The E1B-55kDa protein is known to be phosphorylated on both serine and threonine residues (40, 60, 74). In previous studies with the

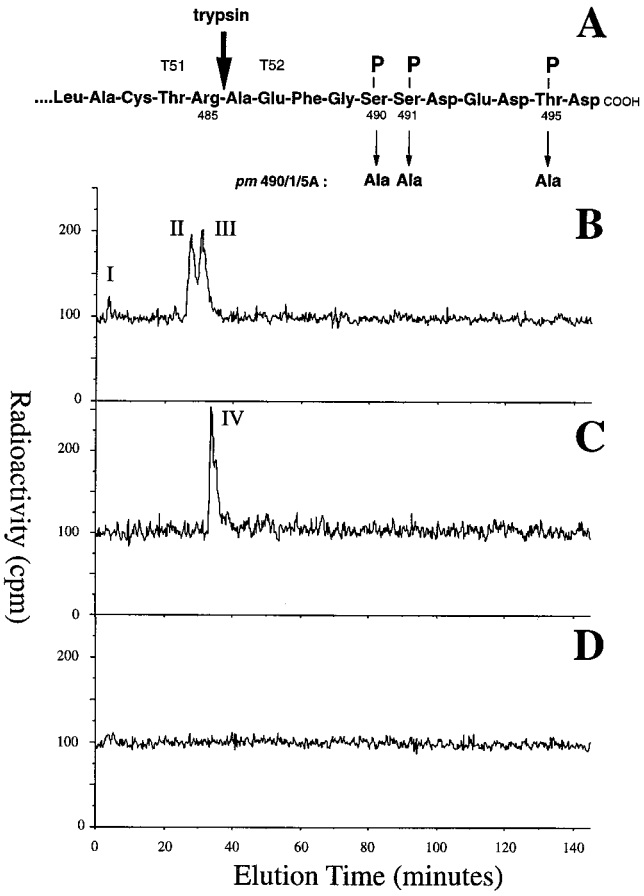

FIG. 1. Analysis of tryptic phosphopeptides from the E1B-55kDa protein by HPLC. KB cells were infected with wt Ad5 or mutant *pm*490/1A or *pm*490/1/5A, and following labeling with [^{32}P]orthophosphate, the 55-kDa protein was purified by immunoprecipitation and SDS-PAGE, digested with trypsin, and analyzed by reverse-phase HPLC, as described in Materials and Methods. (A) Amino acid sequence of the carboxy terminus of the Ad5 E1B-55kDa protein. The position of trypsin cleavage following residue 485 is indicated, as are the resulting tryptic peptides, T51 and T52. Phosphorylation sites at Ser-490, Ser-491, and Thr-495 are noted, as is conversion of these sites to alanine residues to form mutant *pm*490/1/5A. (B to D) Analysis of phosphopeptides of the 55-kDa protein. The HPLC elution profiles from the ^{32}P-labeled 55-kDa protein synthesized by the wt (B), *pm*490/1A (C), and *pm*490/1/5A (D) are shown.

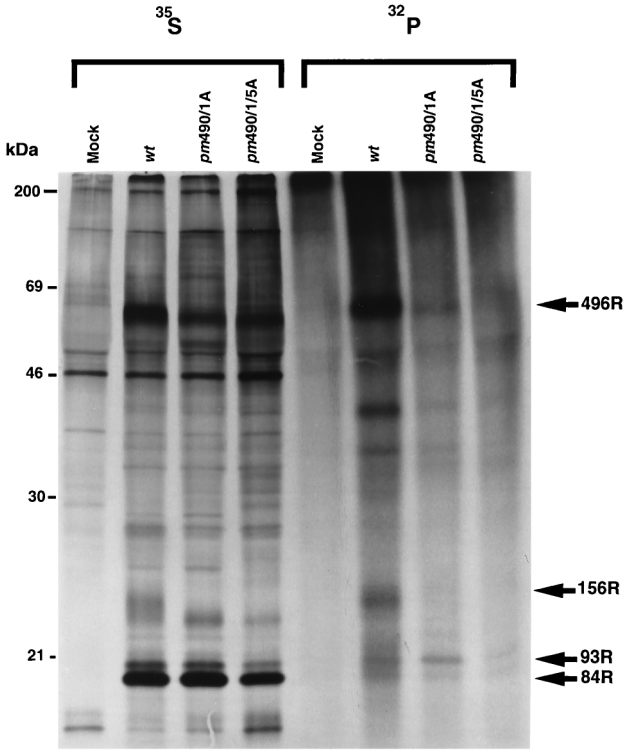

FIG. 2. Synthesis of E1B-55kDa protein by mutant and wt viruses. KB cells were infected with wt Ad5 or mutant *pm*490/1A or *pm*490/1/5A, or they were mock infected, and following labeling with [^{33}S]methionine or [^{32}P]orthophosphate, the 55-kDa protein was immunoprecipitated with 58-N2 antipeptide serum and precipitates were analyzed by SDS-PAGE. The positions of molecular mass markers are on the left, and those of the 55-kDa protein (496R) and the related species 156R, 93R, and 84R are on the right.

55-kDa protein labeled in vivo with [^{32}P]orthophosphate in Ad5-infected KB cells (and as shown in Fig. 1B), we showed that two tryptic phosphopeptides were present, which eluted as species II and III from C_{18} reverse-phase HPLC columns (66, 67). The small amount of label eluting immediately, termed species I, was shown to consist of free phosphate (67). Both phosphopeptides II and III were shown to be recognized by 58-C1 serum prepared against the six-residue peptide corresponding to the carboxy-terminal sequence of the 55-kDa protein (66). In addition, whereas peptide III was shown to contain exclusively phosphoserine, peptide II contained both phosphoserine and phosphothreonine (67). These results strongly suggested that phosphorylation of the 55-kDa protein occurred at serine residues 490 and 491 and at Thr-495, which are the only serine and threonine residues located within the carboxy-terminal T52 tryptic peptide (Fig. 1A). Confirmation of the Ser-490 and -491 sites was obtained through the generation of a mutant, termed *pm*490/1A, which contained alanine residues in place of these serines (67). The 55-kDa protein produced by *pm*490/1A was shown to lack phosphoserine but to retain phosphothreonine, which was present in a single peptide, termed species IV, eluting from C_{18} columns slightly later than peptide III (67). A similar profile obtained with *pm*490/1A is shown in Fig. 1C. To confirm that the remaining phosphothreonine present in peak IV originated from phosphorylation at Thr-495, a new E1B mutant, termed *pm*490/1/5A, that contained Ala-495 in addition to Ala-490 and Ala-491 was produced. HPLC analysis of the tryptic peptides generated from the E1B-55kDa protein isolated from *pm*490/1/5A-infected cells which had been incubated with [^{32}P]orthophosphate indicated no detectable labeled phosphopeptides (Fig. 1D). Thus, Thr-495 appeared to represent a third phosphorylation site in the E1B-55kDa species within tryptic peptide T52. We recognize that such types of studies are indirect and that it remains formally possible that the introduction of mutations at one site may affect phosphorylation at a distant site. Nevertheless, the fact that 58-C1 serum recognizes the phosphoserine- and phosphothreonine-containing peptides strongly suggests that all three sites lie within tryptic peptide T52 at Ser-490, Ser-491, and Thr-495.

To characterize these proteins further, wt and mutant forms of the 55-kDa protein were immunoprecipitated from infected KB cells, which had been labeled with either [^{35}S]methionine or [^{32}P]orthophosphate, by using 58-N2 antipeptide serum, which recognizes the amino terminus of the 55-kDa polypeptide and related E1B species. Figure 2 shows the pattern on SDS-PAGE and indicates that although similar levels of ^{35}S-labeled 55-kDa proteins were produced in wt- and mutant-

infected cells, the level of ^{32}P incorporation with mutant *pm*490/1A was greatly reduced, and that with mutant *pm*490/1/5A was essentially undetectable. Figure 2 also shows that similar reductions were evident in mutant-infected cells for the E1B-156R protein, which contains the same 77-residue carboxy-terminal sequence and migrates on SDS-PAGE as a doublet. Prolonged autoradiography of this gel, and previous studies (67), indicated that 156R synthesized by *pm*490/1A clearly contained low levels of ^{32}P, whereas with that made by *pm*490/1/5A labeling was undetectable. These results confirmed the presence of an additional phosphorylation site at Thr-495 and also suggested that this residue, Ser-490, and Ser-491 appear to be the only phosphorylation sites in the E1B-55kDa protein.

Ser-490 and Ser-491 both lie within classic CKII substrate consensus sequences, and Thr-495 could represent a CKI site (52) (Fig. 1A). We made several attempts to phosphorylate recombinant Ad2 E1B-55kDa which had been purified from insect cells infected with a baculovirus expressing this polypeptide (provided by Arnie Berk), by using purified CKII and CKI. We showed that the 55-kDa protein was labeled efficiently in vitro by both kinases and that following digestion with trypsin some labeled material eluted from C_{18} columns with properties identical to those of peptides II and III (data not shown). However, several other peptides were also labeled, and we were never able to find conditions with either kinase in which peptides II and III were the predominant species. Thus, although serines 490 and 491 appear likely to be sites for CKII, a more precise evaluation of the kinases involved in phosphorylation of the E1B-55kDa protein will have to be made.

Importance of phosphorylation of the E1B-55kDa protein in cell transformation. Both the 19- and 55-kDa E1B proteins are able to cooperate with E1A products to transform rodent cells (5, 8, 46, 72, 76, 81). To analyze the importance in cell transformation of the phosphorylation sites on the 55-kDa species, primary BRK cells were transfected with DNA from plasmids expressing E1A and wt or phosphorylation-defective forms of the 55-kDa polypeptide. Plasmid pXC38 expresses wt E1A and E1B products, whereas pXC490/1A and pXC490/1/5A produce the phosphorylation mutant forms of the 55-kDa protein as well as the E1B-19kDa product. Plasmids p490/1A and p490/1/5A express mutant forms of the 55-kDa protein and E1A products but not the E1B-19kDa product. Mutants p19K− and p55K− are wt and produce E1A but no 19- or 55-kDa proteins. Figure 3 shows a summary of data obtained from three separate studies. The numbers of transformants obtained with both pXC490/1/5A and p490/1A were about 50% of that obtained with the wt. These results indicated that the phosphorylation sites appeared to be important, because, as found previously (46, 72, 81), transformation by E1A with either the 19-kDa (p55K−) or 55-kDa (p19K−) E1B product alone was found to occur at about 25 to 35% of the wt rate. The number of foci obtained by using plasmid p490/1A, which produces no 19-kDa polypeptide, was significantly reduced, to about 4% of that with pXC38 and about 12% of that with p19K−. Almost no foci were obtained with p490/1/5A. These results indicated that removal of the Thr-495 phosphorylation site rendered the 55-kDa protein even more defective for transformation than the Ala-490/Ala-491 mutant. In an attempt to mimic the presence of phosphorylated residues at positions 490 and 491, aspartic acid residues were introduced by site-directed mutagenesis at these sites to create the p490/1D construct. Figure 3 shows that introduction of such negative charges did not reconstitute transforming activity.

Importance of phosphorylation in E1B-55kDa protein-mediated transcriptional repression. Previous work has demonstrated that the transforming activity of the E1B-55kDa protein is dependent upon interactions with p53 (75). One clear effect of such complex formation is the suppression of p53 transactivation activity (77). The 55-kDa polypeptide has been shown to possess a potent transcriptional repression domain which functions independently of p53 and is able to repress transcription from a wide variety of promoters when localized to the promoter region by fusion with GAL4 (77). To determine if conversion of the carboxy-terminal phosphorylation sites of the 55-kDa product affects endogenous repression activity, mutant sequences were introduced into a construct that encodes a fusion product containing the 55-kDa species linked to the DNA-binding region of GAL4 driven by the simian virus 40 promoter (pGAL4-55K) (Fig. 4). The ability of these chimeric proteins to repress transcription was determined in DNA transfection assays with a reporter construct containing five GAL4 DNA-binding sequences linked to the CAT gene under the control of the HSV TK promoter (G5tk-CAT). Figure 4 shows the results of three separate transfection experiments, with data presented relative to the CAT activity of the G_5tkCAT reporter obtained with the GAL4 DNA-binding region alone (pSG424). As observed previously (77), the wt 55-kDa protein was found to repress transcription, by about 85% in these experiments. All three phosphorylation mutants were totally defective for this repression activity, and transcription occurred at least as well as with pSG424 alone. These data suggested that the carboxy-terminal phosphorylation sites are required for transcriptional repression by the 55-kDa protein and thus that phosphorylation may regulate this activity. With mutants *pm*490/1A and *pm*490/1D, CAT activity was comparable to that of the control pSG424 at all concentrations of plasmid DNA tested; however, with *pm*490A/1/5A, a consistent observation was that transfection with increasing amounts of the GAL4-*pm*490/1/5 plasmid DNA resulted in an enhancement of transcriptional activity. These results may suggest that

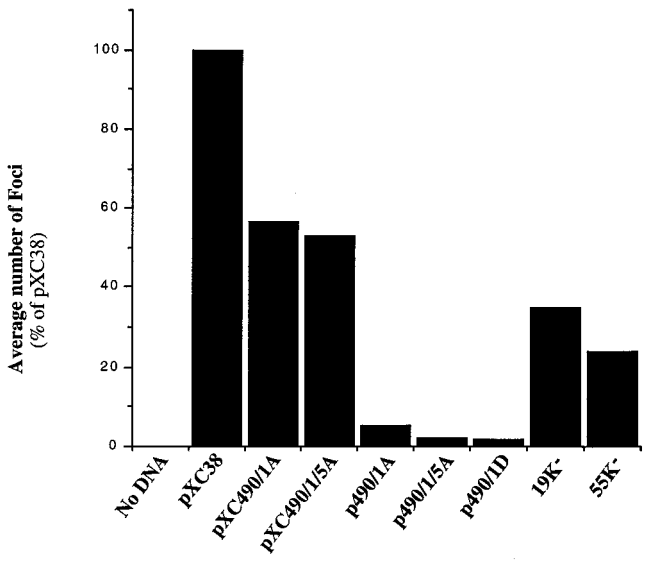

FIG. 3. Transformation of BRK cells by E1A and wt or mutant E1B. Primary BRK cells were transfected with DNA from plasmids expressing E1A and wt or phosphorylation-defective forms of the 55-kDa polypeptide, including pXC38 (wt E1A and E1B), pXC490/1A and pXC490/1/5A (E1A, E1B-19kDa protein, and 490/1A or 490/1/5A forms of the 55-kDa protein), p490/1A and p490/1/5A (E1A and 490/1A or 490/1/5A forms of the 55-kDa protein), p19K− (E1A and the E1B-55kDa protein only), and p55K− (E1A and the E1B-19kDa protein only). Averages of data obtained from three separate studies are presented, with the number of foci obtained with pXC38 (80 to 120 foci per dish) defined as 100%.

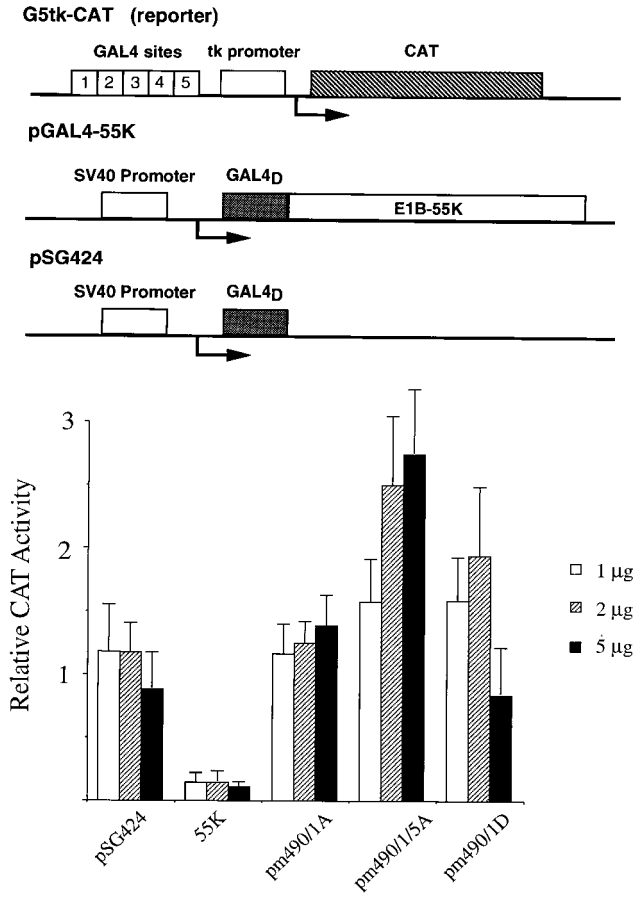

FIG. 4. Analysis of repression activity of the E1B-55kDa protein. Transcriptional repression activity was determined by measuring the ability of the GAL4-55K fusion proteins, relative to that of pSG424 to suppress transcription of a reporter construct, G_5tkCAT (77), containing five GAL4 DNA binding sites linked to the TK promoter and CAT-coding sequence (illustrated at the top). Assays were performed with CHO cells as described in Materials and Methods with 1 (open bars), 2 (striped bars), or 5 (solid bars) μg of wt or mutant GAL4-55K plasmid DNA. Constructs included pSG424 (no 55-kDa protein) and those expressing wt, *pm*490/1A, *pm*490/1/5A, or *pm*490/1D forms of the E1B-55kDa protein. CAT activity was quantified from thin-layer chromatography with a phosphorimager. Results are means ± standard deviations. SV40, simian virus 40.

in the absence of the carboxy-terminal phosphorylation sites, the 55-kDa protein may possess transcriptional activation activity.

Inhibition of p53-dependent apoptosis by the E1B-55kDa protein. It has been shown by several groups that adenovirus E1A proteins induce programmed cell death resulting from p53-dependent apoptosis (15, 38, 68). The E1B-19kDa protein is believed to function in both productive infection and cell transformation by blocking E1A-induced apoptosis via a mechanism similar to that of the cellular proto-oncogene Bcl-2 (10, 14). E1A products are also able to cause cell transformation in cooperation with the E1B-55kDa protein, and it has been speculated that this activity may derive from its interaction with and inactivation of p53. This possibility has never been tested directly, in part due to the problem that human adenoviruses have been observed to induce apoptosis by both p53-dependent and p53-independent mechanisms (65, 68). Ad5 E1A produces two major early E1A mRNAs, of 13S and 12S, that encode proteins of 289 and 243 residues, respectively, which differ only by the presence of a 46-residue internal region responsible for the activation of transcription of early viral genes (reviewed in references 1 and 62). We have demonstrated recently that induction of p53-independent apoptosis requires expression of the product of the 13S E1A mRNA and the subsequent transcriptional activation of early region 4 (41, 68). Under conditions in which adenovirus infection induces p53-independent apoptosis, such as in p53-null cells, we showed that cell death can be blocked by the E1B-19kDa product but not by the 55-kDa protein (41, 68). Our results also indicated that infection by an adenovirus which expresses only 12S E1A mRNA was incapable of inducing apoptosis in p53-null cells but rather produced only p53-dependent apoptosis. Thus, to test if the E1B-55 kDa protein is able to block p53-dependent apoptosis, we created virus mutants (Fig. 5) which express 12S but not 13S E1A mRNA (*dl*520), the E1B-55kDa protein but not the 19-kDa product (*dl*520/55K), or neither E1B protein (*dl*520/E1B−). In addition, viruses expressing the phosphorylation mutant forms of the 55-kDa protein and no 19-kDa polypeptide (*dl*520/490/1A and *dl*520/490/1/5A) were generated to test their ability to block apoptosis induced by p53. Experiments were carried out with these viruses and primary BMK cells to ensure that the p53 being expressed was wt. DNA from virus- and mock-infected cells was extracted and examined on agarose gels to assess the appearance of DNA degradation characteristic of apoptosis. Figure 5 shows that with mock-infected BMK cells and those infected with wt virus, which expresses both major E1A and E1B products, or with *dl*520, which expresses only 12S E1A mRNA but both E1B products, no DNA degradation was apparent. With cells infected with *dl*520E1B−, which produces 12S E1A mRNA only and no E1B products, characteristic nucleosome-sized DNA fragments associated with apoptosis were observed. Such DNA degradation was not observed with mutant *dl*520/55K, indicating that expression of the E1B-55kDa protein blocks p53-dependent apoptosis. However, in cells infected with phosphorylation mutant *dl*520/490/1A (19K−) or *dl*520/490/1/5A(19K−), extensive DNA degradation was apparent. These data indicated that the E1B-55 kDa protein is able to block E1A-induced p53-dependent apoptosis and that the carboxy-terminal phosphorylation sites are essential for this activity.

DISCUSSION

Productive infection of quiescent epithelial target cells by human adenoviruses requires the induction of cellular DNA synthesis machinery to permit replication of viral DNA and production of progeny virions. E1A proteins accomplish this task very efficiently and induce unscheduled cellular DNA synthesis through interactions with two classes of cellular proteins, the retinoblastoma family of growth suppressers and the transcriptional modulator p300 and related proteins (reviewed in reference 6). Such interactions also initiate a process of p53-dependent apoptosis which must be prevented in order to yield progeny virus efficiently or, in the case of a nonproductive infection, to generate stably transformed cells (15, 38). One major mechanism to prevent the untimely death of the host cell is elicited through the E1B-19 kDa protein, which is believed to act in a fashion similar to that of Bcl-2, which appears to block apoptosis, at least in part, through interactions with the Bax polypeptide, a downstream regulator of cell death (13, 30). In the present report we present for the first time direct evidence that the E1B-55kDa protein also blocks E1A-induced cell death by preventing p53-dependent apoptosis. The demonstration of antiapoptotic activity by the E1B-55kDa protein

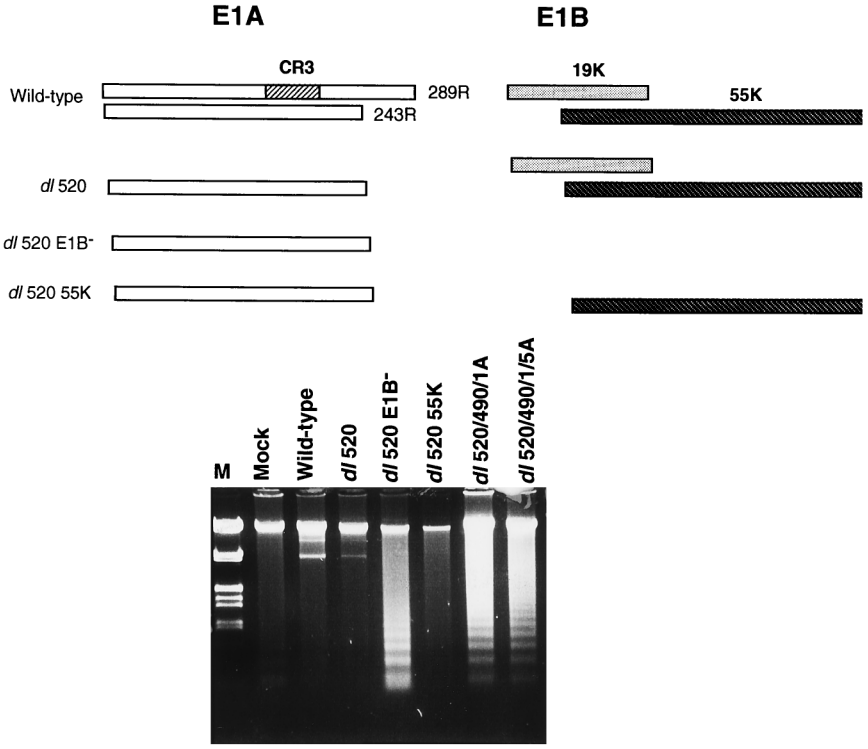

FIG. 5. Effects of wt and mutant E1B-55kDa proteins on E1A-induced p53-dependent apoptosis. Primary BMK cells were infected with wt or mutant Ad5, and then DNA of low molecular mass was isolated and analyzed on 1% agarose gels stained with ethidium bromide. The E1A and E1B products of the viruses used are illustrated at the top, and the gel profiles are presented at the bottom. Lane M, molecular mass markers.

has been technically difficult because adenovirus infection and expression of E1A initiate both p53-dependent and p53-independent apoptosis (65, 68). These two different forms of apoptosis are genetically separable in virus infection with E1A mutants, and the p53-independent process has been shown to require the transactivation of other early viral genes by conserved region 3 of the product of the E1A 13S mRNA (41, 68). The product of the 12S E1A mRNA, which lacks conserved region 3, was shown to induce only apoptosis which is dependent upon p53 (68). Expression of the E1B-55kDa protein in the presence of the E1A 12S mRNA product was clearly able to block apoptosis. This inhibition presumably was induced by its direct interaction with p53. p53 is a transcription factor and has been shown to enhance gene expression of several proteins which could have important roles in induction of growth arrest and cell death. For instance, p53 has been shown to bind to and transactivate the promoters of the *waf-1* gene (18), which encodes an inhibitor of cyclin-dependent kinases, and *bax* (47). In binding to p53, the E1B-55kDa species may superimpose a transcriptional repression region onto the acidic activation domain of p53 and hence block expression of genes which are involved in the induction of cell death. Complex formation between the 55-kDa protein and p53 may mimic the effects of the cellular protein mdm2, which has also been shown to abrogate p53-dependent transcription and, when overexpressed, to induce cell transformation (48, 50, 51).

The ability of the E1B-55kDa species to prevent apoptosis was completely dependent upon its endogenous transcriptional repression activity. There are conflicting reports concerning the requirement for transcriptional activation by p53 in inducing apoptosis. Some studies suggested that p53 is capable of inducing apoptosis in the absence of transcriptional activation (12, 32), whereas others indicated that transcriptional activation is an essential requirement (57). Our results suggest that the transcriptional repression activity of the 55-kDa protein is required, and they thus appear to support a model in which repression of transcription activated by p53 blocks the cell death pathway.

Of continuing interest was the observation that mutations affecting the Ser-490, Ser-491, and Thr-495 carboxy-terminal phosphorylation sites of the E1B-55kDa protein eliminated both the ability to inhibit p53-dependent transcription and apoptosis. Furthermore, such mutants were also defective for transformation of primary rodent cells, a function most likely due to their inability to prevent E1A-induced apoptosis. Although these mutants were defective for these activities, other functions, such as shutoff of host cell protein synthesis and viral replication in cell culture, remained relatively unaffected (67). It is likely that Ser-490 and Ser-491 are both phosphorylated by CKII, as both sites lie within classic substrate consensus sequences (52); however, we were unable to demonstrate such specificity of phosphorylation in vitro by using purified CKII. It is also possible that phosphorylation of Thr-495 occurs via CKI, as this site lies within a CKI substrate consensus sequence (52). Again, however, our attempts to demonstrate such specificity in vitro by using purified CKI yielded uncertain results.

At present it is unclear how phosphorylation regulates the activity of the 55-kDa protein. It does not appear to affect protein stability, as the half-life of the phosphorylation mutant form was shown to be similar to that of wt 55-kDa protein (67). Phosphorylation may provide additional negative charge required for repression activity, or it may alter the conformation of the 55-kDa protein such that the repression domain interacts more efficiently with p53. It was also noted that the E1B-

55kDa protein contains a region which is reasonably homologous to the Kruppel-associated box domain, which mediates the transcriptional repression activity of the Kruppel protein (7, 42). Phosphorylation may regulate a comparable domain in the E1B-55kDa protein which interacts with specific aspects of the transcriptional machinery to repress gene expression. Another possibility is that phosphorylation may regulate some other property of the 55-kDa protein, such as dimerization, which has also been shown to be the case with the Kruppel protein (61). Experiments to analyze the specific role of phosphorylation further are under way.

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REFERENCES

1. **Akusjärvi, G.** 1993. Proteins with transcription regulatory properties encoded by human adenoviruses. Trends Microbiol. **1:**163–170.
2. **Anderson, C. W., R. C. Schmitt, J. E. Smart, and J. B. Lewis.** 1984. Early region 1B of adenovirus 2 encodes two coterminal proteins of 495 and 155 amino acid residues. J. Virol. **50:**387–396.
3. **Babiss, L. E., and H. S. Ginsberg.** 1984. Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. J. Virol. **50:**202–212.
4. **Babiss, L. E., H. S. Ginsberg, and J. E. J. Darnell.** 1985. Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. Mol. Cell. Biol. **5:**2552–2558.
5. **Barker, D. D., and A. J. Berk.** 1987. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology **156:**107–121.
6. **Bayley, S. T., and J. S. Mymryk.** 1994. Adenovirus E1A proteins and transformation. Int. J. Oncol. **5:**425–444.
7. **Bellefroid, E. J., D. A. Poncelet, P. J. Lecocq, O. Revelant, and J. A. Martial.** 1991. The evolutionarily conserved Kruppel-associated box domain defines a subfamily of eukaryotic multifingered proteins. Proc. Natl. Acad. Sci. USA **88:**3608–3612.
8. **Bernards, R., M. G. de Leeuw, A. Houweling, and A. J. van der Eb.** 1986. Role of the adenovirus early region 1B tumor antigens in transformation and lytic infection. Virology **150:**126–139.
9. **Bos, J. L., L. J. Polder, R. Bernards, P. I. Schrier, P. J. van den Elsen, A. J. van der Eb, and H. van Ormondt.** 1981. The 2.2 kb E1b mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell **27:**121–131.
10. **Boyd, J. M., S. Malstrom, T. Subramanian, L. K. Venkatesh, U. Schaeper, B. Elangovan, E. C. D'Sa, and G. Chinnadurai.** 1994. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. Cell **79:**341–351.
11. **Bridge, E., and G. Ketner.** 1990. Interaction of adenoviral E4 and E1b products in late gene expression. Virology **174:**345–353.
12. **Caelles, C., A. Helmberg, and M. Karin.** 1994. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature **370:** 220–223.
13. **Chen, G., P. E. Branton, E. Yang, S. J. Korsmeyer, and G. C. Shore.** 1996. Adenovirus E1B 19-kDa death suppressor protein interacts with Bax but not with Bad. J. Biol. Chem. **271:**24221–24225.
14. **Chiou, S. K., C. C. Tseng, L. Rao, and E. White.** 1994. Functional complementation of the adenovirus E1B 19-kilodalton protein with Bcl-2 in the inhibition of apoptosis in infected cells. J. Virol. **68:**6553–6566.
15. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. **7:**546–554.
16. **Diller, L., J. Kassel, C. E. Nelson, M. A. Gryka, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S. J. Baker, B. Vogelstein, and S. H. Friend.** 1990. p53 functions as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol. **10:**5772–5781.
17. **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.
18. **el Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
19. **Farmer, G., J. Bargonetti, H. Zhu, P. Friedman, R. Prywes, and C. Prives.** 1992. Wild-type p53 activates transcription in vitro. Nature **358:**83–86.
20. **Fields, S., and S. K. Jang.** 1990. Presence of a potent transcription activating sequence in the p53 protein. Science **249:**1046–1049.
21. **Finlay, C. A., P. W. Hinds, and A. J. Levine.** 1989. The p53 proto-oncogene can act as a suppressor of transformation. Cell **57:**1083–1093.
22. **Gingeras, T. R., D. Sciaky, R. E. Gelinas, D. J. Bing, C. E. Yen, M. M. Kelly, P. A. Bullock, B. L. Parsons, K. E. O'Neill, and R. J. Roberts.** 1982. Nucleotide sequences from the adenovirus-2 genome. J. Biol. Chem. **257:**13475–13491.
23. **Ginsberg, D., S. I. Hirai, K. O. Pinhasi, M. Yaniv, and M. Oren.** 1991. Transfected mouse c-jun can inhibit transformation of primary rat embryo fibroblasts. Oncogene **6:**669–672.
24. **Ginsberg, D., F. Mechta, M. Yaniv, and M. Oren.** 1991. Wild-type p53 can down-modulate the activity of various promoters. Proc. Natl. Acad. Sci. USA **88:**9979–9983.
25. **Gorman, C. M., F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell Biol. **2:**1044–1051.
26. **Graham, F. L., J. Smiley, W. C. Russel, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–72.
27. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
28. **Green, M., K. H. Brackmann, M. A. Cartas, and T. Matsuo.** 1982. Identification and purification of a protein encoded by the human adenovirus type 2 transforming region. J. Virol. **42:**30–41.
29. **Halbert, D. N., J. R. Cutt, and T. Shenk.** 1985. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J. Virol. **56:**250–257.

29a. **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.

30. **Han, J., P. Sabbatini, D. Perez, L. Rao, D. Modha, and E. White.** 1996. The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death promoting Bax protein. Genes Dev. **10:**461–477.
31. **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.
32. **Haupt, Y., S. Rowan, E. Shaulian, K. H. Vousden, and M. Oren.** 1995. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. Genes Dev. **9:**2170–2183.
33. **Jelsma, T. N., J. A. Howe, C. M. Evelegh, N. F. Cunniff, M. H. Skiadopoulos, M. R. Floroff, J. E. Denman, and S. T. Bayley.** 1988. Use of deletion and point mutants spanning the coding region of the adenovirus 5 E1A gene to define a domain that is essential for transcriptional activation. Virology **163:**494–502.
34. **Kastan, M. B., Q. Zhan, W. S. el Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett, B. Vogelstein, and A. J. Fornace.** 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell **71:**587–597.
35. **Leppard, K. N., and T. Shenk.** 1989. The adenovirus E1B 55 kd protein influences mRNA transport via an intranuclear effect on RNA metabolism. EMBO J. **8:**2329–2336.
36. **Lewis, J. B., and C. W. Anderson.** 1987. Identification of adenovirus type 2 early region 1B proteins that share the same amino terminus as do the 495R and 155R proteins. J. Virol. **61:**3879–3888.
37. **Logan, J., and T. Shenk.** 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc. Natl. Acad. Sci. USA **81:**3655–3659.
38. **Lowe, S. W., and H. E. Ruley.** 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. **7:**535–545.
39. **Lucher, L. A., K. H. Brackmann, J. S. Symington, and M. Green.** 1984. Antibody directed to a synthetic peptide encoding the NH2-terminal 16 amino acids of the adenovirus type 2 E1B-53K tumor antigen recognizes the E1B-20K tumor antigen. Virology **132:**217–221.
40. **Malette, P., S.-P. Yee, and P. E. Branton.** 1983. Studies on the phosphorylation of the 58,000 dalton early region 1B protein of human adenovirus type 5. J. Gen. Virol. **64:**1069–1078.
41. **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.
42. **Margolin, J. F., J. R. Friedman, W. K. Meyer, H. Vissing, H. J. Thiesen, and F. R. Rauscher.** 1994. Kruppel-associated boxes are potent transcriptional repression domains. Proc. Natl. Acad. Sci. USA **91:**4509–4513.
43. **McGlade, C. J., M. L. Tremblay, S. P. Yee, R. Ross, and P. E. Branton.** 1987. Acylation of the 176R (19-kilodalton) early region 1B protein of human adenovirus type 5. J. Virol. **61:**3227–3234.
44. **McGrory, W. J., D. S. Bautista, and F. L. Graham.** 1988. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. Virology **163:**614–617.
45. **McKinnon, R. D., J. S. Waye, D. S. Bautista, and F. L. Graham.** 1985.

Nonrandom insertion of Tn5 into cloned human adenovirus DNA. Gene **40:**31–38.
46. **McLorie, W., C. J. McGlade, D. Takayesu, and P. E. Branton.** 1991. Individual adenovirus E1B proteins induce transformation independently but by additive pathways. J. Gen. Virol. **72:**1467–1471.
47. **Miyashita, T., and J. C. Reed.** 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell **80:**293–299.
48. **Momand, J., G. P. Zambetti, D. C. Olson, D. George, and A. J. Levine.** 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell **69:**1237–1245.
49. **Nguyen, M., P. E. Branton, P. A. Walton, Z. N. Oltvai, S. J. Korsmeyer, and G. C. Shore.** 1994. Role of membrane anchor domain of Bcl-2 in suppression of apoptosis caused by E1B-defective adenovirus. J. Biol. Chem. **269:**16521–16524.
50. **Oliner, J. D., K. W. Kinzler, P. S. Meltzer, D. L. George, and B. Vogelstein.** 1992. Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature **358:**80–83.
51. **Oliner, J. D., J. A. Pietenpol, S. Thiagalingam, J. Gyuris, K. W. Kinzler, and B. Vogelstein.** 1993. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature **362:**857–860.
52. **Pearson, R. B., and B. E. Kemp.** 1991. Protein kinase phosphorylation site sequences and consensus specificity motifs. Methods Enzymol. **200:**62–81.
53. **Perricaudet, M., G. Akusjärvi, A. Virtanen, and U. Pettersson.** 1979. Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature **281:**694–696.
54. **Pilder, S., M. Moore, J. Logan, and T. Shenk.** 1986. The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. Mol. Cell. Biol. **6:**470–476.
55. **Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer, and E. White.** 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA **89:**7742–7746. (Erratum, **89:**9974.)
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. **Sabbatini, P., J. Lin, A. J. Levine, and E. White.** 1995. Essential role for p53-mediated transcription in E1A-induced apoptosis. Genes Dev. **9:**2184–2192.
58. **Sandler, A. B., and G. Ketner.** 1989. Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. J. Virol. **63:**624–630.
59. **Sarnow, P., P. Hearing, C. W. Anderson, D. N. Halbert, T. Shenk, and A. J. Levine.** 1984. Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. J. Virol. **49:**692–700.
60. **Sarnow, P., C. A. Sullivan, and A. J. Levine.** 1982. A monoclonal antibody detecting the adenovirus type 5-E1b-58Kd tumor antigen: characterization of the E1b-58Kd tumor antigen in adenovirus-infected and -transformed cells. Virology **120:**510–517.
61. **Sauer, F., and H. Jackle.** 1993. Dimerization and the control of transcription by Kruppel. Nature **364:**454–457.
62. **Shenk, T., and J. Flint.** 1991. Transcriptional and transforming activities of the adenovirus E1A proteins. Adv. Cancer Res. **57:**47–85.
63. **Shepherd, S. E., J. A. Howe, J. S. Mymryk, and S. T. Bayley.** 1993. Induction of the cell cycle in baby rat kidney cells by adenovirus type 5 E1A in the absence of E1B and a possible influence of p53. J. Virol. **67:**2944–2949.
64. **Subler, M. A., D. W. Martin, and S. Deb.** 1992. Inhibition of viral and cellular promoters by human wild-type p53. J. Virol. **66:**4757–4762.
65. **Subramanian, T., B. Tarodi, and G. Chinnadurai.** 1995. p53-independent apoptotic and necrotic cell deaths induced by adenovirus infection: suppression by E1B 19K and Bcl-2 proteins. Cell Growth Diff. **6:**131–137.
66. **Takayesu, D., J. G. Teodoro, S. G. Whalen, and P. E. Branton.** 1994. Characterization of the 55K adenovirus type 5 E1B product and related proteins. J. Gen. Virol. **75:**789–798.
67. **Teodoro, J. G., T. Halliday, S. G. Whalen, D. Takayesu, F. L. Graham, and P. E. Branton.** 1994. Phosphorylation at the carboxy terminus of the 55-kilodalton adenovirus type 5 E1B protein regulates transforming activity. J. Virol. **68:**776–786.
68. **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.
69. **Tremblay, M. L., D. J. Dumont, and P. E. Branton.** 1989. Analysis of phosphorylation sites in the exon 1 region of E1A proteins of human adenovirus type 5. Virology **169:**397–407.
70. **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.
71. **Virtanen, A., and U. Pettersson.** 1985. Organization of early region 1B of human adenovirus type 2: identification of four differentially spliced mRNAs. J. Virol. **54:**383–391.
72. **White, E., and R. Cipriani.** 1990. Role of adenovirus E1B proteins in transformation: altered organization of intermediate filaments in transformed cells that express the 19-kilodalton protein. Mol. Cell. Biol. **10:**120–130.
73. **White, E., R. Cipriani, P. Sabbatini, and A. Denton.** 1991. Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. J. Virol. **65:**2968–2978.
74. **Yee, S. P., D. T. Rowe, M. L. Tremblay, M. McDermott, and P. E. Branton.** 1983. Identification of human adenovirus early region 1 products by using antisera against synthetic peptides corresponding to the predicted carboxy termini. J. Virol. **46:**1003–1013.
75. **Yew, P. R., and A. J. Berk.** 1992. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature **357:**82–85.
76. **Yew, P. R., C. C. Kao, and A. J. Berk.** 1990. Dissection of functional domains in the adenovirus 2 early 1B 55K polypeptide by suppressor-linker insertional mutagenesis. Virology **179:**795–805.
77. **Yew, P. R., X. Liu, and A. J. Berk.** 1994. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. Genes Dev. **8:**190–202.
78. **Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren.** 1991. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature **352:**345–347.
79. **Zambetti, G. P., J. Bargonetti, K. Walker, C. Prives, and A. J. Levine.** 1992. Wild-type p53 mediates positive regulation of gene expression through a specific DNA sequence element. Genes Dev. **6:**1143–1152.
80. **Zantema, A., J. A. Fransen, O. A. Davis, F. C. Ramaekers, G. P. Vooijs, B. DeLeys, and A. J. van der Eb.** 1985. Localization of the E1B proteins of adenovirus 5 in transformed cells, as revealed by interaction with monoclonal antibodies. Virology **142:**44–58.
81. **Zhang, S., S. Mak, and P. E. Branton.** 1992. Overexpression of the E1B 55-kilodalton (482R) protein of human adenovirus type 12 appears to permit efficient transformation of primary baby rat kidney cells in the absence of the E1B 19-kilodalton protein. J. Virol. **66:**2302–2309.