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Importance of the Ser-132 Phosphorylation Site in Cell Transformation and Apoptosis Induced by the Adenovirus Type 5 E1A Protein

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The 289-residue (289R) and 243R early region 1A (E1A) proteins of human adenovirus type 5 induce cell transformation in cooperation with either E1B or activated *ras*. Here we report that Ser-132 in both E1A products is a site of phosphorylation in vivo and is the only site phosphorylated in vitro by purified casein kinase II. Ser-132 is located in conserved region 2 near the primary binding site for the pRB tumor suppressor and, in 289R, just upstream of the conserved region 3 transactivation domain involved in regulation of early viral gene expression. Mutants containing alanine or glycine in place of Ser-132 interacted with pRB-related proteins at somewhat reduced efficiency; however, all Ser-132 mutants transformed primary rat cells in cooperation with E1B as well as or better than the wild type when both major E1A proteins were expressed. Such was not the case with mutants expressing only 289R. In cooperation with E1B, the Asp-132 and Gly-132 mutants yielded reduced numbers of smaller transformed foci. With activated *ras*, all Ser-132 mutants were significantly defective for transformation and the rare foci produced were small and contained extensive areas populated by low densities of flat cells. In the absence of E1B, all Ser-132 mutants induced p53-independent cell death more readily than virus expressing wild-type 289R. These results suggested that phosphorylation at Ser-132 may enhance the binding of pRB and related proteins and also reduce the toxicity of E1A 289R, thus increasing transforming activity.

Adenovirus early region 1A (E1A) produces 13S and 12S immediate-early mRNAs that encode highly similar proteins of 289 and 243 residues (289R and 243R, respectively) which differ only by an internal 46-amino-acid sequence (4, 6, 58). E1A polypeptides are acidic nuclear phosphoproteins (30, 64, 83) containing three regions that are highly conserved in all viral serotypes, CR1, CR2, and CR3 (37). Figure 1 shows that CR1 (residues 40 to 80) and CR2 (residues 120 to 139) are present in both 289R and 243R, whereas CR3 (residues 140 to 185) comprises the internal sequence unique to 289R. In combination with adenovirus E1B or activated c-*ras*, E1A transforms primary rodent cells (24, 39, 65) in a process dependent mainly on the amino terminus, CR1 and CR2 (16, 35, 38, 43, 44, 54, 66, 69, 81). Transformation results largely from interactions with two classes of cellular proteins (15, 16, 29, 80–82), including the pRB retinoblastoma tumor suppressor (15, 79) and related proteins p107 (18) and p130 (1, 19) and the transcriptional coactivator p300 and a related p400 protein (1, 2, 14, 29, 82). Complex formation with p300 and p400 depends on the amino terminus and a portion of CR1, whereas all pRB-related proteins interact with a core binding site in CR2 and to some degree rely on CR1 and the amino terminus (1, 2, 16, 74, 81). E1A proteins are toxic and induce both p53-dependent (9, 46) and p53-independent (68, 70) apoptosis. The major role of E1B products in transformation is to suppress the cytotoxic effects induced by E1A, thus allowing the survival of E1A-transformed cells (62, 78). The mechanism by which cells transformed by E1A and activated *ras* survive is not understood.

E1A proteins are known to be phosphorylated at several serine residues. Ser-219 (72, 73) and Ser-89 (12, 71, 72) are substrates for a Cdk-like protein kinase (10). Ser-96 may also be phosphorylated under certain circumstances (11). Other sites also exist in the carboxy-terminal half of E1A products (72, 77). In addition, there is a potential site at Ser-132 (Ser-132-Asp-Asp-Glu-Asp-Glu-Glu) which conforms to a consensus casein kinase II (CKII) substrate site (57). It lies just downstream of the core binding site for pRB-related proteins (Asp-121-<u>Leu</u>-Thr-<u>Cys</u>-His-<u>Glu</u>) which contains the conserved Leu, Cys, and Glu residues found in other pRB-binding proteins, including simian virus 40 and polyomavirus large T antigens and the human papillomavirus E7 product (8, 13, 16, 40, 55, 81). All of these proteins also contain one or two phosphorylation sites (usually CKII sites) just downstream, suggesting that phosphorylation could affect complex formation. In the case of E7 proteins, these sites (Ser-31 and Ser-32) are highly phosphorylated in the high-risk human papillomavirus serotypes (3, 21, 22, 59). Mutational analysis indicated that alteration of the E7 sites caused considerable (22) or some (33) reduction in E7-mediated cell transformation; however, little effect on complex formation with pRB or activation of E2F transcription factors regulated by pRB and related proteins was observed (3, 22, 33, 59). In the case of the E1A 289R protein, this potential CKII site also lies just upstream of the CR3 transactivation domain which activates the transcription of early viral genes and some cellular genes (52, 63). This region consists of at least two functional subdomains, including an activation domain (residues 140 to 170) involved in interactions with the TATA-box-binding protein and containing a zinc finger (42, 75) and a downstream promoter recognition region required for interactions with transcription factors such as ATF-2 (45).

In the present study, Ser-132 of E1A proteins was shown to be phosphorylated by CKII and to play some role in E1A-mediated cell transformation and cell death.

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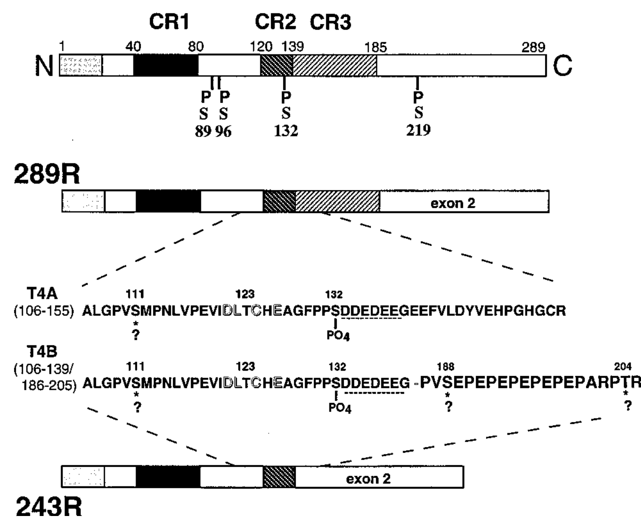

FIG. 1. E1A protein structures and phosphorylation sites. The illustration of the Ad5 E1A protein shows the positions of CR1, CR2, and CR3 (residue numbers shown above) and sites of phosphorylation (P) at serine (S) residues (see text). Below are shown the 289R and 243R proteins, with an expansion of the regions defined by tryptic peptides T4A (from 289R [residues 106 to 155) and T4B (from 243R [residues 106 to 139 linked to 186 to 205 because of splicing of the 12S mRNA]). *, position of Ser or Thr residues which represent potential (?) phosphorylation sites. The CKII consensus substrate sequence is underlined.

MATERIALS AND METHODS

Cell culture, viruses, and plasmids. Human KB cells growing in α-minimal essential medium were infected with wild-type (*wt*) or mutant adenovirus type 5 (Ad5) at 35 PFU per cell, as described previously (64, 82). The Ad5 strain used as the *wt* was *dl*309 (36). Some experiments employed mutant *pm*975, which produces only 13S E1A mRNA and thus 289R but not 243R (52), or *dl*520, which expresses only 12S E1A mRNA and thus 243R but not 289R (27). Additional mutants included *dl*1107 (residues 111 to 123 deleted) and *dl*1109 (residues 128 to 138 deleted) present in either a *dl*309 or *dl*520 background (34); *pm*975/953, which expresses only 289R containing Gly in place of Ser-132 (43); and *dl*313, which contains an in-frame deletion removing the 70 carboxy-terminal residues of both 289R and 243R (7). Mutants containing Ala, Gly, or Asp in place of Ser-132 were prepared by site-directed mutagenesis, as described previously (12). The fidelity of each mutant was confirmed by sequencing and appropriate restriction enzyme digestion. Mutations were introduced into plasmid pXC38, which contains the entire E1A and E1B regions of Ad5 (49), to yield plasmids pXC132A, pXC132G, and pXC132D. DNAs from these plasmids were used to rescue mutations into virus (48) by using either a *dl*309 (mutants ADS132A, ADS132G, and ADS132D) or *pm*975 background (mutants *pm*975/S132A, etc.). Virus stocks were grown in 293 cells that express Ad5 E1A and E1B proteins (25). Mutations were also rescued into two other plasmids, pXC13, which was derived by inserting the E1A region of *pm*975 into pXC38 to yield a construct which expresses E1B and E1A 289R only (mutants pXC13/S132A, etc.), and pH3G-13S, which had been generated previously (43) and expresses E1A 289R alone (mutants pH3G-13/S132A, etc.). Additional plasmids used in some experiments included pH3G-13/953, containing Gly in place of Ser-132; pH3G-13/936, containing Gly in place of Glu-126 within the pRB binding core; and pH3G-13/1112, containing Gly in place of Ser-185 within CR3 (43). pXC89A/96A/132A, which contains Ala residues in place of the Ser-89, -96, and -132 phosphorylation sites, was produced by combining DNA fragments from pAD89A96A (12) and pXC132A. Mutant viruses were also produced by introducing mutations which eliminate the expression of E1B products (50, 70) into *pm*975 and Ser-132 mutant derivatives, yielding *pm*975/E1B−, *pm*975/S132A/E1B−, etc. *dl*520/E1B− has been reported previously (67).

Radioactive labeling. Mock- and Ad5-infected KB cells were labeled from 8 to 12 h postinfection (p.i.) with $^{32}P_i$ (3,000 Ci/mmol; New England Nuclear) in 2 ml of phosphate-free medium or from 10 to 12 h p.i. with 100 μCi of [^{35}S]methionine (1,300 Ci/mmol; Amersham Corp.) in methionine-free medium.

Antiserum and immunoprecipitation. E1A proteins present in cell extracts prepared in standard RIPA lysis buffer (72) containing 500 μM sodium orthovanadate, 50 mM NaF, 1 mM EDTA, and 1 mM β-glycerol phosphate were purified by immunoprecipitation using 5 μl of concentrated M73 mouse monoclonal antibody, which recognizes the E1A carboxy terminus (28), followed by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), as described previously (10, 71). For studies involving the analysis of E1A protein complexes, immunoprecipitations were carried out with cell extracts prepared in lysis buffer consisting of 50 mM Tris-HCl (pH 8.5) containing 250 mM NaCl, 1 mM EDTA, 1% (vol/vol) Nonidet P-40, and 100 KIU of aprotinin per ml and clarified by centrifugation. Extracts were preincubated for 1 h in 150 μl of protein A-Sepharose beads (Pharmacia; resuspended 1 to 2 in lysis buffer) and then for 2 to 3 h with a similar amount of protein A-Sepharose in the presence of the appropriate antibody and 2 mg of bovine serum albumin per ml.

Analysis of tryptic peptides by TLC. ^{32}P-labeled E1A proteins were purified by immunoprecipitation and SDS-PAGE, and after elution, they were digested with trypsin and oxidized, as described previously (72). Peptides were analyzed by two-dimensional thin-layer chromatography (TLC) using Polygram CEL 300 thin layer plates (10).

Analysis of tryptic peptides by HPLC. $^{32}P_i$-labeled E1A proteins purified by immunoprecipitation and SDS-PAGE were extracted and treated with L-(tosylamido 2-phenyl)ethyl chloromethyl ketone (TPCK)-trypsin (Worthington), as described previously (72). Tryptic peptides were separated on a Waters dual pump high-performance liquid chromatography (HPLC) system with a 600E controller using an Ultrasphere octyldecyl silane (C_{18}) reverse-phase column (4.6 by 250 mm) which had been preequilibrated with solution A (5% formic acid in water). Peptides were eluted from the column by a linear (1 to 63%) gradient of solution B (5% formic acid in ethanol) for 95 min at a flow rate of 1 ml/min, as described previously (10). The detection of labeled peptides was achieved by using an on-line LB507A isotope detector (Berthold).

In vitro phosphorylation of E1A proteins by purified CKII. The 289R E1A protein was produced in and purified from *Escherichia coli*, as previously described (17, 20), and resuspended in HB buffer (25 mM morpholinepropanesulfonic acid [MOPS] [pH 7.2] containing 15 mM EGTA, 15 mM *p*-nitrophenyl phosphate, 60 mM β-glycerol phosphate, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1% Triton X-100, and 5 μg [each] of leupeptin and pepstatin per ml). In vitro phosphorylation was carried out in 50 μl of reaction buffer (20 mM morpholineethanesulfonic acid [MES] [pH 6.8] containing 130 mM KCl, 10 mM $MgCl_2$, and 5 mM dithiothreitol) by using 5 μg of E1A 289R and 0.2 μg of purified human CKII purified (obtained from Steve Pellech) in reaction buffer with 1 mM ATP containing 50 μCi of [γ-^{32}P]ATP (10.0 mCi/ml; specific activity, 3,000 Ci/mM; New England Nuclear). The mixture was incubated at 37°C for 5 min, and the reaction was stopped by the addition of an equal volume of double-strength SDS-PAGE sample buffer. Tryptic peptides were prepared from gel-purified labeled E1A proteins.

Quantitative analysis of E1A-binding proteins. To quantify the binding of cellular proteins, equal amounts of immunoprecipitates from extracts prepared by using equal numbers of [^{35}S]methionine-labeled infected KB cells were analyzed by SDS-PAGE. The amount of each protein was then analyzed by one of two methods. In the first, dried gels were fluorographed and then submitted to autoradiography using preflashed Kodak XAR-5 film (41). The intensities of individual bands were quantified by using a Millipore Corp. Bio-Image densitometer system, as described previously (1, 2). In the second, dried gels were analyzed with a Fujix Bas 2000 PhosphorImager. Data from five separate experiments were obtained and averaged. The amounts of radioactivity present in p400, p300, p130, p107, and pRB were determined and then corrected for the amount of cell extract by using the band density of a nonspecific species migrating to about 75 kDa. Previous studies had indicated that because of the considerable excess of E1A proteins in infected cells, complex formation was independent of small variations in E1A products (1).

Transformation of primary baby rat kidney cells. Transformation assays were carried out with primary baby rat kidney cells prepared from 6-day-old Wistar rats, as described previously (12). Cells were transfected with 5 μg of DNA from each of the following plasmids containing *wt* or mutant E1A sequences: pXC38, containing genomic E1A and E1B, pXC13, which expresses only the 289R E1A product plus E1B; and a combination of pH3G which expresses only 289R plus pEJ^{ras}, which expresses activated p21$^{\text{Ha-}ras}$ (11). Five separate experiments using independent DNA preparations were carried out. The numbers of foci on each plate were counted, and the data were used to analyze the efficiency of transformation relative to that of *wt*.

Cell viability assays. Mouse 10(1) cells, which fail to express p53 (31), growing in 24-well plates in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum were infected at about 80% confluence with *wt* or mutant Ad5. At various times after infection, adherent and nonadherent cells were pooled and viability was assessed by trypan blue exclusion. At least 300 cells were counted at each time point.

Measurement of E1A-mediated transactivation of adenovirus E2, E3, and E4 promoters. Transactivation assays using NIH 3T3 or CHO cells plated at a density of 2×10^5 cells on 60-mm-diameter dishes were performed. Reporter plasmids containing one of the Ad5 promoters, E2, E3, or E4, upstream of the chloramphenicol acetyltransferase (CAT) gene included E2-CAT, pKCAT23 and E4-CAT, respectively (76). Transient cotransfections were performed by the calcium phosphate precipitation method (26) using 2.5 μg of reporter plasmid DNA and 2.5 μg of DNA from plasmids expressing *wt* or mutant E1A products. Plasmid pSV2CAT (23) was used as a positive control. In addition, 3 μg of RSVβ-Gal plasmid (60) was included to allow the normalization of transfection efficiency by measuring β-galactosidase activity. NIH 3T3 cells were glycerol shocked after 12 h and then harvested 36 h later. CAT assays using cell extracts containing equal amounts of β-galactosidase activity were performed essentially as described by Gorman et al. (23). The amount of activity was quantified from TLC plates by using a Fujix Bas 2000 PhosphorImager.

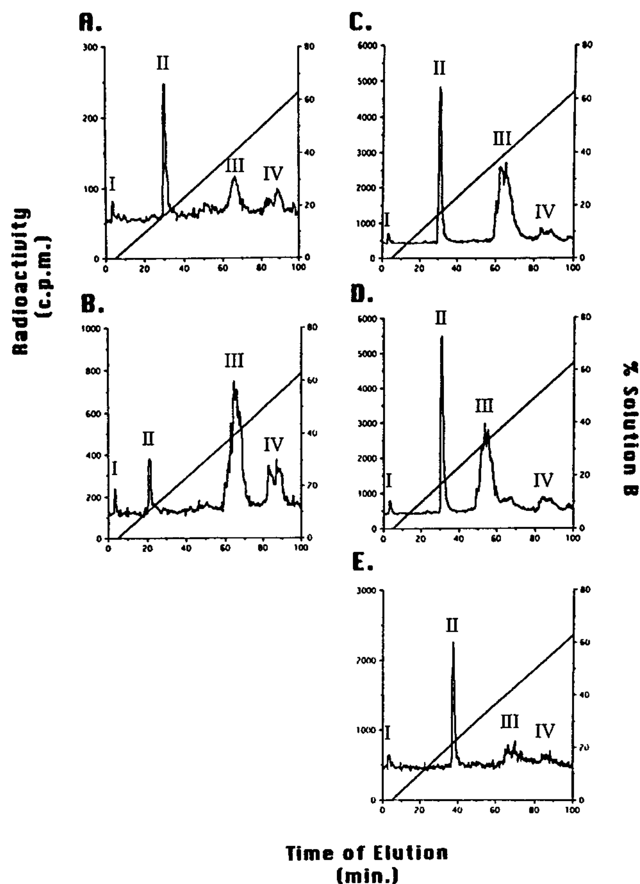

FIG. 2. Analysis of E1A tryptic phosphopeptides by HPLC. E1A proteins labeled with $^{32}P_i$ were isolated by immunoprecipitation and SDS-PAGE, and tryptic peptides were analyzed by HPLC, as described in Materials and Methods. The amount of radioactivity is shown on the ordinate, and the time of column elution (in minutes) is shown on the abscissa. (A) *wt dl*309; (B) *dl*313; (C) *dl*520; (D) *dl*1107/520; (E) *dl*1109/520.

RESULTS

Analysis of E1A tryptic phosphopeptides by HPLC. Previous studies (72) have indicated that Ad5 E1A proteins are phosphorylated at multiple serine residues, including a major site, at Ser-219, present within tryptic peptide T11 (residues 216 to 223). Figure 2A shows tryptic peptides obtained from *wt* Ad5-infected KB cells labeled with $^{32}P_i$ and analyzed by HPLC using reverse-phase chromatography. As shown previously, peptide T11 eluted at about 30 to 35 min as the major peak (peak II) and a small amount of free phosphate eluted immediately at 2 to 3 min as peak I (72). Labeled material also eluted at about 82 to 95 min (peak IV) in the position shown for some of phosphorylated peptide T2 (residues 3 to 97), which contains phosphorylation sites at Ser-89 and possibly Ser-96 (10, 11). (Earlier studies had indicated that most of the phosphorylated form of T2 peptide remained bound to the column, whereas unphosphorylated T2 eluted much earlier [71, 72].) Figure 2A shows that additional labeled material eluted between 60 and 75 min (peak III). Previous studies in which ^{32}P- and [^{35}S]methionine-labeled E1A tryptic peptides were sequenced by automated Edman degradation suggested that the peak III species might originate from peptide T12 (residues 224 to 258), which contains multiple potential phosphorylation sites (72). To analyze the origin of peak III further, E1A tryptic phosphopeptides were examined from mutant *dl*313, which lacks residues 220 to 289 and thus all of T12 and a portion of T11. Figure 2B shows that the T11 peak II phosphopeptide was relatively poorly labeled and eluted earlier (20 to 25 min) than that from the *wt* (Fig. 2A). Peptide T11 from the *wt* consists of Arg-Pro-Thr-Ser-219-Pro-Val-Ser-Arg, whereas in *dl*313 the deletion alters the sequence to Arg-Pro-Thr-Ser-219-Leu-Arg-STOP (7). Thus, it was not surprising that the times of elution of these two peptides differed or that, as Ser-219 is phosphorylated by a Cdk-like protein kinase (10) which requires Pro immediately downstream (57), phosphorylation in *dl*313 was less efficient than that in the *wt*. Phosphorylation of the peak IV T2 peptide was similar to that of the *wt*, as was that of the peak III peptide. Thus, peak III could not have originated from T12, which is absent in the E1A products of *dl*313. To examine the origin of this material further, E1A tryptic phosphopeptides were studied by using mutant *dl*520, which produces only the 243R E1A product, and two E1A deletion mutants present in the *dl*520 background. Figure 2C shows that *dl*520 yielded a pattern virtually identical to that of the *wt* (Fig. 2A). However, *dl*1107/520, which lacks residues 111 to 123, yielded normal T11 (peak II) and T2 (peak IV) peptides, but peak III eluted for the most part at 50 to 60 min, with only low levels appearing at the normal elution time of 60 to 75 min. These results suggested that most of peak III must originate from a tryptic peptide containing amino acids 111 to 123 but that the phosphorylation site(s) is not present within these 13 residues. Figure 1 shows that amino acids 111 to 123 are present within a tryptic peptide, T4, that starts at residue 106 but differs in 289R and 243R at the carboxy-terminal end because of the unique splice donor site in the 12S E1A mRNA. In 289R, tryptic peptide T4A results from cleavage at Arg-155, which is present within CR3. In 243R, tryptic peptide T4B contains resides 106 to 139, which, because of the 12S mRNA splicing pattern, are linked directly to residues 186 to 205. Apart from Ser-111 and Thr-123, which are within the *dl*1107 deletion, the only other potential phosphorylation site is at Ser-132, which lies in the context of a consensus substrate site for CKII (57). In the case of T4B, in addition to Ser-132, other potential sites are present at Ser-188 and Thr-204. Previous studies involving the sequencing of HPLC-purified [^{35}S]methionine- and ^{32}P-labeled E1A tryptic peptides by automated Edman degradation had identified both T4A and T4B and suggested that they were not phosphorylated. However, as described above, our finding that with T2, phosphorylation can induce a major change in the time of elution left open the possibility that Ser-132 might be phosphorylated. To examine this possibility more directly, E1A tryptic phosphopeptides were examined by using mutant *dl*1109, which lacks residues 128 to 138. Figure 2E shows that the T11 (peak II) and T2 (peak IV) peptides were present at levels comparable to those of *wt dl*520 (Fig. 2C), but only a small amount of material was eluted at 60 to 75 min in the position of peak III. These results suggested that much of peak III from the 243R E1A protein originated from peptide T4B and that Ser-132 appeared to be the major phosphorylation site within this peptide. Therefore, the small amount of material which did elute at 60 to 75 min may derive from another as-yet-unidentified peptide.

Analysis of E1A tryptic phosphopeptides by TLC. Tryptic phosphopeptides from the 243R E1A protein encoded by *dl*520 were analyzed by two-dimensional TLC. Figure 3A (top) shows that phosphopeptide T11 from *wt dl*520 migrated toward the cathode as a single major species, whereas T2 appeared as a collection of species, as found previously (10, 11). In addition to several minor species of unknown origin, two or more major phosphopeptides which we believe represent ^{32}P-labeled T4B were also observed. Multiple species could arise from T4B by incomplete oxidation, partial trypsin digestion, or the presence

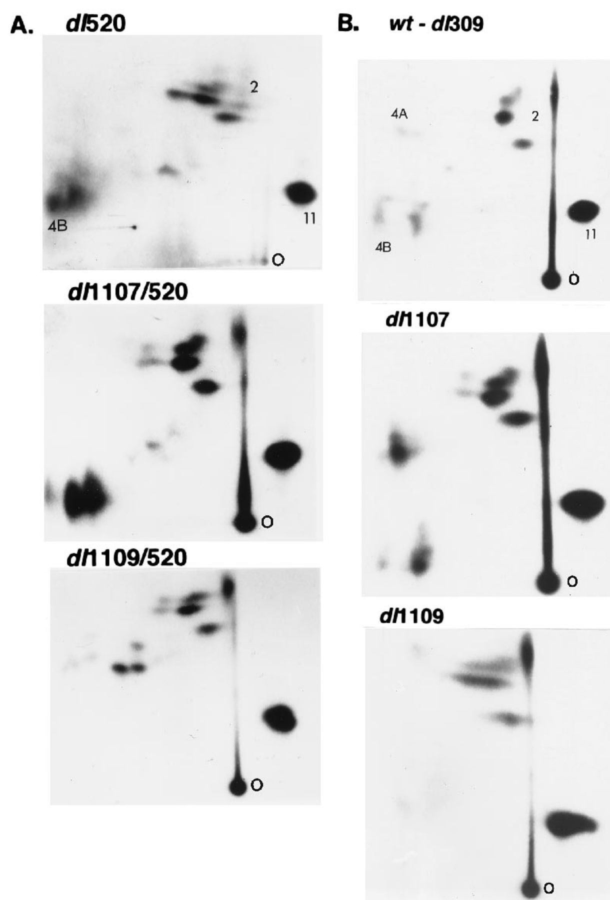

FIG. 3. Analysis of tryptic phosphopeptides of E1A proteins by TLC. E1A proteins isolated from KB cells infected with *dl*520 (A), *dl*309 (B), or mutant derivatives and labeled with $^{32}P_i$ were purified by immunoprecipitation and SDS-PAGE and treated with trypsin, as described in Materials and Methods. The resulting tryptic peptides were separated by TLC, and phosphopeptides were detected by autoradiography. The positions of peptides T11, T2, T4A, and T4B (see text) as well as that of the origin (O) are indicated. Chromatography was from bottom to top, and electrophoresis was from right to left, with the cathode at the right.

of multiple phosphorylation sites. Figure 3A (middle) shows that with *dl*1107, which lacks residues 111 to 123 within T4B, these species were still well labeled, although they did not appear to migrate as far from the origin as did corresponding forms in the *wt*. The remaining phosphopeptide pattern was similar to that of the *wt*. These results paralleled those obtained by HPLC (Fig. 2D), suggesting that these forms originate from peptide T4B. With *dl*1109/520, which lacks resides 128 to 138 and thus Ser-132, these species were absent (Fig. 3A, bottom), although there was evidence of two or three novel species which could represent minor phosphopeptides more highly labeled in this experiment or modified peptide T4B phosphorylated at other sites (see below). Nevertheless, these results also indicated Ser-132 was a site of phosphorylation.

Figure 3B (top) shows that with *wt dl*309, which produces both 289R and 243R in addition to the two species believed to represent T4B, a novel peptide which appeared to originate from peptide T4A was also present. By using *dl*1107 (Fig. 3B, middle), both this species and the T4B peptides migrated closer to the origin, as would be expected as portions of peptides T4A and T4B were deleted. With *dl*1109, which lacks Ser-132, none of these species were apparent. These results suggested that these peptides corresponded to T4A and T4B and that Ser-132 was a site of phosphorylation in both.

Analysis of E1A tryptic phosphopeptides from Ser-132 mutants. To determine if Ser-132 is phosphorylated, ^{32}P-labeled E1A tryptic peptides were analyzed from cells infected with either *pm*975, which produces only 289R (52), and *pm*975/953, which produces only 289R containing Gly in place of Ser-132 (43). Figure 4A (top) shows that *wt pm*975 yielded a pattern similar to that of *wt dl*309, except that the species believed to originate from T4B and to be present only in 243R were absent. In addition to peptides T11, T2, and T4A, two additional minor species which we have recently shown to originate from novel sites in the 289R E1A protein (77) were apparent. Figure 4A (bottom) shows that *pm*975/953 yielded a similar pattern, except that the species believed to originate from T4A was totally absent. These results confirmed both the identity of peptide T4A on TLC and the fact that Ser-132 is the only site of phosphorylation within T4A. To examine phosphorylation at Ser-132 in both 289R and 243R, mutants were produced in a *dl*309 background in which the codons for Ser-132 were converted to Ala (AD132A), Gly (AD132G), or Asp (AD132D). Figure 4B (top left) shows again the pattern obtained with *wt dl*309, in which the T4A peptide was present as a single species whereas the proposed T4B peptide migrated as two or more forms. The patterns produced by all of the Ser-132 mutants (Fig. 4B) were similar. As with *pm*975/953 (Fig. 4A, bottom), the ^{32}P-labeled species corresponding to T4A was not detected. In each case, a cluster of species presumably representing peptide T4B was also present. These results suggested that Ser-132 was the only site of phosphorylation in the T4A peptide from 289R; however, with T4B from 243R, at least one additional site must exist, as suggested above by the results with *dl*1109/520 (Fig. 3A, bottom).

Ser-132 is phosphorylated by CKII. Because Ser-132 exists adjacent to a series of six acidic residues in a classic CKII substrate consensus sequence (Fig. 1), we determined if CKII could phosphorylate this site in vitro. E1A 289R protein synthesized in and purified from *E. coli* was incubated with purified CKII in the presence of [γ-^{32}P]ATP, and tryptic peptides were prepared and analyzed by TLC. Figure 5B shows that a single ^{32}P-labeled species which migrated to the position of peptide T4A labeled in vivo in *pm*975-infected KB cells (Fig. 5A) was evident. Its identity as T4A was confirmed by analyzing a mixture of in vitro- and in vivo-labeled samples (Fig. 5C). Thus, CKII is probably the protein kinase that phosphorylates Ser-132 in vivo.

Importance of the Ser-132 phosphorylation site in E1A protein complex formation. E1A-mediated cell transformation requires the formation of complexes with several cellular proteins, including p300 and a related p400 protein and pRB and related polypeptides, p107 and p130. To determine if phosphorylation at the downstream Ser-132 site affects such interactions, complex formation by E1A proteins with mutations affecting Ser-132 was measured, as described previously (1, 2). Figure 6 shows one of several SDS-PAGE analyses of immunoprecipitates prepared under mild conditions by using E1A-specific M73 antibodies and extracts from [^{35}S]methionine-labeled KB cells infected with *wt* or mutant Ad5. The amounts of each E1A-binding protein were quantified from dried polyacrylamide gels or autoradiographs (such as that shown in Fig. 6) and expressed as percentages of *wt* levels. Table 1 summarizes the results of seven such experiments which examined E1A complex formation by Ser-132 mutants. All mutants interacted with p300 and p400 at levels similar to those of the *wt*. This result was not surprising, as these proteins interact with the amino terminus and CR1 and have little or no requirement

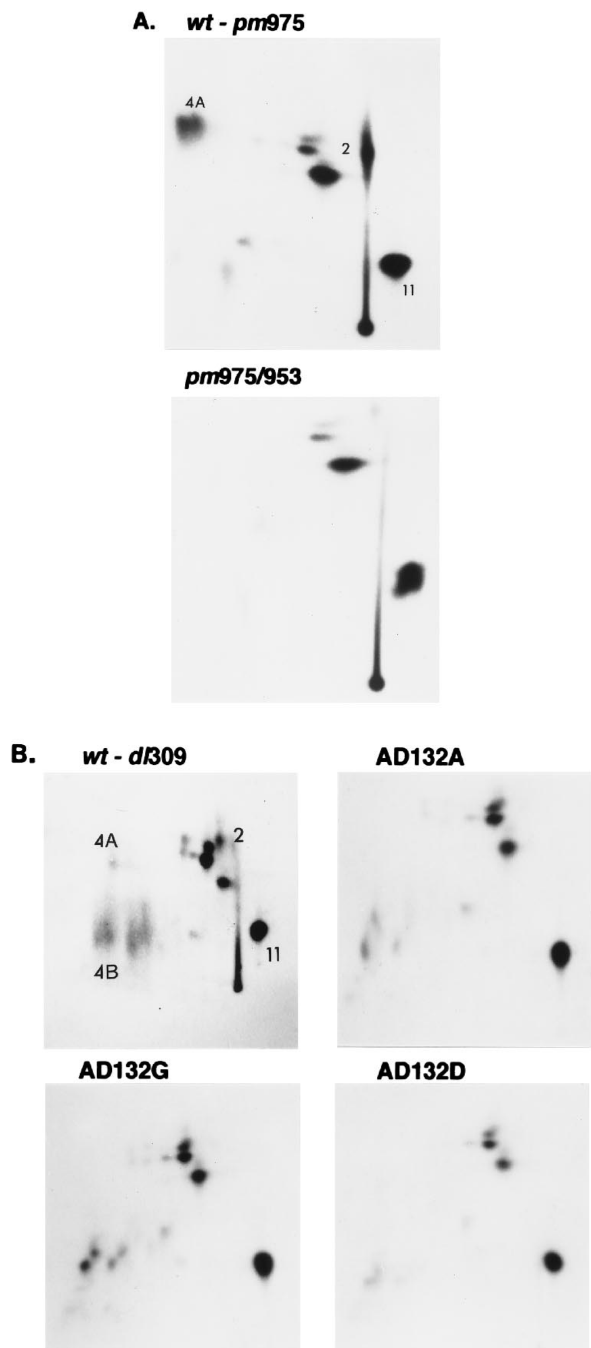

FIG. 4. Analysis of phosphopeptides from E1A 289R and from Ser-132 mutants by TLC. E1A tryptic phosphopeptides were prepared from KB cells infected with the *wt* or Ser-132 mutants and labeled with $^{32}P_i$ as described in the legend to Fig. 3. The positions of peptides T11, T2, T4A, and T4B (see text) are indicated. Mutant *pm*975/953 contains Gly-132.

for CR2 (1, 2, 16, 81). In the case of mutants in the *dl*309 background which express both 289R and 243R, the interactions with p107 were similar in all cases. However, the binding of both pRB and p130 was reduced by 22 to 34% for the Gly-132 and Ala-132 mutants (ADS132G and ADS132A), although that of the Asp-132 mutant (ADS132D) was similar to that of the *wt*. In the case of mutants in the *pm*975 background which express 289R only, including *pm*975/953, which was generated by others and contains Gly-132 (43), the binding of pRB, p130, and p107 was reduced by 17 to 30% for the S132A and S132G mutants; with the S132D mutant, interactions were only modestly affected. Thus, conversion of the Ser-132 site of the E1A 289R protein to Gly or Ala (but apparently less so to an acidic Asp residue) appeared to reduce the binding of all pRB-related proteins by about 20 to 30%.

Effects of Ser-132 mutations on E1A-mediated cell transformation. To determine if E1A transforming activity is affected by the reduction in complex formation with pRB-related proteins or some other effect resulting from the alteration of the Ser-132 phosphorylation site, transformation assays using primary baby rat kidney cells transfected with DNA from plasmids expressing *wt* or mutant E1A and E1B were conducted. Table 2 shows that all Ser-132 mutants actually transformed somewhat more efficiently than did the *wt*. These results suggested that elimination of the phosphorylation site at Ser-132, at least under the conditions employed, did not affect E1A-mediated cell transformation. An independent study by another group also showed that an Ala-132 E1A mutant was able to transform rodent cells (53). However, an additional study reported that an E1A plasmid expressing only the 289R protein containing Gly-132 was defective for transformation in cooperation with activated c-Ha-*ras* (43). These different outcomes could have resulted from differences in experimental conditions, including the use of *ras* instead of E1B or the expression of 289R alone rather than expression of both E1A products. To clarify this discrepancy, we introduced our Ser-132 mutants into plasmid pXC13, which expresses E1A 289R only plus E1B, and pH3G-13, which expresses 289R alone in

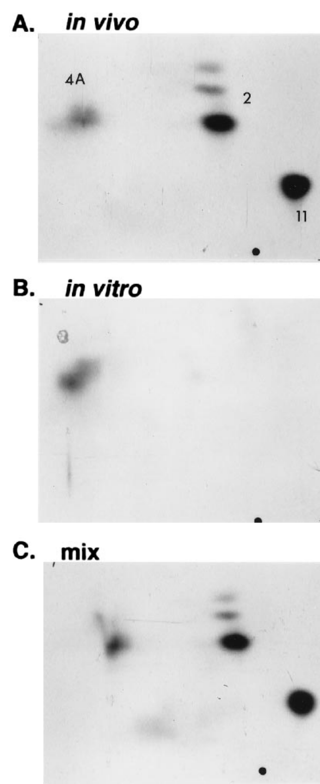

FIG. 5. Analysis of phosphopeptides from E1A products labeled with ^{32}P in vivo and in vitro. E1A 289R protein synthesized in and purified from *E. coli* was incubated in vitro with [γ-^{32}P]ATP and purified CKII, and the tryptic phosphopeptide pattern determined by TLC was compared with that of 289R labeled in vivo in *pm*975-infected cells. (A) 289R labeled in vivo; (B) 289R labeled in vitro with purified CKII; (C) an equal mixture (mix) of samples from panels A and B.

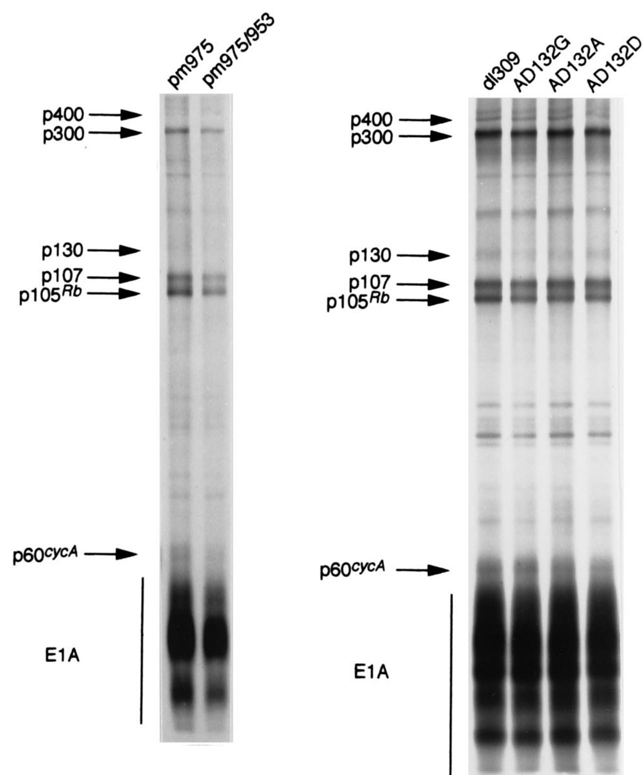

FIG. 6. Complex formation by mutant and *wt* E1A proteins. KB cells infected with either *pm*975, *dl*309, or Ser-132 E1A mutants were labeled with [^{35}S]methionine, and cell extracts were immunoprecipitated under mild conditions with M73 serum, as described in Materials and Methods. The migration positions of E1A-binding proteins are shown on the left.

the absence of E1B. Transformation experiments using mutant and *wt* pXC13 constructs and pH3G-13 constructs in combination with activated Ha-*ras* were carried out. Table 3 shows that pXC13 transformed cells efficiently but consistently worse than did pXC38. These data suggested that in cooperation with E1B, transformation was more efficient in the presence of both major E1A products than of 289R alone. With the Ser-132 mutants, although pXC13/S132A was similar to the *wt*, both pXC13/S132G and pXC13/S132D showed reduced numbers of transformants. Table 3 also shows that the numbers of transformed foci obtained with pXC13 were greater than those obtained with pH3G-13 plus *ras*, indicating that for E1A 289R alone the transforming efficiency was higher with E1B than with activated *ras*. No transformation was observed with *ras* alone. The morphologies of transformed foci were also of some interest. Figure 7A and B show typical foci produced by pXC38 and pXC13, respectively, which consist of extremely dense concentrations of rounded cells typical of E1A- and E1B-transformed colonies. Figure 7C shows a typical focus produced by pH3G-13 and *ras*, which is characterized by lower numbers of cells and several regions which appear to be devoid of all cells. Such foci never reached the size or density of those produced with E1B even after longer periods in culture. These results suggested that relative to transformation with E1B, transformation with *ras* led to lower numbers of poorly developed foci in which cell death occurred at a high rate. Table 3 shows that the Ser-132 mutants induced even fewer transformants than did the *wt*; as shown in Fig. 7D through F, these rare colonies were also small and contained extensive areas populated by low densities of flat cells. Table 3 shows that similarly low levels of transformation were also obtained with the Gly-132 mutant pH3G-13/953 of Lillie et al. (43) as well as with two other mutants produced by this group containing Ala residues in place of Glu-126 in the pRB binding core, which greatly reduces the binding of pRB (pH3G-13/936), and Ser-185 in CR3, which blocks E1A transactivation activity, thus reducing the levels of E1A mRNA (pH3G-13/1112). In summary, the data indicated that removal of the Ser-132 phosphorylation site in E1A 289R resulted in a large reduction in transforming activity and the production of foci with morphologies typical of poorly transformed cells.

Effects of Ser-132 mutations on E1A-induced cell death. One explanation for the reductions in transformation could be related to the toxicities of E1A products. The expression of E1A proteins in the absence of E1B products is cytotoxic and induces apoptosis. A major role of the 55- and 19-kDa E1B products in infection and transformation is to block E1A-induced cell death, thus allowing the establishment of permanently transformed foci. The expression of E1A alone normally results in abortive transformation, and thus few foci develop because most transformants die because of apoptosis. The mechanism by which *ras* complements E1A is not known, but the lower transforming efficiencies relative to that of E1B could be due to increased E1A-induced cell death. E1A induces cell death by at least two mechanisms, one dependent on p53 and induced by either 289R or 243R and a second independent of p53 and induced only by 289R (70). To examine the relative toxicities of E1A proteins, mouse 10(1) cells were infected with *wt* or Ser-132 mutant virus and cell viability was measured at various times after infection by a trypan blue exclusion assay, as described previously (70). 10(1) cells fail to express p53 and thus are susceptible only to p53-independent apoptosis induced by E1A-289R (70). Figure 8A shows that cells remained viable for up to 80 h after infection with either *wt* virus *dl*309 or *pm*975 which expresses E1B products. Infection with mutant *pm*975/E1B− led to extensive cell death, commencing at about 40 h. Such was not seen with *dl*520/E1B−, which expresses 243R but not 289R. All three Ser-132 mutants present in a *pm*975/E1B− background, especially those containing Ala-132 and Gly-132, induced cell death more efficiently than did the *wt*. These results indicated that removal

TABLE 1. Binding of cellular proteins and phosphorylation at Ser-132[a]

Virus	Amt (%)				
	p300	p400	p105^{Rb}	p107	p130
Expressing 289R and 243R					
*dl*309 (*wt*)	100	100	100	100	100
ADS132A	92 ± 38	98 ± 65	68 ± 20	90 ± 18	66 ± 23
ADS132G	91 ± 25	92 ± 24	78 ± 19	93 ± 27	70 ± 19
ADS132D	93 ± 17	88 ± 40	99 ± 33	93 ± 28	90 ± 18
Expressing 289R only					
*pm*975	100	100	100	100	100
*pm*975/953	98 ± 23	94 ± 17	72 ± 19	78 ± 21	79 ± 20
*pm*975/S132A	97 ± 14	104 ± 40	75 ± 14	78 ± 10	78 ± 15
*pm*975/S132G	93 ± 13	90 ± 15	70 ± 19	75 ± 12	83 ± 16
*pm*975/S132D	103 ± 24	98 ± 25	87 ± 11	93 ± 30	91 ± 17

[a] The amounts of cellular proteins that coimmunoprecipitate with *wt* and Ser-132 mutant E1A proteins were assessed by SDS-PAGE in seven separate studies, including the one shown in Fig. 6. The amounts of each protein were quantified from autoradiographs or with a PhosphorImager, as described in Materials and Methods, and then expressed as mean percentages ± standard deviations of the levels obtained with *wt* virus *dl*309 or *pm*975.

TABLE 2. Transformation of baby rat kidney cells in cooperation with E1B by E1A mutants with defects affecting phosphorylation at Ser-132[a]

Plasmid	Avg no. of foci per plate[b]					Avg % ± SD of *wt* result
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	
pXC38 (*wt*)	13.3 (100)	13.1 (100)	5.3 (100)	46.6 (100)	54.9 (100)	100
pXC132A	17.9 (135)	21.3 (162)	5.4 (108)	77.8 (167)	70.5 (128)	130 ± 27
pXC132G	49.6 (372)	47.3 (261)	8.5 (187)	70.8 (152)	92.4 (160)	223 ± 82
pXC132D	32.4 (244)	31.8 (243)	5.3 (100)	77.9 (167)	86.9 (158)	162 ± 59

[a] Primary baby rat kidney cells were transfected with 5 μg of DNA from plasmids expressing either *wt* Ad5 E1A and E1B proteins (pXC38) or mutants in which the codon for Ser-132 of E1A products had been altered to Ala, Gly, or Asp. Usually five plates of baby rat kidney cells were used for each construct.
[b] Parenthetical data are percentages for mutants relative to the results for *wt* pXC38. A different DNA preparation was used for each experiment.

of the Ser-132 phosphorylation site made E1A 289R more toxic, thus reducing E1A transforming activity in cooperation with *ras*. However, the Gly-132 and Asp-132 mutants also transformed less efficiently in cooperation with E1B (Table 3). To determine if differences in E1A toxicity were evident in the presence of E1B products, a similar cell killing experiment using 10(1) cells and viruses containing Ser-132 mutations but expressing E1B was conducted. It should be remembered that in the presence of the E1B 19-kDa protein, infected cells survive for much longer times. Figure 8B shows this to be the case. As in Fig. 8A, cells infected with *pm*975/E1B− died starting at about 48 h p.i. and death was complete by 75 h p.i. With both *wt dl*309 and *pm*975, cell death was not at all evident until after 120 h p.i. and continued beyond 168 h p.i. *pm*975, which produces E1A 289R only, appeared to be somewhat more toxic than was *dl*309, which produces both 289R and 243R. Of greatest interest was the finding that both *pm*975/S132G and *pm*975/S132D were somewhat more toxic than was *pm*975. These results suggested that increased cell toxicity may account for the reduced transforming efficiencies of these mutants in the presence of E1B. The results obtained with the S132A mutant were more puzzling. Figure 8B shows that although in the absence of E1B this mutant was more toxic than was the *wt*, such was not the case when E1B proteins were present. Nevertheless, these results correlated with the data in Table 3 which indicated that transformation by the S132A mutant was quite defective in cooperation with *ras* but was similar to that of the *wt* with E1B.

Effects of Ser-132 mutations on E1A transactivation activity. p53-independent apoptosis is induced by E1A 289R but not by 243R (47, 70). It was possible therefore that removal of the Ser-132 phosphorylation site affected the toxicity of 289R by affecting the activity of the CR3 transactivation domain, which resides just downstream, perhaps by affecting interactions with the TATA-binding protein or other transcription factors. This possibility was tested in DNA transfection experiments in which the transactivation activities of *wt* and mutant 289R were determined by measuring the levels of CAT expressed from plasmids containing CAT under the control of the Ad5 E2, E3, and E4 promoters. No obvious differences were apparent between the *wt* and mutants with any CAT construct (data not shown). Such was the case even with triple mutant pXC89A/132A, in which the phosphorylation sites at Ser-89, -96, and -132 were all converted to Ala. Thus, at least by this approach, alteration of the Ser-132 site appeared to have little effect on the transactivating activity of 289R.

DISCUSSION

The present studies have identified Ser-132 as a phosphorylation site in Ad5 E1A proteins. Previous work had indicated that purified CKII could phosphorylate a synthetic peptide consisting of E1A residues 125 to 139 (5), and here we have shown that it phosphorylates full-length E1A 289R in a tryptic peptide containing Ser-132. Both genetic and biochemical evidence indicated that such phosphorylation also occurs in vivo.

TABLE 3. Transformation of baby rat kidney cells by E1A 289R mutants with E1B or activated *ras*

Plasmid	Avg no. of foci per plate[a]					Avg % of *wt* result
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	
pXC38 (289R/243R + E1B)	97 (149)	110 (190)	105 (154)			164
pXC13 (*wt* 289R + E1B)	65 (100)	58 (100)	68 (100)			100
pXC13/S132A	82 (126)	59 (102)	60 (88)			105
pXC13/S132G	35 (54)	30 (52)	26 (38)			48
pXC13/S132D	17 (26)	14 (24)	19 (28)			26
pXC13 (E1A + E1B)				66 (287)	51 (182)	235
ras alone				0 (0)	0 (0)	0
pH3G-13 (*wt* 289R) + *ras*				[23] (100)	[28] (100)	100
pH3G-13/S132A + *ras*				[5] (22)	[10] (36)	29
pH3G-13/S132G + *ras*				[2] (9)	[5] (18)	14
pH3G-13/S132D + *ras*				[3] (13)	[4] (14)	14
pH3G-13/053 + *ras*				[2] (9)	[3] (11)	10
pH3G-13/936 + *ras*				[1] (4)	[0] (0)	2
pH3G-13/1112 + *ras*				[5] (22)	[10] (36)	29

[a] Primary baby rat kidney cells were transfected with 5 μg of DNA from plasmids expressing *wt* or mutant E1A proteins and E1B (experiments 1 through 3) or 5 μg of DNA expressing *wt* or mutant 289R E1A protein plus 5 μg of *ras* plasmid DNA (experiments 4 and 5). Usually five plates of cells were used for each construct. A different DNA preparation was used for each experiment. Parenthetical data are percentages for mutants relative to the results for the *wt*. Brackets around data indicate that the colonies observed were small and had the unusual phenotype described in the text.

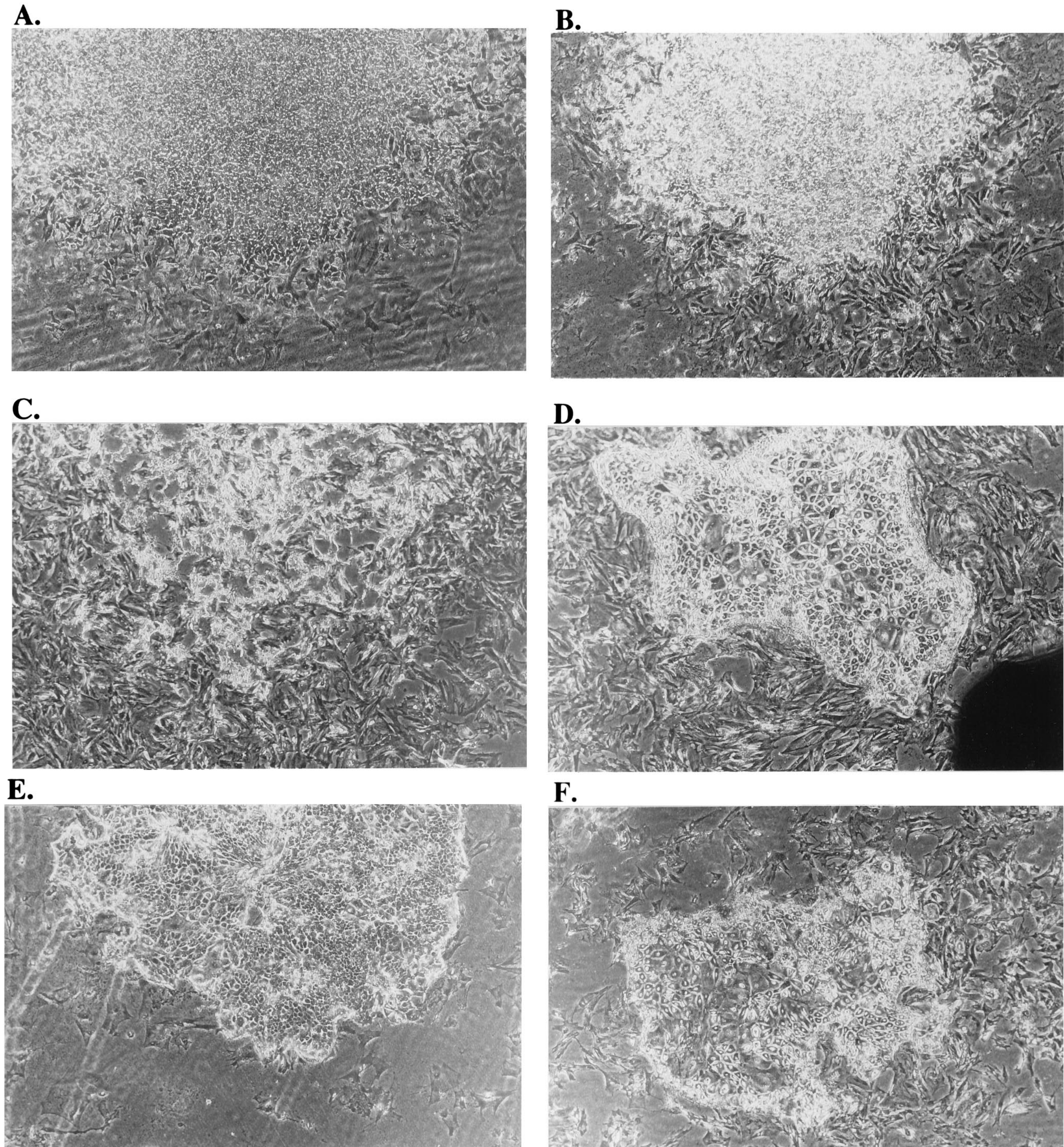

FIG. 7. Morphologies of E1A-transformed cells. Representative transformed cell foci generated in DNA transfection experiments (Table 3) are presented. (A) pXC38; (B) pXC13; (C) pH3G-13 + *ras*; (D) pH3G-13/S132A + *ras*; (E) pH3G-13/S132G + *ras*; (F) pH3G-13/S132D + *ras*.

This site was not identified in our previous studies (72) because the phosphorylated and unphosphorylated forms of T4 tryptic peptides containing Ser-132 elute from reverse-phase columns at very different times, as found previously for the T2 E1A phosphopeptide (10). The detection of high levels of unphosphorylated T4 peptides (72) suggests that only 5 to 10% of E1A molecules may be phosphorylated at Ser-132.

It is likely that Ser-132 is the only phosphorylation site in the T4A tryptic peptide from E1A 289R. Such does not appear to be the case for the T4B peptide from 243R, which differs from T4A at the carboxy terminus because of the 12S mRNA splicing pattern. T4B migrated during TLC as multiple species which were still somewhat ^{32}P labeled with the Ser-132 mutants, suggesting the existence of one or more additional phosphorylation sites. T4B contains two other possible sites, Ser-188 and Thr-204, and we now have direct biochemical and genetic evidence that Ser-188 is phosphorylated in the E1A 289R protein (77), suggesting that multiple T4B peptides could

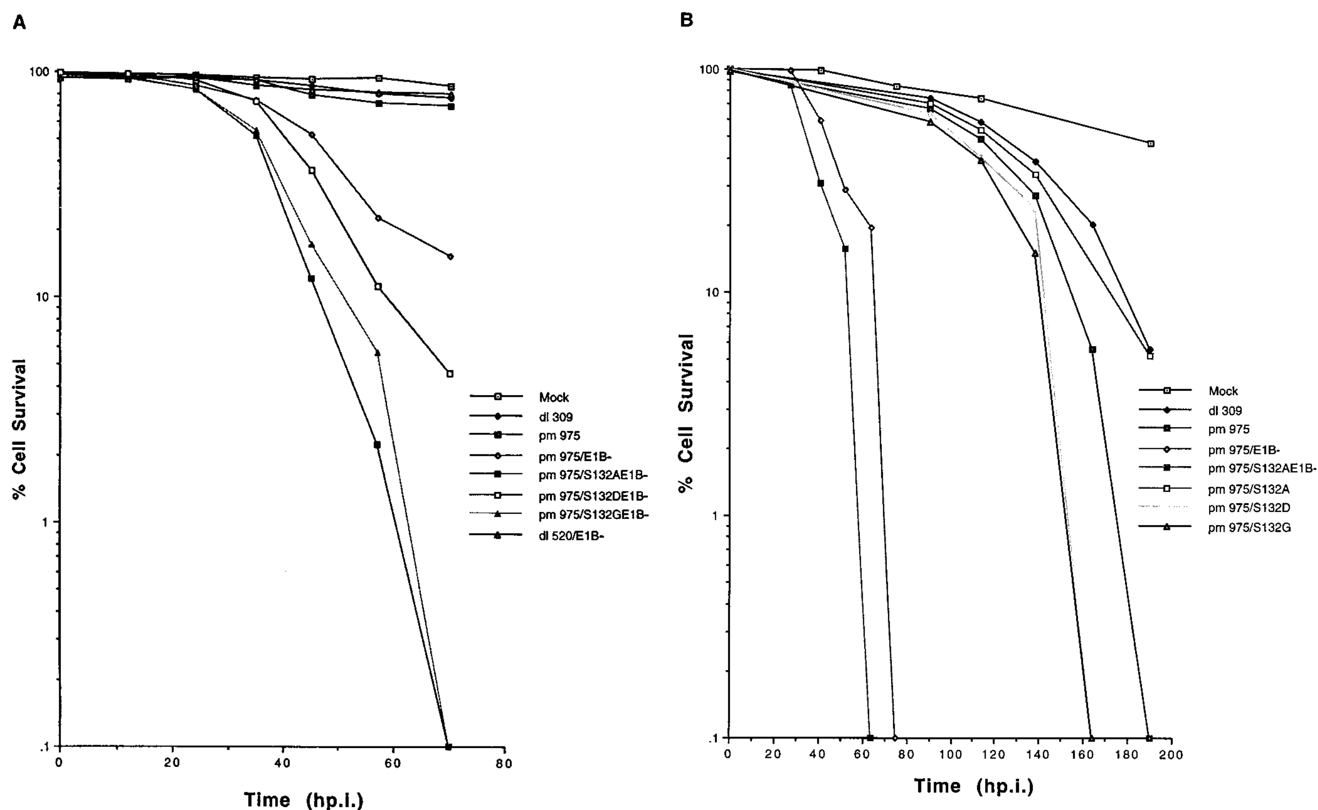

FIG. 8. Analysis of the viabilities of 10(1) cells infected with *wt* and mutant Ad5. p53-deficient mouse 10(1) cells were mock infected or infected with various Ad5 mutants and were tested for viability by a trypan blue exclusion assay at various times p.i., as described in Materials and Methods. The averages of two separate experiments are presented as the logarithm of the percentage of viable cells. (A) Mutants lacking E1B; (B) mutants expressing E1B.

result from phosphoisomers of the Ser-132 and Ser-188 sites. Partial proteolysis enhanced by the presence of Pro-207 just downstream of the Arg-205 trypsin cleavage site could also be a cause.

Phosphorylation at Ser-132 appeared to have significant biological consequences in terms of cell transformation, but the observed biochemical effects were more subtle. Lillie et al. found that a mutant expressing E1A 289R containing Gly-132 was defective for the repression of enhancer activity (43). However, Ser-132 lies just downstream of the major E1A binding site for pRB and related proteins at residues 121 to 126. As discussed above, many pRB-binding proteins contain CKII phosphorylation sites downstream of the binding core in a position similar to that of Ser-132. With the human papillomavirus E7 protein, mutagenesis of the corresponding sites at Ser-31 and Ser-32 reduced E7-mediated transformation (22, 33) but had little effect on complex formation with pRB (3, 22, 33, 59). The overall importance of downstream sequences in E1A proteins is illustrated by Ad5 mutant *dl*1109, which lacks residues 128 to 138 and is defective for cell transformation in cooperation with either E1B or activated *ras* (15, 16, 35). In the present studies, Ad5 viruses expressing 289R and 243R E1A proteins containing Ala or Gly in place of Ser-132 formed complexes with pRB and p130 about 20 to 30% less efficiently than did the *wt*, although interactions with p107 were relatively normal. It had been shown previously that the binding of pRB and p130 was affected by mutations within or downstream of the binding core, whereas interactions with p107 remained relatively unaffected (1, 2, 8, 16). Reductions of 20 to 30% in the binding of pRB, p130, and in this case p107 were also observed with mutants expressing only the E1A 289R protein, including *pm*975/953 (Gly-132) and newly created mutants *pm*975/S132G and *pm*975/S132A. The replacement of Ser-132 by Asp resulted in normal or slightly reduced levels of complex formation, suggesting that a negative charge at residue 132 may enhance interactions.

In terms of E1A-mediated cell transformation, we showed previously that E1A mutants which form complexes with pRB at reduced levels are also less efficient in the transformation of primary rat kidney cells (8). Mutations of the Ser-132 codon in plasmids expressing E1B and both 289R and 243R E1A proteins had no apparent effect on transformation, suggesting that reductions in the binding of pRB and p130 were of little or no consequence under these conditions. These results were in general agreement with an earlier analysis of an Ala-132 E1A mutant (53) but differed from those of Lillie et al. (43), who used activated Ha-*ras* and a plasmid expressing only E1A 289R containing Gly-132. In analyzing these differences further, we found that in cooperation with E1B, transformation with 289R alone was less efficient than when both E1A products were expressed, as found previously (43, 51, 54). This decrease in transforming efficiency could be related to the levels of E1A expression or differences in complex formation by or toxicity of the 289R and 243R E1A proteins. Transformation by 289R alone in cooperation with *ras* was less efficient than was transformation with E1B. With plasmids which express E1A 289R only, the Gly-132 and Asp-132 mutants transformed less efficiently than did the *wt* in cooperation with either E1B or *ras*. With the Ala-132 mutant, this defect was apparent only with *ras*. Nevertheless, transformation by Ser-132 mutants was generally reduced.

We believe that there may be two reasons for the defect in transformation by Ser-132 mutants. The first relates to the reduction in complex formation with pRB, p130, and, in the

case of 289R, p107. However, the decreases in binding were relatively modest and did not always correlate exactly with transforming activity. The second and probably more important reason concerns the toxicities of E1A products. Both 289R and 243R cause apoptosis via a p53-dependent pathway induced by complex formation with either pRB or p300 (56, 61, 70, 78). However, 289R is more toxic than is 243R, as it also induces p53-independent cell death in virus-infected cells (70). In the present study, we analyzed the ability of Ser-132 mutants to kill $p53^{-/-}$ cells by this p53-independent apoptosis pathway. In the absence of E1B, all Ser-132 mutants were more toxic than was the *wt*; this property could explain the reduction in transforming efficiency in cooperation with *ras* and the production of colonies containing extensive regions of both dead cells and flat cells characteristic of a partially transformed or revertant phenotype. Such cells could survive because they express lower levels of E1A or have acquired mutations in cell death pathways. However, even in the presence of the E1B 19-kDa protein (which suppresses apoptosis), the S132G and S132D mutants were shown to be somewhat more toxic than was the *wt*; this characteristic may explain the reduced levels of transformation in cooperation with E1B. Although the differences in toxicity exhibited here were modest, they could have profound effects on cell survival over the many cell generations required to form transformed foci. An intriguing result was that the S132A mutant transformed like the *wt* and did not exhibit increased toxicity in the presence of E1B. It may be that the cytotoxicity induced by the Ala-132 form of E1A protein is more easily suppressed by E1B products.

What is the basis of the increased toxicity of Ser-132 mutants? Previous studies indicated that the induction of p53-independent apoptosis is dependent upon CR3-mediated transactivation activity and, in the case of virus-infected cells, on products of one or more additional early adenovirus genes (47, 70). It is possible that phosphorylation negatively regulates CR3 transactivation activity and that the conversion of Ser-132 results in enhanced activity and thus increased toxicity. One possible mechanism for such regulation comes from the results of a recent study which suggested that the binding of pRB-related proteins prevents the interaction of E1A proteins with the TATA-binding protein (32). Thus, mutant E1A products which interact with pRB at reduced efficiency could possess higher levels of transactivation activity. However, we were unable to demonstrate any convincing difference between mutant and *wt* E1A proteins in the stimulation of CAT activity by using constructs with CAT under the control of the E2, E3, or E4 promoter. In addition, these DNA-mediated transformation experiments were conducted in the absence of other early Ad5 genes. It is possible that E1A toxicity could also result from the transactivation of cellular genes involved in apoptosis and that our transactivation assays failed to reflect effects on meaningful cellular promoters. Future studies involving E1A CR3 mutants expressed under heterologous promoters will be required to pursue this issue.

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REFERENCES

1. **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.
2. **Barbeau, D., R. C. Marcellus, S. Bacchetti, S. T. Bayley, and P. E. Branton.** 1992. Quantitative analysis of regions of adenovirus E1A products involved in interactions with cellular proteins. Biochem. Cell Biol. **70:**1123–1124.
3. **Barbosa, M. S., C. Edmonds, C. Fisher, J. T. Schiller, D. R. Lowy, and K. H. Vousden.** 1990. The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. EMBO J. **9:**153–160.
4. **Berk, A. J., and P. A. Sharp.** 1978. Structure of the adenovirus 2 early mRNAs. Cell **14:**695–711.
5. **Carroll, D., N. Santoro, and D. R. Marshak.** 1988. Regulating cell growth: casein kinase-II-dependent phosphorylation of nuclear oncoproteins. Cold Spring Harbor Symp. Quant. Biol. **53:**91–95.
6. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early regions of adenovirus 2. J. Mol. Biol. **141:**249–265.
7. **Colby, W. W., and T. Shenk.** 1981. Adenovirus type 5 virions can be assembled in vivo in the absence of detectable polypeptide IX. J. Virol. **39:**977–980.
8. **Corbeil, H. B., and P. E. Branton.** 1994. Functional importance of complex formation between the retinoblastoma tumor suppressor family and adenovirus E1A proteins as determined by mutational analysis of E1A conserved region 2. J. Virol. **68:**6697–6707.
9. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. **7:**546–554.
10. **Dumont, D. J., and P. E. Branton.** 1992. Phosphorylation of adenovirus E1A proteins by the $p34^{cdc2}$ protein kinase. Virology **189:**111–120.
11. **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.
12. **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.
13. **Dyson, N., R. Bernards, S. H. Friend, L. R. Gooding, J. A. Hassell, E. O. Major, J. M. Pipas, T. Vandyke, and E. Harlow.** 1990. Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. J. Virol. **64:**1353–1356.
14. **Eckner, R., M. E. Ewen, D. Newsome, M. Gerdes, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston.** 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adapter. Genes Dev. **8:**869–884.
15. **Egan, C., S. T. Bayley, and P. E. Branton.** 1989. Binding of the *Rb1* protein to E1A products is required for adenovirus transformation. Oncogene **4:**383–388.
16. **Egan, C., T. N. Jelsma, J. A. Howe, S. T. Bayley, B. Ferguson, and P. E. Branton.** 1988. Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. Mol. Cell. Biol. **8:**3955–3959.
17. **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 **160:**292–296.
18. **Ewen, M. E., Y. Xing, J. Bentley-Lawrence, and D. M. Livingston.** 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell **66:**1155–1164.
19. **Faha, B., E. Harlow, and E. Lees.** 1993. The adenovirus E1A-associated kinase consists of cyclin E-$p33^{cdk2}$ and cyclin A-$p33^{cdk2}$. J. Virol. **67:**2456–2465.
20. **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.
21. **Firzlaff, J. M., D. A. Galloway, R. N. Eisenman, and B. Lüscher.** 1989. The E7 protein of human papillomavirus type 16 is phosphorylated by casein kinase II. New Biol. **1:**44–53.
22. **Firzlaff, J. M., B. Lüscher, and R. N. Eisenman.** 1991. Negative charge at the casein kinase II phosphorylation site is important for transformation but not for Rb protein binding by the E7 protein of human papillomavirus type 16. Proc. Natl. Acad. Sci. USA **88:**5187–5191.
23. **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.
24. **Graham, F. L., H. L. Heijneker, and A. J. van der Eb.** 1974. Size and location of the transforming region in human adenovirus DNA. Nature (London) **251:**687–691.
25. **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.
26. **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.
27. **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.
28. **Harlow, E., B. R. Franza, Jr., and C. Schley.** 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in

early region 1A products. J. Virol. **55:**533–546.

29. **Harlow, E., P. Whyte, B. R. Franza, Jr., and C. Schley.** 1986. Association of adenovirus early-region 1A proteins with cellular polypeptides. Mol. Cell Biol. **6:**1579–1589.
30. **Harter, M. L., and J. B. Lewis.** 1978. Adenovirus type 2 early proteins synthesized in vivo and in vitro: identification in infected cells of the 38,000- to 50,000-molecular-weight protein coded by the left end of the adenovirus type 2 genome. J. Virol. **26:**736–749.
31. **Harvey, D. M., and A. J. Levine.** 1991. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. Genes Dev. **5:**2375–2385.
32. **Hateboer, G., H. T. M. Timmers, A. K. Rustgi, M. Biaaud, L. J. van'T Veer, and R. Bernards.** 1993. TATA-binding protein and the retinoblastoma gene product bind to overlapping epitopes on c-Myc and adenovirus E1A protein. Proc. Natl. Acad. Sci. USA **90:**8489–8493.
33. **Heck, D. V., C. L. Yee, P. M. Howley, and K. Münger.** 1992. Efficiency of binding the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses. Proc. Natl. Acad. Sci. USA **89:**4442–4446.
34. **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.
35. **Jelsma, T. N., J. A. Howe, J. S. Mymryk, C. M. Evelegh, N. F. A. Cuniff, and S. T. Bayley.** 1989. Sequences in E1A proteins of human adenovirus 5 required for cell transformation, repression of a transcriptional enhancer, and induction of proliferating cell nuclear antigen. Virology **171:**120–130.
36. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host-range deletion mutants defective for transformation of rat embryo cells. Cell **17:**683–689.
37. **Kitchingman, G. R., and H. Westphal.** 1980. The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. J. Mol. Biol. **137:**23–48.
38. **Kuppuswamy, M. N., and G. Chinnadurai.** 1987. Relationship between the transforming and transcriptional regulatory functions of adenovirus 2 E1a oncogene. Virology **159:**31–38.
39. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304:**596–602.
40. **Larose, A., N. Dyson, M. Sullivan, E. Harlow, and M. Bastin.** 1991. Polyomavirus large T mutants affected in retinoblastoma protein binding are defective in immortalization. J. Virol. **65:**2308–2313.
41. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56:**335–341.
42. **Lee, W. S., 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 **67:**365–376.
43. **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.
44. **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.
45. **Liu, F., and M. Green.** 1994. Promoter targeting by adenovirus E1a through interaction with different cellular DNA-binding domains. Nature (London) **368:**520–525.
46. **Lowe, S. W., and H. E. Ruley.** 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus E1A in mouse thymocytes. Genes Dev. **7:**535–545.
47. **Marcellus, R. C., J. G. Teodoro, T. Wu, D. E. Brough, G. Ketner, G. S. Shore, and P. E. Branton.** Adenovirus 5 early region 4 is responsible for E1A-induced p53-independent apoptosis. Submitted for publication.
48. **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.
49. **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.
50. **McLorie, W., C. J. McGlade, D. Takayesu, and P. E. Branton.** 1991. Individual adenovirus E1A proteins induce transformation independently but by additive pathways. J. Gen Virol. **72:**1467–1471.
51. **Montell, C., G. Courtois, C. Eng, and A. J. Berk.** 1984. Complete transformation by adenovirus 2 requires both E1A proteins. Cell **36:**951–961.
52. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1982. Resolving the functions of overlapping genes by site-specific mutagenesis at a mRNA splice site. Nature (London) **295:**380–384.
53. **Moran, E.** Unpublished data.
54. **Moran, E., T. Grodzicker, R. J. Roberts, M. B. Mathews, and B. Zerler.** 1986. Lytic and transforming functions of individual products of the adenovirus E1A gene. J. Virol. **57:**765–775.
55. **Münger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley.** 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. **8:**4099–4105.
56. **Mymryk, J. S., K. Shire, and S. T. Bayley.** 1994. Induction of apoptosis by adenovirus type 5 E1A in rat cells requires a proliferation block. Oncogene **9:**1187–1193.
57. **Pearson, R. B., and B. E. Kemp.** 1991. Protein kinase phosphorylation site sequences and consensus specificity motifs. Methods Enzymol. **200:**62–81.
58. **Perricaudet, M., G. Aküsjarvi, A. Virtanen, and U. Pettersson.** 1979. Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature (London) **281:**694–696.
59. **Phelps, W. C., K. Münger, C. L. Yee, J. A. Barnes, and P. M. Howley.** 1992. Structure-function analysis of the human papillomavirus type 16 E7 oncoprotein. J. Virol. **66:**2418–2427.
60. **Pöpperl, H., and M. S. Featherstone.** 1992. An autoregulatory element of the murine *Hox*-4.2 gene. EMBO J. **11:**3673–3680.
61. **Querido, E., J. G. Teodoro, and P. E. Branton.** Unpublished data.
62. **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.
63. **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:**1621–1625.
64. **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.
65. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304:**602–606.
66. **Schneider, J. F., F. Fisher, C. Gooding, and N. C. Jones.** 1987. Mutational analysis of the adenovirus E1A gene: the role of transcriptional regulation in transformation. EMBO J. **6:**2053–2060.
67. **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.
68. **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 Differ. **652:**131–137.
69. **Svensson, C., M. Bondesson, E. Nyberg, S. Linder, N. Jones, and G. Akusjärvi.** 1991. Independent transformation activity by adenovirus-5 E1a-conserved regions 1 or 2 mutants. Virology **182:**553–561.
70. **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.
71. **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.
72. **Tremblay, M. L., C. J. McGlade, G. E. Gerber, and P. E. Branton.** 1988. Identification of the phosphorylation sites in the early region 1A proteins of adenovirus type 5 by analysis of proteolytic peptides and amino acid sequencing. J. Biol. Chem. **263:**6375–6383.
73. **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.
74. **Wang, H.-G. H., Y. Rikitake, M. C. Carter, P. Yaciuk, S. E. Abraham, B. Zerler, and E. Moran.** 1993. Identification of specific adenovirus E1A N-terminal residues critical to the binding of cellular proteins and to the control of cell growth. J. Virol. **67:**476–488.
75. **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.
76. **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.
77. **Whalen, S. G., R. C. Marcellus, D. Barbeau, and P. E. Branton.** Unpublished data.
78. **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.
79. **Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow.** 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature (London) **334:**124–129.
80. **Whyte, P., H. E. Ruley, and E. Harlow.** 1988. Two regions of the adenovirus early region 1A proteins are required for transformation. J. Virol. **62:**257–265.
81. **Whyte, P., N. M. Williamson, and E. Harlow.** 1989. Cellular targets for transformation by the adenovirus E1A proteins. Cell **56:**67–75.
82. **Yee, S.-P., and P. E. Branton.** 1985. Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. Virology **147:**142–153.
83. **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 **46:**1003–1013.