JOURNAL OF VIROLOGY, Sept. 1996, p. 6207–6215 Vol. 70, No. 9
0022-538X/96/$04.00+0

Adenovirus Type 5 Early Region 4 Is Responsible for E1A-Induced p53-Independent Apoptosis

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Received 1 April 1996/Accepted 14 June 1996

In the absence of E1B, the 289- and 243-residue E1A products of human adenovirus type 5 induce p53-dependent apoptosis. However, our group has shown recently that the 289-residue E1A protein is also able to induce apoptosis by a p53-independent mechanism (J. G. Teodoro, G. C. Shore, and P. E. Branton, Oncogene 11:467–474, 1995). Preliminary results suggested that p53-independent cell death required expression of one or more additional adenovirus early gene products. Here we show that both the E1B 19-kDa protein and cellular Bcl-2 inhibit or significantly delay p53-independent apoptosis. Neither early region E2 or E3 appeared to be necessary for such cell death. Analysis of a series of E1A mutants indicated that mutations in the transactivation domain and other regions of E1A correlated with E1A-mediated transactivation of E4 gene expression. Furthermore, p53-deficient human SAOS-2 cells infected with a mutant which expresses E1B but none of the E4 gene products remained viable for considerably longer times than those infected with wild-type adenovirus type 5. In addition, an adenovirus vector lacking both E1 and E4 was unable to induce DNA degradation and cell killing in E1A-expressing cell lines. These data showed that an E4 product is essential for E1A-induced p53-independent apoptosis.

Replication of human adenoviruses in terminally differentiated epithelial cells requires an efficient mechanism to induce cellular DNA synthesis to permit replication of viral DNA and production of progeny virus. Products of early region 1A (E1A) induce cell DNA synthesis and are largely responsible for cell transformation by adenoviruses (for reviews, see references 2, 6, and 14 and references therein). E1A produces two major mRNAs of 13 and 12S which encode proteins of 289 and 243 residues (289R and 243R, respectively) that are identical except for a central 46-amino-acid sequence, termed conserved region 3 or CR3 (48). Two additional regions present in the common sequence encoded by exons 1 of both E1A mRNAs are also conserved in all human serotypes and have been termed CR1 and CR2 (see Fig. 1A). E1A products induce DNA synthesis by two mechanisms: (i) complex formation with Rb and related p107 and p130 proteins through interactions with CR1 and CR2 and (ii) complex formation with the transcriptional modulator p300 and possibly related proteins utilizing the amino terminus and CR1 of E1A (3, 4, 26–29, 38, 56, 87–90). E1A 289R also activates expression of the early viral transcription units E2, E3, and E4 and certain cellular genes at least in part through interactions with transcription factors and basal transcription machinery requiring CR3 (for reviews, see references 1, 6, 12, 32, 60, and 72). In addition to CR3, transactivation of the E4 promoter has also been shown to rely to some degree on two regions encoded by the second exon of 13S mRNA, termed auxiliary regions 1 and 2, or AR1 and AR2 (10). Production of stably transformed cells requires E1B, which encodes polypeptides of 19 and 55 kDa that are individually capable of cooperating with E1A via separate but additive pathways (5, 7, 55, 84, 92, 95).

Considerable evidence indicates that a major function of E1B proteins in lytic infection and cell transformation is to suppress cytotoxic effects and apoptosis induced by expression of E1A. Without E1B, the toxicity of E1A products results in the death of E1A-transformed cells and a reduction in the yield of progeny due to the early demise of productively infected cells. E1A proteins can cause apoptosis by a process mediated by the tumor suppressor p53 (22, 52), which controls growth arrest and programmed cell death pathways (23, 31, 34, 94). Expression of E1A products results in the elevation of p53 levels (13, 52, 66). The 55-kDa E1B protein binds to p53 (71, 91) and blocks both p53-mediated activation of gene expression (77, 93) and apoptosis (76). The 19-kDa E1B protein appears to suppress apoptosis by a mechanism that is functionally analogous to that of the cellular proto-oncogene product Bcl-2 (11, 61, 67, 86). Cells infected with adenovirus mutants which fail to express the 19-kDa protein display enhanced cytotoxicity and extensive degradation of both cellular and viral DNAs into nucleosome size fragments (30, 61, 63, 74, 85, 86).

Recently, both our group (78) and Subramanian et al. (74) showed that in the absence of E1B, E1A products also induce p53-independent apoptosis. Our results indicated that such apoptotic cell death was induced only by the 289R E1A protein. Furthermore, when p53-null mouse cells constitutively expressing E1A products were infected by an adenovirus vector lacking the entire E1A and E1B coding regions but containing early regions E2, E3, and E4, rapid cell death was observed (78). Such cell death exhibited all of the hallmarks of apoptosis, including degradation of DNA to nucleosome-sized fragments, extensive chromosomal condensation, and formation of cytoplasmic vacuoles (78). These results indicated that the role of E1A 289R may be to transactivate expression of an additional early transcript whose product actually induces p53-independent apoptosis. In the present studies we report that

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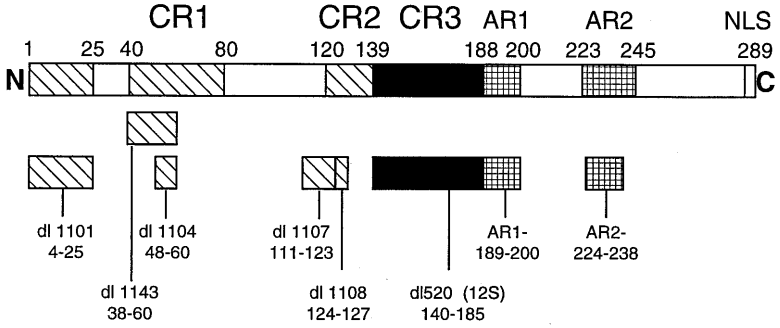
A



B

Virus Mutant	Description
wt Ad5	wt E1A (12S &13S mRNAs), wt E1B. In some cases *dl* 309 which has a partial deletion of E3 was used
12S / E1B-	12S E1A mRNA only, no E1B expression
13S / E1B-	13S E1A mRNA only, no E1B expression
E1B / 55K-	wt E1A (12S &13S mRNAs), no E1B 55K expression, wt E1B 19K
E1B / 19K-	wt E1A (12S &13S mRNAs), no E1B 19K expression, wt E1B 55K
dl 1101 / E1B-	12S/13S E1A mRNAs, E1A mutation as in fig. 1A, no E1B expression
dl 1104 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
dl 1107 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
dl 1108 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
dl 1143 / 08 / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR1- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR2- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AR1- / AR2- / E1B-	12S/13S E1A mRNAs, E1A mutation as in Fig. 1A, no E1B expression
AD147VL / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
AD171CS / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
AD185SG / E1B-	13S E1A only, E1A point mutation in CR3, no E1B expression
dl 1019	wt E1A, E1B, E2 and E3, no E4 expression
AdLacZ	no E1A or E1B expression, wt E2, E3 and E4
Ad5*dl*70-8	no E1A, E1B or E3 expression, wt E2 and E4
AdRSVβ-gal.11	no E1A, E1B or E4 expression, wt E2 and E3

FIG. 1. (A) Ad5 E1A mutants. Proteins encoded by some of the mutants used in the present studies are diagrammed, and the residues removed in deletion mutants are indicated. (B) Other adenovirus mutants.

one or more E4 gene products appear to be responsible for such cell killing.

MATERIALS AND METHODS

Cells and viruses. Human SAOS-2 cells (ATCC HTB 85) and 10(1) mouse embryo fibroblast-derived cells (40), which are both deficient for p53 expression, were cultured on 60-mm-diameter dishes (Corning Glass Works, Corning, N.Y.) in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum, as were both NIH 3T3 cells and CHO cells. The cell line Saos-2/Bcl-2(3g4), which stably expresses Bcl-2, was derived for this study from SAOS-2 cells by selection with G418, as previously described (20), as was the control line Saos-2/neo(2a2). A1.A3, A1.A6, and A1.A12 mouse embryo fibroblast lines expressing Ad5 E1A proteins and Hy.A3 hygromycin-selected control lines have been described previously (51) and were cultured in Dulbecco's modified minimal essential medium containing 10% fetal calf serum and 100 μg of hygromycin per ml. Normally, cells were infected with mutant or wild-type (wt) adenovirus type 5 (Ad5) at a multiplicity of 100 PFU per cell, as described previously (77). The virus used as wt has been described by Harrison et al. (39), although in many cases the wt strain was *dl*309 (47), which also was used in the preparation of E1A and E1B mutants, which were propagated in 293 cells (36). Ad5 E1A mutants are illustrated in Fig. 1A and include deletion mutants *dl*1101 (residues 4 to 25 deleted), *dl*1143 (residues 38 to 60 deleted), *dl*1107 residues 111 to 123 deleted), *dl*1108 (residues 124 to 127 deleted), *dl*1143/08 (residues 38 to 60 plus 124 to 127 deleted), and *dl*1132 (residues 224 to 238 deleted), which have all been described previously (46). A new E1A mutant, AR1$^-$/E1B$^-$, which lacks the entire AR1 region (residues 189 to 200) and also fails to express E1B products, was constructed as described previously (25). Mutant AR2$^-$/E1B$^-$ was generated by introducing *dl*1132 (58), which lacks residues 224 to 238, into a background that fails to express E1B proteins. Mutant AR1$^-$/AR2$^-$/E1B$^-$ represents a combination of the latter two mutants. Additional E1A mutants containing single amino acid substitutions at various sites within CR3 were produced by subcloning appropriate restriction enzyme fragments from mutant E1A cDNA plasmids (81) into genomic viral DNA and then rescuing them into virus by the method of McGrory et al. (54) to form mutants AD147VL (Val-147 converted to Leu), AD177CS, and AD185SG. All other mutants are described in Fig. 1B. Two were produced previously by our group (55) and fail to express E1B proteins of 19 kDa (originally termed *pm*1716/2072 but now called E1B/19K$^-$) and 55 kDa (originally *pm*2019/2250, now E1B/55K$^-$). Mutant 12S/E1B$^-$ (originally *dl*520E1B$^-$) produces only the E1A 243R protein encoded by the 12S mRNA and no E1B products (73). Mutant E1B$^-$, which expresses both major E1A products but neither the 19-kDa or the 55-kDa E1B species, was described previously (78), and a similar mutant that expresses only 289R in the absence of E1B, termed 13S/E1B$^-$, was prepared for the present studies. A series of E1A mutants (*dl*1101/E1B$^-$, *dl*1107/E1B$^-$, AD147VL/E1B$^-$, etc.) which express no E1B products was also produced by introducing E1A mutations into mutant E1B$^-$, which expresses both 289R and 243R E1A products but no E1B (78). The presence of mutations in all mutants was confirmed by DNA sequencing, restriction enzyme digestion, or Southern blotting, as described previously (25). Ad5 vectors used in this study included AdLacZ, in which the E1 (E1A plus E1B) region was replaced with the *Escherichia coli* gene *lacZ* under the control of the cytomegalovirus promoter (2), and Ad5dl70-8, which was generated by cotransfection of plasmids pAB7 and pBHG10 (9) and which lacks both E1 and the entire E3 region (8). These vectors and other E1A and E1B mutants were grown on human 293 cells (36). Adenovirus vector AdRSVβgal.11, which lacks the entire E1 and

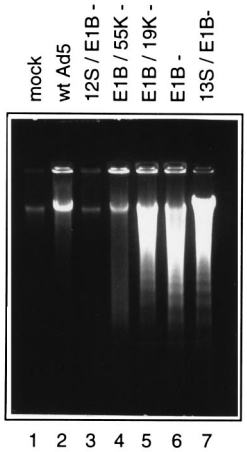


FIG. 2. Induction of DNA fragmentation by Ad5 mutants in the absence of p53. 10(1) cells, which fail to express p53 were infected with various Ad5 mutants, or they were mock infected, and at 40 h postinfection low-molecular-weight DNA was analyzed by agarose gel electrophoresis.

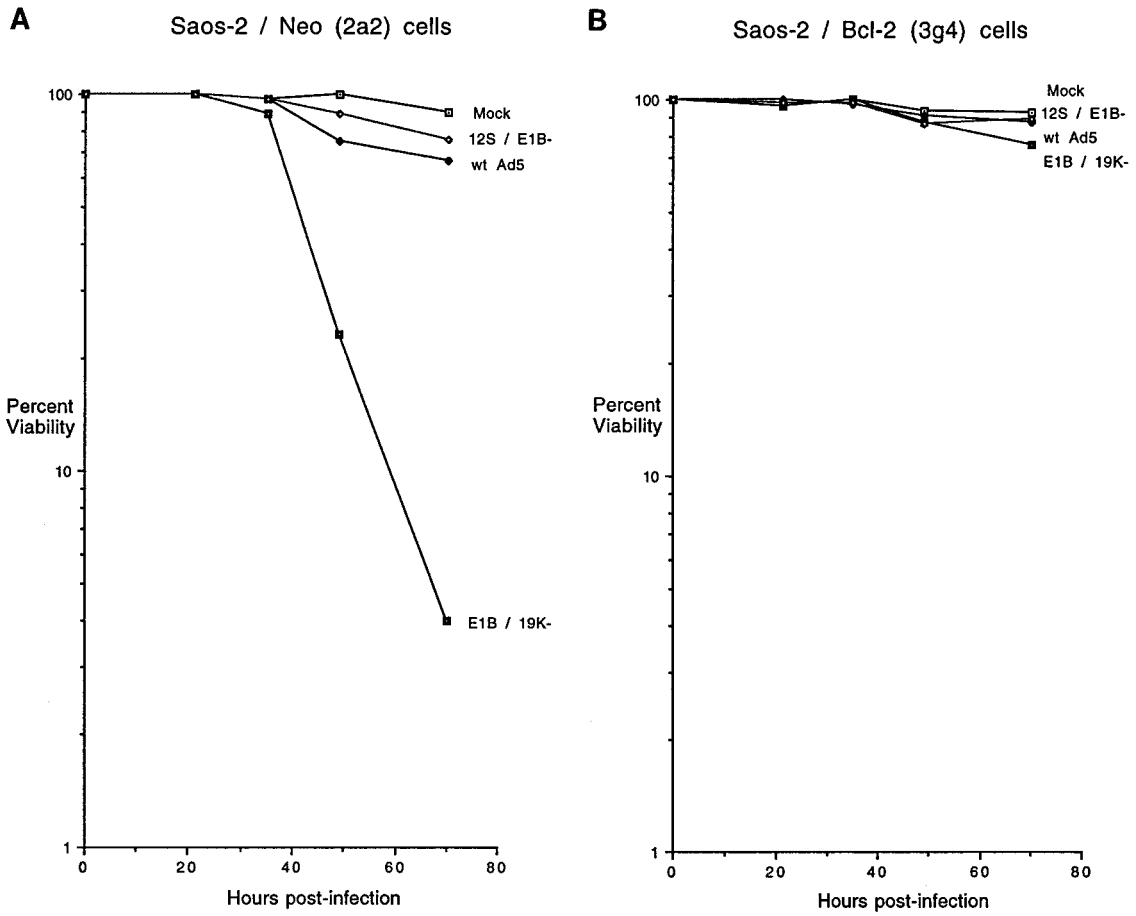


FIG. 3. Viability of infected normal and Bcl-2-expressing SAOS-2 cells. p53-deficient human Saos-2/neo(2a2) cells (A) or Saos-2/Bcl-2(3g4) cells, which express human Bcl-2 constitutively (B), were mock infected or infected with wt, E1B/19K$^-$, or 12S/E1B$^-$ and were tested for viability by a Trypan Blue exclusion assay at various times following infection, as described in Materials and Methods. The data are log percent viable cells.

E4 regions, was prepared and propagated as described elsewhere (18). In addition, some experiments were carried out with human Ad2 mutant *dl*1019, which contains deletions that eliminate expression of all E4 products and which was propagated on W162 monkey cells, as described previously (15).

DNA fragmentation. Low-molecular-weight DNA was isolated from mock- or Ad5-infected cells by a modified Hirt extraction procedure (42). For such experiments, 60-mm-diameter plates of cells were harvested at 40 h postinfection and lysed in pronase lysis buffer composed of 10 mM Tris-HCl (pH 8) containing 5 mM EDTA, 100 mM NaCl, and 1 mg of pronase per ml, to which sodium dodecyl sulfate 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 then incubated overnight at 4°C and centrifuged at 15,000 × *g* for 30 min. Extracted nucleic acids were treated with RNase A and analyzed on 1% agarose gels stained with ethidium bromide. In all cases DNAs extracted from equal numbers of cells were analyzed.

Cell viability assays. Cells were infected with wt or mutant Ad5 in 24-well plates containing cells at about 80% confluence. At various times following 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 the adenovirus E4 promoter. Transactivation assays were performed with NIH 3T3 or CHO cells plated at a density of 2×10^5/60-mm-diameter dish. The E4 reporter plasmid was E4-CAT containing the E4 promoter upstream of the chloramphenicol acetyltransferase (CAT) gene (82). Transient cotransfections were performed by the calcium phosphate precipitation method (37) using 2.5 μg of reporter plasmid DNA and 2.5 μg of DNA from plasmids expressing wt or mutant E1A products. The plasmid pSV2CAT (35) was used as a positive control. In addition, 3 μg of the RSVβ-Gal plasmid (65) was included to allow normalization of transfection efficiency by measuring β-galactosidase activity. Cells were glycerol shocked after 12 h and then harvested 36 h later. CAT assays were performed with cell extracts containing equal amounts of β-galactosidase activity, essentially as described previously (35). The amount of activity was quantified on thin-layer chromatography plates with a Fujix Bas 2000 Phosphorimager.

RESULTS

E1A-induced p53-independent apoptosis is inhibited by both the E1B 19-kDa protein and cellular Bcl-2. Previous studies indicated that, whereas both major Ad5 E1A products could induce apoptosis in cells expressing p53, only the 289R E1A protein could do so in cells lacking p53 (78). Figure 2 shows the pattern of DNA fragmentation in p53-deficient mouse 10(1) cells infected by various Ad5 mutants. Extracts from mock-infected cells (lane 1) and those infected with wt Ad5 (lane 2), which expresses E1B products, displayed reduced levels of extracted DNA of low molecular mass and little or no degraded DNA, as did those from cells infected with mutant E1B/55K$^-$ (lane 4), which produces the E1B 19-kDa protein but not the E1B 55-kDa product. With cells infected with mutant E1B$^-$, which synthesizes both the 289R and the 243R E1A proteins but which produces no E1B products (lane 6), large amounts of DNA were extracted and high levels of nucleosome-sized DNA fragments were evident. Similar results were also obtained with cells infected with E1B/19K$^-$ (lane 5), which produces the E1B 55-kDa species but not the 19-kDa protein. Induction of DNA degradation in these p53-deficient cells did not occur following infection with 12S/E1B$^-$ (lane 3), which produces only E1A 243R and no E1B, but it did occur with 13S/E1B$^-$ (lane 7), which yields only E1A 289R in the absence of E1B products. Thus, as shown previously, E1A 289R, but not 243R, induces p53-independent apoptosis in the

absence of E1B proteins. In addition, the results indicated that the E1B 19-kDa polypeptide, but not the 55-kDa E1B product, is able to protect against apoptosis induced by E1A in the absence of p53.

To examine the specificity of inhibition of apoptosis further, studies were conducted to determine if the cellular Bcl-2 protein is also able to prevent p53-independent apoptosis, as several previous studies had shown that Bcl-2 and the E1B 19-kDa protein may be functionally similar (11, 61, 67, 86). Human SAOS-2 cells, which are defective for synthesis of p53, were transfected with cDNAs encoding the human Bcl-2 protein and the neomycin resistance marker, and several cell lines were selected with G418 (see Materials and Methods). One such Bcl-2-expressing clone, Saos-2/Bcl-2(3g4), and a control SAOS-2 clone, Saos-2/neo(2a2), selected only for resistance to G418, were infected with wt Ad5 or mutant 12S/E1B$^-$ or E1B/19K$^-$ or were mock infected, and cell viability assays were conducted at various times after infection. Figure 3A shows that Saos-2/neo(2a2) control cells were killed by the E1B/19K$^-$ virus that expresses E1A 289R, but those infected with wt or 12S/E1B$^-$ remained almost as viable as mock-infected cells during the test period. Figure 3B shows that, with Saos-2/Bcl-2(3g4), cells, which stably express high levels of Bcl-2 (data not shown), little cell death was induced by the E1B/19K$^-$ virus. Similar results were obtained with three other control and Bcl-2-producing SAOS-2 cell lines (data not shown). Thus, like the E1B 19-kDa protein, Bcl-2 also blocks E1A-induced p53-independent apoptosis.

Role of E1A domains in p53-independent apoptosis. To investigate the regions of E1A products involved in causing p53-independent cell death, p53-deficient mouse 10(1) cells were infected with Ad5 mutants which fail to express E1B and which harbor a variety of defects at various regions of the E1A molecule. Extracts were harvested and analyzed on gels to determine the extent of degradation of low-molecular-weight DNA. The levels of E1A expression were analyzed in parallel by Western blotting (immunoblotting) using E1A-specific M73 monoclonal antibody and were found to be comparable in all cases (data not shown). Figure 4 shows that, again, mutant E1B/19K$^-$ (lane 3) induced DNA degradation characterized by the appearance of high levels of extracted DNA and nucleosome-sized DNA fragments whereas this did not occur with wt Ad5 (lane 2) or mock-infected cells (lane 1). Mutants which affected the E1A transactivation function associated with CR3 all failed to induce DNA degradation. These included 12S/E1B$^-$ (lane 8) and point mutants AD147VL/E1B$^-$, AD171CS/E1B$^-$, and AD185SG/E1B$^-$ (lanes 9 to 11, respectively), which carry single-residue substitutions at critical residues in CR3 that eliminate E1A transactivation activity (81). In addition, deletion of AR1 or both AR1 and AR2 (AR1$^-$/E1B$^-$ and AR1$^-$/AR2$^-$/E1B$^-$ [lanes 12 and 14]) also eliminated DNA degradation, whereas removal of AR2 alone (AR2$^-$/E1B$^-$ [lane 13]) had little effect. Interestingly, mutations in CR2 which eliminate complex formation with pRB and related proteins (*dl*1107/E1B$^-$ and *dl*1108/E1B$^-$ in lanes 5 and 6) had no effect on the induction of DNA degradation, whereas those that eliminated binding of p300 by removal of the N terminus (*dl*1101/E1B$^-$ [lane 4]) or a portion of CR1 as well as the pRB binding site (*dl*1143/08/E1B$^-$ [lane 7]) no longer caused this effect. These results suggested that E1A-induced p53-independent apoptosis required the CR3 transactivation domain, AR1, and the regions necessary for binding of p300 but not pRB-related proteins. Figure 5 shows that similar results were obtained with these mutants in cell killing assays. Cell death was induced by the E1B/19K$^-$ virus, which expresses both E1A products, and by *dl*1107/E1B$^-$. Mutant

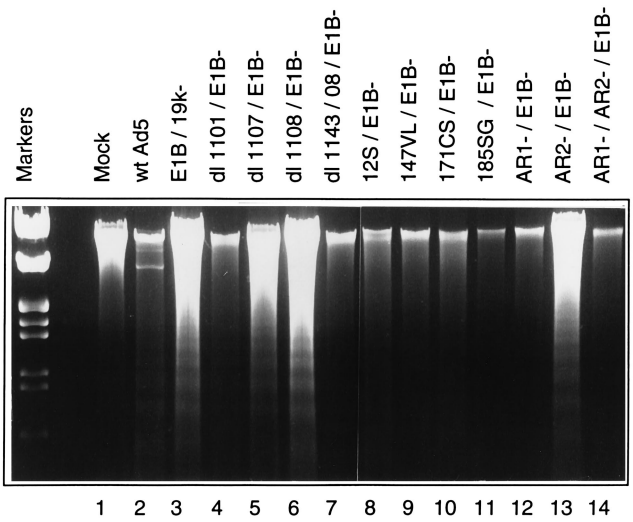


FIG. 4. Induction of DNA fragmentation by E1A mutants. An experiment with p53-deficient 10(1) cells similar to that in Fig. 2 was performed with a series of Ad5 E1A mutants defective in expression of E1B products.

AR2$^-$/E1B$^-$, which lacks AR2, also killed but was consistently less toxic than the former viruses. All other mutants affecting CR3, AR1, and the p300 binding sites failed to kill significantly during the test period.

Activation of E4 expression and apoptosis. The requirement for AR1 suggested that E4 products might somehow be involved in the induction of p53-independent apoptosis, as this region is not important in the activation of other early viral transcription units (10). Studies were therefore carried out to examine the pattern of E1A transactivation of the E4 promoter in which plasmid DNA encoding various mutant forms of E1A 289R was cotransfected into NIH 3T3 or CHO cells along with DNA from E4-CAT, a construct that encodes CAT under the control of the Ad5 E4 promoter (82). Table 1 shows that, in addition to CR3, activation of the E4 promoter required AR1 and to some extent AR2. In addition, regions at the N terminus and in CR1 involved in binding of p300 also were of some importance. These results closely paralleled the pattern of E1A-induced p53-independent apoptosis and suggested that E4 products might be involved.

E2 and E3 products are not required for apoptosis. It was unlikely that E2 products were responsible for the induction of p53-independent apoptosis for two reasons. First, in addition to CR3, complex formation involving CR2 and the pRB family of proteins activates E2 expression (50), and CR2 was shown to be of little importance in cell killing. Second, reasonably high levels of expression of E2 proteins are known to be induced by the E1A 243R protein (79), which is completely unable to induce p53-independent apoptosis. Thus, experiments were carried out to determine if any E3 products were involved. The A1.A3 mouse embryo fibroblast line lacking p53 but expressing Ad5 E1A proteins and Hy.A3 hygromycin-selected p53-deficient control cells (51) were infected with wt Ad5, the E1B/19K$^-$ virus, adenovirus vector AdlacZ, which contains *lacZ* in place of E1A and E1B, or vector Ad5dl70-8, which lacks both the entire E1 region and the entire E3 region. Cell extracts were assayed for the presence of degraded DNA as before. Figure 6 shows that high levels of DNA degradation were induced in A1.A3 cells with the E1B/19K$^-$ mutant (lane 3) as well as both adenovirus vectors AdlacZ (lane 4) and Ad5dl70-8 (lane 5). Similar results were also obtained with two other

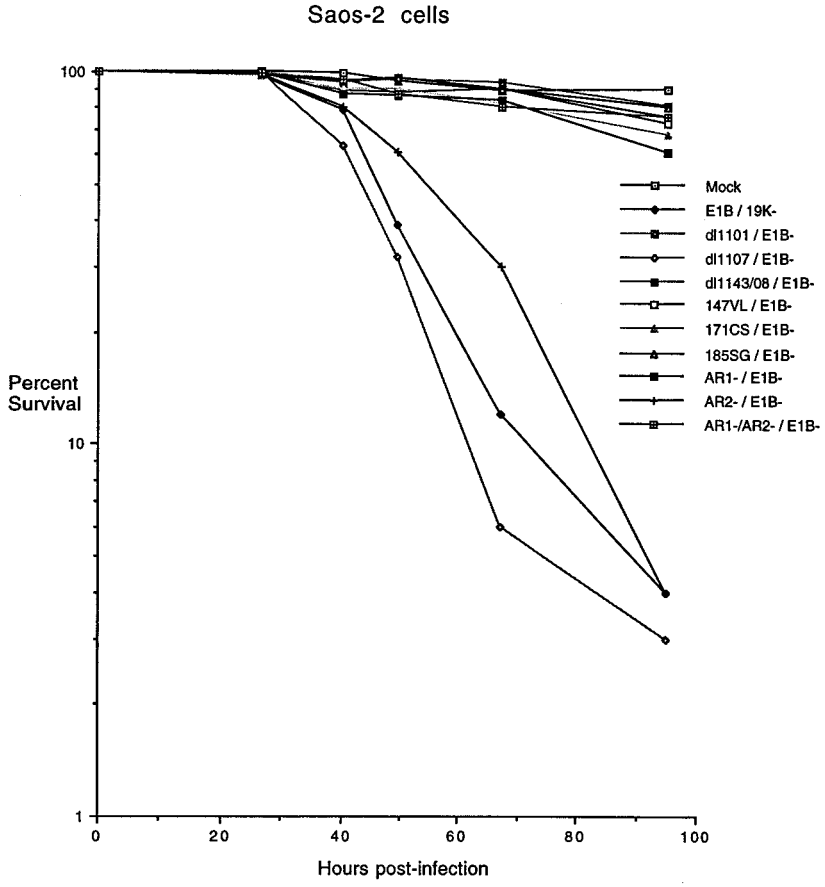


FIG. 5. Analysis of viability of SAOS-2 cells infected with E1A mutants. An experiment similar to that in Fig. 3 was carried out with SAOS-2 cells infected with various E1A mutants defective in expression of E1B products. The data are log percent viable cells.

comparable p53-null, E1A-expressing cell lines, A1.A6 and A1.A12 (data not shown). Figure 6 also shows that, in the control cells lacking constitutive E1A expression, only the E1B/19K$^-$ virus (lane 8) induced DNA degradation. These results indicated that E3 products were not required for induction of p53-independent apoptosis by E1A under these conditions.

E4 proteins are essential for p53-independent apoptosis. To determine directly if E4 products are involved in the induction of cell death, as suggested by experiments described above, two approaches were taken. In the first, human p53-deficient SAOS-2 cells were infected with wt Ad5 or Ad2 or with Ad2 mutant *dl*1019, which produces no E4 proteins (15), or they were mock infected. Although such viruses express E1B proteins and thus are protected from E1A-induced apoptosis, it was thought that, if E4 products were essential for p53-independent cell death, some difference in long-term cell survival might be observed, and thus, at various times up to 10 days, infected cultures were tested for cell viability. Figure 7 shows that cells infected by wt Ad5 virus (or Ad2 [data not shown]) began to die at about 100 h postinfection and by 240 h postinfection almost all of the cells were dead. Such was not the case with *dl*1019-infected cells, which remained almost as viable as mock-infected cells even 10 days after infection. These results indicated that an E4 product was required for cell killing in the absence of p53. This idea was confirmed in experiments involving infection of E1A-expressing p53-deficient A1.A3 cells with the adenovirus vector AdRSVβgal.11, in which both the E1 and the E4 regions had been completely deleted (18). Figure 8 shows that, in control Hy.A3 p53-deficient cells, which do not express E1A, only the E1B/19K$^-$ Ad5 mutant caused DNA degradation (lane 3), and neither the wt, the 12S/E1B$^-$ mutant (lane 4), nor the AdRSVβgal.11 vector (lane 5) had any significant effect. With A1.A3 cells, both the E1B/19K$^-$ (lane 8) and the 12S/E1B$^-$ (lane 9) viruses induced DNA degradation. Importantly, with the AdRSVβgal.11 vector (lane 10), even though somewhat higher levels of DNA were present, little or no nucleosome-sized DNA fragments were apparent. Similar

TABLE 1. E4 transactivation by E1A mutants

Virus	Residues deleted	Region affected	E4 CAT activity (% wt ± SD)[a]
wt	None	None	100
*dl*1101	4–25	N terminus	30 ± 11
*dl*1104	48–60	CR1	40 ± 5
*dl*1107	111–123	CR2	85 ± 5
*dl*1108	124–127	CR2	81 ± 14
*dl*520	140–185	CR3	10 ± 7
AR1$^-$	189–200	AR1	25 ± 7
AR2^{-b}	224–238	AR2	64 ± 16

[a] CHO or NIH 3T3 cells were transfected with plasmid DNA encoding the virus and CAT under the control of the Ad5 E4 promoter. Cell extracts were assayed for CAT activity as described in Materials and Methods. Three independent assays were done for each mutant.

[b] *pm*1132.

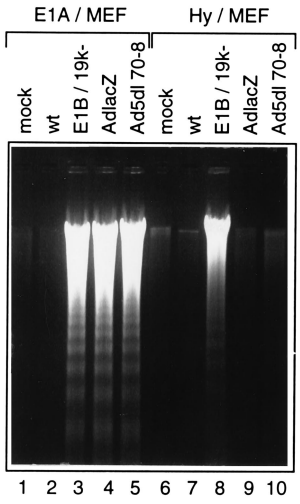


FIG. 6. Induction of DNA degradation in the absence of E3. Cell lines expressing 289R and 243R E1A proteins constitutively or the Hy.A3 nonexpressing control cells were mock infected or infected with wt Ad5 or adenovirus vector AdLacZ or Ad5dl70-8. After 40 h, DNA was extracted and analyzed by agarose gel electrophoresis. MEF, mouse embryo fibroblasts.

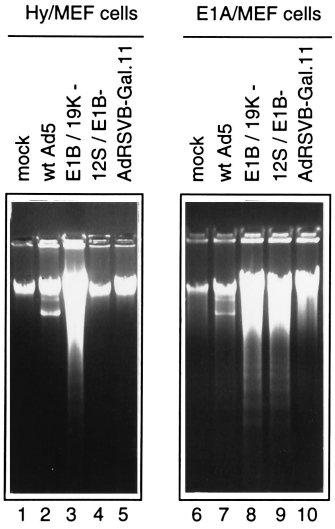


FIG. 8. Analysis of DNA degradation in the absence of E4. Cell lines expressing 289R and 243R E1A proteins constitutively or the Hy.A3 nonexpresser control cells were mock infected or infected with wt Ad5, E1B/19K$^-$, 12S/E1B$^-$, or the adenovirus vector AdRSVβgal.11, which lacks both E1 and E4. After 40 h, DNA was extracted and analyzed by agarose gel electrophoresis. MEF, mouse embryo fibroblast.

results were obtained with the other two sister cell lines, A1.A6 and A1.A12, discussed above (data not shown). The ability of this virus to induce apoptosis in A1.A3 cells was analyzed further in cell killing experiments. Figure 9A shows that, in the Hy.A3 control cells, only the E1B/19K$^-$ virus induced cell death, whereas in A1.A3 cells both the E1B/19K$^-$ and the 12S/E1B$^-$ viruses did so. However, in both cases the AdRSVβgal.11-infected cells remained as fully viable as mock-infected cultures. In addition, these cultures were seen by light microscopy to be virtually indistinguishable from mock-infected cells and did not exhibit the rounded morphology and loss of adhesion typical of A1.A3 cells infected by E1B/19K$^-$ and 12S/E1B$^-$ viruses. These data thus confirmed that an E4 product is responsible for E1A-induced p53-independent cell death.

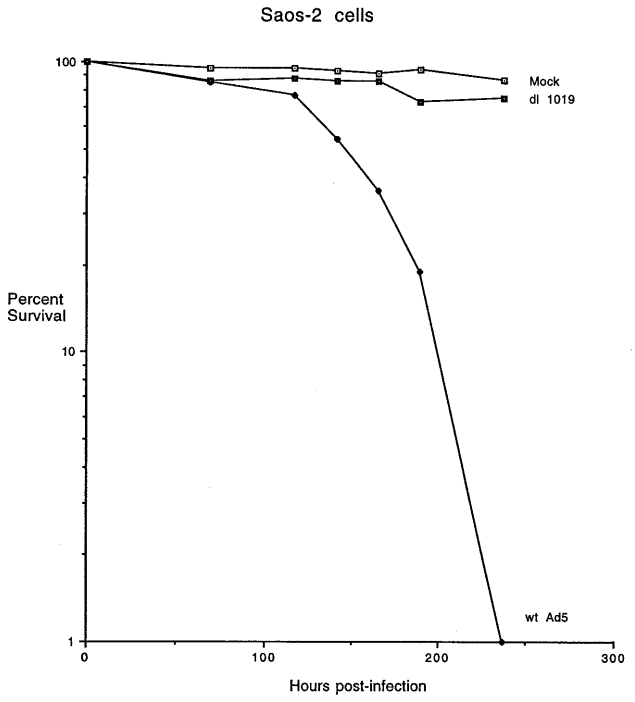


FIG. 7. Role of E4 products in p53-independent cell killing. SAOS-2 cells were mock infected or infected with wt Ad5 or with *dl*1019, which expresses no E4 products. At various times up to 10 days, cell viability was assessed by Trypan Blue exclusion.

DISCUSSION

It has been known for some time that adenovirus E1A products induce DNA degradation, rapid cell death, and other hallmarks of apoptosis when expressed in the absence of E1B products whose major role in lytic infection and transformation is to suppress E1A toxicity. Both the 289R and the 243R E1A proteins are able to induce apoptosis through p53-dependent pathways (22, 52). This function appears to require regions of E1A proteins involved in complex formation with the pRB and p300 families of proteins and may be linked with the induction of cell DNA synthesis (66, 83, 85) and increases in levels of p53 (13, 52, 66). This form of apoptosis is effectively blocked by expression of either the 19-kDa (30, 61, 63, 74, 85, 86) or the 55-kDa (76) E1B protein. Thus, infected or transformed cells are efficiently protected against this form of E1A-induced cell death.

E1A proteins also induce apoptosis in cells lacking p53 (75, 78). We found that this p53-independent apoptosis was elicited only by the 289R E1A protein, and preliminary evidence suggested that expression of one or more additional early viral genes regulated by E1A 289R was required (78). The present experiments indicated that the E1B 55-kDa protein is unable to block this effect, but both the E1B 19-kDa product and the cellular suppressor of apoptosis Bcl-2 significantly inhibited this response.

The major goal of this work was to identify which early viral transcription units were required to induce cell death in the absence of p53. Results obtained with E1A mutants clearly indicated that CR3 is important. Furthermore, CR3-mediated transactivation activity appeared to be required, as several mutants with point mutations in CR3 which were known to

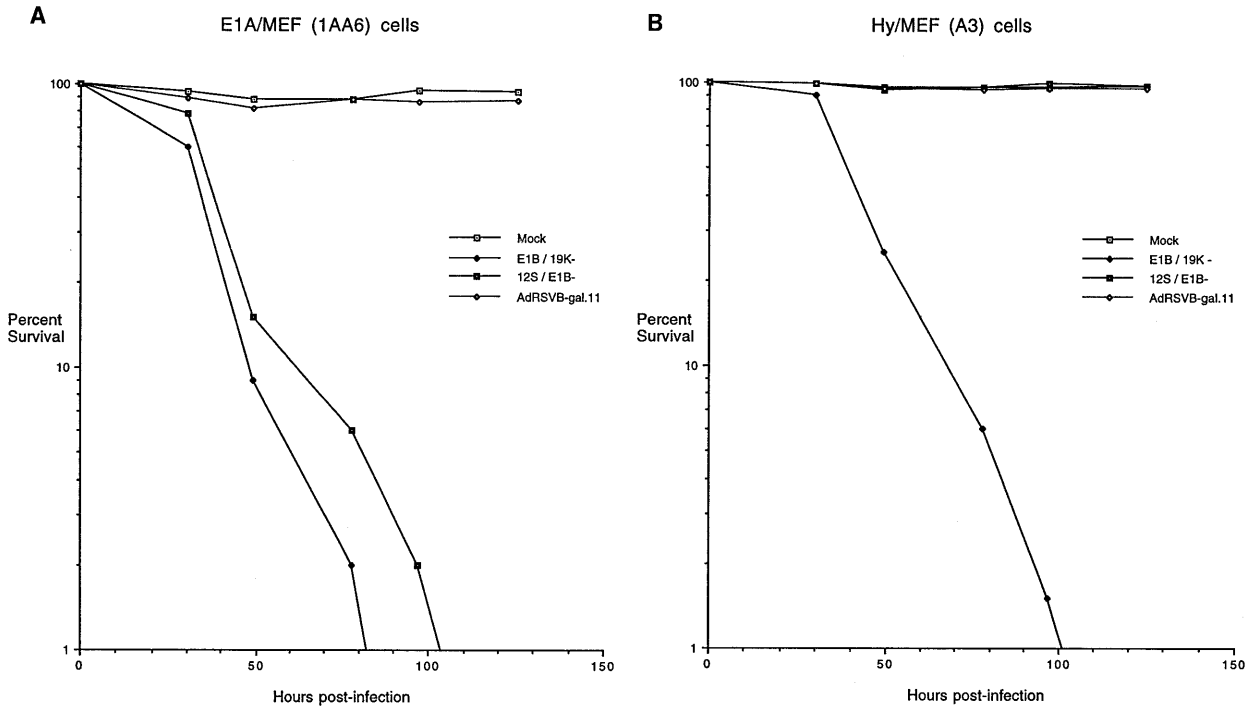


FIG. 9. Analysis of p53-independent cell killing in the absence of E4 products. Cell lines expressing 289R and 243R E1A proteins constitutively (A) or the Hy.A3 nonexpresser control cells (B) were mock infected or infected with wt Ad5, E1B/19K$^-$, 12S/E1B$^-$, or the adenovirus vector AdRSVβgal.11. At various times, cell viability was assessed by Trypan Blue exclusion. MEF, mouse embryo fibroblasts.

eliminate transactivation of target genes were defective for induction of DNA degradation and cell killing. Of great interest were results obtained with mutants with defects outside CR3. Mutant *dl*1108, which lacks the core binding site for pRB, and related proteins induced p53-independent apoptosis like the wt. However, mutant *dl*1101, which binds pRB at reasonably normal levels but fails to bind the p300 transcriptional modulator (3, 4), was totally defective. These results may suggest that interactions between p300 and 289R are essential to institute cell death pathways. Another possibility was offered by results obtained with two additional mutants with defects in the AR1 and AR2 regions encoded by the second exon of the 13S E1A mRNA. The AR1-defective mutant was unable to induce p53-independent apoptosis, and that lacking AR2 was somewhat impaired. These results corresponded exactly to the relative abilities of these mutant E1A molecules to transactivate the E4 promoter. We also found that *dl*1101 was partially defective for transactivation of E4, thus suggesting both that E4 products might be involved in induction of cell death and that interactions of 289R with p300 may reflect more a requirement for transactivation of E4 transcription than a direct role in apoptosis. This question will be answered only by further experiments using a new series of mutants.

Early regions E2, E3, and E4 encode a variety of products which could play some role in cell death. E2 proteins are largely involved in viral DNA synthesis (43). Although we did not examine the specific requirement for E2 products directly, it is unlikely that any play an essential role in cell death. First, E2 transcription requires not only CR3 but also the formation of complexes with pRB which result in the activation of the E2F family of transcription factors and E2 gene expression. Our results clearly indicated that complex formation with pRB was not essential for apoptosis. Second, the adenovirus vector AdRSVβgal.11 contains a wt E2 region and yet was defective for induction of p53-dependent apoptosis in E1A-expressing cells. The E3 region encodes several proteins which affect virus-host interactions; however, the adenovirus vector Ad5dl70-8 was fully capable of inducing apoptosis in E1A expressing p53-deficient cells. As discussed above, the pattern of apoptosis observed with E1A mutants suggested that the early viral proteins associated with cell death are encoded by E4. Direct evidence that an E4 protein is responsible was obtained from experiments in which the pattern of death was observed in p53-null SAOS-2 cells infected by wt virus or a mutant defective in E4 expression. Because E1B products were expressed by these viruses, cell death occurred only at late times, but the observation that E4 mutant-infected cells displayed considerably retarded death clearly implicated an E4 product in the death process. It was possible that cell death did not occur because E4 products are required for virus replication (17, 69). However, induction of E1A-induced p53-dependent apoptosis does not require replication, as the effect has been demonstrated to occur in the absence of the E1B 55-kDa protein, which is essential for replication, and in rodent cells in which replication does not occur. Final confirmation came from results with the AdRSVβgal.11 adenovirus vector, which was defective for cell killing. This virus was unable to induce DNA degradation or cell killing in p53-deficient cells expressing E1A.

At present, we do not know which Ad5 E4 products are involved in the induction of p53-independent apoptosis. E4 is believed to encode seven proteins as determined by identification of open reading frames and sequencing of cloned E4 cDNAs (33, 41, 80). The E4orf6 product is a 34-kDa protein known to associate with the E1B 55-kDa polypeptide and to be involved with host shutoff and transport of viral late mRNAs to the cytoplasm (16, 19, 64, 69, 70). It is unlikely that induction of apoptosis is the result of host cell shutoff, as cell death was

induced efficiently in the absence of the E1B 55-kDa protein, which is absolutely required for this effect (16, 64, 69, 70). The E4orf3 protein is an 11-kDa species associated with the nuclear matrix and involved in accumulation of late viral mRNA (24, 71) and, along with E4orf4 and E4orf6, appears to affect viral DNA synthesis (17). E4orf6/7 is a 17-kDa protein that binds as a homodimer to transcription factor E2F to promote E2 promoter activity by ensuring correct spacing and orientation (21, 44, 53, 59, 62, 68). The E4orf4 protein is a 13-kDa species that binds to and activates protein phosphatase 2A (49) and may have a role in the regulation of DNA synthesis (17) and AP-1 activity (57). Mutants defective in E4orf4 show increased cytotoxicity (57). Little is known about the Ad5 E4orf1, E4orf2, or E4orf3/4 products, although one or more products in the region encoding these proteins in the Ad9 serotype cooperates with E1A in cell transformation (45). Any of the E4 species could be linked to apoptosis, and studies designed to identify which species are involved and to determine the precise mechanism of induction of p53-independent apoptosis are ongoing.

ACKNOWLEDGMENTS

We are indebted to Andy Bett and Frank Graham for the gift of adenovirus vectors AdLacZ and Ad5dl70-8; to Stan Bayley for mutants *dl*520(12S)/E1B$^-$ and *dl*1132 and other E1A mutants; to Arnold Levine for 10(1) cells; to Scott Lowe for cell lines Hy.A3, A1.A3, A1.A6, and A1.A12; to Rob Ricciardi for E1A plasmids containing point mutations in CR3, which allowed construction of some CR3 mutants; and to Tom Shenk for *dl*309. We also thank Rachelle Charbonneau and Dennis Takayesu for some technical assistance.

This work was supported by grants to P.E.B. from the National Cancer Institute of Canada and the Medical Research Council of Canada. J.G.T. holds a Research Studentship from the NCIC, and R.C.M. received a Student Research Award from the Glaxo/Burroughs-Wellcome Corp.

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