VIROLOGY 220, 152–162 (1996)
ARTICLE NO. 0295

ANN E. TOLLEFSON,* JAN S. RYERSE,† ABRAHAM SCARIA,*[1] TERRY W. HERMISTON,* and WILLIAM S. M. WOLD*[,2]

*Department of Molecular Microbiology and Immunology and †Department of Pathology, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, Missouri 63104

Received January 23, 1996; accepted April 8, 1996

We have reported that an 11,600-Da nuclear membrane glycoprotein named adenovirus death protein (ADP), encoded by the E3 region, is required for the efficient death (lysis) of adenovirus (Ad)-infected cells. We postulated that ADP mediates the release of virions from cells at the conclusion of replication. Here we provide further characterization of cells infected by *adp*$^+$ and *adp*$^-$ Ads. Using virus mutants with deletions in the individual E3 genes, we show that only mutants that lack ADP have small plaques that are slow to develop. Mutants in the *adp* gene replicated as well as wild-type Ad, but the cells lysed much more slowly. Cell lysis and viability were determined by plaque size, cell morphology, trypan blue exclusion, the release of lactate dehydrogenase, and the MTT assay for mitochondrial activity. ADP is required for efficient lysis of human A549, KB, 293, and MCF-7 cells. A549 cells infected with *adp*$^+$ Ads began to die at 2–3 days postinfection and were dead by 6 days. With *adp* mutants, >80% of cells remained viable for 5–6 days; when the medium was changed, >80% of cells were viable after 7 days and 10–20% after 14 days. When the MTT assay was used, there was an increase in mitochondrial activity, suggesting that Ad infection stimulates respiratory metabolism. Nearly all nuclei from wild-type Ad-infected cells lacked DAPI-stained DNA by 7 days, whereas with an *adp* mutant nearly all nuclei stained brightly after 15 days. Nuclei from *adp* mutant-infected cells were extremely swollen and full of virus, and appeared to have an intact nuclear membrane. Cells infected with wild-type Ad had many vacuoles and perhaps a disrupted nuclear membrane; they did not display features typical of apoptosis.

INTRODUCTION

Apoptosis (programmed cell death) is a regulated biochemical cell death process (reviewed in Korsmeyer, 1995; Kumar, 1995; Martin and Green, 1995; Reed, 1994; Steller, 1995). Apoptosis may be a defense against virus infections, i.e., the cell precludes virus replication by committing suicide. Viruses, in turn, might be expected to regulate cell viability, inhibiting apoptosis during early stages of replication, then promoting apoptosis (or another form of cell lysis) late in infection so that virus can be released from the cell. Indeed, there are several viral proteins that inhibit apoptosis (reviewed in Vaux *et al.,* 1994; Shen and Shenk, 1995; Wold *et al.,* 1995a). The cowpox virus CrmA protein, a serpin, prevents apoptosis in CrmA-transfected cells by inhibiting the interleukin-1β-converting enzyme (ICE) (Gagliardini *et al.,* 1994; Wang *et al.,* 1994) and/or the ICE family member Yama/CPP32 (Tewari *et al.,* 1995). Another serpin, SPI-1, is required to inhibit apoptosis in rabbit poxvirus-infected cells (Brooks *et al.,* 1995). The Epstein–Barr virus BHRF1 protein is structurally related to cellular Bcl-2, and in common with Bcl-2, BHRF1 inhibits apoptosis (Henderson *et al.,* 1993; Tarodi *et al.,* 1994). The African swine fever virus *LMW5-HL* gene is similar to *bcl-2* and *BHRF1* (Neilan *et al.,* 1993). The herpes simplex virus-1 $\gamma_1$34.5 protein is required to preclude premature death in neuronal cells (Chou and Roizman, 1992; He *et al.,* 1996). The human cytomegalovirus IE1 and IE2 proteins block apoptosis induced by tumor necrosis factor (TNF) or adenovirus (Ad) E1A expression (Zhu *et al.,* 1995). Baculoviruses encode two proteins, p35 and IAP, that inhibit apoptosis (Clem *et al.,* 1991; Kamita *et al.,* 1993; Clem and Miller, 1994; Hershberger *et al.,* 1994). p35 may block apoptosis by directly inhibiting ICE (Bump *et al.,* 1995; Xue and Horvitz, 1995).

In Ad, the immediate early E1A proteins induce apoptosis as a consequence of their ability to deregulate the cell cycle (reviewed in Bayley and Mymryk, 1994; Moran, 1994; White, 1994). The Ad E1B-19K protein inhibits apoptosis induced by E1A (Rao *et al.,* 1992; White *et al.,* 1992; White, 1994) or the DNA-damaging agents cisplatin and UV light (Subramanian *et al.,* 1993; Tarodi *et al.,* 1993). E1B-19K may be the Ad functional equivalent of BHRF1 and Bcl-2 (Tarodi *et al.,* 1993; Chiou *et al.,* 1994). E1B-19K, Bcl-2, and BHRF1 physically interact with common cellular proteins (Boyd *et al.,* 1994; Farrow *et al.,* 1995), and E1B-19K and Bcl-2 share limited sequence similarity (Tarodi *et al.,* 1993; Chiou *et al.,* 1994; Subramanian *et al.,* 1995a). E1B-19K may prevent apoptosis by alleviating repression by p53 of cellular survival proteins

[1] Present address: Genzyme Corporation, 1 Mountain Road, Framingham, MA 01701.

[2] To whom correspondence and reprint requests should be addressed. Fax: (314) 773-3403; E-mail: WOLDWS@SLUVCA.SLU.EDU.

0042-6822/96 $18.00

(Sabbatini *et al.,* 1995). The Ad E1B-55K protein also is hypothesized to inhibit E1A-induced apoptosis by binding to and inactivating p53 (Yew and Berk, 1992; Lowe *et al.,* 1994).

Ad also has proteins that inhibit cell death induced by other agents (reviewed in Gooding, 1994; Laster *et al.,* 1994; Wold *et al.,* 1994, 1995a,b). E1B-19K (Gooding *et al.,* 1991a; White *et al.,* 1992), the E3-14.7K protein (Gooding *et al.,* 1988, 1990; Horton *et al.,* 1991; Ranheim *et al.,* 1993), and the E3-10.4K/14.5K complex of proteins (Gooding *et al.,* 1991b) protect cells from cytolysis by TNF. E1B-19K also blocks apoptosis induced by cross-linking the Fas/APO-1 antigen, a receptor related to the p55 TNF receptor (Chiou *et al.,* 1994; Hashimoto *et al.,* 1991). The E3-gp19K protein forms a complex with major histocompatibility class I antigens in the endoplasmic reticulum and blocks their transport to the cell surface; accordingly, E3-gp19K prevents Ad-infected cells from being killed by cytotoxic T-lymphocytes (reviewed in Wold *et al.,* 1995a,b).

We have described a novel Ad protein that, rather than inhibiting cell death as is the case for the proteins discussed above, is required for the efficient death (lysis) of Ad-infected cells (Tollefson *et al.,* 1996). This protein, named adenovirus death protein (ADP), was previously named E3-11.6K (Wold *et al.,* 1984). We have proposed that ADP mediates the release of Ad from the cell after the infectious cycle is complete. ADP is synthesized in small amounts from the E3 transcription unit during early stages of infection, but in very large amounts from the major late transcription unit at very late stages of infection (Tollefson *et al.,* 1992). ADP is a type III ($N_{exo}C_{cyt}$) bitopic N-linked O-linked glycoprotein that localizes predominantly to the nuclear membrane and Golgi at very late stages of infection (Scaria *et al.,* 1992). Here we report further characterization of cells infected with *adp* mutants.

MATERIALS AND METHODS

Cells and viruses

A549, KB, 293, and MCF-7 cells were grown as monolayers in Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum (FBS). Virus stocks were prepared in suspension cultures of KB cells, banded in CsCl, and titered on A549 cells as described by Green and Wold (1979).

The viruses used in this study (see Fig. 1A), *rec*700 (Wold *et al.,* 1986), *dl*712 (ADP^-) (Deutscher *et al.,* 1985), *dl*722 ($12.5K^-$, ADP^+), *dl*731 ($12.5K^-$, ADP^+), *dl*762 ($14.7K^-$, ADP^+), *dl*739 ($6.7K^-$, ADP^+) (Brady *et al.,* 1992), *dl*764 ($14.5K^-$, ADP^+) (Tollefson *et al.,* 1990), *dl*753 ($10.4K^-$, ADP^+) (Brady and Wold, 1987), *dl*704 ($gp19K^-$, ADP^+) (Bhat and Wold, 1987), and *dl*7001 ($E3^-$) (Ranheim *et al.,* 1993), have been described. *rec*700 is an Ad5–Ad2–Ad5 recombinant consisting of the Ad5 *Eco*RI A (map position 0–76), Ad2 *Eco*RI D (76–83), and Ad5 *Eco*RI B (83–100) fragments (Wold *et al.,* 1986). *rec*700 is the equivalent of Ad2 and Ad5 in inducing cell lysis (Tollefson *et al.,* 1996), and it is the wild-type control for the studies described here. *dl*712 deletes the entire *adp* gene (Deutscher *et al.,* 1985). *pm*734.1 (Δ1–48, i.e. lacking residues 1–48 in ADP) has two missense mutations at Met_1 and Met_{41} such that only residues 49–101 in ADP (Fig. 1B) can be synthesized; these mutations were made by oligonucleotide mutagenesis, as will be described elsewhere. The presumptive 49–101 ADP polypeptide cannot be detected by immunoprecipitation with the antipeptide antiserum against residues 87–101 in ADP (unpublished results). *pm*734.1 and *dl*712 have an indistinguishable *adp* mutant phenotype, and so the mutants are used interchangeably. *dl*7001 ($E3^-$) lacks all E3 genes (Ranheim *et al.,* 1993).

Plaque development assay for cell viability

Plaque assays were carried out on A549 cells as described by Green and Wold (1979). Plaques were counted at 2- to 3-day intervals until 4 weeks postinfection (p.i.).

Trypan blue exclusion assay for cell viability

Cells were infected at 20, 25, or 100 PFU/cell (as indicated in figure legends) in 1 ml serum-free DME. At 1 hr p.i., DME (10% FBS) was added to each dish to a final serum concentration of 5 to 8%. At indicated times, the supernatant was removed, cells were trypsinized, the supernatants and cells were combined, and trypan blue (GibcoBRL, Gaithersberg, MD) was added to a final concentration of 0.02%. Cells were counted on a hemacytometer (a total of 600 to 1000 cells per point). For Fig. 4B the medium was changed every 2 days during the course of infection. The floating cells were collected by centrifugation, then resuspended in DME containing 8% FBS.

Lactate dehydrogenase (LDH) release assay for cell viability

A549 cells (2.3×10^6 cells/60-mm dish), 293 cells (1.8×10^6 cells/35-mm dish), KB (ATCC) cells (2.2×10^6 cells/60-mm dish), or MCF-7 cells (9.9×10^5 cells/35-mm dish) were infected at 100 PFU/cell in serum-free medium except for 293 cells which were infected in DME (2% FBS). At 1 hr p.i. DME (10%) was added to a final serum concentration of 5% (MCF-7), 6% (293), or 8% (A549, KB). Twenty-microliter samples were removed at the indicated times and assayed in triplicate for LDH release by the Cytotox 96 assay (Promega Corp., Madison, WI). Samples were read on an EL340 microplate reader (BioTec Instruments, Inc.) at 490 nm.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay for cell viability

A549 cells were infected at 20 PFU/cell and 2.2×10^4 cells were plated per well in 96-well plates at 4 hr p.i.

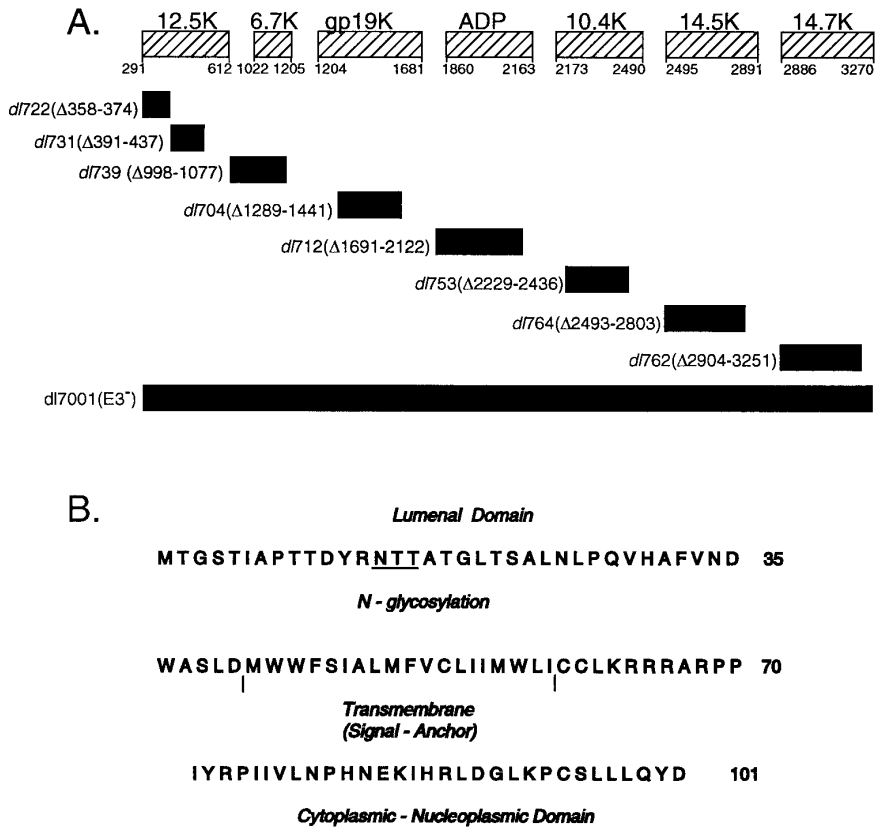

FIG. 1. Schematic of the E3 proteins of *rec*700, the deletions in the virus mutants used, and the amino acid sequence of ADP. (A) E3 proteins (reviewed in Wold *et al.,* 1995b) and the mutant deletions. Although ADP is coded by the E3 transcription unit, ADP is in fact primarily a late protein expressed from the major late promoter as part of the major late transcription unit (Tollefson *et al.,* 1992). The numbers refer to nucleotide numbers in the E3 transcription unit of *rec*700, with 1 being the transcription initiation site. *rec*700 is an Ad5–Ad2–Ad5 recombinant. Numbers for the Ad2 E3 region are used from nucleotide 1 to 2437, and numbers for the Ad5 E3 region are used downstream of nucleotide 2437 (see Cladaras and Wold, 1985). (B) Amino acid sequence of ADP of Ad2 (Hérissé *et al.,* 1980; Wold *et al.,* 1984).

At indicated times p.i., 25 μl of MTT (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS) (5 mg/ml) was added to each well (Mosmann, 1983; Hansen *et al.,* 1989). After 2 hr, lysis buffer (20% sodium dodecyl-sulfate in 50:50 dimethyl formamide:ddH_2O, pH 4.7) was added to each well and plates were incubated overnight at 37°. Plates were read on a microplate reader at 570 nm. Samples were in triplicate.

4,6-Diamidino-2-phenylindole (DAPI) staining

Infected cells were collected in a microfuge, washed with PBS, then resuspended in methanol containing DAPI (Sigma) (2 μg/ml) to fix and stain the cells. After 10 min, cells were pelleted and methanol/DAPI was removed. Cells were resuspended in methanol to remove excess DAPI (10 min), centrifuged in a microfuge, resuspended in a small volume of methanol, and spread on glass slides. When dry, mounting medium and coverslips were applied. Immunofluorescence was viewed by epifluorescence on a Nikon Optiphot microscope using an UV cube.

Electron microscope cytology

For electron microscopy, cells at 4 days p.i. were gently trypsinized, pelleted, and, after the supernatant was discarded, fixed with glutaraldehyde and postfixed with osmium tetroxide in sodium cacodylate buffer. After washing, the tissue was stained *en bloc* with uranyl acetate, dehydrated through graded ethanols and propylene oxide, and infiltrated, embedded, and polymerized in Polybed resin. Sections were cut from the tissue blocks with a Reichert Ultracut E ultramicrotome using a diamond knife, collected on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and viewed and photographed with a JEOL 100 CX electron microscope at 60 kV.

RESULTS

Virus mutants in the *adp* gene, and no other E3 gene, have small plaques that are slow to develop

The E3 region encodes seven known proteins (Fig. 1A). Mutants in the *adp* gene, and none of the other E3 genes, have small plaques (Fig. 2). The difference in plaque sizes can be quantitated based upon the rate at which the plaques develop, as shown in Fig. 3. The data are presented as the number of plaques seen on any given day of the plaque assay as a percentage of the final number of plaques seen at the end of the assay

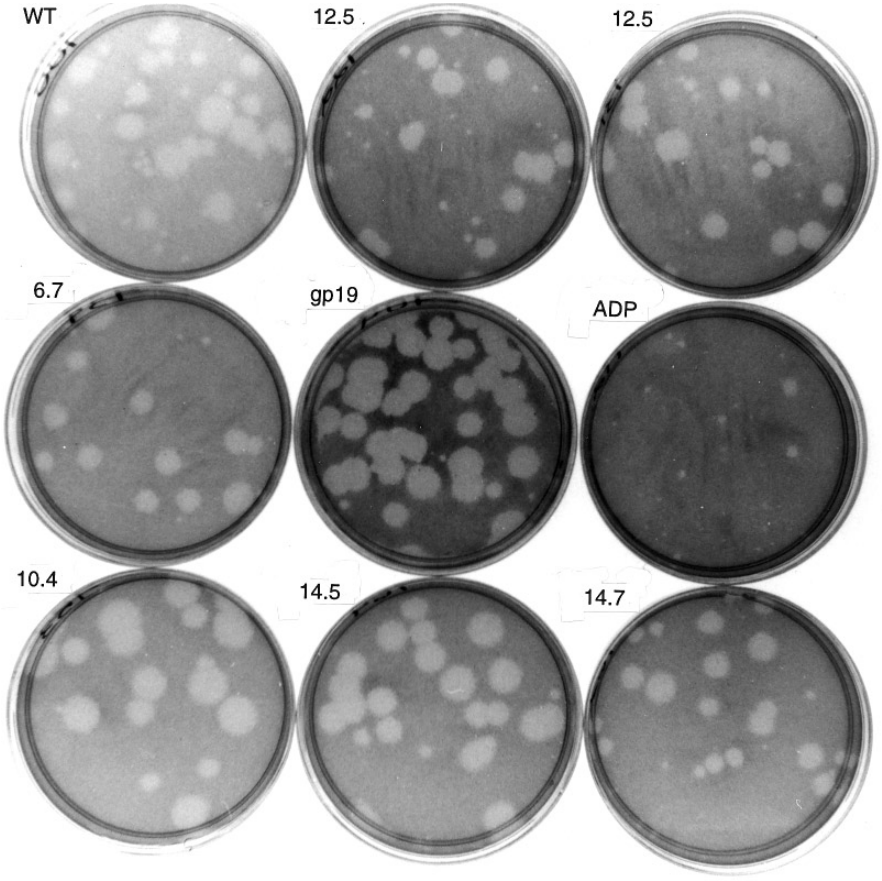

FIG. 2. Plaques on A549 cells for Ad mutants that delete the individual E3 genes. The genes deleted are indicated. The mutants used (left to right, top to bottom) are *rec*700 (wild-type), *dl*722 (12.5K$^-$), *dl*731 (12.5K$^-$), *dl*739 (6.7K$^-$), *dl*704 (gp19K$^-$), *dl*712 (ADP$^-$), *dl*753 (10.4K$^-$), *dl*764 (14.5K$^-$), and *dl*762 (14.7K$^-$). Plaques were photographed at 14 days p.i. Only the mutant that deletes the *adp* gene (*dl*712) has small plaques.

versus the number of days of the assay. At 10 days, 10% of the final plaques was observed with the *adp* mutant (*dl*712), whereas approximately 60–88% of the final plaques was observed with the other E3 mutants. Clearly,

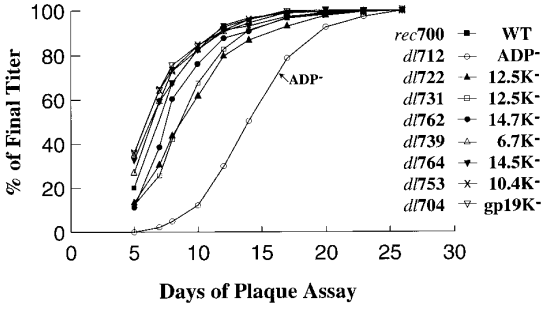

FIG. 3. Plaque development assay for Ad mutants deleted in the individual E3 genes. The *y* axis shows the number of plaques observed on any given day of the plaque assay (the *x* axis) as a percentage of the total number of plaques that were observed on the final day (Day 26) of the plaque assay. (A) The virus mutants used and the genes deleted are indicated at the right. Only *dl*712, deleted in the *adp* gene, has plaques that are slow to develop. The final titers of these CsCl-banded virus stocks were as follows: 2.8×10^{11} for *rec*700, 1.4×10^{11} for *dl*722, 1.4×10^{11} for *dl*731, 7.8×10^{10} for *dl*739, 2.4×10^{11} for *dl*704, 1.1×10^{11} for *dl*712, 1.1×10^{11} for *dl*753, 1.4×10^{11} for *dl*764, 1.6×10^{11} for *dl*762.

the *dl*712 plaques develop slowly compared to the other mutants.

The final titers of all these CsCl-banded virus stocks were similar (legend to Fig. 3). These data, together with wild-type Ad and *adp* mutant growth data (Tollefson *et al.,* 1996), indicate that *adp* mutants are not defective in growth, only in efficient lysis of cells and the release of virus. The slow release of virus explains why the plaques are small.

Cells infected with *adp* mutants remain viable much longer than cells infected with wild-type adenovirus

As described by Tollefson *et al.* (1996), when cell viability was examined using a LDH release assay, a trypan blue exclusion assay, or the MTT assay for mitochondrial activity, cells infected with *rec*700 (wild-type) began to die at 2–3 days p.i. and most were dead by 6 days, whereas cells infected with *dl*712 (ADP$^-$) did not begin to die until 6 days p.i. Here we provide additional features of the death of Ad-infected cells. Figure 4A shows a typical trypan blue exclusion experiment with *rec*700 or *pm*734.1 (Δ1–48 in ADP) using 20 PFU/cell; about 90% of *rec*700-infected cells were dead at 5 days, whereas 95% of *pm*734.1-infected cells were alive. *pm*734.1-in-

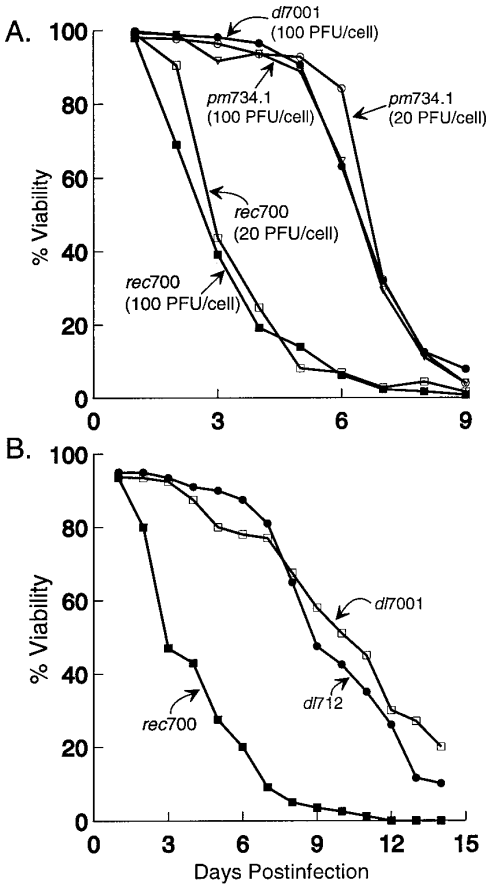

FIG. 4. Trypan blue exclusion assay for cell viability in A549 cells infected at different multiplicities of infection with *rec*700 (wild-type), *pm*734.1 (Δ1–48 in ADP), and *dl*7001 (E3$^-$), without and with medium changes. (A) Cells (1.3×10^6 cells/60-mm dish) were infected at a multiplicity of 20 or 100 PFU/cell. (B) Cells (7.6×10^5 cells/35-mm dish) were infected at 25 PFU/cell. The medium (DME plus 5% FBS) was changed every 2nd day.

fected cells died at the same rate as *dl*7001 (E3$^-$)-infected cells, supporting the results in Figs. 2 and 3 indicating that no other E3 protein plays a significant role in cell death. The cell death kinetics were only slightly faster when 100 PFU/cell of virus was used (Fig. 4A); thus, cell death is not due to toxicity caused by high multiplicities of infection.

In the above experiments, the medium was not changed following infection. When the medium was changed every 2 days there was an even more pronounced difference in the viability of infected cells. Cells infected with *rec*700 died at the same rate as when the medium was not changed, but cells infected with *dl*712 (ADP$^-$) or *dl*7001 (E3$^-$) were 80% viable at 7 days and 10–20% viable at 14 days (Fig. 4B).

The different rates of cell death can also be observed morphologically. Cells infected with *rec*700 began to die at 2 days p.i. and cell death progressed from Days 4–8. With *dl*712 (ADP$^-$), the cells were nearly all intact at 5 and 6 days, and only at Days 7 and 8 were dead cells observed (data not shown).

Thus, cells infected with *adp* mutants stay alive much longer than cells infected with *adp*$^+$ viruses.

Nuclei of *adp* mutant-infected cells are enlarged, full of virus, and appear to have an intact nuclear membrane

The nuclei in *dl*712-infected cells were very enlarged as is apparent in electron micrographs of typical *dl*712-infected cells at 4 days p.i. (Figs. 5A and 5B), a time when 90% of cells were alive. The nuclei were packed with virus and the nuclear membrane appeared to be intact. Virus was not detected in the cytoplasm. Figures 5C and 5D show examples of different types of *rec*700-infected cells at 4 days p.i., when >80% of cells were dead. The cells contained many vacuoles and often had poorly stained structures of unknown origin (the upper cells in Figs. 5C and 5D), or they were totally lysed (bottom cell in Fig. 5D). The upper cells in Figs. 5C and 5D also contained virus, but the nuclear membrane did not seem to be intact as was the case with *dl*712-infected cells. Condensed chromatin and membrane blebs, diagnostic of apoptosis, were not observed.

DNA remains associated with nuclei in cells infected with an *adp* mutant but not with wild-type adenovirus

We showed previously by agarose gel electrophoresis and the TUNEL (terminal deoxynucleotidyltransferase end labeling) assay that DNA is degraded sooner in cells infected with wild-type Ad compared to an *adp* mutant (Tollefson *et al.,* 1996). With *rec*700, significant detectable DNA degradation began at 3 days p.i., but it was delayed by 2 days with *dl*712 (ADP$^-$). When DNA was examined within nuclei using DAPI to stain DNA, a dramatic difference was seen between *rec*700 and *pm*734.1 (Δ1–48 in ADP) (Fig. 6). At 7 days p.i. with *rec*700, most nuclei were not stained with DAPI, giving them a ghost-like appearance (Fig. 6A). A small fraction of cells was brightly stained, and others displayed a speckled pattern. With *pm*734.1, most nuclei were brightly stained throughout, although some nuclei were brighter on the rim (Fig. 6B). All the *rec*700-infected cells and 70% of *pm*734.1-infected cells were lysed by 7 days. With *rec*700 at 9 and 13 days p.i. (data not shown), and at 15 days p.i. (Fig. 6C), there were few brightly stained or speckled nuclei, and only the rims of the nuclei were visible. With *pm*734.1 at 15 days (Fig. 6D), the nuclei were brightly stained, similar to the nuclei at 7 days. Thus, the DNA remains associated with the nucleus much longer in *adp* mutant-infected cells.

Adenovirus infection stimulates mitochondrial activity prior to inducing cell death

Figures 7A and 7B show parallel cell viability assays using MTT and trypan blue exclusion. MTT is a tetrazolium salt whose tetrazolium ring is cleaved to formazan (dark blue) by dehydrogenases, primarily in active mito-

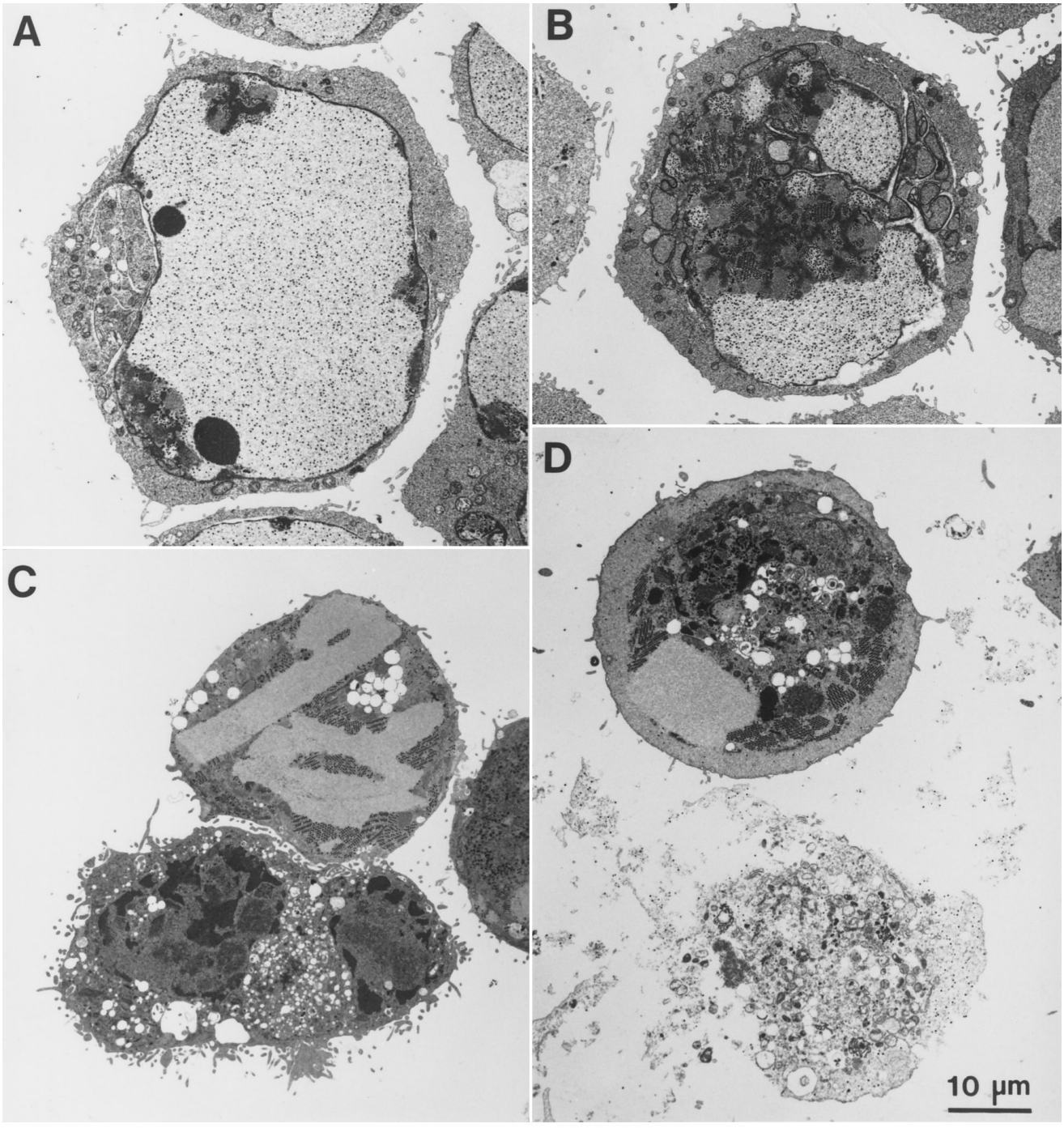

FIG. 5. Electron micrographs of A549 cells infected with *rec*700 (wild-type) or *dl*712 (ADP^-) at 4 days p.i. Typical morphologies are shown. Original magnification was 1320×. (A and B) *dl*712. (C and D) *rec*700.

chondria (Mosmann, 1983; Hansen *et al.,* 1989). The amount of formazan generated per cell is proportional over a wide range to the number of living cells. It is also proportional to the level of energy metabolism in cells, as shown by comparing resting lymphocytes with lymphocytes stimulated with concanavalin A (Mosmann, 1983). Thus, MTT can be used as an indicator of the number of viable cells as well as the energy metabolism in cells. With *rec*700, there was a sharp increase in mitochondrial activity from 1 to 2 days p.i. (Fig. 7A), a period when nearly all the cells were intact (Fig. 7B). Mitochondrial activity declined at 3 days as the cells began to die. With *dl*712 (ADP^-) or *pm*734.1 (Δ1–48), mitochondrial activity continued to increase until 4 or 5 days (Fig. 7A), when most of the cells are alive (Fig. 7B), then began to decline at 6 days, coincident with the onset of cell death. Prolonged metabolic activity in *dl*712- or *pm*734.1-infected cells, compared to *rec*700-infected cells, was also indicated by the pH of the medium which was 6.5 with *adp* mutants and 7.2–7.4 with *rec*700 at 5 days p.i. (data

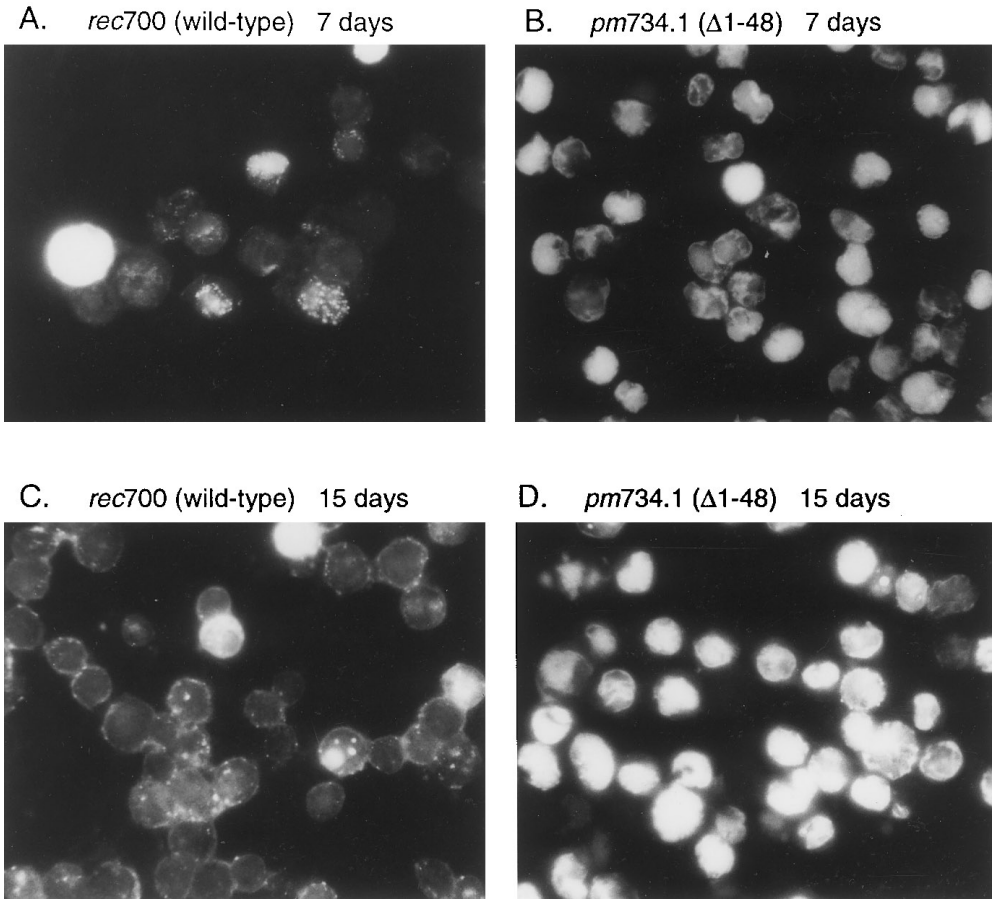

FIG. 6. Immunofluorescence of DNA from cells infected with *rec*700 (wild-type) and *pm*734.1 (Δ1–48 in ADP). A549 cells were infected with 20 PFU/cell of virus, then at 7 and 15 days p.i. were fixed and stained with DAPI. With *rec*700-infected cells, many nuclei can be seen, but only a few were stained by DAPI. With *pm*734.1, most of the nuclei contained DAPI-stainable DNA.

not shown). This decrease in pH occurred even though the medium was buffered.

It is important to emphasize that the virus-infected cells ceased to divide after infection, so the increase in mitochondrial activity was not due to an increase in cell number. This contrasts with the increase in mitochondrial activity seen with mock-infected cells (Fig. 7A), where the cells continued to multiply during the experiment. At 5 days, there were 4–6 times more mock-infected cells than virus-infected cells. Despite the larger cell number, the medium from mock-infected cells was not as acidic as the medium from *adp* mutant-infected cells.

These results suggest that Ad has a function that stimulates mitochondrial activity. This function is manifested prior to the onset of cell death.

ADP is required for efficient cell death in the human A549, KB, 293, and MCF-7 cell lines

The experiments in Figs. 2–7 were performed in A549 cells. In Figs. 8 and 9, the LDH release and trypan blue exclusion cell viability assays were used to compare the death kinetics of *rec*700- and *pm*734.1 (Δ1–48)-infected A549 cells with three other cell lines. When infected with *rec*700, A549 cells died perhaps slightly more rapidly than KB, 293, or MCF-7 cells. When infected with *pm*734.1 or *dl*712, A549 cells seemed to remain viable about 1 day longer than the other cell lines. Results obtained with primary human foreskin fibroblasts were similar to those with A549 cells (data not shown). It is clear that ADP is required for efficient death in A549, KB, 293, and MCF-7 cells.

DISCUSSION

We have shown that Ad *adp* mutants have small plaques that develop slowly. Mutants that lack other E3 genes have essentially normal plaques. The *adp* mutants grow as well as wild-type Ad, but the cells lyse much more slowly so the plaques are small. A549 cells infected with wild-type Ad begin to die at 2–3 days p.i. and are dead by 6 days p.i. With *adp* mutants, most cells do not begin to die until 6 days p.i. Cell lysis was assayed by trypan blue exclusion, LDH release, cell morphology, DAPI staining of DNA, and the MTT assay for mitochondrial activity. Cell viability was examined previously using agarose gel electrophoresis of degraded DNA and RNA, the TUNEL assay for nicked DNA, and protein synthesis

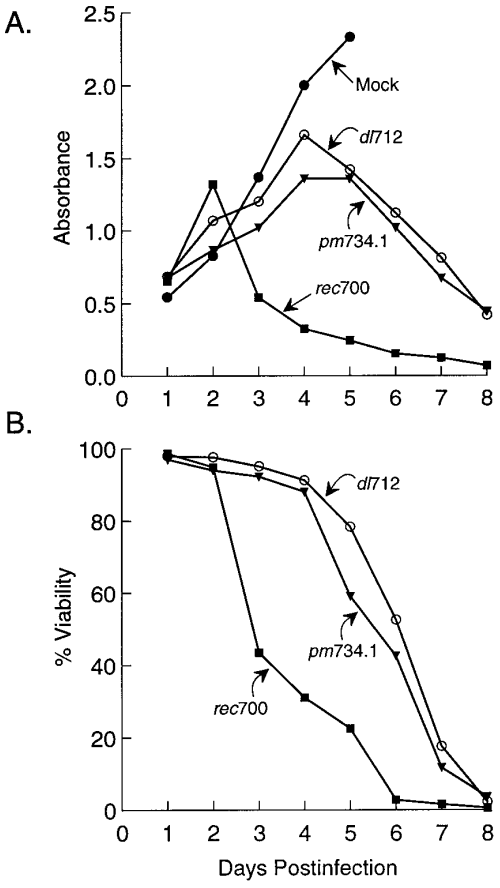

FIG. 7. MTT assay for mitochondrial activity and cell viability. A549 cells were infected with 20 PFU/cell of virus *rec*700 (wild-type), *dl*712 (ADP$^-$), or *pm*734.1 (Δ1–48 in ADP), then the cells were examined using (A) the MTT or (B) the trypan blue exclusion assay at different days p.i.

(Tollefson *et al.,* 1996). With wild-type Ad, cells detach and disperse, many have a ballooned plasma membrane, the DNA is highly degraded, and protein synthesis declines by 2 days p.i. and is nonexistent by 3 days (Tollefson *et al.,* 1996). Virus is released into the culture supernatant beginning at 2–3 days p.i., coincident with cell lysis. The degraded DNA does not remain associated with the nuclei in lysed cells (Fig. 6). With *adp* mutants, the DNA remains in the nucleus until at least 15 days p.i. (Fig. 6), long after the cell has lysed (Fig. 4A), suggesting that the DNA is less degraded than in wild-type-infected cells. Synthesis of Ad proteins continues robustly until 4 days p.i. (Tollefson *et al.,* 1996). Cellular protein synthesis is shut off as in normal infection, and the cells show Ad cytopathic effect (CPE) in that they detach into grape-like clusters (Tollefson *et al.,* 1996). The CPE is probably due to disruption of the cytoskeleton by the Ad L3 protease and the inability of the cell to repair the cytoskeleton via synthesis of new cytokeratins (Chen *et al.,* 1993; Zhang and Schneider, 1994). Despite this CPE, the *adp* mutant-infected cells do not begin to lyse and release virus until 6 days p.i.

Since cells infected with *adp* mutants remain viable much longer than wild-type, ADP apparently functions to promote cell death and the release of Ad from the infected cell (Tollefson *et al.,* 1996). This ADP function represents a novel concept in viral pathogenesis, i.e., by promoting rapid cell lysis after virus replication is complete, ADP facilitates the spread of virus and reduces the chances of virus being destroyed by cell-mediated immunity.

Prior to cell lysis, the nuclei of *adp* mutant-infected cells were remarkable in that they were extremely swollen and full of virus. In some cells, crystals of virus were apparent. Although not investigated in detail, the nuclear membrane appeared to be intact. Consistent with this, virus was not observed in the cytoplasm. With wild-type Ad, it was difficult to discern a nuclear membrane. ADP localizes to the nuclear membrane (and Golgi) at very late stages of infection (>30 hr) (Scaria *et al.,* 1992). Therefore, a manifestation of ADP function may be the disruption of the nuclear membrane and the liberation of virus from the nucleus. However, a mechanism must also exist that leads to lysis of the entire cell. In this regard, E1B-19K (White *et al.,* 1984) and Bcl-2 (Lithgow *et al.,* 1994; Reed, 1994) localize, in part, to the nuclear membrane. These proteins inhibit both apoptosis and necrosis (Subramanian *et al.,* 1995b). Perhaps ADP induces cell lysis by abrogating the ability of E1B-19K and/or Bcl-2 to inhibit apoptosis and/or necrosis.

A number of cellular and viral proteins have been identified that inhibit apoptosis, and some cellular proteins promote apoptosis (see Introduction). ADP is not obvi-

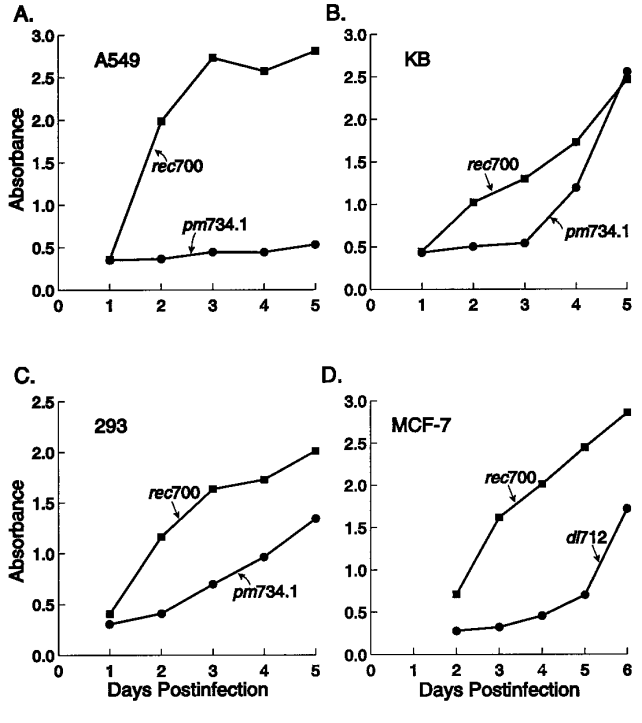

FIG. 8. Lactate dehydrogenase release cell viability assay of different human cell lines infected at 100 PFU/cell of *rec*700 (wild-type), *pm*734.1 (Δ1–48 in ADP), or *dl*712 (ADP$^-$).

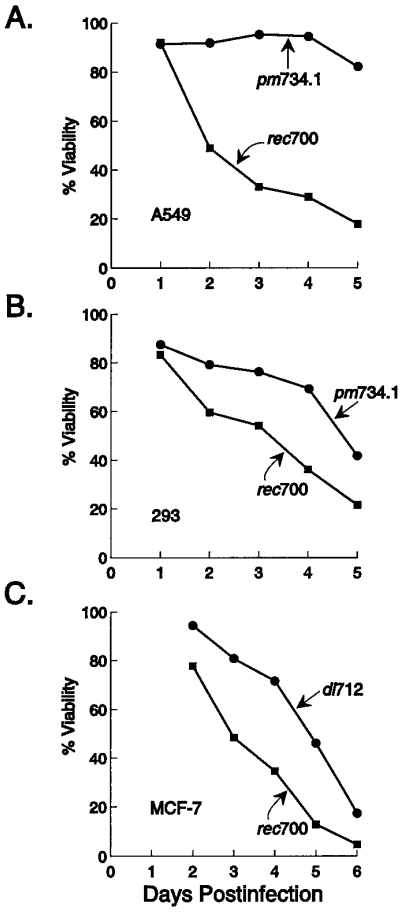

FIG. 9. Trypan blue exclusion cell viability assay of different human cell lines infected at 100 PFU/cell of *rec*700 (wild-type), *pm*734.1 (Δ1–48 in ADP), or *dl*712 (ADP$^-$). The data are from the same experiment as in Fig. 8.

ously related to any of these proteins (Feinstein *et al.,* 1995), so it probably functions in a novel manner. The death of Ad-infected cells, mediated by ADP, did not have features characteristic of apoptosis, i.e., we did not observe condensed chromatin, blebbed membranes (Fig. 5), or a DNA ladder after agarose gel electrophoresis (Tollefson *et al.,* 1996). Thus, ADP may promote a novel form of programmed cell death. It is possible that ADP promotes nonspecific necrosis, but this seems unlikely from a teleological point of view because necrosis generates an inflammatory response which would be disadvantageous to the virus.

When the medium was changed every 2nd day, wild-type Ad-infected cells died at about the same rate as when the medium was not changed. However, the viability of *adp* mutant-infected cells was significantly prolonged by medium changes. This suggests that the death of *adp* mutant-infected cells is caused, at least in part, by the exhaustion of a rate-limiting nutrient, or perhaps by the accumulation of a toxic metabolite. This contrasts with wild-type Ad-infected cells, where ADP presumably activates a specific cell death program.

ADP is required for efficient lysis of infected A549, KB, 293, and MCF-7 cells. The KB, 293, and MCF-7 cells died at a slightly faster rate than the A549 cells. This probably reflects, in part, inherent differences in the fragility of cells or their ability to survive crowded conditions. For example, uninfected KB, 293, and MCF-7 cells died more rapidly in dishes than A549 cells. This suggests that these cells overgrow to a greater extent, deplete nutrients more rapidly, or produce higher levels of toxic metabolic products. This would result in earlier death for ADP mutant-infected cells that is not directly determined by viral infection or viral proteins. Human primary foreskin fibroblasts showed a cell death pattern most similar to A549 cells (data not shown), consistent with longer term stability of the cell monolayer. On the other hand, these cell types may differ in expression of proteins, e.g., members of the Bcl-2 or ICE families, which could play a role in ADP-induced cell death.

Interestingly, as judged by the MTT assay, Ad-infection stimulates mitochondrial activity. This was particularly apparent in cells infected with *adp* mutants because they remain viable for so long. The increased MTT activity in Ad-infected cells at 1–2 days p.i. is consistent with the classical observation that Ad-infected cells initially produce more acid than mock-infected cells as evidenced by the media changing to an orange-yellow color. The increase in mitochondrial activity occurred in cells that had been exponentially growing in complete DME containing 10% FBS; these cells would be expected to be fully activated. Therefore, Ad may encode a function that superinduces mitochondrial activity. This should be a useful function for the virus, inasmuch as high levels of ATP must be required for the prodigious synthesis of DNA, RNA, and protein that occurs in Ad-infected cells. It will be interesting to determine whether this putative mitochondrial-stimulated function is an Ad protein, and, if so, how the protein functions.

ACKNOWLEDGMENTS

This work was supported by Grant CA24710 from the National Institutes of Health. We thank Lora Wold for technical assistance, Vishva Dixit for the MCF-7 cells, and Jayma Mikes for preparation of the manuscript and figures.

REFERENCES

Bayley, S. T., and Mymryk, J. S. (1994). Adenovirus E1A proteins and transformation. *Int. J. Oncol.* 5, 425–444.

Bhat, B. M., and Wold, W. S. M. (1987). A small deletion distant from a splice or polyadenylation site dramatically alters pre-mRNA processing in region E3 of adenovirus. *J. Virol.* 61, 3938–3945.

Boyd, J. M., Malstrom, S., Subramanian, T., Venkatesh, L. K., Schaeper, U., Elangovan, B., D'Sa-Eipper, C., and Chinnadurai, G. (1994). Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. *Cell* 79, 341–351.

Brady, H. A., and Wold, W. S. M. (1987). Identification of a novel sequence that governs both polyadenylation and alternative splicing in region E3 of adenovirus. *Nucleic Acids Res.* 15, 9397–9416.

Brady, H. A., Scaria, A., and Wold, W. S. M. (1992). Map of cis-acting sequences that determine alternative pre-mRNA processing in the E3 complex transcription unit of adenovirus. *J. Virol.* 66, 5914–5923.

Brooks, M. A., Ahmad, N., Turner, P. C., and Moyer, R. W. (1995). A rabbitpox virus serpin gene controls host range by inhibiting apoptosis in restrictive cells. *J. Virol.* 69, 7688–7698.

Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenberg, A. H., Miller, L. K., and Wong, W. W. (1995). Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. *Science* 269, 1885–1888.

Chen, P. H., Ornelles, D. A., and Shenk, T. (1993). The adenovirus L3 23-kilodalton proteinase cleaves the amino-terminal head domain from cytokeratin 18 and disrupts the cytokeratin network of HeLa cells. *J. Virol.* 67, 3507–3514.

Chiou, S. K., Tseng, C. C., Rao, L., and White, E. (1994). Functional complementation of the adenovirus E1B 19-kilodalton protein with Bcl-2 in the inhibition of apoptosis in infected cells. *J. Virol.* 68, 6553–6566.

Chou, J., and Roizman, B. (1992). The γ134.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc. Natl. Acad. Sci. USA* 89, 3266–3270.

Cladaras, C., and Wold, W. S. M. (1985). DNA sequence of the early E3 transcription unit of adenovirus 5. *Virology* 140, 28–43.

Clem, R. J., and Miller, L. K. (1994). Control of programmed cell death by the baculovirus genes p35 and iap. *Mol. Cell. Biol.* 14, 5212–5222.

Clem, R. J., Fechheimer, M., and Miller, L. K. (1991). Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science* 254, 1388–1390.

Deutscher, S. L., Bhat, B. M., Pursley, M. H., Cladaras, C., and Wold, W. S. M. (1985). Novel deletion mutants that enhance a distant upstream 5′ splice in the E3 transcription unit of adenovirus 2. *Nucleic Acids Res.* 13, 5771–5788.

Farrow, S. N., White, J. H., Martinou, I., Raven, T., Pun, K. T., Grinham, C. J., Martinou, J. C., and Brown, R. (1995). Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. *Nature* 374, 731–733.

Feinstein, E., Kimchi, A., Wallach, D., Boldin, M., and Varfolomeev, E. (1995). The death domain: A module shared by proteins with diverse cellular functions. *Trends Biochem. Sci.* 20, 342–344.

Gagliardini, V., Fernandez, P. A., Lee, R. K., Drexler, H. C., Rotello, R. J., Fishman, M. C., and Yuan, J. (1994). Prevention of vertebrate neuronal death by the crmA gene. *Science* 263, 826–828.

Gooding, L. R. (1994). Regulation of TNF-mediated cell death and inflammation by human adenoviruses. *Infect. Agents Dis.* 3, 106–115.

Gooding, L. R., Elmore, L. W., Tollefson, A. E., Brady, H. A., and Wold, W. S. M. (1988). A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. *Cell* 53, 341–346.

Gooding, L. R., Sofola, I. O., Tollefson, A. E., Duerksen-Hughes, P., and Wold, W. S. M. (1990). The adenovirus E3-14.7K protein is a general inhibitor of tumor necrosis factor-mediated cytolysis. *J. Immunol.* 145, 3080–3086.

Gooding, L. R., Aquino, L., Duerksen-Hughes, P. J., Day, D., Horton, T. M., Yei, S. P., and Wold, W. S. M. (1991a). The E1B 19,000-molecular-weight protein of group C adenovirus prevents tumor necrosis factor cytolysis of human cells but not of mouse cells. *J. Virol.* 65, 3083–3094.

Gooding, L. R., Ranheim, T. S., Tollefson, A. E., Aquino, L., Duerksen-Hughes, P., Horton, T. M., and Wold, W. S. M. (1991b). The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all mouse cell lines against lysis by tumor necrosis factor. *J. Virol.* 65, 4114–4123.

Green, M., and Wold, W. S. M. (1979). *In* "Methods in Enzymology" (W. B. Jakoby and I. H. Pastan, Eds.), pp. 425–435. Academic Press, New York.

Hansen, M. B., Nielsen, S. E., and Berg, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods* 119, 203–210.

Hashimoto, S., Ishii, A., and Yonehara, S. (1991). The E1b oncogene of adenovirus confers cellular resistance to cytotoxicity of tumor necrosis factor and monoclonal anti-Fas antibody. *Int. Immunol.* 3, 343–351.

He, B., Chou, J., Liebermann, D. A., Hoffman, B., and Roizman, B. (1996). The carboxyl terminus of the murine MyD116 gene substitutes for the corresponding domain of the gamma1 34.5 gene of herpes simplex virus to preclude the premature shutoff of total protein synthesis in infected human cells. *J. Virol.* 70, 84–90.

Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G., and Rickinson, A. (1993). Epstein–Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. *Proc. Natl. Acad. Sci. USA* 90, 8479–8483.

Hérissé, J., Courtois, G., and Galibert, F. (1980). Nucleotide sequence of the EcoRI D fragment of adenovirus 2 genome. *Nucleic Acids Res.* 8, 2173–2192.

Hershberger, P. A., LaCount, D. J., and Friesen, P. D. (1994). The apoptotic suppressor P35 is required early during baculovirus replication and is targeted to the cytosol of infected cells. *J. Virol.* 68, 3467–3477.

Horton, T. M., Ranheim, T. S., Aquino, L., Kusher, D. I., Saha, S. K., Ware, C. F., Wold, W. S. M., and Gooding, L. R. (1991). Adenovirus E3 14.7K protein functions in the absence of other adenovirus proteins to protect transfected cells from tumor necrosis factor cytolysis. *J. Virol.* 65, 2629–2639.

Kamita, S. G., Majima, K., and Maeda, S. (1993). Identification and characterization of the p35 gene of *Bombyx mori* nuclear polyhedrosis virus that prevents virus-induced apoptosis. *J. Virol.* 67, 455–463.

Korsmeyer, S. J. (1995). Regulators of cell death. *Trends Genet.* 11, 101–105.

Kumar, S. (1995). ICE-like proteases in apoptosis. *Trends Biochem. Sci.* 20, 198–202.

Laster, S. M., Wold, W. S. M., and Gooding, L. R. (1994). Adenovirus proteins that regulate susceptibility to TNF also regulate the activity of PLA_2. *Semin. Virol.* 5, 431–442.

Lithgow, T., van Driel, R., Bertram, J. F., and Strasser, A. (1994). The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. *Cell Growth Differ.* 5, 411–417.

Lowe, S. W., Jacks, T., Housman, D. E., and Ruley, H. E. (1994). Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. *Proc. Natl. Acad. Sci. USA* 91, 2026–2030.

Martin, S. J., and Green, D. R. (1995). Protease activation during apoptosis: Death by a thousand cuts? *Cell* 82, 349–352.

Moran, E. (1994). Mammalian cell growth controls reflected through protein interactions with the adenovirus E1A gene products. *Semin. Virol.* 5, 327–340.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–64.

Neilan, J. G., Lu, Z., Afonso, C. L., Kutish, G. F., Sussman, M. D., and Rock, D. L. (1993). An African swine fever virus gene with similarity to the proto-oncogene bcl-2 and the Epstein–Barr virus gene BHRF1. *J. Virol.* 67, 4391–4394.

Ranheim, T. S., Shisler, J., Horton, T. M., Wold, L. J., Gooding, L. R., and Wold, W. S. M. (1993). Characterization of mutants within the gene for the adenovirus E3 14.7-kilodalton protein which prevents cytolysis by tumor necrosis factor. *J. Virol.* 67, 2159–2167.

Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S., and White, E. (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.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.* 124, 1–6.

Sabbatini, P., Chiou, S. K., Rao, L., and White, E. (1995). Modulation of p53-mediated transcriptional repression and apoptosis by the adenovirus E1B 19K protein. *Mol. Cell Biol.* 15, 1060–1070.

Scaria, A., Tollefson, A. E., Saha, S. K., and Wold, W. S. M. (1992). The E3-11.6K protein of adenovirus is an Asn-glycosylated integral

membrane protein that localizes to the nuclear membrane. *Virology* 191, 743–753.

Shen, Y., and Shenk, T. E. (1995). Viruses and apoptosis. *Curr. Opinion Genet. Dev.* 5, 105–111.

Steller, H. (1995). Mechanisms and genes of cellular suicide. *Science* 267, 1445–1449.

Subramanian, T., Tarodi, B., Govindarajan, R., Boyd, J. M., Yoshida, K., and Chinnadurai, G. (1993). Mutational analysis of the transforming and apoptosis suppression activities of the adenovirus E1B 175R protein. *Gene* 124, 173–181.

Subramanian, T., Boyd, J. M., and Chinnadurai, G. (1995a). Functional substitution identifies a cell survival promoting domain common to adenovirus E1B 19 kDa and Bcl-2 proteins. *Oncogene* 11, 2403–2409.

Subramanian, T., Tarodi, B., and Chinnadurai, G. (1995b). p53-independent apoptotic and necrotic cell deaths induced by adenovirus infection: Suppression by E1B 19K and Bcl-2 proteins. *Cell Growth Differ.* 6, 131–137.

Tarodi, B., Subramanian, T., and Chinnadurai, G. (1993). Functional similarity between adenovirus E1B 19K gene and Bcl2 oncogene: Mutant complementation and suppression of cell death induced by DNA damaging agents. *Int. J. Oncol.* 3, 467–472.

Tarodi, B., Subramanian, T., and Chinnadurai, G. (1994). Epstein–Barr virus BHRF1 protein protects against cell death induced by DNA-damaging agents and heterologous viral infection. *Virology* 201, 404–407.

Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 81, 801–809.

Tollefson, A. E., Krajcsi, P., Pursley, M. H., Gooding, L. R., and Wold, W. S. M. (1990). A 14,500 MW protein is coded by region E3 of group C human adenoviruses. *Virology* 175, 19–29.

Tollefson, A. E., Scaria, A., Saha, S. K., and Wold, W. S. M. (1992). The 11,600-MW protein encoded by region E3 of adenovirus is expressed early but is greatly amplified at late stages of infection. *J. Virol.* 66, 3633–3642.

Tollefson, A. E., Scaria, A., Hermiston, T. W., Ryerse, J. S., Wold, L. J., and Wold, W. S. M. (1996). The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J. Virol.* 70, 2296–2306.

Vaux, D. L., Haecker, G., and Strasser, A. (1994). An evolutionary perspective on apoptosis. *Cell* 76, 777–779.

Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994). Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* 78, 739–750.

White, E. (1994). Function of the adenovirus E1B oncogene in infected and transformed cells. *Semin. Virol.* 5, 341–348.

White, E., Blose, S. H., and Stillman, B. W. (1984). Nuclear envelope localization of an adenovirus tumor antigen maintains the integrity of cellular DNA. *Mol. Cell. Biol.* 4, 2865–2875.

White, E., Sabbatini, P., Debbas, M., Wold, W. S. M., Kusher, D. I., and Gooding, L. R. (1992). The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor α. *Mol. Cell. Biol.* 12, 2570–2580.

Wold, W. S. M., Cladaras, C., Magie, S. C., and Yacoub, N. (1984). Mapping a new gene that encodes an 11,600-molecular-weight protein in the E3 transcription unit of adenovirus 2. *J. Virol.* 52, 307–313.

Wold, W. S. M., Deutscher, S. L., Takemori, N., Bhat, B. M., and Magie, S. C. (1986). Evidence that AGUAUAUGA and CCAAGAUGA initiate translation in the same mRNA region E3 of adenovirus. *Virology* 148, 168–180.

Wold, W. S. M., Hermiston, T. W., and Tollefson, A. E. (1994). Adenovirus proteins that subvert host defenses. *Trends Microbiol.* 2, 437–443.

Wold, W. S. M., Tollefson, A. E., and Hermiston, T. W. (1995a). "Viroreceptors, Virokines, and Related Mechanisms of Immune Modulation by DNA Viruses" (G. McFadden, Ed.), pp. 145–183. R. G. Landes Co., Texas.

Wold, W. S. M., Tollefson, A. E., and Hermiston, T. W. (1995b). "The Molecular Repertoire of Adenoviruses" (W. Doerfler and P. Bohm, Eds.), pp. 237–274. Springer-Verlag, Heidelberg.

Xue, D., and Horvitz, R. (1995). Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature* 377, 248–251.

Yew, P. R., and Berk, A. J. (1992). Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature* 357, 82–85.

Zhang, Y., and Schneider, R. J. (1994). Adenovirus inhibition of cell translation facilitates release of virus particles and enhances degradation of the cytokeratin network. *J. Virol.* 68, 2544–2555.

Zhu, H., Shen, T., and Shenk, T. (1995). Human cytomegalovirus IE1 and IE2 proteins block apoptosis. *J. Virol.* 69, 7960–7970.