

# A Model System for the Design of Armed Replicating Adenoviruses Using *p53* as a Candidate Transgene<sup>1</sup>

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## Abstract

**Cancer gene therapy endeavors to overcome the low therapeutic index of currently available therapeutic modalities via the efficient and safe delivery of genetic material into tumor cells. However, despite promising preclinical results, replication-deficient viral vectors have demonstrated a limited efficacy in the clinical setting. To increase vector efficiency, replication-competent viruses have been proposed. Clinical trials have shown the safety of locally injected, conditionally replicative adenoviruses (Ads) but have underscored the need for improved potency. To further increase the therapeutic effect of replicating viral vectors, armed therapeutic viruses (ATVs) have recently been used for high-efficiency transgene expression. However, interference with cellular signaling and viral production by constitutive transgene expression may be counterproductive for ATV replication, thereby hindering the therapeutic outcome. Consequently, studies are equivocal with regard to the potential benefits of ATVs. To address this issue, we hypothesized that induction of replication of an Ad expressing *p53* may be a useful strategy in the context of ATV because *p53* does not interfere with Ad replication and may even increase its cytolytic effect. We show that in our *in vitro* ATV model system, E1 transcomplementation of a replication-deficient Ad encoding *p53* resulted in dramatic augmentation of cell killing and circumvented resistance to apoptosis. Correlation was found between the degrees of cell killing and apoptosis induction, rather than with viral burst. Furthermore, both Ad5 E1B 55kDa and E4 orf6 genes were required to enhance the cell killing. In conclusion, our *p53*-ATV model system demonstrates the potential utility of therapeutic transgene expression**

**by a replicating Ad after a rational selection of a candidate transgene.**

## Introduction

Gene therapy has been suggested as a novel strategy to improve the therapeutic index of cancer therapy. Whereas replication-deficient viral vectors have demonstrated great promise as anticancer agents in preclinical studies, this has not been translated into patient benefit in the clinical setting (1). As a natural extension, replication-competent viruses have been suggested as a means to address the multidimensional biological aspects of tumors (2). To date, replication-competent viruses used in cancer clinical trials have included Ads<sup>3</sup> and, to a lesser extent, HSVs. Whereas replication-competent vectors have been shown to be safe and potentially beneficial for therapy of localized tumors, their potency clearly needs to be improved (3). Therefore, “armed” replicative viruses, incorporating therapeutic transgenes, have been introduced for cancer gene therapy (1, 4, 5). These ATVs embody two potential advantages. First, they exhibit a capacity for up to 3 orders of magnitude higher levels of transgene expression relative to their replication-defective vector counterparts, in selected instances (1) (6). Second, incorporated transgenes may provide a fail-safe mechanism to abolish viral replication by the induction of toxic cell death (6, 7). There are, however, potential limitations to the use of ATVs. In this regard, constitutive gene expression by Ad vectors may interfere with cellular signaling and result in premature cellular toxicity. Consequently, early apoptosis may impair viral replication (8), confounding the antitumor effects linked to oncolysis.

Accordingly, the utility of ATVs for cancer gene therapy is uncertain. In three studies, the inclusion of suicide/prodrug gene therapy with HSVtk/GCV in a replicating Ad did not augment antitumor efficacy *in vitro* or *in vivo* (9–11). In contrast, other studies have shown that combined oncolysis, caused by a replicating virus and suicide/prodrug gene therapy with HSVtk/GCV, is complementary in improving outcome *in vivo* (4, 5). Furthermore, a replicating Ad with double suicide gene therapy containing the cytosine deaminase/5-FC (*cd/5FC*) and HSVtk fusion gene markedly enhanced the CPE relative to the isolated viral effect (7, 12). Of note, the role of E1B 55kDa deletion in the context of ATV is also inconclusive (9, 12).

To address these inconsistencies, we have developed a strategy that induces replication of transgene-expressing, replication-deficient Ad vectors. As a proof of principle, we

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<sup>3</sup> The abbreviations used are: Ad, adenovirus; ATV, armed therapeutic virus; HSV, herpes simplex virus; tk, thymidine kinase; GCV, ganciclovir; CMV, cytomegalovirus; MOI, multiplicity of infection; CPE, cytopathic effect.

have selected a replication-deficient Ad vector encoding *p53*. Because the inhibition of viral replication by HSVtk/GCV or *cd/5FC* may counterbalance the therapeutic effect of ATV (13), *p53* may be a useful therapeutic transgene in the context of ATV because it does not interfere with Ad replication (14) and may even increase its cytolytic effect (15).

Furthermore, selection of *p53* to indirectly induce apoptosis may circumvent transgene effects that induce apoptosis downstream of *p53* and thus do not allow effective production and lateralization of the Ad vector (16, 17).

To this end, we induced replication of Ad vector encoding *p53* by a variety of Ad mutants and evaluated cell killing and viral kinetics. Our studies show that ATV has a potential for higher cancer cell killing rates *in vitro* in the context of a transgene that is not counterproductive for viral replication.

We further observed that the burst of replicating Ad did not fully correlate with cell killing in the context of ATV. Finally, we found that both the Ad *E1B 55kDa* and *E4 orf6* genes were essential for the enhanced therapeutic effect of ATV encoding *p53*. These findings are highly consequential for an understanding of the efficacy of replicating Ad agents and for a rational design of ATV.

## Materials and Methods

**Recombinant Ads.** A replication-deficient Ad expression vector for the delivery of wild-type human *p53* cDNA has been reported previously (18). This vector expresses human wild-type *p53* under the transcriptional control of the CA promoter comprising a CMV enhancer and the chicken  $\beta$ -actin promoter (AdCAp53). Ad338 is an Ad mutant lacking 524 bp within the *E1B 55kDa* gene (19). Ad355 is deleted for the *E4 orf6* gene (20). As a control for AdCAp53, we used Ad5luc1, a replication-deficient Ad (*E1/E3* deleted) expressing the *luciferase* gene from the *E1* region. As a wild-type equivalent we used Ad5luc3, a replication-competent Ad (*E1* intact, *E3* deleted) expressing the *luciferase* gene from the *E3* region. Both these viruses were constructed and propagated in our laboratory.

**Cells, Transfections, and Infections.** A549 and H460, human lung cancer cell lines with intact *p53*, were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were grown at 37°C in RPMI 1640 with 2 mM L-glutamine, supplemented with 10% fetal bovine serum. Infections were performed 24 h after seeding  $2 \times 10^5$  cells/well in 12-well plates. For infections, growth medium was replaced by serum-free medium with the index virus at the indicated MOI. An hour later, the infection medium was removed, cells were rinsed with PBS, and 5% fetal bovine serum growth medium was restored. The medium was not replaced thereafter during the experiment and was sampled daily for determination of Ad *E1* or *E4* gene copy numbers.

For transient transfections, cells were seeded on 12-well plates and transfected with the indicated plasmids (1  $\mu$ g/well) at a confluence in the range of 70%, using the Superfect (Qiagen, Santa Clarita, CA) method, according to the instructions of the manufacturer. Expression vectors used for transfection were constructed as follows; pCMVE1 was derived from the shuttle plasmid (pShuttle) of the "Adeasy" system (21) by cloning the consecutive Ad E1 region extending from

position 489 to 5789 of the Ad genome into the multicloning site, thereby deleting the right arm of pShuttle. Next, the CMV promoter/enhancer was cloned into the *XhoI* and *EcoRV* restriction sites of the recombinant plasmid. pCMVluc was derived from cloning of the CMV promoter/enhancer into the mammalian expression vector pGL3 basic vector (Promega, Madison, WI) upstream of the *luciferase* gene.

**Apoptosis Assay.** To determine whether apoptosis or necrosis was the underlying mechanism of cellular death, we infected A549 cells with AdCAp53 at a MOI of 20, and 48 h later, we coinfecting the cells with either the replication-competent Ad5luc3 or the *E1B 55kDa*-deleted Ad338, at a MOI of 5. Control wells were initially infected with the replication-deficient control virus Ad5luc1 and coinfecting 48 h later with Ad5luc3. Forty-eight h after the second infection, cells were harvested, washed in cold PBS, and adjusted for a cell density of  $\sim 1 \times 10^6$  cells/ml in PBS.

To detect apoptosis or necrosis, cells were stained with the dyes of the Vybrant Apoptosis Assay (Molecular Probes, Eugene, OR). This assay allows the detection of three groups of cells under a fluorescence microscope equipped with the appropriate filters for fluorescein or rhodamine. Whereas live cells show only a low level of fluorescence, apoptotic cells show green fluorescence, and necrotic cells show both red and green fluorescence (and therefore show yellow fluorescence when merged).

**TaqMan PCR Assay.** The *E1a* copy number was determined for each medium sample obtained as of the first day after infection. Genomic DNA was isolated and cleaned using a Qiagen Tissue Kit (Qiagen), following the instructions of the manufacturer. The concentration of isolated DNA was determined by spectrophotometry. TaqMan primers and probe design, the forward primer, the reverse primer, and the 6-carboxyfluorescein-labeled probe to amplify the *E1a* and *E4* genes were designed by the Primer Express 1.0 software (Perkin-Elmer, Foster City, CA) following the recommendations of the manufacturer. The sequences of the forward and the reverse *E1a* primers were AACCAGTTGCCGTGAGAGTTG (anneals between residues 966 and 986) and CTCGTTAAGCAAGTCCTCGATACAT (anneals between residues 1033 and 1009), respectively, whereas the TaqMan probe was CACAGCCTGGCGACGCCA (anneals between residues 988 and 1006). The sequences of the forward and the reverse *E4* primers were GGAGTGCGCCGAGACAAC (anneals between residues 816 and 833 of the *E4 orf6* open reading frame) and ACTACGTCCGGCGTTCCAT (anneals between residues 883 and 865), respectively.

The sequence of the TaqMan probe was TGGCATGACAC-TACGACCAACACGATCT (anneals between residues 836 and 863). With optimized concentration of primers and probe, the components of real-time PCR mixture were designed to result in a master mix with a final volume of 10  $\mu$ l/reaction containing 1 $\times$  Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM forward primer, 100 nM reverse primer, 1 nM probe, and 0.025% BSA. For the assay, 1  $\mu$ l of extracted DNA sample was added to 10  $\mu$ l of PCR mixture in each reaction capillary. A no-template control received 10  $\mu$ l of reaction mixture with 1  $\mu$ l of water. All capillaries were then sealed and centrifuged using LC Car-

ousel Centrifuge (Roche Molecular Biochemicals, Indianapolis, IN) to facilitate mixing. All PCR reactions were carried out using a LightCycler System (Roche Molecular Biochemicals). The thermal cycling conditions were 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

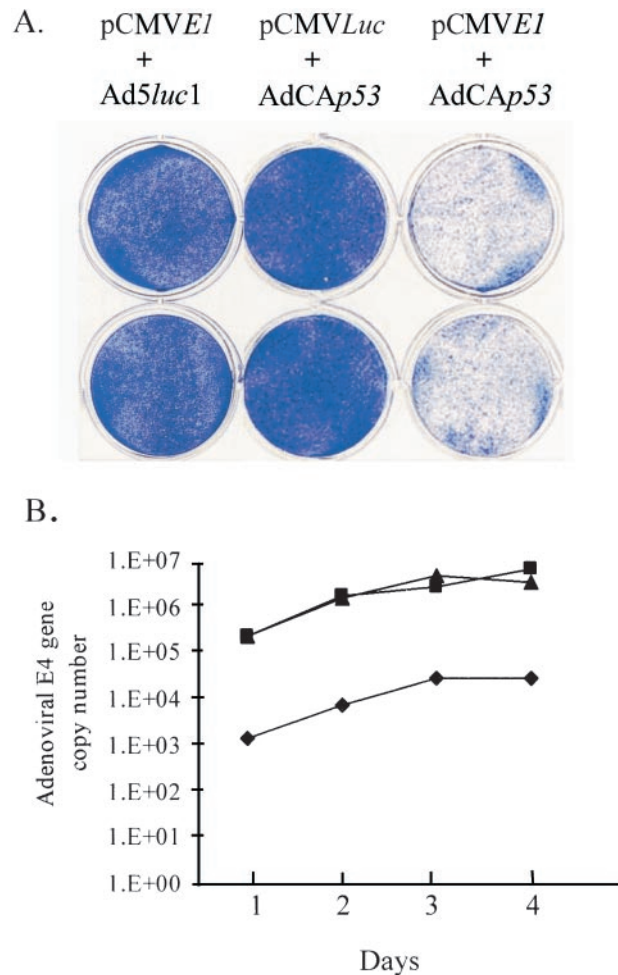
**Statistical Analysis.** Data were initially tested for normality by the Shapiro-Wilk test. All abnormal tests were further tested for significance by the Wilcoxon scores test. Results are expressed as a mean of at least three samples. Results were considered statistically significant for  $P < 0.05$ .

## Results

**Induction of Replication of AdCAp53 by E1 Transfection to Determine Cell Killing of ATV.** Expression of *p53* has been reported to be detrimental for Ad replication and cellular transformation (22), suggesting that Ad *E1B 55kDa* mutants may not grow in normal tissues (23). In contrast, others have shown that *p53* may in fact be essential for productive Ad infection (15) and that *p53* overexpression does not interfere with Ad replication (14). Based on these considerations, we first determined the effect on viral replication and cell killing of heterologous *p53* expression. In a previous study, we have confirmed that AdCAp53 expresses *p53*, induces apoptosis, and inhibits the growth of selected lung cancer cell lines *in vitro* and *in vivo* (18).

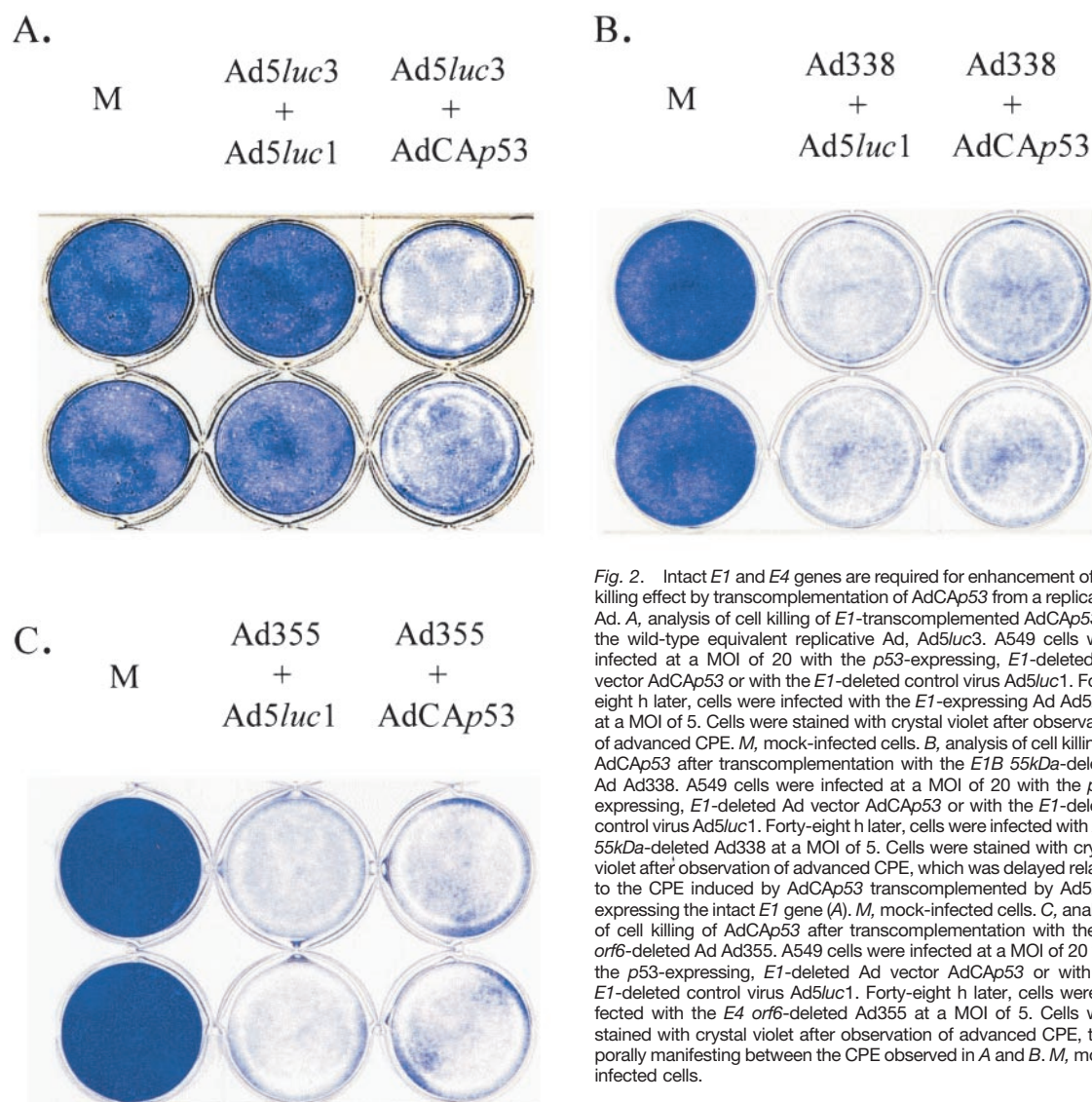
To evaluate the effect of *E1* transfection on AdCAp53 replication and cell killing, we first confirmed that at a MOI of 20 plaque-forming units/cell, AdCAp53 does not replicate or cause significant cell killing. Specifically, A549 cells infected with either the replication-defective Ad5*luc1* or with AdCAp53 remained viable for more than 12 days after infection. After this period, cells began to degrade but did not manifest any overt signs of viral CPE. Additionally, Ad *E1a* gene copy levels, as determined by quantitative PCR, were at the background level (data not shown). These results indicate that AdCAp53 does not replicate and does not cause a significant CPE in A549 cells. Next, we induced replication of AdCAp53 by transcomplementation with an intact *E1* gene. For this study, A549 cells were plated in each well of 12-well plates. After reaching 70% confluence, cells were transfected in triplicates with either pCMVE1 or pCMV*luc*.

Twenty-four h later, cells were infected with either Ad5*luc1* or AdCAp53 at a MOI of 20. An advanced CPE was observed 3 days after infection only for the *E1*-transfected, AdCAp53-infected cohort (Fig. 1A). Transfection with pCMV*luc* followed by infection with AdCAp53 did not induce any CPE, whereas transfection with pCMVE1 followed by infection with Ad5*luc1* resulted in delayed and low CPE relative to *E1*-transcomplemented AdCAp53. These data suggest that in our *p53*-ATV model, cell killing by *p53* overexpression after induction of replication of AdCAp53 is more efficient relative to Ad-mediated oncolysis or relative to cell killing of nonreplicating AdCAp53. Because previous studies have shown that transgene expression may impair viral oncolysis and that *E1* has an independent apoptotic effect (24), we next evaluated the kinetics of viral replication and burst relative to CPE. To this end, we assayed Ad *E4* gene copies in the medium and found that kinetics of Ad DNA accumulation in the medium of *E1*-transfected, AdCAp53-infected cells indi-



**Fig. 1.** Transfection of the adenoviral *E1* gene enhances the cell killing effect of AdCAp53 in an oncolytic-independent fashion. **A**, the human lung adenocarcinoma cell line A549 was transfected in triplicates with the adenoviral *E1* expression vector pCMVE1 or with a control plasmid expressing luciferase (pCMV*luc*). Twenty-four h later, cells were infected with *E1*-deleted, replication-incompetent adenoviral vectors at a MOI of 20 plaque-forming units/cell. Vectors studied included a control vector expressing the luciferase reporter gene (Ad5*luc1*) and a *p53*-expressing vector (AdCAp53). Cells were stained with crystal violet after observation of advanced CPE. **B**, analysis of replication of Ad vectors induced by *E1* transcomplementation. A549 cells were transfected with pCMVE1 or pCMV*luc* and infected 24 h later with Ad5*luc1* or AdCAp53, exactly as described in **A**. Media samples were collected in triplicates from the different cohorts and subjected to quantitative PCR analysis of Ad *E4* copy number as an index of Ad replication and burst. Experimental groups included pCMVE1 + Ad5*luc1* (▲), pCMVE1 + AdCAp53 (■), and pCMV*luc* + AdCAp53 (◆).

cated that viral replication was similar to the viral replication of *E1*-transfected, Ad5*luc1*-infected cells (Fig. 1B). Therefore, *p53* overexpression by AdCAp53 does not significantly inhibit or support Ad replication and burst. These patterns were corroborated by the corresponding intracellular Ad DNA levels (data not shown). To account for enhanced cell killing, a synergistic effect of *E1* and *p53* proteins, independent of Ad replication, is unlikely because it would be counterproductive for viral replication. Thus, it appears that the enhanced killing effect of *E1*-transcomplemented AdCAp53



**Fig. 2.** Intact *E1* and *E4* genes are required for enhancement of cell killing effect by transcomplementation of AdCAp53 from a replicative Ad. **A**, analysis of cell killing of *E1*-transcomplemented AdCAp53 by the wild-type equivalent replicative Ad, Ad5luc3. A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCAp53 or with the *E1*-deleted control virus Ad5luc1. Forty-eight h later, cells were infected with the *E1*-expressing Ad Ad5luc3 at a MOI of 5. Cells were stained with crystal violet after observation of advanced CPE. **M**, mock-infected cells. **B**, analysis of cell killing of AdCAp53 after transcomplementation with the *E1B 55kDa*-deleted Ad Ad338. A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCAp53 or with the *E1*-deleted control virus Ad5luc1. Forty-eight h later, cells were infected with *E1B 55kDa*-deleted Ad338 at a MOI of 5. Cells were stained with crystal violet after observation of advanced CPE, which was delayed relative to the CPE induced by AdCAp53 transcomplemented by Ad5luc3 expressing the intact *E1* gene (**A**). **M**, mock-infected cells. **C**, analysis of cell killing of AdCAp53 after transcomplementation with the *E4 orf6*-deleted Ad Ad355. A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCAp53 or with the *E1*-deleted control virus Ad5luc1. Forty-eight h later, cells were infected with the *E4 orf6*-deleted Ad355 at a MOI of 5. Cells were stained with crystal violet after observation of advanced CPE, temporally manifesting between the CPE observed in **A** and **B**. **M**, mock-infected cells.

is caused by induction of AdCAp53 replication and efficient transgene expression, rather than by the isolated effects of Ad oncolysis or the toxic protein effect of the combination of *E1* and *p53*.

**Intact *E1* and *E4* Transcomplementation Are Required to Enhance the Cell Killing Effect of AdCAp53.** To further evaluate the enhancement of cell killing by AdCAp53 in the context of a replicating Ad, we transcomplemented AdCAp53 or Ad5luc1 with a wild-type equivalent Ad vector (Ad5luc3) or with Ad vectors deficient in either the *E1B 55kDa* or the *E4 orf6* genes. We reasoned that evaluation of distinct deletions of the Ad genome would allow the identification of Ad genes that are essential to achieve enhanced cell killing in the context of ATV. First, we validated that the fidelity of *E1* transcomplementation of AdCAp53 is maintained at the viral level and that it results in augmentation of cell killing. To this end, A549 cells were infected with AdCAp53 or Ad5luc1 at a MOI of 20. Forty-eight h later, cells were infected with

Ad5luc3 at a MOI of 5. Three days after infection with Ad5luc3, CPE was evident only for cells coinfecting with AdCAp53 and Ad5luc3, whereas CPE for cells coinfecting with Ad5luc1 and Ad5luc3 was delayed (Fig. 2A). To confirm the enhanced cell killing potency of *E1* transcomplementation of AdCAp53 in the context of a replicating virus, we performed the same experiment with the human lung adenocarcinoma H460 cell line. These cells have a significantly lower infectivity rate by Ad, and because they express wild-type *p53*, they are also relatively resistant to apoptosis induced by the replication-deficient AdCAp53 (18). We infected H460 exactly as described above for Fig. 2A, and we found that AdCAp53 transcomplemented by *E1* from Ad5luc3 induced cell killing efficiently. As in A549 cells, transcomplementation of Ad5luc1 by Ad5luc3 resulted in delayed CPE, whereas AdCAp53 alone had no effect at this MOI (data not shown).

A plausible interpretation of the capacity of *E1*-transcomplemented AdCAp53 to circumvent the resistance

of A549 and H460 to AdCAp53 would be that the therapeutic effect of the replication-deficient AdCAp53 is limited due to the low efficiency of infection of the initial viral inoculum and the inherent resistance of these *p53*-positive cells to heterologous *p53* expression. In contrast, the potential therapeutic advantages afforded by *E1* transcomplementation involve augmented transgene expression, viral replication, and spread of the viral progeny to infect neighboring tumor cells. Thus, *E1* transcomplementation with an Ad vector encoding *p53* results in highly efficient killing of resistant cancer cells.

To further evaluate the indispensability of candidate Ad genes for the enhancement of the therapeutic effect of AdCAp53, we evaluated the interaction of several Ad mutants with AdCAp53. To this end, we used Ad338 and Ad355, Ad vectors with deleted *E1B 55kDa* or *E4 orf6* genes, respectively. First, we infected A549 cells with AdCAp53 or Ad5luc1, as described above for Fig. 2A. After 48 h, cells were coinfecting with the *E1B 55kDa*-deleted Ad338 at a MOI of 5. Plates were stained with crystal violet after the observation of advanced CPE (Fig. 2B). In this instance, transcomplementation of AdCAp53 by Ad338 was distinct from transcomplementation by the *E1*-intact Ad5luc3 in two ways. First, CPE induced by Ad338 transcomplementation of AdCAp53 was delayed in comparison with the efficient CPE after Ad5luc3 transcomplementation of AdCAp53. Second, there was no difference in the cell killing patterns of AdCAp53 or Ad5luc1 after coinfection with Ad338. These findings were corroborated by coinfecting AdCAp53 with another *E1B 55kDa*-deleted Ad vector constructed in our laboratory.

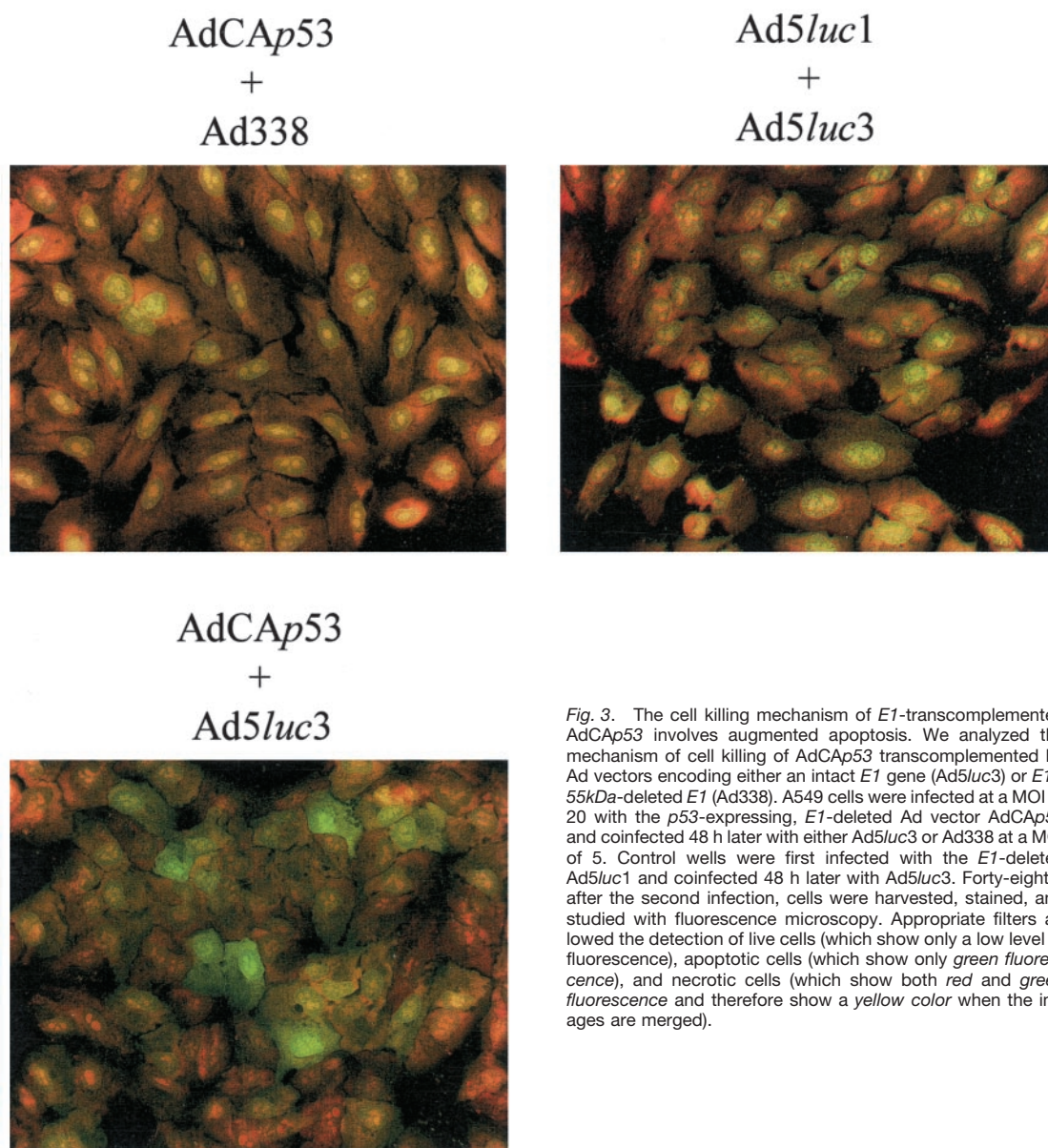
Thus, deletion of the *E1B 55 kDa* gene prevents the enhancement of cell killing observed for transcomplementation of AdCAp53 by an intact *E1* gene. Furthermore, these results support previous reports regarding the significance of *E1B 55kDa-p53* interaction for efficient Ad-mediated cell killing (15). Because the *E1B 55kDa* protein functions in concert with the *E4 orf6* gene product during the late Ad infection phase, we hypothesized that the latter is also essential to augment the effect of AdCAp53 in the context of ATV. To this end, we infected A549 cells with AdCAp53 or Ad5luc1, as described above for Fig. 2A. Forty-eight h later, we coinfecting cells with the *E4 orf6*-deleted Ad355 at a MOI of 5. As for Ad338, transcomplementation of AdCAp53 with Ad355 did not increase cell killing to a level greater than that achieved by Ad oncolysis alone (Fig. 2C). However, cell killing induced by Ad355 was observed before CPE was detected in Ad338-infected cells. Of note, whereas *E4 orf6* is intact in AdCAp53, coinfection with Ad355 and AdCAp53 was comparable with coinfection with Ad355 and Ad5luc1, implying that the primary *E4 orf6* mutation in the complementing Ad negated its capacity to significantly enhance the therapeutic effect of AdCAp53. These studies, taken together with the important role of the *E1B 55kDa* and *E4 orf6* genes in the replicative life cycle of Ad (25, 26), suggest that transformation of the replication-deficient AdCAp53 into an ATV may depend on intact *E1B 55kDa* and *E4 orf6* genes.

**The Cell Killing Mechanism of *E1*-transcomplemented AdCAp53 Involves Augmented Apoptosis.** To further delineate the mechanism of the augmentation in cell killing after

*E1* transcomplementation of AdCAp53, we evaluated parameters of apoptosis and necrosis. Ads have developed distinct strategies to counteract cellular responses to viral infection by blocking cellular apoptosis at critical junctions in the death-signaling cascade (27). On the other hand, wild-type *p53* may enhance the ability of Ad to induce cell death (28). In the absence of *E1B 55kDa*, cell death is delayed similar to that observed for *p53*-deficient cells infected with a wild-type Ad (15). Based on these considerations, we hypothesized that the combination of heterologous *p53* and intact *E1* expression, in the context of our ATV model, would be optimal in terms of apoptosis induction. To this end, we infected A549 cells with AdCAp53 at a MOI of 20, and 48 h later, we coinfecting the cells with either the replication-competent Ad5luc3 or Ad338 at a MOI of 5. Control wells were initially infected with the replication-deficient vector Ad5luc1 and coinfecting 48 h later with Ad5luc3. Forty-eight h after the second infection, cells were stained to detect apoptosis or necrosis. Whereas necrosis was detected in all cohorts, significant apoptosis was detected only in cells coinfecting with AdCAp53 and Ad5luc3 (Fig. 3). The *E1B 55kDa* deletion at Ad338 abolished the apoptosis-enhancing potency of *E1*-transcomplemented AdCAp53. In addition, under these conditions, we could not detect significant apoptosis for the replicating Ad5luc3 alone. Thus, these data indicate that the augmented cell killing induced by intact *E1* transcomplementation of AdCAp53 is mediated by efficient apoptosis induction.

**Enhancement of AdCAp53-induced Cell Killing Is Related to the Replication and Burst Kinetics of the Transcomplementing Ad Vectors.** One interpretation of the above-mentioned studies would be that in this model system of *p53*-ATV, AdCAp53 transcomplementation by *E1* results in dramatically higher cell killing by virtue of increased transgene expression.

Alternatively, it could be suggested that *p53* overexpression supports the oncolytic effect of Ad5luc3, thereby inducing Ad burst as the primary cause of cell death. To address this issue, we assayed the burst kinetics of Ad5luc3, Ad338 and Ad355. We selected the measurement of the Ad *E1a* gene as a specific indicator of the burst kinetics of the complementing viruses because it is absent from AdCAp53. To this end, we sampled daily the media from wells infected as described above for Fig. 2 and determined the Ad *E1a* gene copies for the relevant combination of viruses (Fig. 4A). Whereas media sampling is a direct method to evaluate viral burst of replication-competent viruses (2), it indicates replication only indirectly. Therefore we also evaluated intracellular Ad DNA parameters (Fig. 4B). When evaluating the kinetics of the transcomplementing vectors Ad5luc3, Ad338, and Ad355, without the effect of heterologous *p53* overexpression, the gene copy levels of the wild-type equivalent Ad5luc3 in the media were the highest as of the first day after infection, indicating efficient primary replication and burst of this vector. In contrast, Ad355 levels were lower than those of Ad5luc3, and Ad338 levels were the lowest. These patterns are in accord with the major role of *E1B 55kDa* in the Ad life cycle (25), and its role in supporting AdCAp53-mediated cell killing in the context of ATV. Likewise, the impaired

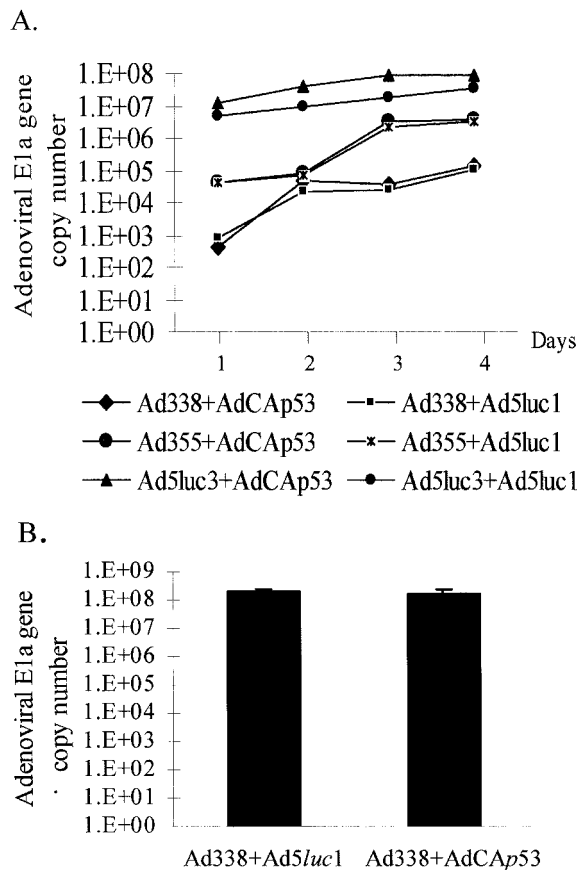


*Fig. 3.* The cell killing mechanism of *E1*-transcomplemented AdCAp53 involves augmented apoptosis. We analyzed the mechanism of cell killing of AdCAp53 transcomplemented by Ad vectors encoding either an intact *E1* gene (Ad5luc3) or *E1B* 55kDa-deleted *E1* (Ad338). A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCAp53 and coinfecting 48 h later with either Ad5luc3 or Ad338 at a MOI of 5. Control wells were first infected with the *E1*-deleted Ad5luc1 and coinfecting 48 h later with Ad5luc3. Forty-eight h after the second infection, cells were harvested, stained, and studied with fluorescence microscopy. Appropriate filters allowed the detection of live cells (which show only a low level of fluorescence), apoptotic cells (which show only green fluorescence), and necrotic cells (which show both red and green fluorescence and therefore show a yellow color when the images are merged).

replication and gene expression of *E4 orf6* mutants (26, 29) may have negated the effect of Ad355 on both AdCAp53 replication and cell killing. Consequently, it appears that the functional *E4 orf6* from AdCAp53 could not have complemented Ad355 to support viral replication and cell killing. Therefore, only the wild-type equivalent Ad5luc3 could achieve both high replication and burst rates and enhance the cell killing of AdCAp53.

A striking finding of the evaluation of Ad5luc3 burst after coinfection with AdCAp53 was that despite the clear augmentation of cell killing upon coinfection with AdCAp53, the kinetics of the Ad5luc3 burst did not differ significantly from the burst kinetics assayed for coinfection of Ad5luc3 with Ad5luc1. A trend toward earlier burst of Ad5luc3 was observed after coinfection of Ad5luc3 with AdCAp53, possibly

indicating the effect of *p53* overexpression and earlier cell death on viral burst. However, the slightly earlier burst is clearly distinct from the unequivocally higher cell killing effect induced by coinfection with these two viruses. Because the burst of the wild-type equivalent, Ad5luc3, was not significantly affected by coinfection with AdCAp53, an earlier Ad burst, possibly induced by transgene expression, cannot completely account for the enhanced cell killing potency after coinfection with a replicative Ad and the *p53*-expressing Ad. Rather, *E1* transcomplementation of AdCAp53 may have resulted in an augmented effect of the toxic transgene, as has been recently demonstrated for HSVtk/GCV expressed by a replicative Ad (6). Thus, because differences in burst kinetics cannot explain the enhanced cell killing, timely and efficient transgene expression by the *E1*-transcomple-



**Fig. 4.** Wild-type equivalent Ad and E1B and E4 mutant Ad vectors differ in their replication and burst kinetics. **A**, analysis of replication and burst of Ad vectors as a function of *E1* and *E4* status and as a function of *p53* overexpression. A549 cells were infected with AdCap53 or Ad5luc1 at a MOI of 20. Forty-eight h later, cells were infected with Ad5luc3, Ad338, or Ad355. Media samples were collected daily in triplicates from the different cohorts and analyzed with quantitative PCR for Ad *E1a* gene copy numbers. **B**, direct analysis of Ad338 DNA replication as a function of *p53* status. A549 cells were infected as described in Fig. 3A with AdCap53 or Ad5luc1 and coinfecting 48 h later with Ad338. Seventy-two h after the second infection, the cellular fraction was harvested and analyzed for Ad DNA.

mented Ad vector AdCap53 may account for the augmented cell killing in this ATV model system.

## Discussion

A major limitation of cancer gene therapy is the inadequacy of replication-deficient Ad vectors to efficiently infect tumors (30). To address this problem, replicating vectors have been suggested as a means to amplify an initial infection event (31). However, despite the safety of these viruses deriving from the tumor selectivity of replication dynamics, their efficacy is limited (3). Although incorporation of a therapeutic transgene into a replicating Ad to form an ATV has the potential to enhance the potency of replication-competent vectors, recent studies could not resolve this issue. Specifically, expression of incorporated transgenes may directly compromise the goal of viral replication and thus indirectly interfere with antitumor oncolysis (32).

In this study, we hypothesized that alleviation of the replication restriction on AdCap53 would form a platform for studying the therapeutic effects and viral kinetics of ATV. First, we validated that intact *E1* induces replication of AdCap53 and that Ad replication is not affected by *p53* overexpression. These findings validated the recent documentation of Ad replication despite *p53* overexpression (14). Next, we showed that induction of replication of AdCap53, mimicking an ATV, results in a higher therapeutic effect than the oncolytic effect of replicative Ad alone. However, this effect depended on Ad transcomplementation by intact *E1B 55kDa* and *E4 orf6*. In this regard, although one might expect faster killing of cells infected with an *E1B 55kDa*-deleted Ad because of unbalanced up-regulation of *p53* by *E1a*, this is not the case (15, 33, 34). Rather, both *E1B 55kDa* and *E4 orf6* genes are important for productive Ad infection and viral oncolysis (24, 35). Furthermore, the cell killing potency of both *E1B 55kDa* and *E4 orf6* may in fact depend on cellular expression of *p53* (15, 28, 36).

Our findings of the enhanced cancer cell killing potency after *p53* overexpression, in the context of intact *E1B 55kDa* and *E4 orf6* genes, question the utility of ATV derived from *E1B 55kDa*-deleted or *E4 orf6*-deleted Ad genomes, at least for vectors encoding *p53*, and require additional studies. Because a number of previous studies have shown that ATVs with HSVtk/GCV are not more oncolytic than replicating Ad alone, stringent selection of the therapeutic transgene is clearly essential for the design of ATV. In this regard, our ATV model allows screening of therapeutic transgenes *in vitro*. Careful scrutiny of transgenes according to this model may select for ATV expressing transgenes that are not counterproductive for Ad replication.

A pertinent finding of this study involves the mechanism of the induction of cell death by adenoviral vectors. In this ATV model, it appears that earlier viral burst may have been secondary to the primary transgene therapeutic effect, as indicated by the selective and extensive apoptosis in cells coinfecting with Ad5luc3 and AdCap53 and by the borderline induction of an earlier burst of Ad5luc3 by AdCap53. In this regard, the role of intact Ad genes in the context of an ATV has been recently demonstrated by augmented transgene expression and oncolytic effect *in vitro* and *in vivo* (6). However, in the context of cancer cell killing by an ATV, earlier viral burst may act in concert with transgene overexpression to expedite infection of neighboring cells. Therefore, means to support burst and lateralization of replicative Ad are also warranted to achieve efficient infection of all tumor cells.

In conclusion, we have developed an *in vitro* model for the evaluation of therapeutic transgenes in the context of ATV. A replication-deficient Ad vector encoding *p53* was induced to function as an ATV and shown to be superior to replicative Ad alone by virtue of its enhanced apoptotic cell killing effect. In view of the need to improve vector efficacy for cancer gene therapy, we suggest this model system to allow for a rational design of ATV.

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