

## Development and Characterization of Novel Empty Adenovirus Capsids and Their Impact on Cellular Gene Expression

Jackie L. Stilwell,<sup>1</sup> Douglas M. McCarty,<sup>1</sup> Atsuko Negishi,<sup>2</sup> Richard Superfine,<sup>2</sup> and R. Jude Samulski<sup>1,3\*</sup>

*UNC Gene Therapy Center,<sup>1</sup> Department of Physics and Astronomy,<sup>2</sup> and Department of Pharmacology,<sup>3</sup> University of North Carolina, Chapel Hill, North Carolina 27599*

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**Adenovirus (Ad) has been extensively studied as a eukaryotic viral vector. As these vectors have evolved from first-generation vectors to vectors that contain either very few or no viral genes (“gutless” Ad), significant reductions in the host innate immune response upon infection have been observed. Regardless of these vector improvements an unknown amount of toxicity has been associated with the virion structural proteins. Here we demonstrate the ability to generate high particle numbers ( $10^{11}$  to  $10^{12}$ ) of Ad empty virions based on a modification of Cre/lox gutless Ad vectors. Using a battery of analyses (electron microscopy, atomic force microscopy, confocal images, and competition assays) we characterized this reagent and determined that it (i) makes intact virion particles, (ii) competes for receptor binding with wild-type Ad, and (iii) enters the cell proficiently, demonstrating an ability to carry out essential steps of viral entry. To further study the biological impact of these Ad empty virions on infected cells, we carried out DNA microarray analysis. Compared to that for recombinant Ad, the number of mRNAs modulated upon infection was significantly reduced but the expression signatures were similar. This reagent provides a valuable tool for studies of Ad in that researchers can examine the effect of infection in the presence of the virion capsid alone.**

Adenovirus (Ad) vectors have evolved from first-generation vectors where the transgene replaces only the E1 region to vectors that contain either very few or no viral genes. In the first-generation vectors, the deletion of E1 prohibits transactivation of the viral genes that are required for viral replication. Unfortunately, even in the absence of the E1 gene products, there is residual expression of the remaining viral genes. This expression results in an innate cytokine response and an antigen-dependent immune response that includes cell-mediated destruction of transduced cells (reviewed in reference 29), resulting in limited transgene expression. These initial observations have led to the development of vectors containing deletions of the E1 and E2 and/or E4 genes (1, 6, 19, 22) that have shown reduced toxicity in animals. The adverse event in a gene therapy trial using these vectors that resulted in a fatal outcome (28) has demonstrated the importance of further modifications before the vectors can be considered safe.

The major emphasis in the quest to develop Ad vectors that demonstrate less toxicity has been to remove as much of the viral genome as possible. Recent studies with Ad vectors that do not contain any of the viral genome, “gutless” vectors, demonstrate prolonged transgene expression with reduced immune response against the virus itself (4, 5, 9, 21, 25). However, other studies have shown that psoralen- or UV-inactivated viruses, with no *de novo* viral protein expression, can still induce a cellular immune response (2, 15, 26). There are still several possible mechanisms for the activation of adverse cellular response after infection with these modified viruses. These are (i) the primary process of infection, including bind-

ing to the cellular receptor, (ii) escape from the endosome by capsid components, which reach the cytosol and enter the major histocompatibility complex class I antigen-processing pathway, and (iii) the presence of foreign DNA in the nucleus. While data are accumulating, the exact role of each of these components in the activation of immune response has yet to be established.

Ideally, viral or vector gene expression and viral infection should be uncoupled to understand the impact of Ad vectors on cells and organisms. To achieve this goal, the ability to generate a high-titer Ad vector completely devoid of viral DNA is required. Genetic approaches to generating empty Ad particles have been identified (8, 11, 12). The reagents involved (temperature-sensitive and genetic mutants) have been extremely important for understanding Ad assembly. In fact these mutants, although difficult to grow to extremely high titer, provided the impetus to look for alternative ways to produce high-titer empty particles. We have developed a method for producing high-titer empty capsid Ad (eAd) based on the Cre/lox gutless Ad vector production method (10, 23). In addition, we have characterized the resulting eAd viral capsids and determined that they (i) are nearly identical in physical characteristics, (ii) compete for receptor binding with the wild type (wt), and (iii) enter the cell proficiently. Based on these characterizations, these eAd viruses should be useful for understanding the issues surrounding Ad vector infection. One specific use for this type of reagent is to provide the ability to monitor the cellular response to Ad vectors by DNA arrays, comparing for the first time the role of viral infection in viral gene expression.

Several methods for the production of eAd, including using a mutant Ad with a temperature sensitivity mutation in the L1 52/55-kDa protein coding region (ts369), were evaluated (8,

\* Corresponding author. Mailing address: UNC Gene Therapy Center, University of North Carolina, CB# 7352, Chapel Hill, NC 27514. Phone: (919) 962-3285. Fax: (919) 966-0907. E-mail: rjs@med.unc.edu.

12). At the nonpermissive temperature empty capsids are produced, but in our hands not at very high titers, so a second method was chosen. Empty Ad type 5 particles were made by using a Cre/lox recombination system, specifically utilizing the  $\Psi$ 5 virus (it contains loxP sites flanking the DNA packaging signal) typically used to make gutless vectors by supplying Ad functions in *trans* (10). In CRE8 cells, expressing Cre recombinase, the DNA packaging site in  $\Psi$ 5 is deleted, thereby preferentially packaging recombinant Ad (rAd) vector genomes into newly formed virions. To make a high-titer empty capsid, the helper virus was used to infect CRE8 cells at a multiplicity of infection (MOI) of 5 in the absence of coinfecting rAd. After 48 h cells were harvested and lysates were subjected to a step gradient with 1.2 and 1.4 g of CsCl/cm<sup>3</sup> and ultracentrifuged at 20,000  $\times$  *g* for 2 h. In contrast to what was found for wt Ad, two bands were observed with the majority of material in the upper portion of the gradient. Both were harvested and characterized. Refractive indexes of the minor peak indicated a density of approximately 1.32 g/cm<sup>3</sup>, and the density at the major peak was 1.29 g/cm<sup>3</sup>, consistent with that of wt and empty Ad capsids (12). The peak at 1.29 g/cm<sup>3</sup> was placed on a continuous gradient at 1.295 g/cm<sup>3</sup> and centrifuged at 38,000  $\times$  *g* overnight. The band from this gradient was purified on a third gradient and then dialyzed into phosphate-buffered saline (PBS). To quantify yields of these empty capsid Ad preparations, protein levels were measured by bicinchoninic acid protein assay (Pierce) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and compared to an Ad preparation of known titer. The rAd used in these experiments (Ad-LacZ and Ad-green fluorescent protein [GFP]) was isolated as previously described and purified on a CsCl gradient (14). Based on these analyses we were able to obtain nearly identical amounts of empty and full virions ( $3.6 \times 10^{12}$  particles of empty virions/ml compared to  $4.2 \times 10^{12}$  particles of full virions/ml).

Further physical characterization of these empty particles demonstrated the presence of all expected structural proteins. Capsid proteins were separated by SDS-PAGE as described previously (13). Virus was boiled in sample buffer for 10 min and loaded onto a 12.5% polyacrylamide SDS-PAGE gel. The gel was silver stained with the Silver Stain Plus kit (Bio-Rad) according to manufacturer's directions. In these preparations it was apparent that polypeptides V and VII were missing from eAd capsids (data not shown). These polypeptides are part of the core protein of Ad, associated with the packaged viral DNA, so it was not surprising that they are missing from the empty particles. The rest of the capsid components appeared to be present. A dot blot and ethidium bromide-stained agarose gel were produced as previously described, and the results indicated that DNA was not present in the eAd preparations (data not shown).

Since both core proteins and viral DNA are missing from the eAd preparations, both electron microscope (EM) and atomic force microscope (AFM) images of the virus were made to insure that intact virions were produced. The EM image (Fig. 1A) indicates that the empty capsids are predominantly intact; however, there are much more viral debris and defective viral particles than for rAd (Fig. 1B), indicating that the empty capsid virus shell may be less stable than virus containing DNA and core proteins. Based on sized and shape, the debris ap-

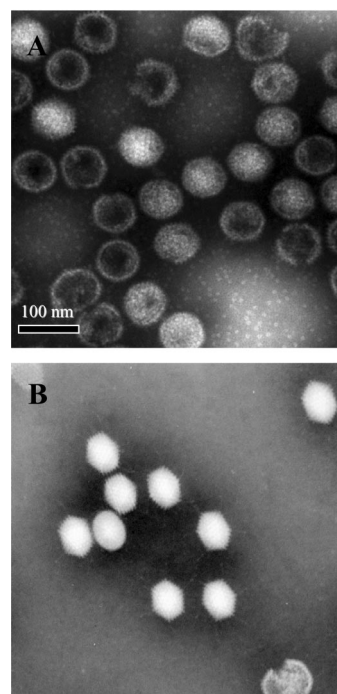


FIG. 1. EM of rAd and eAd. Suspensions of virus were examined by transmission EM using a negative-stain technique. A glow-discharged Pioloform-film-coated 400 mesh nickel grid was placed film side down onto a 10- $\mu$ m drop of viral suspension for 10 min to allow for particle adsorption. The grid was rinsed briefly to remove buffer salts by floating on 2 drops of distilled H<sub>2</sub>O and stained by transferring it to a drop of 2% aqueous uranyl acetate, pH 3.5 (or 2% potassium phosphotungstate, pH 7.0), for 1 min. The stained grids were air dried and examined at an accelerating voltage of 80 keV in a LEO EM-910 transmission EM (LEO Electron Microscopy, Inc., Thornwood, N.Y.). Magnification,  $\times 160,000$ . Two populations of virus are apparent here. (A) Empty particles; (B) rAd.

pears to be composed mostly of hexon subunits. The empty capsids also appear to be much more rounded and much less compact (12). Since the core proteins and the DNA are missing from the empty capsids, these proteins may serve to stabilize and provide structure to the virus by electrostatic interactions with other capsid components, leading to its characteristic icosahedral appearance, density, and stability. This is consistent with observations of viral protein exchanges concomitant with DNA entry (20). AFM was performed to obtain information about surface characteristics of the Ad virions and a more accurate size measurement. In the AFM images the eAd is slightly larger, based on the amount of debris, and appears more fragile than the rAd (Fig. 2), similar to what was observed in the EM images. The average height of the eAd was 68 nm versus 55 nm for the rAd (Fig. 2). The debris in both images indicates that the eAd may break apart more readily than the Ad, which may be an artifact of preparation for EM and AFM. Regardless, these images demonstrate the production of predominantly intact virus by this method.

To demonstrate that empty particles infect cells, we showed that eAd competitively inhibits the transduction of cells by rAd-GFP, indicating that some of its interactions with the cell are similar to those of rAd. The eAd was mixed in increasing concentrations with rAd-GFP to determine if eAd could block

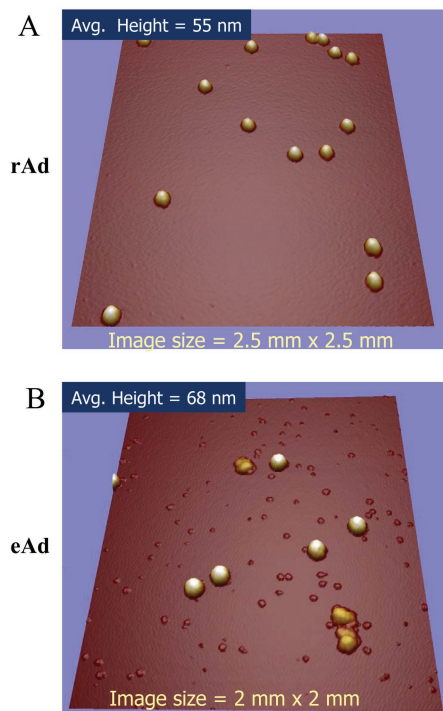


FIG. 2. AFM of rAd and eAd. The silicon substrate surface with intact native oxide was prepared by UV cleaning, rinsing with distilled H<sub>2</sub>O (dH<sub>2</sub>O), and drying under a filtered-nitrogen stream. Approximately 15  $\mu$ l of solution of 10<sup>11</sup> Ad particles/ml was deposited on the above surface, and viruses were allowed to adsorb for 15 min. Unbound viruses were removed by washing with dH<sub>2</sub>O and then dried under a nitrogen stream. Imaging was performed in air with a ThermoMicroscopes Explorer (Veeco/TM, Sunnyvale, Calif.) with a 100- $\mu$ m tripod air scanner calibrated with a NT-MDT calibration grid. Silicon tips (Nanosensors FESP; Digital Instruments, Santa Barbara, Calif.) with spring constant of  $\sim$ 2 N/m and resonance frequency of  $\sim$ 70 kHz were used for imaging in intermittent-contact mode. (A) rAd; (B) empty particles.

the entry of normal recombinant virus at high concentrations (Fig. 3). This was measured by evaluating transduction of embryonic lung fibroblasts by the rAd-GFP in the presence of competing virus. These data were compared to experiments where particle numbers of rAd-LacZ equal to particle numbers of eAd were also used for competition. After 24 h the number of GFP-expressing cells was evaluated and was shown to decrease as more eAd or rAd-LacZ was added to the virus mixture (Fig. 3). This was expressed as percent inhibition of transduction with rAd-GFP and is the ratio of transduced cells with competitor virus present to the control with no competing virus. The results indicate that the eAd can compete with rAd-GFP for receptor binding and reduce its ability to transduce cells. When rAd-LacZ was used as the competitor the results were similar, although the rAd-LacZ appears to be a slightly more effective competitor (by less than twofold; Fig. 3). This experiment provides a demonstration of competitive inhibition of binding to the cellular receptor but does not supply direct evidence that the empty particle can enter cells.

To obtain direct evidence that eAd enters cells, eAd was labeled with Cy3 and used to infect lung fibroblasts. A Cy3 fluorescent label was conjugated to the empty capsid Ad (Cy3-

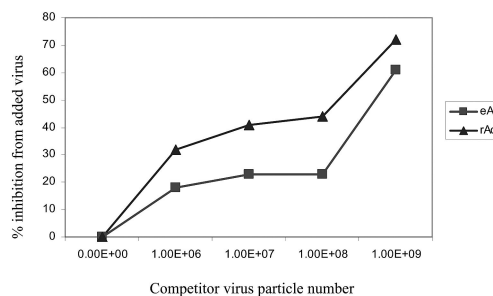


FIG. 3. Competition of eAd with rAd. Ad mixtures with approximately  $5 \times 10^5$  rAd-GFP particles were incubated with 10<sup>5</sup> IMR-90 cells at 4°C for 1 h to allow virus attachment in cold DMEM with 2% FCS. The cells were then washed three times in ice-cold PBS to remove unattached virus. DMEM with 10% FCS was then added, and cells were placed at 37°C in 5% CO<sub>2</sub>. At 24 h cells were examined for GFP expression by fluorescence microscopy with a fluorescein isothiocyanate filter. GFP-expressing cells were counted, and duplicate experiments yielded similar results. IMR-90 cells were demonstrated to express the Ad receptor, CAR, on their surface by immunohistochemistry (data not shown).

eAd), purified, and then used to infect cells. Approximately  $5 \times 10^{12}$  empty particles were dialyzed into PBS containing 10% glycerol with three changes of buffer. Two hundred microliters of a 1-mg/ml solution of an *N*-hydroxysuccinimide ester of FluoroLink Cy3 (Amersham Life Science) was made up in 0.1 M carbonate buffer, pH 9.3, and added to 1 ml of Ad. This

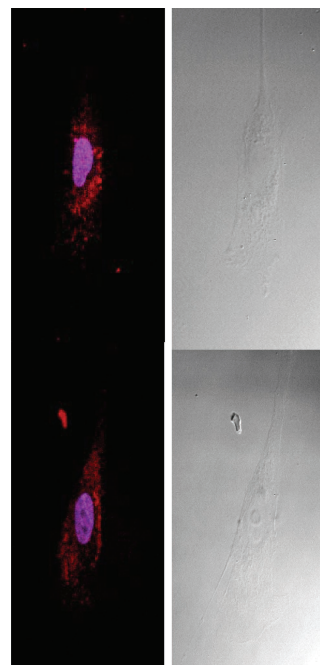


FIG. 4. Internalization of eAd. For internalization studies IMR-90 cells were grown on eight-well chamber slides (LabTech II) and then infected with Cy3-labeled Ad (red) at approximately 10,000 particles per cell. Cells were fixed at 6 h p.i. with 4% paraformaldehyde in PBS for 15 min. They were then mounted with Vectashield containing DAPI to label nuclei (blue) (Vector Laboratories, Burlingame, Calif.). Internalization was assessed by confocal microscopy with a Zeiss CLSM 210 confocal laser scanning microscope with a UV laser.



TABLE 1. Genes modulated by eAd and rAd at 24 h p.i.

Encoded protein	Genbank accession no.	Class or function <sup>b</sup>	Fold change <sup>a</sup> in expression due to:	
			eAd	rAd
GTP-binding protein	U10550	immediate early response	2.3	2.7
Transcription factor 7	X59871	immune response	2.2	~3.1
Interferon-induced protein 56	M24594	immune response	1.8	5.9
Zinc finger protein 161	D28118	cellular defense resp.	1.7	1.8
c-Jun	J04111	immediate early response	~2.0	~5.8
JunB	X51345	immediate early response	2	
Early growth response 1	X52541	immediate early response	2	4.7
MEKK4	D86968	mediator of stress	2.1	1.7
Growth arrest damage-inducible 153	S40706	induced by stress	~5.4	~42.5
Hypoxia-inducible factor 1	U22431	induced by stress	1.7	2
Heat shock protein 70 kDa 1A	M11717	heat shock protein	1.9	2.8
Zinc finger protein mel-18	D13969	transcription factor	2.3	
Zinc finger protein 36	X78992	transcription factor	8.8	
Zinc finger protein 91	L11692	transcription factor	2	
Splicing factor 1	Y08766	transcription co-repressor	2.2	
DEAD/H box polypeptide 21	U41387	RNA helicase	2.6	
DEAD/H box polypeptide 38	U90426	RNA helicase	2	
Splicing factor 3a	X85237	mRNA processing	2.1	

<sup>a</sup> A ~ in front of a value indicates that the transcripts in one of the samples did not register as present and therefore the change was calculated against background.

<sup>b</sup> Red, immune response; blue, stress response; green, transcriptional regulation; purple, RNA processing.

mixture was incubated at room temperature for 2 h in the dark (17). This solution was then dialyzed into a solution containing 10 mM Tris, pH 7.8, 140 mM NaCl, 1 mM MgCl<sub>2</sub>, and 10% glycerol. Virus was removed from the dialysis chamber, aliquoted, and stored at -20°C. Previous studies have determined that these modifications reduce Ad infectivity titer by less than 10-fold, so MOIs were adjusted accordingly (16, 24). Embryonic lung fibroblast cells were exposed to Cy3-eAd for 1 h at 4°C for viral binding and then washed extensively before incubation at 37°C for 6 h. As previously described (17) the cells were then fixed and viewed in cross sections by confocal microscopy (Fig. 4). The nucleus was counterstained with DAPI (4',6'-diamidino-2-phenylindole). Labeled virus was observed in the centers of the infected cells but not in the nuclei as previously described (7, 27), providing direct evidence that these empty particles are capable of entering cells. Taken together these results indicate that eAd can bind the cell surface in a receptor-mediated manner and enter IMR-90 cells, indicating that these particles are a suitable reagent for studying the impact of the Ad virion shell during infection.

To evaluate these particles for impact of cellular function in the absence of viral DNA, we analyzed IMR-90 cells after infection using Affymetrix GeneChip arrays. Cells were infected with rAd while they were below confluence at an MOI of infectious virus of 250 to 500. Since particle numbers were determined by protein measurement and since EM analysis

indicated the presence of viral debris, we used a higher relative MOI of empty particles in these experiments (MOI, 2,500). Additionally, the competition experiment indicated that if MOIs of rAd-LacZ of 250 to 500 were used, approximately 10-fold-higher levels of eAd were required to achieve the same percentage of competition with rAd-GFP. Comparable volumes of material from mock virus preparations were added to cells for controls. Cells were placed in Dulbecco's modified Eagle medium (DMEM) with 2% fetal calf serum (FCS) during infection for 1 h at 4°C for virus binding, and then cells were washed, the appropriate growth medium was added, and the cells were placed at 37°C in 5% CO<sub>2</sub>. Infected cells were collected at 24 h postinfection (p.i.) and lysed, and total RNA was isolated with an RNeasy kit according to manufacturer's directions (Qiagen). Approximately 10 µg of isolated total RNA was used to generate biotin-labeled cRNA as described previously (18). This was fragmented and hybridized to GeneChip arrays and then installed in a fluidics station (Affymetrix) for washing and staining as previously described. Arrays were read twice with a Hewlett-Packard GeneArray scanner. Data were collected and analyzed with GeneChip expression analysis software. The Affymetrix GeneChip used for this analysis was the HL6800 containing 5,600 human expressed sequence tags. Details of the data analysis have been previously described (18).

When differential levels of cellular gene expression in eAd-

and rAd-infected cells were compared, we observed that the removal of the viral genome eliminated the modulation in expression of many cellular genes (147 and 476, respectively, were modulated by twofold or more; for the entire data set go to <http://www.med.unc.edu/genether/>). When IMR-90 cells were infected with rAd, two clusters of mRNAs related to the immune response and stress response increased in concentration. A subset of these increased in concentration in eAd-infected cells (Table 1). Two other clusters, related to transcription regulation and RNA-processing genes, were observed in eAd-infected cells but not in rAd-infected cells. This is of interest because one would expect that the Ad capsid would only modulate a subset of genes observed in rAd infection. However, this difference may represent an activity of the wt virus or vector, through expression of its genome, to repress some of the events that the viral capsid sets in motion. This type of phenomenon has been observed with human cytomegalovirus (HCMV), where viral gene products block antiviral pathways that are triggered by HCMV binding and entry (3).

This reagent provides a critical tool for studies of Ad where researchers can examine the effect the virion shell on viral infection alone. In the past psoralen- and UV-treated viruses have been used to eliminate viral gene expression for these types of studies. It is not clear if all viral gene expression is eliminated by these treatments or if the treatments themselves altered the virus in ways that obscure the influence of viral capsid components or infection. The eAd reagent is not only useful for studies like the one described here but also has the potential to become a beneficial tool for animal studies designed to examine the toxicity of the capsid components of Ad. A database established from *in vitro* and *in vivo* studies using empty capsid Ad particles will be essential for future vector design where capsid mutations intended to mitigate unwanted effects are assayed.

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