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Construction of an Adenovirus Type 7a E1A^{-} Vector

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A strategy for constructing replication-defective adenovirus vectors from non-subgroup C viruses has been successfully demonstrated with adenovirus type 7 strain a (Ad7a) as the prototype. An E1A-deleted Ad7a reporter virus expressing the chloramphenicol acetyltransferase (CAT) gene from the cytomegalovirus promoter enhancer was constructed with DNA fragments isolated from Ad7a, an Ad7a recombination reporter plasmid, and the 293 cell line. The Ad7a-CAT virus particle transduces A549 cells as efficiently as Ad5-based vectors. Intravenous infections in a murine model indicate that the Ad7a-CAT virus infects a variety of tissues, with maximal levels of CAT gene expression found in the liver. The duration of Ad7a-CAT transgene expression in the liver was maximally maintained 2 weeks postinfection, with a decline to baseline activity by the week 4 postinfection. Ad7a-CAT represents the first example of a non-subgroup C E1A^{-} adenovirus gene transfer vector.

First-generation adenovirus vectors include virus constructs with deletions in the E1 domain and can include constructs with deletions in the E3 regions. To date, all E1-deficient adenovirus gene transfer vectors have been constructed from subgroup C viruses, with serotype 5 being the most common base virus. Serotype 5 adenovirus (Ad5) vectors demonstrate very efficient gene transfer in vitro and in vivo (for reviews, see references 1 and 30). The humoral immune response to an Ad5 infection results in the generation of a variety of anti-Ad5 antibodies, some of which are able to neutralize infection when the host is exposed to a second administration of an Ad5-based virus or vector. By definition, an antibody capable of neutralizing an Ad5 virus infection cannot neutralize an adenovirus of a different serotype (9). A distinct adenovirus serotype is sensitive only to type-specific neutralizing antibodies determined by serotype-specific viral capsid protein epitopes. Currently, 49 distinct adenovirus isolates fulfill the serotype criteria (9, 22). Based on additional features, including G+C content, tumorigenicity, and hemagglutination characteristics, each serotype is catalogued into a subgroup (13, 28). By comparing the biological characteristics of different serotypes, especially those in different subgroups, differences in tissue tropism, tumorigenicity, and virus attachment proteins have been identified (18). These differences are a direct result of the genetic variation among the adenovirus serotypes, and the molecular basis of these differences is not well understood. Generating replication-defective vectors from non-subgroup C viral backbones offers a simple strategy to create a library of distinct serotype vectors which could be used to allow repeat administration of adenovirus vectors without relying on suppression of the immune system. In addition to offering an alternative to the Ad5-based serotype to evade the humoral immune response in a repeat administration strategy, defective adenovirus vectors from different subgroups may provide unforeseen advantages over the current subgroup C vectors with respect to tissue tropism, altered presentation of the infected cell to the host immune system, or an enhanced transduction efficiency.

Although replication-competent vaccine strains have been constructed from subgroup B viruses (5, 23, 24), there are no published reports which describe construction of a non-subgroup C replication-defective adenovirus vector. In our view, the biggest impediment to constructing non-subgroup C replication-defective adenovirus vectors is the lack of serotype-specific complementing cell lines. If each serotype requires serotype-specific or subgroup-specific E1 functions, the construction of non-subgroup C vectors becomes a considerable task. However, if there is sufficient cross-serotype complementation between the Ad5 E1 gene products and an E1-deleted virus from a different subgroup, than cell lines expressing an Ad5 E1 gene, such as 293 cells, can be used for construction of a non-subgroup C virus. Precedents for cross-serotype E1 region complementation have been demonstrated within subgroup C viruses (17) and outside of the subgroup C viruses by growth of an Ad12 (subgroup A) E1A mutant in the 293 cell line expressing Ad5 E1A and E1B (2). Furthermore, comparison of E1 DNA sequence identities among a variety of serotypes indicates strong homology in both the E1A (26) and E1B (16, 25) gene products. These findings suggested that a strategy based on Ad5 E1 complementation would be successful for construction of non-subgroup C vectors.

Construction of Ad7a-CAT. We chose Ad7 strain a (Ad7a) as our prototype virus for constructing a replication-defective non-subgroup C vector. Ad7a is a subgroup B virus which is responsible for a variety of respiratory diseases (20, 27). It infects cells via a different receptor than that used by the group C viruses (6, 10), and wild-type (wt) Ad7a will grow in 293 cells. The strategy for construction of the Ad7a-chloramphenicol acetyltransferase gene (Ad7a-CAT) vector (as indicated in Fig. 1) requires isolation of an appropriate Ad7a DNA fragment (viral backbone) which is deleted of left-end elements required for virus replication and packaging. Generating such a fragment from Ad7a is complicated by the fact that the majority of the Ad7a DNA sequence has not been determined (7, 8, 15). By analyzing the available Gomen strain Ad7 left-end sequence, we identified a battery of restriction endonucleases which were able to digest Ad7 DNA at an appropriate site at

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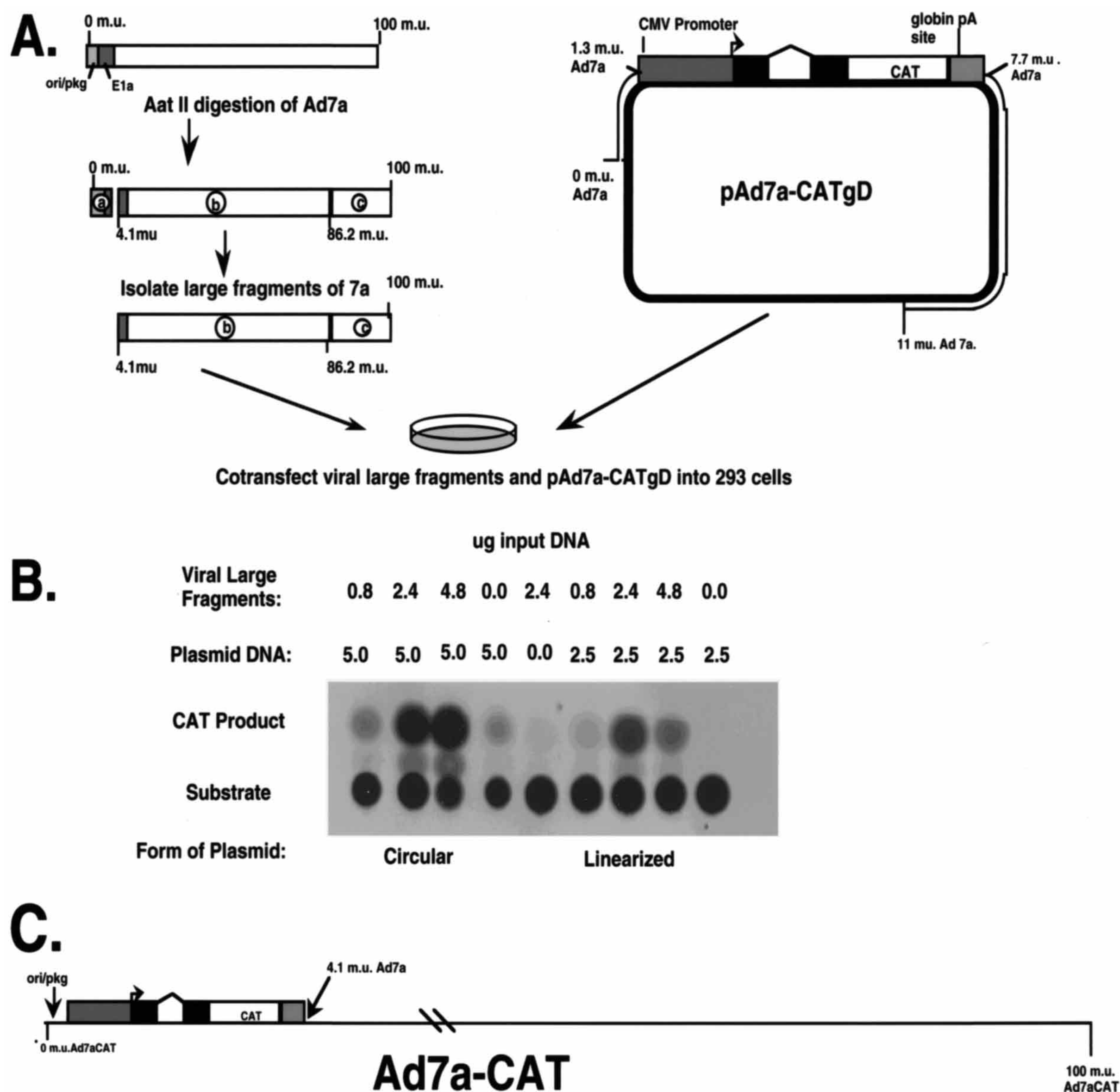

FIG. 1. Construction of Ad7a-CAT. (A) Schematic representation of the methods used to generate Ad7a-CAT. E1a-defective Ad7a DNA was generated, and a plasmid containing the CAT reporter gene was constructed. Digestion of wt Ad7a DNA with the restriction endonuclease *Aat*II produced three fragments: a small left-end fragment (a), a middle fragment 29.5 kb in length (b), and a right-end fragment 5 kb in length (c). pAd7a-CATgD was constructed by insertion of three elements—Ad7a ori/pkg, a reporter minigene cassette containing the CMV promoter; CAT gene, and Ad7a sequence from 7.7 map units (m.u.) (begins in the E1B region) to 11 m.u.—as described in Materials and Methods. The viral large fragments (b and c) were cotransfected with pAd7a-CMV-CATgD into 293 cells. (B) Detection of recombinant virus Ad7a-CAT following transfection into a 293 cell monolayer of the Ad7a large fragment with different forms and concentrations of pAd7a-CATgD. Lysate from each transfection was used to infect a monolayer of A549 cells. Twenty-four hours later, the A549 cells were harvested and lysates were prepared. The lysates were assayed for CAT reporter gene activity as previously described (10). (C) Diagram of Ad7a-CAT DNA.

the left end. Ad7a DNA was digested with this battery of enzymes, individually, to identify one which would allow the isolation of a right-end fragment of adenovirus DNA (large fragment) lacking the left-end origin and packaging sequences as well as the E1A transcription unit. The enzyme which most closely accommodated our needs was *Aat*II. *Aat*II has two digestion sites in Ad7a (indicated in Fig. 1A). The first is at position 1483, which is toward the left end of the Ad7 E1A gene, and the second site is approximately at position 31,000, which by our estimate is located just 5′ of the Ad7 fiber gene, somewhere in the E3 transcription unit. The large fragments, the 29.5- and 5-kb pieces, were copurified from the 1,483-bp fragment by centrifugation on a continuous 10 to 20% sucrose gradient. The gradient was fractionated, and the fractions were pooled into four groups. A group which contained the 5-kb fragment in a twofold excess over the large fragment was selected. The pooled large fragments were then ethanol precipitated and resuspended in 10 mM Tris–1 mM EDTA, pH 7.5. This mixture of viral fragments, through end-to-end joining and overlap recombination with an appropriately constructed plasmid, should be able to generate a recombinant E1-defective virus.

The recombination vector plasmid pAd7a-CATgD (Fig. 1A) was constructed by combining Ad7a DNA fragments gener-

ated by PCR with the CAT reporter gene expressed from the cytomegalovirus (CMV) promoter in a Bluescript plasmid. The left-end Ad7a sequence corresponding to nucleotide positions 1 to 475 includes the left-end origin of replication and sequences estimated to include the viral DNA packaging domain, based on their similarity to Ad5 sequences (11, 12, 21). Additionally, the Ad7a sequence from nucleotide position 2800 to 4000 (or 7.7 to 11 map units) was PCR amplified and cloned into our base plasmid. This sequence provides an Ad7 sequence homologous to a sequence in the Ad7a large fragment necessary for overlap recombination. The plasmid, as constructed, is deleted of the Ad7a sequence from nucleotide 476 to 2799. This deletion corresponds to removal of all of the Ad7 sequence coding for E1A and the majority of the E1B coding region. Inserted into the region of the E1 deletion is a CMV promoter-driven CAT gene, as well as RNA processing elements that have been used in Ad5 reporter constructs (10).

The combination of Ad7a fragments, when cotransfected with pAd7a-CATgD, has the ability to recombine to form an E1-defective Ad7a virus. Based on the ability of Ad7a to grow in cell line 293, we felt there was a reasonable probability that the E1-complementing functions of 293 cells would be able to complement an E1 defect in a non-subgroup C virus such as Ad7a. Cotransfection of pAd7a-CMV-CATgD and the Ad7a large-fragment fraction into 293 cells was carried out under several conditions, using different concentrations of viral large fragments and using both linearized and circular forms of the pAd7a-CMV-CATgD plasmid (as indicated in Fig. 1B). Seven days posttransfection, cells were harvested and prepared as virus lysates. Aliquots from each transfection were used to infect noncomplementing A549 cells; 24 h postinfection, cell lysates were prepared for CAT assays (Fig. 1B). Based on the CAT activity, it was evident that a recombinant Ad7a-CAT virus had been successfully generated. Virus was produced from transfections which used either circular or linearized pAd7a-CATgD plasmid. From each transfection, the resultant crude lysate, containing recombinant virus, was further expanded. Viral DNA was isolated from infected 293 cells; analysis by *Aat*II restriction digestion indicated that a mixture of recombinant and wt Ad7a DNA was present in each cell lysate, with wt being dominant.

Purification and characterization of Ad7a-CAT. We attempted to plaque purify the recombinant virus on 293 cells. All of our efforts to isolate Ad7a-CAT failed to produce an isolate that was free of contaminating wt Ad7a. Continued passage of the virus mixture resulted in a decrease in the Ad7a-CAT/wt Ad7a titer ratio. Based on our inability to plaque purify the Ad7a-CAT virus in 293 cells, it appeared that 293 cells were not able to fully complement the Ad7a-CAT virus and that in the mixed lysate the presence of wt Ad7a virus potentially contributed required E1 complementation functions.

By design, the Ad7a recombinant virus should be compromised in both E1A and E1B functions. All of the known E1A interactions are protein-protein interactions with host factors (4, 19, 32); therefore, we did not consider any of the Ad5 E1A gene products as the primary candidate for the protein functioning to limit complementation of Ad7a-CAT in cell line 293 infections. On the other hand, E1B gene products interact with both host and viral proteins (specifically with the E4 open reading frame [ORF] 4 and ORF 6 gene products [3, 29]); therefore, serotype-specific E1B interactions seemed more likely to be responsible for our inability to isolate a pure Ad7a-CAT virus in 293 cells. Since cell lines which express E1A, E1B, and E4 gene products have recently been developed, we attempted plaque isolation of Ad7a-CAT on one of them, 293-ORF6 cells (4). In comparison to those on 293 cells, plaque assays on the 293-ORF6 cell line resulted in an increase in plaque number and an improved plaque morphology from the Ad7a mixed lysate. Twenty isolates were expanded, and 2 of the 20 exhibited high levels of CAT activity when lysates were assayed for infectivity on A549 cells as previously described. Neither of the positive isolates exhibited Ad7 E1A contamination when subjected to PCR analysis. Both positive isolates were subjected to a second round of plaque purification, yielding isolates which did not contain detectable Ad7 E1A (or Ad5 E1A) as judged by PCR analysis. Virus was grown on a large scale and purified through three CsCl bandings. The final virus yield was approximately 2 ml of virus at 10^{12} particles/ml. This is essentially the same yield one would expect from similar large-scale Ad5 preparations.

Viral DNA was isolated from a portion of the large-scale virus preparation and characterized by restriction digestion and PCR analysis. Using a variety of restriction enzymes, it was discovered that the Ad7a-CAT virus had an unexpected left-end configuration. The expected homologous recombination event should have occurred somewhere between positions 2800 and 4000 of Ad7a, resulting in a deletion of the E1 region sequence from bp 476 to bp 2799 as previously described. Based on the restriction digestion pattern of Ad7a-CAT, the actual configuration of the left end includes sequence in the E1B region which should have been deleted. PCR amplification of the junction domain and characterization of the junction indicated that there was complete conservation of the Ad7a large-fragment DNA sequence. A nonhomologous recombination event occurred in pAd7a-CATgD at the end of the gD sequence in the plasmid adjoining the Ad7a large fragment (as shown in Fig. 1C). When we examined the second plaque isolate from the original 293-ORF6 cell line, it was found to have the same genomic configuration. The final isolate is deleted of Ad7a sequence between positions 476 and 1483, which is the coding domain of E1A, but retains the coding region for E1B. We have also found that purified Ad7a-CAT can grow in 293 cells but requires input of a large number of virus particles per cell and, as expected, is growth restricted in cells which do not complement the E1 defect (data not shown).

In vitro transduction efficiency of Ad7a-CAT. By using the Ad7a-CAT virus in a transduction assay, we determined the efficiency of vector entry into the cell and the establishment of a transcriptionally active DNA template. A549 cells were infected with one of three viruses, *dl*Ad5-NCAT, *dl*Ad5-NCAT-F7, and Ad7a-CAT, at a concentration of 10^3 particles/cell. *dl*Ad5-NCAT is a standard E1- and E3-deleted CAT-expressing vector. *dl*Ad5-NCAT-F7 is a derivative of *dl*Ad5-NCAT which contains the Ad7a fiber gene in place of the Ad5 fiber gene; it therefore binds to cells via the same fiber receptor as Ad7a (10). Four-well chamber slides were seeded with A549 cells in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum, and the cells were cultured for 16 h at 37°C. Cell cultures were then infected with 1,000 particles of Ad7a-CAT, *dl*Ad5-NCAT, or *dl*Ad5-NCAT-F7 per cell (10) in 100 μl of DMEM for 1 h at 37°C. The virus-containing medium was then removed and replaced with 0.5 ml of DMEM containing 10% calf serum, and cultures were incubated for 24 h at 37°C. Cultures were then rinsed in phosphate-buffered saline (PBS), pH 7.4, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then washed in PBS. The fixed cultures were then permeabilized with 2.5% Nonidet P-40 in PBS for 10 min at room temperature, washed with PBS, and blocked in PBS containing 1.5% goat serum (Vector Laboratories, Inc., Burlingame, Calif.) for 15 min at room tempera-

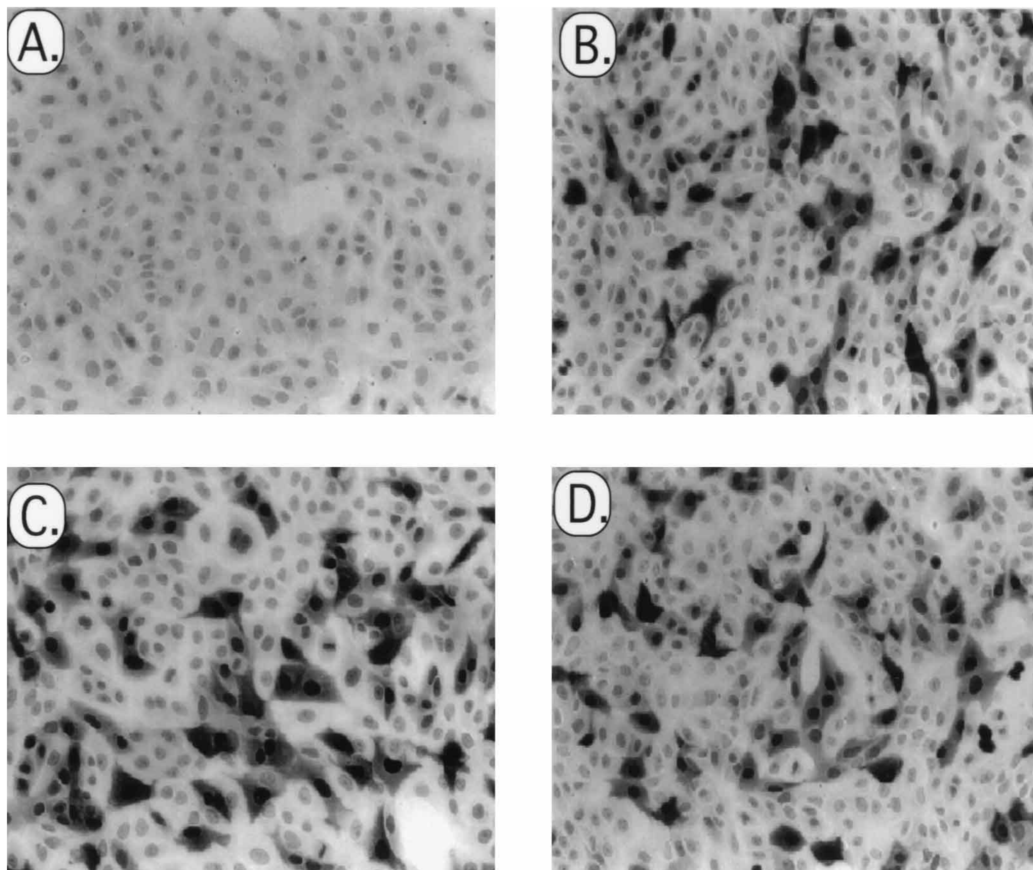

FIG. 2. Transduction efficiencies of Ad7a-CMV-CATgD, *dl*Ad5-NCAT, and *dl*Ad5-NCAT-F7 in a human lung epithelial cell line. Shown are bright-field images of control (uninfected) cells (A) and A549 cells immunostained for CAT activity 1 day after infection with 1,000 particles of *dl*Ad5-NCAT-F7 (B), Ad7a-CAT (C), or *dl*Ad5-NCAT (D) per cell. Magnifications, ×200.

ture. After being washed in PBS, the cultures were incubated for 1 h at 37°C with a rabbit anti-CAT antibody (5 Prime→3 Prime Inc., Boulder, Colo.) at a dilution of 1:750 in 1.5% goat serum–PBS. Cultures were then washed in PBS prior to being immunolabeled and stained with the reagents of the rabbit immunoglobulin G Vectastain Elite ABC kit and the peroxidase substrate DAB kit (Vector Laboratories) in accordance with the instructions of the manufacturer (Fig. 2). By comparing the percentages of cells exhibiting CAT gene expression, we found that the overall transduction efficiencies for the three different viruses were essentially identical. These data indicate that gene transfer by Ad7a-CAT virus particles is as efficient as that by an Ad5-based vector. In support of this conclusion, a time course of CAT gene expression in A549 cells (Fig. 3A) and a dose-response curve (Fig. 3B) indicate that the overall kinetics of gene expression mediated by the Ad7a-CAT vector are identical to those of the Ad5-based vectors. A virus dose-CAT expression assay response study in another human cell line, HEpG2 hepatoma, demonstrated identical efficacies of gene transduction and CAT gene expression for the Ad7a and Ad5 vectors (data not shown).

In vivo transduction of Ad7a-CAT. Based on our in vitro studies of Ad7a-CAT, we have determined that Ad7a functions as an efficient gene transfer vector compared to Ad5-based vectors. As an initial characterization of in vivo Ad7a-CAT gene transduction, Ad7a-CAT was administered intravenously to BALB/c mice as previously described (31). Two aspects of in vivo gene transfer were examined, tissue distribution and duration of CAT gene expression in liver tissue. CAT gene expression in liver tissue was determined over a 4-week experimental time course (Fig. 4A). Peak levels of CAT activity were found on days 1 through 12, and the levels then declined to baseline by day 28. Using CAT gene activity as an assay of in vivo virus dissemination, uptake of virus into a target organ, and gene expression once the virus is in the target cell (Fig. 4B), we found that the highest overall level of CAT gene expression occurred in the liver and that lower levels of viral gene transduction occurred in the spleen, kidney, lung, and heart. These results indicate that a variety of tissue types are susceptible to transduction by the Ad7 serotype.

This study was carried out to determine the feasibility of constructing an E1-defective non-subgroup C adenovirus vector in a cell line transformed with the Ad5 E1 region. The strategy we chose was essentially that used to construct the more traditional Ad5-based vectors, overlap recombination between an Ad7a reporter plasmid and an Ad7a viral backbone deleted of essential left-end elements. Using this strategy, we were able to successfully construct and isolate an E1A-defective Ad7a vector in cell lines expressing Ad5 E1 gene products. Although the Ad7a-CAT virus demonstrates properties of compromised growth under certain conditions, the transduction efficiency of Ad7a-CAT is essentially identical to that of Ad5-based vectors. The combination of efficient gene transduction and a more restricted growth phenotype is attractive from the perspective of gene transfer vectors. Additionally, the ability to propagate the virus in the Ad5-transformed cell line

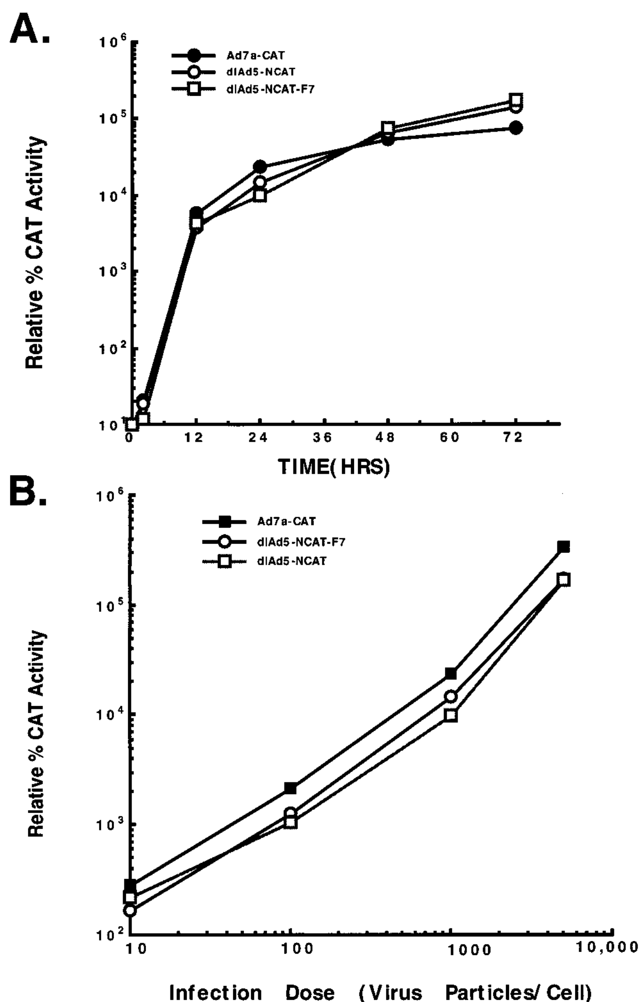

FIG. 3. Dose-response curve and kinetics of Ad7a-CAT-mediated gene transfer to a human lung epithelial cell line: comparison to those of Ad5-based vectors. (A) Time courses for Ad7a-CAT, *dl*Ad5-NCAT-F7, and *dl*Ad5-NCAT-mediated transgene expression in A549 cells. Cultures were incubated with serum-free medium containing 1,000 particles of Ad7a-CAT, *dl*Ad5-NCAT, or *dl*Ad5-NCAT-F7 per cell for 2 h at 37°C. The virus-containing medium was then removed and replaced with serum-containing medium. The cells were then harvested either immediately or 12, 24, 48, or 72 h after the start of the infection and were assayed for CAT activity. (B) Viral dose-response curves of Ad7a-CAT, *dl*Ad5-NCAT-F7, and *dl*Ad5-NCAT-mediated gene expression in A549 cells. Cultures were incubated with serum-free medium containing 10, 100, 1,000, or 5,000 particles of Ad7a-CAT, *dl*Ad5-NCAT, or *dl*Ad5-NCAT-F7 per cell for 2 h at 37°C; the virus-containing medium was then removed, and cultures were incubated with serum-containing medium. Twenty-four hours after the start of the infection, cultures were harvested and assayed for CAT activity as previously described (10).

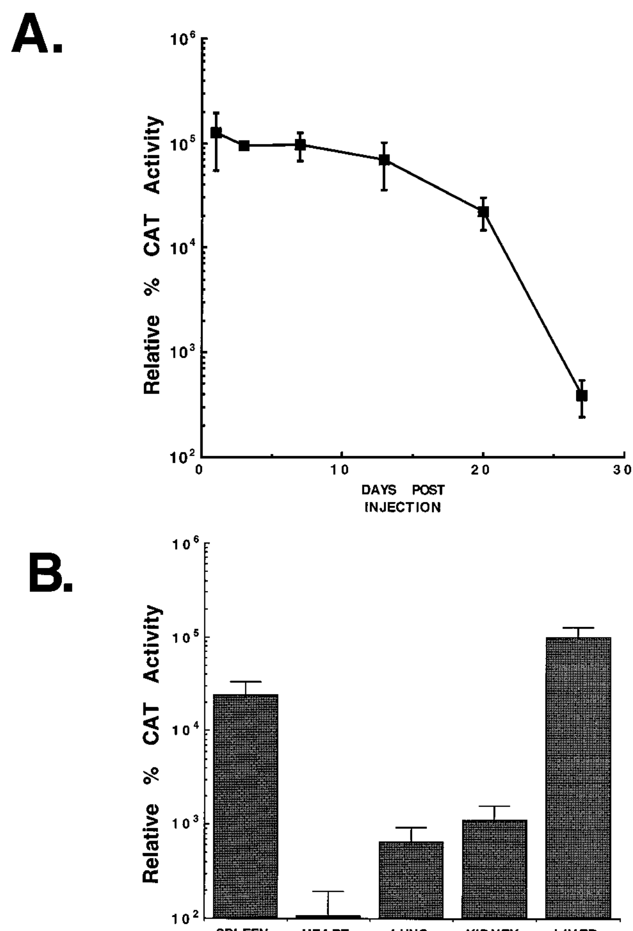

FIG. 4. Ad7a-mediated transgene expression following intravenous administration. (A) Time course of recombinant Ad7a-mediated gene expression in mouse livers following intravenous administration of Ad7a-CAT (as described in reference 31). Suspensions of Ad7a-CAT (5×10^{10} particles) were injected into the jugular veins of mice, which were sacrificed immediately or 3, 7, 14, 28, or 35 days postinfection. Livers were evaluated for CAT gene expression by a [^{14}C]chloramphenicol assay. Values are the means (± 1 standard deviation) of data for 10 to 15 animals from two to three separate experiments. (B) Tissue distribution of CAT gene expression. Suspensions of Ad7a-CAT (5×10^{10} viral particles) were injected into the jugular veins of mice; 3 days later, mice were necropsied and spleens, hearts, lungs, kidneys, and livers were evaluated for CAT gene expression by a [^{14}C]chloramphenicol assay. Values represent the mean CAT activities (± 1 standard deviation) for the various organs.

implies that there will be a decreased likelihood of recombination between the host Ad5 E1 elements and the Ad7a-CAT vector, another desirable trait in a first-generation adenovirus vector. The use of cell lines expressing both adenovirus E1 and E4 gene products proved to be a critical element that we had not originally anticipated.

The Ad7a-CAT constructs that have been characterized to date have the same E1A-negative, E1B-positive genotype, which resulted from end joining of the Ad7a backbone to the essential elements of the reporter plasmid. Based on this observation and the difficulty we had in isolating the virus from a wt background, a more efficient strategy for constructing a first-stage non-subgroup C virus would involve inclusion of pertinent E1B sequences which could enhance the homologous recombination efficiency. With the Ad7a-CAT vector in hand, we are now in a position to determine if the complementation functions of existing cell lines can extend to the production of further deletions in the non-subgroup C viruses, paying particular attention to the E1B and E4 elements without wt background interference. Based on the limited in vivo assays that have been performed in this study, we are inclined to believe that the host immune response to Ad7a-CAT is essentially the same as that seen with Ad5-based vectors.

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