

Adenovirus Type 5 and 7 Capsid Chimera: Fiber Replacement Alters Receptor Tropism without Affecting Primary Immune Neutralization Epitopes

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The efficient uptake of adenovirus into a target cell is a function of adenovirus capsid proteins and their interaction with the host cell. The capsid protein fiber mediates high-affinity attachment of adenovirus to the target cell. Although the cellular receptor(s) for adenovirus is unknown, evidence indicates that a single receptor does not function as the attachment site for each of the 49 different serotypes of adenovirus. Sequence variation of the fiber ligand, particularly in the C-terminal knob domain, is associated with serotype-specific binding specificity. Additionally, this domain of fiber functions as a major serotype determinant. Fiber involvement in cell targeting and its function as a target of the host immune response make the fiber gene an attractive target for manipulation, both from the perspective of adenovirus biology and from the perspective of using adenovirus vectors for gene transfer experiments. We have constructed a defective chimeric adenovirus type 5 (Ad5) reporter virus by replacing the Ad5 fiber gene with the fiber gene from Ad7A. Using the chloramphenicol acetyltransferase reporter gene, we have characterized this virus with respect to infectivity both *in vitro* and *in vivo*. We have also characterized the role of antifiber antibody in the host neutralizing immune response to adenovirus infection. Our studies demonstrate that exchange of fiber is a strategy that will be useful in characterizing receptor tropism for different serotypes of adenovirus. Additionally, the neutralizing immune response to Ad5 and Ad7 does not differentiate between two viruses that differ only in their fiber proteins. Therefore, following a primary adenovirus inoculation, antibodies generated against fiber do not constitute a significant fraction of the neutralizing antibody population.

The pathway of adenovirus entry into mammalian cells is an important aspect of virus biology which contributes to virus tropism as well as the overall efficiency of infection. The efficiency of adenovirus infection has been largely responsible for its popularity as a vector for gene transfer. Aspects of adenovirus biology which contribute to its high efficiency as a gene transfer vector *in vivo* center on the attachment and internalization of the virus particle. Adenovirus binds to a specific cellular receptor with a high affinity, 9×10^9 to 20×10^9 M⁻¹ per site for adenovirus serotypes 2 and 3 (Ad2 and Ad3) (5, 22), via the fiber protein (16, 24) which extends from each vertex of the icosahedral protein capsid. The virus particle is subsequently endocytosed, undergoes capsid conformational changes, penetrates the cytosol by escaping from the endosome, and delivers its DNA to the nucleus (7, 15, 28–30). Once in the nucleus, the viral genome attaches to the nuclear matrix through terminal protein (26, 39). By using replication defective adenoviruses containing reporter genes, it has been shown that the viral genome can be stably maintained *in vivo* for periods exceeding 6 months (4, 12).

There are three viral interactions with the host cell entry pathway which can be influenced by serotypic variations in the capsid proteins: (i) different serotypes of adenovirus bind through different cell surface receptors (5, 27), (ii) the secondary virus-cell interaction mediated through penton base varies by serotype (1, 2, 17, 33), and (iii) escape from the endosome may involve distinct mechanisms of vesicle disruption, again in

a serotype-dependent manner (5, 32). These variations in the general pathway of adenovirus uptake indicate that capsid proteins and capsid structure can, in theory, contribute to serotype cell tissue tropism. One way to determine the contribution of the capsid proteins to the tropism seen with different serotypes of adenovirus is by a gene replacement strategy.

The strategy of capsid gene replacement would also provide a method of further characterizing targets of the immune response generated against a primary infection with adenovirus or adenovirus vectors. The humoral immune response to capsid proteins is responsible for limiting adenovirus gene transfer to a single administration. This neutralizing antibody response is by definition serotype specific and is responsible for blocking successful readministration of the current generation of adenovirus gene transfer vectors (which are primarily based on Ad5) (4, 12, 38). At least two points along the pathway of gene transfer by adenovirus are susceptible to blockage by circulating antibodies, blocking the fiber protein-cellular receptor interaction and escape from the endosome. Escape of the viral genome from the endosome can be inhibited by capsid-bound antibodies against both hexon, the major capsid component, and penton base (36, 37). Antihexon antibody is considered to be the dominant neutralizing antibody in response to adenovirus infection (34–36). Although antifiber antibodies can neutralize adenovirus infection (3, 9, 18, 34, 35, 37), there are reports claiming that fiber is not an important immunogen (14, 23, 31). Additionally, the immunogenicity of fiber varies; it is a relatively weak immunogen if it is delivered as a purified protein and a relatively strong immunogen if whole virus particles are used as an immunogen (18). However, none of these studies were able to directly test if antifiber antibodies produced in

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response to adenovirus infection play a significant role in neutralizing second administrations of adenovirus.

The objective of our study was first to demonstrate the feasibility of exchanging the fiber gene of one serotype with the fiber gene of another and then to determine the functional impact on the biological activity of the resulting chimeric virus, specifically altered target receptor specificity and antigen profile. We have constructed a chimeric virus consisting of an Ad5 backbone with the Ad7 fiber gene in place of the Ad5 fiber gene. Here we present data demonstrating that the Ad5 capsid is compatible with the Ad7 fiber protein, that changing the fiber protein is sufficient to alter receptor utilization, and that fiber is not a strong neutralization determinant *in vivo*.

MATERIALS AND METHODS

Adenovirus stocks and characterization of virus infections. Wild-type Ad7A was obtained from the American Type Culture Collection and grown in HeLa monolayer cells for expansion. Following three passages, high-titer stocks were generated and used to infect HeLa suspension cells for large-scale production. All viruses were purified by using standard procedures, which include two CsCl gradients. We have found that the measurement of virus particles by spectrophotometry is in good correlation to the amount of viral protein used in each administration. Because the spectrophotometric quantitation of virus best represents the input viral protein, all infections were standardized by particle number.

E1-defective viruses were constructed and grown in monolayer 293 human embryonic kidney cells (6); for large-scale virus preparations, suspension 293 cells grown in S minimal essential medium (Joklik modified)-5% horse serum were used. Virus stocks were characterized by purifying viral DNA by using a modified Hirt procedure, followed by restriction endonuclease digestion and Southern blot characterization of viral DNA to verify purity. Additionally, viral proteins were characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Construction of the chimeric virus, *dAd5NCAT-F7*. The starting plasmid used in construction of the chimeric viruses was pAd70-100, which contains wild-type Ad5 sequence from map units (m.u.) 70 to 100. pAd70-100 was converted to pAd70-100dIE3 as follows. Partial digestion of the plasmid with restriction enzyme *MunI* was followed by insertion of a *MunI-BamHI* smart linker and circularization. The plasmid containing a *BamHI* site at m.u. 91 was selected for subsequent cloning steps. Deletion of E3 sequences from m.u. 78.6 to 85.8 and insertion of a unique *PacI* site was accomplished by PCR amplification of DNA from m.u. 76.2 to 78.6 (fragment 1) and 85.8 to 87.0 (fragment 2). *PacI* sites were present at the 78.6 and 85.8 ends of fragments 1 and 2, respectively. Fragment 1 was digested with *SrfI* and *PacI*; fragment 2 was digested with *PacI* and *SphI*. pAd70-100(Bam) was digested with *SrfI* and *SphI*. A three-fragment ligation between fragments 1 and 2 and the digested pAd70-100(Bam) resulted in pAd70-100dIE3 (see Fig. 2A).

On the basis of available sequence of Ad7A in the region of E3, fiber(IV), and E4 (10), oligonucleotides 1 (5'-gcgaagcttttaataactctctctaccacaa) and 2 (5'-tgccgataccaattgattttaataaacaagtaaa) were synthesized for PCR amplification. The expected size of the PCR product was 1,083 bp, and it contained *PacI* and *BamHI* sites at the left and right ends. Following PCR amplification, the fiber 7 gene was subcloned into plasmid pAd70-100dIE3 (Fig. 1) at the unique *PacI* and *BamHI* restriction enzyme sites to generate pAd70-100dIE3F-7. The parent virus, Ad5NCAT, was made by using a right-end large fragment from an Ad5 derivative, 5B1-Xba(-), as was a similar construct, Ad5CAT (11, 12). 5B1 virus (a generous gift from Hamish Young) is phenotypically wild-type Ad5 with a partial deletion of E3 (m.u. 79.6 to 84.8); the Xba(-) version lacks *XbaI* restriction enzyme sites. Ad5NCAT contains the neomycin resistance gene driven by the Rous sarcoma virus promoter and the chloramphenicol acetyltransferase (CAT) gene driven by the cytomegalovirus promoter in the E1 region. The large *SrfI* fragment of Ad5NCAT corresponding to 76.8 m.u. of the left-hand end was cotransfected with either pAd70-100dIE3 or pAd70-100dIE3-F7 into HEK-293 cells to produce the recombinant viruses *dAd5NCAT* and *dAd5NCAT-F7*, respectively.

Virus-cell binding competition assay. Approximately 15,000 cpm of ³H-labeled adenovirus (~750 particles per cell) was mixed with competitor virus and applied to a monolayer of approximately 5 × 10⁵ A549 cells. After 1 h at 37°C, the medium was collected and cells were washed three times with cold phosphate-buffered saline (PBS), solubilized in 0.6 N NaOH, neutralized with 1.2 N HCl, and counted in a scintillation counter.

Inhibition assay. Unlabeled competitor virus was preincubated at 4°C with approximately 5 × 10⁵ A549 monolayer cells for 30 min. Then 20,000 cpm of ³H-Ad7a or ³H-*dAd5NCAT-F7* (~800 particles per cell) or 50,000 cpm of ³H-*dAd5NCAT* (~1,700 particles per cell) was applied to the cell monolayer for an additional 30 min at 4°C. The cells were harvested as in the competition assay.

Injection of adenovirus into adult rats. Sprague Dawley rats (~200 g) were

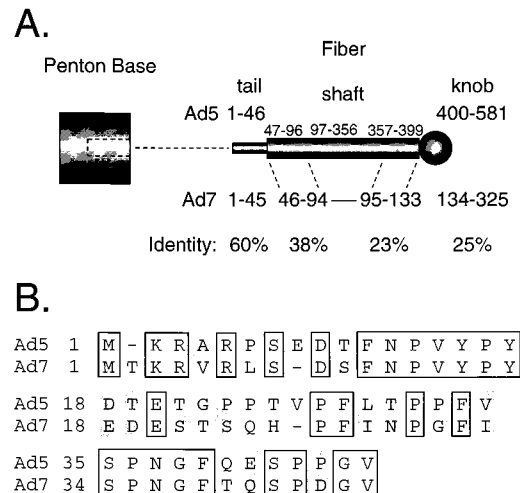


FIG. 1. (A) Schematic representation of penton. Penton consist of a homopentamer of penton (penton base) and a homotrimer of the fiber protein. The fiber protein is divided into three regions: the N-terminal tail, the midsection shaft domain, and the C-terminal knob domain (8). Numbers correspond to amino acids used for identity comparison of Ad5 and Ad7 fiber protein regions. (B) Amino acid sequence alignment of the fiber protein tail region delineated in panel A, produced by the Wisconsin Genetics Computer Group package (1994). Identical residues are boxed; similar residues are shaded.

used for all experiments. Each injection included 6 × 10⁹ particles of virus. Intraperitoneal injections were done in a volume of 100 μl of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ [pH 7.4]). Intramyocardial muscle injections were performed into the apex of the left ventricle in a volume of 50 μl of PBS. Each assay point includes five animals. At the endpoint of the experiment, tissues were harvested, weighed, and homogenized in a Tissuemizer (Tekmar) in 1 ml of homogenization buffer (25 mM glycylglycine [pH 7.8], 15 mM MgSO₄, 4 mM EGTA [pH 8.0], 1 mM dithiothreitol) per 0.5 g (wet weight) of tissue. CAT assays were performed as previously described (11). Initially 5% of the total tissue lysate was assayed. When values were out of the range of the assay (generally >70% acetylated chloramphenicol), lysates were diluted in homogenization buffer plus 0.1 mg of bovine serum albumin (BSA) per ml.

Neutralization assays. Ninety-six-well plates were seeded with 2 × 10⁴ A549 cells grown in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum and grown for 18 to 24 h. For all infections, an estimated multiplicity of infection of 1 was diluted into a final volume of 45 μl in DMEM. Diluted virus was incubated with 5 μl of serum for 1 h at 37°C. Mixtures were then added to designated wells in the 96-well plates and incubated for 1 h at 37°C, and 150 μl of DMEM-5% calf serum was added. All assays were done in triplicate. Plates were incubated at 37°C in 5% CO₂. For neutralization assays of wild-type viruses, the plates were observed daily for cytopathic effect. Control samples underwent 100% cytopathic effect after approximately 4 days of incubation. At this point, cells were fixed and stained with 50 μl of crystal violet-formaldehyde (0.004% crystal violet, 10% methanol, and 7% formaldehyde in PBS) for 20 min and washed with water three times. Neutralization of E1-deleted viruses was scored with a CAT immunohistochemical assay. At 24 h postinfection, cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. The cells were then washed with PBS, permeabilized with 2.5% Nonidet P-40 in PBS for 10 min, washed with PBS, and blocked with 1% BSA-PBS three times for 5 min each at room temperature. The cells were then incubated for 1 h at 37°C with an anti-CAT antibody (5 Prime→3 Prime, Inc.) at a dilution of 1:750 in 1% BSA-PBS and washed three times with BSA-PBS. The cells were then incubated with a goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (Amersham) at 1:200 in PBS-BSA for 30 min. After three 5-min washes in PBS, the peroxidase reaction was developed with a Vecastain DAB kit (Vector Laboratories).

RESULTS

Construction of an Ad5/fiber 7 chimeric virus. Modeling of Ad5 fiber amino acid sequence (8) has allowed the protein to be divided into three domains: the N-terminal tail domain, which includes the first 45 to 50 amino acids of the protein; the shaft domain, which consists of various numbers of a 15-amino-acid pseudo-repeat element; and the knob domain, which in-

cludes the receptor binding domain of the virus (Fig. 1A). Within a viral subgroup in which DNA sequence homology between serotypes is 95 to 99%, there can be considerable sequence and functional conservation of these domains (13, 19, 20). Comparison of the homology of fiber domains outside of a viral subgroup shows that similarity between elements is greatly reduced (13). Of the three domains, the tail region is consistently the most conserved when amino acid sequence are compared between serotypes (Fig. 1B compares fibers from Ad5 and Ad7A) (13). Since the tail region is the domain which anchors the fiber polypeptide to the penton base pentamer at each of the icosahedral vertices, the structure-function demands placed on this portion of fiber are common to all adenovirus serotypes. This is in contrast to functions of the shaft and knob domains of the virus. The shaft appears to function as a spacer between the capsid body and the knob. The length of fiber is a direct result of the number of pseudo-repeat elements in the shaft domain, and while there are conserved positional biochemical characteristics, there is low amino acid sequence identity in this domain of the fiber gene. The knob domain functions both to target the virus to a designated cell surface receptor (5, 9, 27) and to confer serotype-specific epitopes on adenovirus (21); it also has low sequence identity in comparison of fibers from different serotypes. In a study characterizing *tsAd7* mutants (25), Praszkiel and Ginsberg demonstrated that a variety of *tsAd7* viruses could at some level, complement an Ad5 virus which contained a temperature-sensitive mutation in fiber (*ts142*). In these experiments, complementation by Ad7 could have involved fiber alone, fiber and penton, or in some instances fiber, hexon, and penton. Because of the similarity between the N-terminal tail fiber domains and the lack of conservation in the shaft and knob domains, there is a reasonable probability that fiber genes from different serotypes are structurally interchangeable and that they would be functionally distinct with respect to virus targeting and antigenicity.

A simple strategy to dissect functional aspects of capsid proteins such as fiber is to construct reporter virus constructs able to receive a designated capsid protein. As a first step in capsid protein exchange, we have created a strategy which will allow replacement of the Ad5 fiber gene with the Ad7A fiber gene. Ad7 is normally tropic for the lower respiratory tract and, as judged from affinity blotting, binding competition, and immunofluorescence assays, enters target cells through a receptor pathway different from that used by Ad5 (5). The fiber 7 gene was PCR amplified from isolated Ad7A DNA by using oligonucleotides which inserted a *PacI* restriction site upstream of the coding region of fiber. We chose this region for modification because it is in the E3 region of adenovirus, which is dispensable for virus growth, and it allows use of the normal splice acceptor of the fiber mRNA (which incidentally overlaps with the translation initiation codon). We chose a 3' end for our PCR strategy to initiate downstream of the L5 poly(A) signal sequence, in an area that theoretically would not perturb the E4 transcript or reading frame which is expressed on the opposite strand of the genome downstream from the fiber region. The termination codon for fiber is integrated into the AAUAAA sequence of the L5 poly(A) signal. Therefore, the Ad7A PCR product included both 5' and 3' RNA processing elements of the major late transcription unit L5 exon, as well as the complete coding region of the fiber gene. The parent plasmids pAd70-100dIE3 and pAd70-100dIE3-F7 are schematically shown in Fig. 2A. The strategy for virus construction involved isolation of the viral left-end subgenomic fragment (Fig. 2B) from the replication-defective virus Ad5NCAT by digestion with *SrfI* and isolation of the large fragment by sucrose gradient

fractionation. Recombinant virus was isolated following cotransfection of large fragment and pAd70-100dIE3 or pAd70 dIE3-F7 into 293 cells. Lysates were screened for recombinant virus by using Hirt-isolated DNA. The viral lysates containing successful recombinants were used in subsequent plaque purifications. Individual plaque isolates were screened by Hirt DNA characterization, and those isolates which yielded a predicted restriction digestion pattern (Fig. 3A) were grown in large scale. With identical conditions for large-scale preparations of *dAd5NCAT* and *dAd5NCAT-F7*, the final preparations resulted in similar yields of virus (approximately 2.5×10^{13} and 2.0×10^{13} particles, respectively) per 4×10^8 293 cells, as determined by optical density at 260 nm. A portion of virus yield from the large-scale preparation was used to characterize viral DNA by restriction enzyme digestion (Fig. 3B). The final recombinant viruses *dAd5NCAT* and *dAd5NCAT-F7* were verified by Southern blot characterization (Fig. 3C) to be free of contaminating background virus.

On the basis of the successful construction and isolation of the chimeric virus *dAd5NCAT-F7* and a lack of any observed growth or packaging defect, we conclude that the Ad7A fiber gene can complement the fiber requirements of Ad5. The population of virion proteins present in the chimeric construct as determined by SDS-polyacrylamide gel electrophoresis indicates that the protein expressed by the chimeric virus is identical in size to the fiber protein present in Ad7 and that the band corresponding to Ad5 fiber is missing (Fig. 4). These data support the notion that the Ad7 fiber has been left intact and is fully functional in an Ad5 background.

In vitro characterization of the chimeric virus. Following large-scale purification of the chimeric virus, we found that the particle-to-PFU ratio for the Ad5/fiber 7 chimeric construct was similar to that characteristic of the parental Ad5-based viruses. In contrast, the particle-to-PFU ratio associated with wild-type Ad7 can be increased by 1 to 2 log units, depending on the assay (5a). From the particle-to-PFU ratio obtained with the chimeric virus, we conclude that the receptor attachment pathway is not the basis of the discrepancy in infectious particles between Ad7 and Ad5. Further verification of this conclusion was found in a functional assay of virus uptake and localization of the viral genome to the nucleus, i.e., activity of reporter gene product (CAT) generated in cells infected by the virus. A549 lung epithelial cells were infected at 10 or 100 viral particles per cell with either the original Ad5NCAT virus, *dAd5NCAT*, or *dAd5NCAT-F7*. As shown in Fig. 5, the relative amounts of CAT gene expression were essentially identical at equal particle-to-cell ratios. As expected, increasing the dose of virus on a per-cell basis gave a proportional increase in CAT gene expression. Thus, at the level of virus uptake and CAT gene expression in the lung epithelial cell line, the chimeric virus and its Ad5 counterpart are not functionally distinguishable.

To characterize the cell entry pathway of *dAd5NCAT-F7*, we used two forms of virus binding competition assays. In a direct competition assay (Fig. 6A to C), cells were exposed to a mixture of virus (both the ^3H -labeled virus and unlabeled competitor) for 60 min at 37°C, after which they were harvested and processed as described in Materials and Methods. The assay carried out in this manner allows for maximal virus binding at 37°C as well as internalization of bound virus. The effect of increasing concentrations of unlabeled virus on binding and uptake of [^3H]thymidine-labeled target virus to A549 cells was determined by using Ad7A, *dAd5NCAT*, and *dAd5NCAT-F7* (Fig. 6A to C). For all experimental variations, a 100-fold excess of homologous competitor virus resulted in a near-maximal inhibition of ^3H -Ad binding, whereas

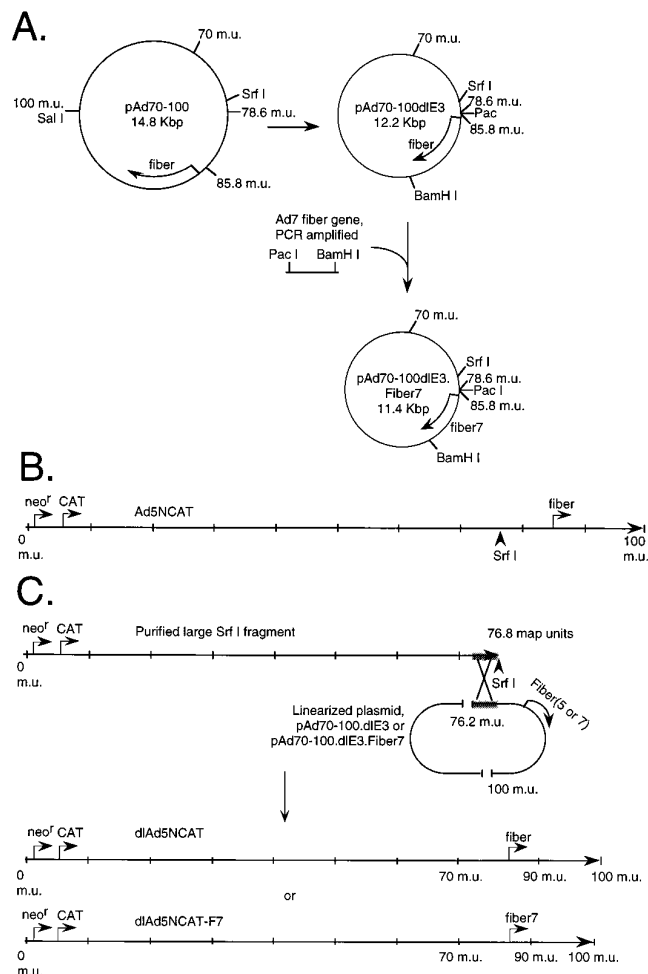


FIG. 2. Construction of recombinant adenoviruses *dlAd5NCAT* and *dlAd5NCAT-F7*. (A) Plasmid pAd70-100 contains wild-type Ad5 from m.u. 70 to 100. pAd70-100dlE3 was constructed by deleting E3 sequence from m.u. 78.6 to 85.8 and by PCR cloning Ad5 fiber at *PacI* and *BamHI* sites. Insertion of PCR-amplified fiber 7 sequence (10) in place of Ad5 fiber creates pAd70-100dlE3-Fiber7. (B) Ad5NCAT viral backbone used for construction of viruses *dlAd5NCAT* and *dlAd5NCAT-F7*. The E1 region is deleted between m.u. 1 to 7.8 with insertion of tandem reporter genes corresponding to neomycin resistance (*neo^r*) and CAT. Digestion of viral DNA with *SrfI* releases the right-end fragment from m.u. 76.8 to 100. (C) Schematic of overlap recombination events between the left-hand end fragment of Ad5NCAT and linearized plasmid DNA in panel A to yield the recombinant viruses *dlAd5NCAT* and *dlAd5NCAT-F7*.

a minimal effect was seen when a 100-fold excess of heterologous virus was used. The competition profile of *dlAd5NCAT-F7* mirrors that of the wild-type Ad7A virus and not that of an Ad5-based vector. The results from this assay support the notion that Ad5 and Ad7 bind to lung epithelial A549 cells through different receptors. One possible caveat to this conclusion is raised in the experiment in which a 500-fold excess of Ad7A was found to have an effect on ^3H -Ad5CAT binding (Fig. 6A). Because the reciprocal experiments did not demonstrate a similar effect when high concentrations of Ad5 were used to compete with Ad7 (Fig. 6B) or *dlAd5NCAT-F7* (Fig. 6C), the phenomenon of cross-serotype competition at high concentration of virus appears to be unidirectional.

The results from a second type of assay, inhibition of ^3H -labeled virus binding at 4°C by prebinding unlabeled competitor virus for 30 min, demonstrate receptor specificity by blocking ^3H -labeled virus binding in the absence of virus internalization

(Fig. 6D to F). This less sensitive assay requires saturation of cell surface receptors for maximal effect (estimated at between 5×10^3 and 7×10^3 per A549 cell [5a, 27]). The 100-fold-excess competitor condition corresponds to approximately 10 input virus particles per receptor. With 500-fold-excess unlabeled competitor, we clearly see that a serotype-specific inhibition of ^3H -labeled virus is occurring. In agreement with experiments characterizing receptor specificities of subgroup C and subgroup B viruses (5, 27), the direct binding inhibition assay demonstrates that Ad7A and Ad5 do not compete for the same receptor on A549 cells.

The two types of competition assays have revealed differences in competition between Ad7 and Ad5, particularly at a 500-fold excess of competitor: Ad7 is able to inhibit binding and uptake of an Ad5 virus at 37°C but not at 4°C. It is not clear what accounts for these differences. There may be competition for the same receptor, which is revealed only at the more optimal binding temperature of 37°C, or competition could occur only at the higher concentration of competitor, indicating a lower ligand affinity, or there may be secondary effects on uptake of ^3H -Ad5NCAT that result from saturating concentrations of Ad7A virus binding to A549 cells (3.75×10^5 particles per cell).

These experiments clearly demonstrate that the chimeric virus does not compete effectively with ^3H -Ad5CAT binding to A549 cells. The competition profile of the chimeric virus is essentially identical to that found for Ad7A. Therefore, by replacing the fiber gene in the chimeric construct, we have redirected the virus to a high-affinity receptor which is normally used for attachment by Ad7A. Combining the data from Fig. 5 and 6, we infer that the receptor numbers, binding affinities, and virus entry pathways are functionally similar for *dlAd5NCAT* and *dlAd5NCAT-F7*.

In vivo infection with *dlAd5NCAT-F7*. The functional significance of replacing the fiber gene in *dlAd5NCAT-F7* may be most clearly illustrated when an in vivo assay is used to assess infectivity and immune response of the host to the chimeric virus. The latter issue is particularly important to the use of adenovirus vectors as gene transfer vectors, since the inhibition of virus uptake in a repeat administration of adenovirus is the result of the humoral immune response against the three major capsid proteins, hexon, penton, and fiber. Antibody generated against hexon is considered to be most effective in neutralization (34–36), but reports indicate that antibody against fiber is sufficiently strong to block a virus infection and that antifiber antibody is a predominant class of antibody produced following an initial infection (34).

In vivo infectivity of the chimeric virus was determined by injecting the virus into the myocardium of Sprague-Dawley rats. Previous experience with direct myocardial injection has shown this route of administration to be an efficient method of infecting heart tissue as well as allowing widespread dissemination of the virus to other organs, including the liver and lung (12). Five days after 6×10^9 particles of *dlAd5NCAT* or *dlAd5NCAT-F7* were injected into rat myocardium, levels of CAT gene expression from heart lysates infected with *dlAd5NCAT* and *dlAd5NCAT-F7* were identical (Fig. 7A). Because the human viruses efficiently infect rat cells, the cellular receptor for Ad7A is conserved across species as is the Ad5 receptor.

Interestingly, there was a considerable difference in the levels of CAT gene expression in the lung and liver tissues from animals which had received the direct myocardial injection (Fig. 7A). In both tissues, infection with *dlAd5NCAT* resulted in 10- to 20-fold-greater relative CAT expression than in the lung and liver tissues from animals which received *dlAd5NCAT-F7*. In

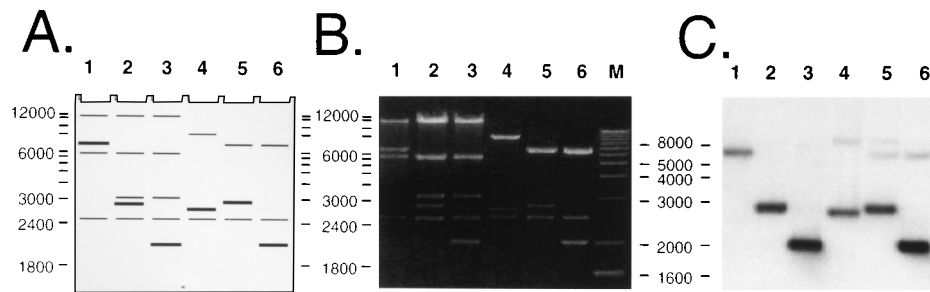


FIG. 3. Characterization of purified viral genomic DNA by restriction endonuclease digestion and Southern blot analysis. (A) Predicted *EcoRI-BamHI* digestion pattern (8a) of Ad5NCAT (lane 1), *dlAd5NCAT* (lane 2), and *dlAd5NCAT-F7* viral DNAs or pAd70-100 (lane 4), pAd70-100dlE3 (lane 5), and pAd70-100dlE3-Fiber7 (lane 6) plasmid DNAs. Boldface bands represent bands containing sequence homologous to the probe used in panel C. (B) Observed *EcoRI-BamHI* digestion pattern. Following restriction enzyme digestion, samples were electrophoresed through a 0.8% agarose gel and stained with ethidium bromide. Lane M, molecular weight markers. Lane numbering is as indicated for panel A. (C) Determination of homogeneity of virus preparations by Southern hybridization analysis. The agarose gel in panel B was blotted and hybridized to a ^{32}P -labeled probe spanning the entire Ad7 fiber gene and approximately 1 kbp of Ad5 sequence upstream (2,066-bp *EcoRI-BamHI* fragment of pAd70-100.dlE3-Fiber7 [lane 6]).

separate experiments (data not shown) in which *dlAd5NCAT-F7* virus was introduced directly into the lung by intratracheal administration, CAT expression levels resulting from *dlAd5NCAT* and *dlAd5NCAT-F7* were found to be equal. We conclude from this latter observation that the difference in gene expression found in the liver and lung (Fig. 7A) is not due to the presence or absence of Ad7A fiber receptors in lung epithelial cells.

To determine the contribution of the fiber protein to inducing an immune response capable of neutralizing subsequent adenovirus infections, a sequential administration protocol was performed in adult Sprague-Dawley rats (Fig. 7B). Wild-type Ad5 or wild-type Ad7A (6×10^9 particles in 100 μl of PBS) was administered intraperitoneally as a primary inoculation. Seventeen days after the primary inoculation, serum samples were taken and then 6×10^9 particles of the indicated reporter virus were administered to each animal. At sacrifice 5 days after administration of the reporter virus, serum and heart tissue were harvested, and heart tissue was processed for CAT expression assays. In animals that received Ad5 as the primary inoculation, reporter gene expression from both *dlAd5NCAT* and *dlAd5NCAT-F7* was reduced by more than 2 orders of magnitude compared with the mock-infected controls. The in vivo efficiency of Ad5-mediated neutralization, as determined by CAT gene expression, has not been altered by changing fiber. In animals which had been exposed to Ad7 prior to administration of the reporter viruses, the level of CAT activity was identical to that found in mock-infected animals. The implication from this experiment is that fiber epitopes do not contribute in a functionally significant manner to the adenovirus neutralization antibody that results from a primary inoculation of wild-type virus.

In vitro neutralization assays support these in vivo observations (Table 1). Serum samples from animals (Fig. 7B) in groups 1 and 2 had circulating neutralizing antibodies by 17 days after the administration of wild-type Ad5 (before the reporter virus was administered). Likewise, the sera from all animals in groups 3 and 4 contained Ad7 neutralizing antibodies at day 17. The group 1 and 2 sera from day 17 neutralized *dlAd5NCAT* and *dlAd5NCAT-F7*, while sera from groups 3 and 4 did not neutralize *dlAd5NCAT*. The sera from groups 3 and 4 had the ability to neutralize *dlAd5NCAT-F7* at a comparatively low level (Table 1). The weak neutralization of *dlAd5NCAT-F7* by anti-Ad7 serum indicates that antifiber antibodies are present under these experimental conditions, but they are not of sufficient strength or quantity to effectively block a virus infection in vivo (Fig. 7C). Additionally, sera

from group 6 rats (Fig. 7B), which were exposed to only *dlAd5NCAT-F7* for 5 days, were able to neutralize an Ad5 infection but not Ad7 infection in vitro (Table 1; group 6, day 22). Taken together, the in vivo infectivity results and the in vitro neutralization data suggest that the fiber protein is not a strong neutralizing determinant in vivo.

DISCUSSION

In this study, we have used a strategy of gene replacement to define specific functions of the capsid protein, fiber, in adenovirus infections. We have shown that amino acid homology between the tail regions of Ad5 and Ad7 is sufficient to allow functional replacement of the Ad5 fiber with Ad7 fiber. The predominant functional change in virus infectivity which results from fiber gene replacement is altered binding affinity for the target receptor on the surface of the cell membrane. We have also shown that in vivo, replacement of fiber has minimal impact on the neutralization of subsequent infections.

The majority of wild-type adenovirus serotypes are able to be cultured in common human cell lines, including HeLa, A549, and 293. By definition, any cell which is permissive for virus infection should contain the receptors that are required

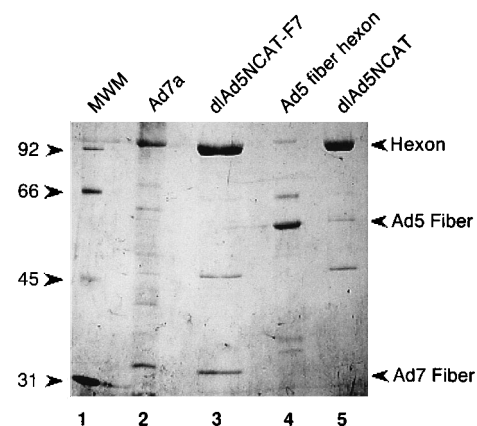


FIG. 4. Analysis of virion capsid polypeptides. A total of 10^{11} particles of cesium chloride-banded Ad7A (lane 2), *dlAd5NCAT-F7* (lane 3), or *dlAd5NCAT* (lane 5) or 100 ng of partially purified adenovirus fiber capsid proteins (hexon and fiber) (lane 4) was electrophoresed on an SDS-10% polyacrylamide gel and silver stained. Lane 1, molecular weight markers (MWM). Numbers on the left represent molecular masses in kilodaltons. Arrowheads denote position of Ad7 fiber, Ad5 fiber, and hexon.

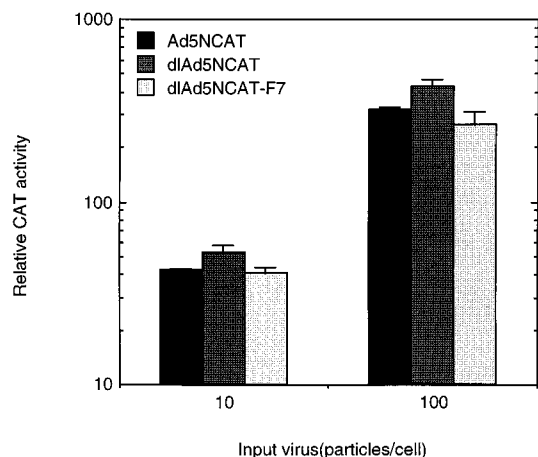


FIG. 5. Infection and CAT gene expression in A549 lung fibroblasts. A549 cells were infected with 10 or 100 particles of Ad5NCAT, *dlAd5NCAT*, or *dlAd5NCAT-F7* per cell. After 24 h, cell lysates were generated and CAT gene expression was determined for each infection. CAT activity is expressed as the calculated total CAT expression (percent conversion of substrate to product in a linear-range assay) present in the prepared lysate.

for virus uptake. Given the similarity of the fiber tail domains and the successful construction of *dlAd5NCAT-F7*, it is likely that the strategy used in this study can be extended to replace the Ad5 fiber with fiber from any given serotype.

By combining capsid gene replacement with the CAT reporter gene assay in replication-defective viruses, we are able to determine infectivity as a function of virus entry and uptake to the nucleus. Competition assays indicate that Ad5 and Ad7 enter A549 cells through different primary receptors. When competition assays were done at 37°C at high concentrations of Ad7 and *dlAd5NCAT-F7*, we found competition with Ad5NCAT, whereas the reciprocal experiment did not show this effect. Two possible explanations for this observation include a specific low affinity of Ad7 fiber for the Ad5 receptor at 37°C or saturation of a common internalization pathway. Virus internalization depends not only on the fiber gene for attachment but also on the penton protein and its interaction with specific integrin subsets. Since the group C (Ad5) and group B (Ad7) adenoviruses used in this study use $\alpha_v\beta_{(3)(5)}$ integrins for internalization (17), it is conceivable that at 37°C we are seeing competition at the level of virus internalization and not binding. If this hypothesis were correct, we would predict that a similar phenomenon would occur when Ad5 was used to compete for uptake against Ad7. Since this did not occur, we are left with the initial explanation that there is a slight affinity of Ad7 fiber for Ad5 receptor at 37°C but Ad5 does not demonstrate a similar affinity for Ad7 receptor under these conditions.

In the case of *dlAd5NCAT-F7*, any tropism or differences in CAT gene expression are directly a result of fiber and should not be the result of secondary internalization effects. Our studies on gross infections of the heart, liver, and lung suggest that the majority of cells available for infection in all likelihood contain receptors for both Ad5 and Ad7 present in functionally equal numbers. Assuming that future *in vivo* studies support the data presented in this study with respect to a strong cellular colocalization of Ad5 and Ad7 receptors, differences in tissue tropism for wild-type Ad 5 and Ad7 must be due to events subsequent to virus attachment and entry. Alternatively, there may be subpopulations of lung epithelial cells which we have not yet identified that are exclusively vulnerable to

Ad7 or Ad5 infection. We have found rat epithelial cell lines that are refractory to *dlAd5NCAT-F7* but can be infected by *dlAd5NCAT* (data not shown). We are currently trying to identify a population or subpopulation of lung epithelial cells that demonstrate receptor tropism for *dlAd5NCAT-F7*.

How fiber exchange is involved in the decrease in CAT gene expression in secondary sites of infection (Fig. 7A) is not clearly understood. Factors which may contribute to the observed lower level of CAT expression include (i) the dose of the chimeric virus that is available in the secondary tissues is less than that of Ad5, or (ii) there is an inherent instability of the chimeric virus *in vivo*. These hypotheses are currently under investigation. If the chimeric virus is legitimately limited in its ability to infect secondary sites, it provides an important method to limit an adenovirus infection to a specified target tissue.

The second major conclusion from the use of the fiber chimera was our ability to more clearly define the contribution of the antifiber humoral immune response against adenovirus

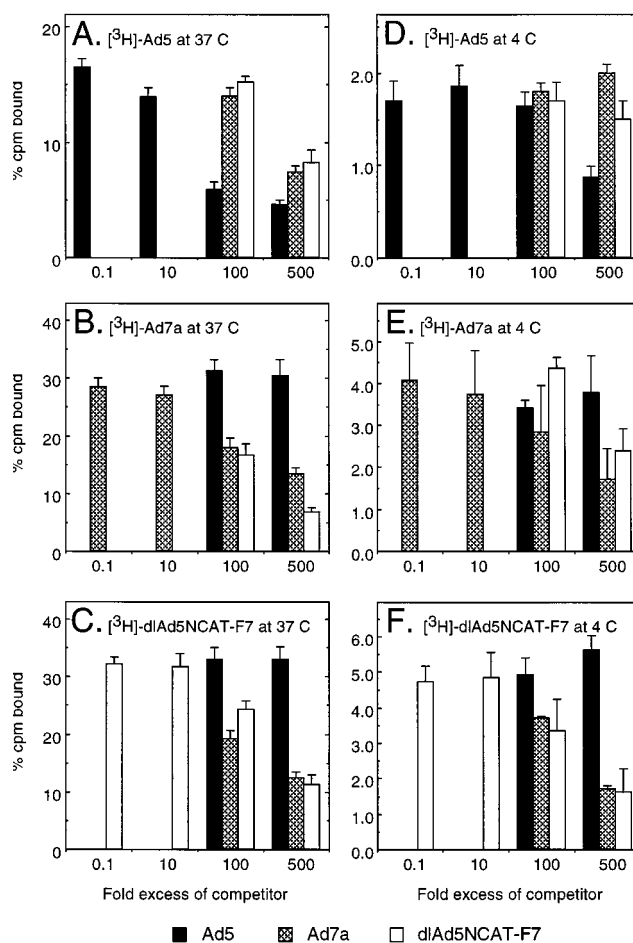


FIG. 6. Binding of ³H-labeled adenovirus to A549 cells in the presence of competitor virus. In direct competition assays carried out at 37°C, competitor virus was premixed with a fixed number of ³H-Ad5NCAT (A), ³H-Ad7 (B), or ³H-*dlAd5NCAT-F7* (C) viral particles. After 1 h of incubation at 37°C, virus binding and uptake were measured as described in Materials and Methods. Alternatively, competition blocking assays were done in which competitor virus, *dlAd5NCAT*, *dlAd5NCAT-F7*, or Ad7, was prebound to A549 cells at 4°C prior to addition of ³H-Ad5NCAT (D), ³H-Ad7 (E), or ³H-*dlAd5NCAT-F7* (F). This assay does not allow virus internalization and is therefore dependent solely on the specificity of virus ligand attachment to the target cell receptor. Virus binding was measured as described in Materials and Methods.

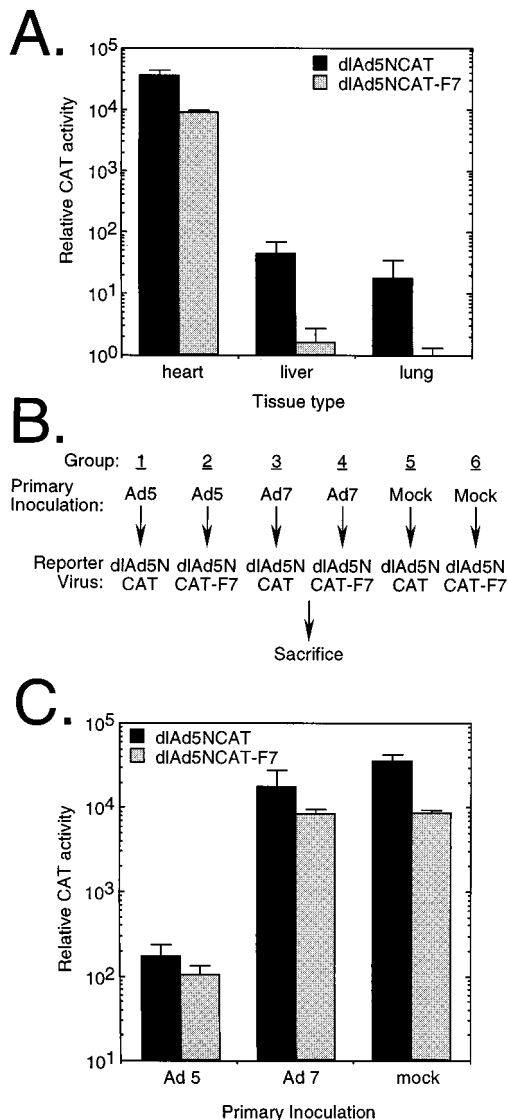


FIG. 7. Characterization of *dlAd5NCAT* and *dlAd5NCAT-F7* infection in vivo. (A) Normalized CAT gene expression in heart, liver, and lung rat tissues following an intramyocardial injection of 5×10^8 adenovirus particles corresponding to *dlAd5NCAT* or *dlAd5NCAT-F7*. Values are means of five animals \pm 1 sample standard deviation. (B) Strategy for successive administrations of adenovirus. Adult female Sprague-Dawley rats received wild-type Ad5 (*sub360*), Ad7a, or PBS intraperitoneally, and 17 days later reporter virus was delivered via an intramyocardial injection. (C) Effect of prior exposure to adenovirus on reporter virus gene expression. Heart tissue samples harvested at sacrifice (22 days after primary inoculation) were assayed for CAT activity. Values are means of five animals \pm 1 sample standard deviation.

infection. From previous studies, it was clear that antihexon antibody is the most effective in neutralizing an adenovirus infection. However, it was also clear that a substantial blocking antibody could be developed against fiber, and depending on the assay system and the immunization protocol, antifiber antibody was considered significant. From the point of view of dissecting the contribution of antifiber antibody to the overall neutralization of adenovirus vectors in vivo gene transfer studies, the chimeric virus clearly demonstrated that under standard repeat administration conditions, the antibody generated against the fiber gene is inconsequential with regard to neutralizing function. Extrapolating from this observation, we

TABLE 1. Neutralizing antibody against adenovirus variants following a single inoculation of adenovirus

Serum ^a	Group ^b	Neutralizing antibody titer ^c against:			
		Ad5	Ad7	<i>dlAd5NCAT</i>	<i>dlAd5NCAT-F7</i>
d.17	1	+++	—	+++	+++
	2	+++	—	+++	+++
	3	—	+++	—	+
	4	—	+++	—	+
	5	—	—	ND	ND
	6	—	—	ND	ND
d.22	6	+++	—	ND	ND

^a d.17 and d.22, serum samples taken at 17 and 22 days after primary inoculation, respectively.

^b Group refers to strategy illustrated in Fig. 7B.

^c Titer (inverse of dilution giving 50% inhibition of infection). +++, >100; +, <100; —, <10; ND, not determined.

would argue that the only capsid protein that is functioning as a dominant neutralizing epitope is the hexon trimer.

If adenovirus neutralizing antibody epitopes functionally reside in a single capsid polypeptide, hexon, then replacement and or mutation of hexon should result in depleted Ad5-neutralizing immune response which cannot be replaced by antibody developed against fiber. Because hexon is involved in a variety of complex protein interactions as the major structural protein of adenovirus, it remains to be seen if the replacement strategy can work in the same fashion as fiber. However, we are currently testing epitopes that have been mapped and found to be likely candidates for partial replacement. The strategy of capsid modification should allow us to further characterize the contribution of the neutralizing epitopes present on the virus capsid and develop strategies which will allow readministration of adenovirus vectors to immunocompetent hosts.

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