Pseudopackaging of Adenovirus Type 5 Genomes into Capsids Containing the Hexon Proteins of Adenovirus Serotypes B, D, or E

PHILOMENA OSTAPCHUK AND PATRICK HEARING*

Department of Molecular Genetics and Microbiology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794-5222

Received 19 July 2000/Accepted 25 September 2000

Adenoviruses (Ad) show promise as a vector system for gene delivery in vivo. However, a major challenge in the development of Ad vectors is the circumvention of the host immune responses to Ad infection, including both the host cytotoxic T-cell response and the humoral response resulting in neutralizing antibodies. One method to circumvent the effect of neutralizing antibodies against an Ad vector is to use different Ad serotypes to deliver the transgene of interest. This approach has been demonstrated with Ad genomes of highly related members of subgroup C. However, it is not known whether an Ad5-based vector DNA molecule can be packaged into capsids of evolutionarily more divergent adenoviruses. The aim of these studies was to determine if capsids containing hexon proteins from other Ad subgroups could package the Ad5 genome. A genetic approach utilizing an Ad5 temperature-sensitive (*ts*) mutant with a mutation in the hexon protein was used. When grown at the nonpermissive temperature, Ad5 *ts*147 replicates normally, providing a source of Ad5 DNA for virus assembly, but does not produce virus particles due to the hexon protein mutation. Coinfection of Ad5 *ts*147 with a wild-type virus of other Ad serotypes (Ad3, Ad4, or Ad9), which supply functional hexon proteins, resulted in the pseudopackaging of the Ad5 DNA genome. Furthermore, the pseudopackaged Ad5 DNA virions obtained in the coinfections were infectious. Therefore, switching hexons did not impair the infectivity or uncoating process of the pseudopackaged virion. Since hexon protein is a major antigenic determinant of the Ad capsid, this approach may prove useful to reduce the antigenicity of therapeutic Ad vectors and allow repeated vector administration.

Considerable progress has been made toward the development of viral vectors for the short- and long-term treatment of disease by gene therapy. Adenoviruses (Ad) show promise as a vector system for gene delivery in vivo for a number of reasons (3, 5, 26). Ad has a wide host cell range and infects both dividing and nondividing cells in vitro and in vivo. Thus, it is possible to transduce quiescent and differentiated cells with Ad to direct the expression of a therapeutic gene. The Ad life cycle has been well characterized, its genome may be readily manipulated in the laboratory, and it is easily grown to high titers in cultured cells. Finally, Ad is a relatively benign human virus that is associated with mild disease and, importantly, is not associated with the development of any human malignancy.

A major challenge in the development of Ad vectors for gene therapy, however, is the circumvention of the host immune responses to viral infection. The host cytotoxic T-cell response to antigens presented by virus infection leads to clearance of Ad-infected cells and thus significantly reduces the efficacy of the therapy. Experiments using animal model systems including nonhuman primates have demonstrated that both antigen-specific and nonspecific immune responses to Ad infection extinguish the expression of a transgene included in the viral vector (22, 43–45, 47). Furthermore, the humoral response of the host to the initial virus inoculum is a significant

barrier to readministration of an Ad vector, which largely limits the utility of the therapy to a single administration (6, 17, 22, 44, 48). Similarly, the results of analyses with human subjects receiving Ad therapeutic vectors suggest that a critical factor which determines the extent of the humoral response of the recipient to Ad inoculation reflects the initial humoral immune state of the host (8, 17). Neutralizing antibodies that prevent the Ad vector from competently infecting its target cell pose a major problem in obtaining a therapeutic effect of vector delivery.

Several different approaches have been tested to escape the limitations set by the host immune response to Ad vectors. The host may be targeted, for example, by oral tolerization (19) or suppression of the immune response by blocking CD4⁺ T-cell activation (20, 25, 46). The viral vector may be targeted, for example, by physically masking the capsid from neutralizing antibodies by encapsidation in polyacid copolymers (2) or covalently attaching a polymer of polyethylene glycol (34). Alternatively, the effect of neutralizing antibodies against an Ad gene therapy vector may be circumvented through transient immunosuppression (4, 9–11, 21, 27) or by utilizing different Ad serotypes to deliver the transgene of interest (23, 28, 29, 32). The latter approach may be accomplished through the development of the genomes of many different Ad serotypes to carry a transgene of interest or through the use of a single viral transgene with encapsidation into the capsids of different Ad serotypes. This has been demonstrated with an Ad5 genome packaged into an Ad2 capsid (15, 35) and offers the facility of having a single vector DNA backbone whose capsid immuno-

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, SUNY at Stony Brook, Stony Brook, NY 11794-5222. Phone: (631) 632-8813. Fax: (631) 632-8891. E-mail: phearing@ms.cc.sunysb.edu.

genicity may be changed by packaging into alternative capsid structures. At present, this approach is limited to the Ad5 and Ad2 serotypes, two very closely related viruses of Ad subgroup C. It is not known whether an Ad5-based vector DNA molecule could be packaged into a more divergent Ad capsid. The necessity of having a wider distribution of Ad vectors from different serotypes is further exemplified by epidemiological studies. One study of a 10-year period from 1967 to 1976 showed that the absolute distribution of antibodies in the worldwide population to the more than 49 serotypes of adenovirus in decreasing order was Ad2, Ad1, Ad7, Ad3, Ad5, Ad6, Ad4, and Ad8, with antibodies against the other serotypes being found on a much less frequent basis (40). Taken together, these results demonstrate that a therapeutic Ad vector system that permitted the exchange of the exterior capsid, and hence the immunogenicity of the virus, with a single viral genome carrying a transgene of interest would be very useful.

Most current Ad vectors are based on serotypes Ad5 and Ad2 because of the extensive research with these viruses, including their genomic sequences. Earlier attempts to exchange the hexon protein of an Ad5-based vector with that of a more distantly related serotype, Ad7, were unsuccessful (15). The authors suggested that incompatibilities of the Ad7 hexon protein with other proteins of Ad5, for example the 100-kDa protein (100K protein) which is involved in hexon assembly and transport to the nucleus or other Ad capsid proteins that interact with hexon in the virus particle, may explain these results. We have tested if the Ad5 genome may be packaged into virus particles that contain hexon proteins of another Ad serotype. Using viruses that represent subtypes B (Ad3), D (Ad9), and E (Ad4), we demonstrate that Ad5 DNA can be packaged in the capsids that contain the hexons of more divergent subtypes. Since hexon is the major antigenic component of the Ad capsid (14, 33), this approach may prove useful to reduce the antigenicity of therapeutic Ad vectors and allow repeated vector administration.

MATERIALS AND METHODS

Viruses and cells. 293 cells (16) and A549 cells (American Type Culture Collection) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% bovine calf serum and the antibiotics penicillin and streptomycin. Virus stocks of Ad3, Ad5, Ad2, Ad4, and Ad9 (American Type Culture Collection) were generated by infection of either 293 or A549 cells at a multiplicity of infection of 200 particles/cell for 1 h at 37°C. Fresh medium was added, and the infected cells were maintained at 37°C until the appearance of a cytopathic effect, usually ~72 h postinfection. A stock of Ad5 *ts*147 (24) was generated following infection of A549 cells by maintenance of the infected cells at 32°C until the appearance of a cytopathic effect. For the preparation of virions, infected cell lysates were prepared by suspension of cells in Tris-buffered saline solution followed by three freeze-thaw cycles and centrifugation at $3,000 \times g$ at 4°C for 15 min. Purified virus particles were prepared by centrifugation over a CsCl₂ step gradient (1.4 to 1.25 g of CsCl₂ per cc) and final purification on a CsCl₂ equilibrium gradient (1.33 of CsCl₂ per cc). Virus particles were quantified by measurement of absorbance at 260 nm, taking 1 optical density unit at 260 nm to be equivalent to 10^{12} particles.

For virus coinfection experiments, A549 cells were infected with Ad5 *ts*147 and the different wild-type Ad serotypes at 200 particle of each virus per cell for 1 h at 37°C, and the cultures were then maintained at the nonpermissive temperature for Ad5 *ts*147 of 39.5°C for 60 to 72 h. Virus particles were purified from coinfecting cells as described above. Secondary infection of A549 cells with mixed populations of virus particles obtained from primary coinfections of Ad5 *ts*147 with the different wild-type Ad serotypes was performed at 1,000 particles per cell to facilitate the detection of both the Ad5 *ts*147 and wild-type genomes in the replication assay.

Determination of packaging efficiencies. A Southern filter hybridization assay was used to quantify the relative amounts of the Ad5 *ts*147 and wild-type DNAs in the coinfection experiments. Ad5 *ts*147 was distinguished from the wild-type viruses by restriction enzyme digestion of the DNAs: *Sph*I for Ad2 and Ad9 or *Mlu*I for Ad3 and Ad4. The products were separated on a 0.8% agarose gel with $1 \times$ TBE (90 mM Tris borate, 2 mM EDTA) and 0.5 μ g of ethidium bromide per ml. The probe for hybridization was a 160-nucleotide (nt) PCR product extending from Ad5 nt 19839 to 19999 within a relatively conserved region of the hexon gene among the different serotypes. The DNA probe was labeled using the AlkPhos Direct labeling system from Amersham Life Sciences; this resulted in the covalent attachment of alkaline phosphatase molecules to the DNA. Filters from the hybridizations were incubated with a fluorescent substrate for alkaline phosphatase, ATTOPHOS (JBL Scientific Inc./Promega). The substrate yields a fluorescent product upon cleavage by alkaline phosphatase, and the signals were imaged on a Storm 860 instrument (Molecular Dynamics) and quantified using Imagequant software version 1.2 (Molecular Dynamics). A titer determination of Ad5 DNA was included in each experiment to ensure that the fluorescent signals obtained were in the linear range of the assay. Since the DNAs of different Ad serotypes have several nucleotide mismatches with the Ad5 probe sequences, a correction factor for quantification of each serotype was determined empirically by averaging the fluorescent signals in several experiments from hybridization of the Ad5 probe to several concentrations of DNA from the other serotypes. The relative correction factor multiplied by the signal (minus background) equals the corrected value for hybridization of the Ad5 probe to a different serotype. The correction factors used in the calculations were as follows: Ad5, 1; Ad2, 2.4; Ad3, 5.5; Ad4, 5.3; and Ad9, 4.7.

The packaging efficiencies of Ad5 *ts*147 in coinfections with different wild-type Ad serotypes were determined by quantifying the relative amounts of packaged DNA isolated from purified virus particles in comparison to the relative amounts of total viral nuclear DNA, as described previously (39). The replication ratio is expressed as the signal from the Ad5 *ts*147 DNA divided by the sum of the signals of viral DNAs of Ad5 *ts*147 and the different wild-type viruses in coinfections of A549 cells. Similarly, the packaged ratio is expressed as the signal from the Ad5 *ts*147 viral DNA divided by the sum of the signals from viral DNAs of Ad5 *ts*147 and the different wild-type viruses in co-infections. The percent packaging efficiency reflects the relative level of Ad5 *ts*147 viral genomes packaged in a coinfection relative to the amount of Ad5 *ts*147 viral genomes replicated. These results are expressed as follows: percent packaging efficiency = [(*ts*147 packaged viral DNA/total packaged viral DNA)/(*ts* replicated DNA/total replicated viral DNA)] $\times$ 100%.

Protein analyses. Hexon protein monomers in virus particles from single infections and coinfections were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining of proteins. Viral proteins from approximately 1×10^9 to 4×10^9 particles from the coinfections and from 0.4×10^{10} to 2×10^{10} particles from single infections were solubilized in a final concentration of $1 \times$ sample buffer (30 mM Tris [pH 6.8], 3% SDS, 5% glycerol, 0.002% bromophenol blue, 5% β -mercaptoethanol). The proteins were separated by electrophoresis on an SDS-7.5% polyacrylamide gel as described previously (37) and stained with silver using an abbreviated version of the method of Merrill et al. (31). Briefly, proteins were fixed in the polyacrylamide gel by incubation in 50% methanol-10% acetic acid followed by successive incubation of the gel in 10% methanol-5% acetic acid, 0.0326 mM dithiothreitol, and 12 mM silver nitrate. The signal was developed by incubation in 2% potassium carbonate containing 0.044% formaldehyde, and development of the staining was stopped by incubation in 1% acetic acid.

RESULTS

A genetic approach was used to address whether an Ad5 DNA genome could be packaged into the capsids of other, more divergent serotypes of Ad. Specifically, we chose to examine whether the hexon protein, one of the major serotype-specific antigenic determinants of Ad (14, 33), could be switched from Ad5 subtype C to other Ad subtypes (B, D, or E). Ad5 packaging is of particular interest since this is the major serotype of Ad used for the generation of Ad vectors for gene therapy (3, 5, 26). We took advantage of Ad5 *ts* mutants with mutations in the hexon protein. When grown at the nonpermissive temperature, *ts* hexon mutants replicate normally to provide a source of Ad5 DNA for virus assembly but do not

FIG. 1. Diagnostic restriction endonuclease fragments of viral DNAs from different Ad serotypes. Southern blot analysis was used to distinguish viral DNAs of different Ad serotypes. Purified viral DNAs were digested with *Mlu*I (lane 1, Ad3; lane 2, Ad4; lane 3, Ad5 *ts*147) or *Sph*I (lane 4, Ad2; lane 5, Ad5 *ts*147; lane 6, Ad9). The blot was hybridized with a 160-nt Ad5 probe corresponding to coding sequences for hexon protein.

produce virus particles due to the hexon protein mutation (24). We surmised that assembly of the Ad5 DNA genome into infectious virus particles could occur by pseudopackaging, that is, the packaging of Ad5 DNA into virus particles provided by coinfection with a wild-type virus of another Ad serotype that would supply a functional hexon protein. The hexon mutant used was Ad5 *ts*147 (24). At the permissive temperature of 32°C, this virus grows to wild-type levels; at the nonpermissive temperature of 39.5°C, the infectious-virus yield of Ad5 *ts*147 is reduced by 5 log units (24) and the virus does not make detectable virus particles (data not shown) because the hexon protein synthesized cannot be transported to the nucleus (24).

An assay was developed to distinguish the Ad5 *ts*147 DNA genome from that of a coinfecting wild-type virus of another Ad serotype in order to measure packaging efficiencies in coinfection experiments. A quantifiable Southern blot assay was used. A series of restriction endonucleases were tested empirically for their ability to generate DNA fragments that distinguish the genomes of different Ad serotypes. Viral DNA fragments were hybridized with a probe derived from Ad5 that corresponds to a relatively conserved region of the hexon protein and cross-hybridizes with the genomes of different Ad serotypes. The mobilities of these diagnostic fragments on an agarose gel are shown in Fig. 1. The genome of Ad5 *ts*147 could be separated from that of wild-type Ad3 and Ad4 by digestion of the DNAs with the restriction endonuclease *Mlu*I. Similarly, Ad2 and Ad9 were separated from Ad5 *ts*147 by digestion with *Sph*I. The relative intensities of the hybridization signals from each Ad serotype in this assay were used to normalize the efficiency of cross-reactivity of the Ad5 DNA probe with the DNAs of the other Ad serotypes. These correction values (see Materials and Methods) were used in subsequent experiments to measure viral DNA replication and packaging efficiencies in coinfections of cells with Ad5 *ts*147 and different wild-type Ad serotypes.

A549 cells, a lung epithelial cell line permissive for infection by different Ad serotypes, were coinfecting with an equal number of particles of Ad5 *ts*147 and either Ad3 (subgroup B), Ad4 (subgroup E), or Ad9 (subgroup D). Additionally, Ad5 *ts*147

FIG. 2. Ad5 *ts*147 DNA is packaged into virions containing hexon proteins of other Ad serotypes. Southern blot analysis was used to quantify replicated and packaged viral DNAs from coinfections of A549 cells with Ad5 *ts*147 and different wild-type Ad serotypes. Lanes 1 to 5, dilution of Ad5 DNA (lane 1, 495 ng; lane 2, 158 ng; lane 3, 59 ng; lane 4, 20 ng; lane 5, 10 ng); lanes 6 and 11, Ad5 *ts*147 DNA digested with *Sph*I or *Mlu*I, respectively; lanes 7, 8, 12, and 13, replicated viral DNAs present in total nuclear DNA isolated from cells coinfecting with Ad5 *ts*147 plus Ad2 (lane 7), Ad9 (lane 8), Ad3 (lane 12), and Ad4 (lane 13); lanes 9, 10, 14, and 15, packaged viral DNAs present in purified virus particles isolated from cells coinfecting with Ad5 *ts*147 plus Ad2 (lane 9), Ad9 (lane 10), Ad3 (lane 14), and Ad4 (lane 15). The asterisks indicate the Ad5 *ts*147 fragment in each coinfection. replic., replicated; pack., packaged.

was coinfecting with Ad2, which is a member of the same subgroup, C. A549 cells were chosen for these analyses since the different Ad serotypes under study all productively infect this cell line and produce comparable levels of infectious virus particles on a per cell basis. Infected cells were maintained at the nonpermissive temperature (39.5°C) for the Ad5 *ts*147 mutant (24). At this temperature, the wild-type viruses of different Ad serotypes were only moderately reduced (ca. two- to threefold) in yield when grown individually, in comparison to the virus yields obtained at 37°C (data not shown). After 3 days of infection, the entire cell population was infected as judged by the complete cytopathic effect of the cell monolayer cultures. Infected cells were collected, and a fraction of the cells were used to isolate total nuclear DNA to examine the replication levels of the coinfecting viruses. Virus particles were isolated from the remaining cells by CsCl₂ equilibrium centrifugation, and the relative packaging efficiencies of Ad5 *ts*147 in comparison to the different wild-type Ad serotypes were determined. The results of the Southern blot analysis of a representative coinfection are shown in Fig. 2. The results of three independent coinfection experiments were quantified and are presented in Table 1.

As expected, replication of Ad5 *ts*147 at the restrictive temperature of 39.5°C was readily detected (Fig. 2, lanes 6 and 11) in a single infection while no virus assembly was observed (data not shown). In coinfection experiments, Ad5 *ts*147 and the different wild-type Ad serotypes all replicated, although to somewhat variable levels depending on the coinfecting viruses

TABLE 1. Replication and packaging efficiency of Ad5 *ts147* in coinfections with different wild-type Ad serotypes

Coinfecting virus	Replication ratio ^a	Packaged ratio ^b	% Packaging efficiency ^c
Ad2	0.30 ± 0.07	0.11 ± 0.04	37
Ad3	0.27 ± 0.12	0.017 ± 0.008	6.3
Ad4	0.59 ± 0.09	0.06 ± 0.01	10
Ad9	0.72 ± 0.06	0.032 ± 0.019	4.4

^a The replication ratio is the level of Ad5 *ts147* viral DNA divided by the level of total viral DNA in coinfecting A549 cells.

^b The packaged ratio is the level of packaged Ad *ts147* DNA divided by the level of total packaged viral DNA.

^c The percent packaging efficiency = [(Ad5 *ts147* packaged viral DNA/total packaged viral DNA)/(Ad *ts147* replicated DNA/total replicated viral DNA)] × 100%.

(lanes 7, 8, 12, and 13). Most importantly, the Ad5 *ts147* genome was rescued for packaging by coinfection with wild-type viruses of different Ad serotypes (Fig. 2, lanes 9, 10, 14, and 15). The results of quantifying the relative levels of Ad5 *ts147* genomes packaged with the different wild-type viruses are shown in Table 1 (percent packaging efficiency). Coinfection of Ad5 *ts147* with the closely related virus Ad2 resulted in efficient packaging of the Ad5 genome (37% of Ad2 levels). Significant but less efficient packaging of Ad5 *ts147* DNA was evident with the other Ad serotypes tested, where a four- to ninefold reduction in packaged Ad5 *ts147* DNA was observed with Ad3, Ad4, and Ad9 in comparison to coinfection with Ad2. Thus, we conclude that the hexon proteins of Ad2, Ad3, Ad4, and Ad9 provide the necessary functional properties to allow the encapsidation of an Ad5 genome into virus particles.

The hexon proteins present in the virus particles obtained from coinfection experiments were examined. The hexon composition of virus particles obtained from two different coinfection experiments is shown in Fig. 3. Hexon monomers of Ad5 *ts147* may be separated from hexon monomers of Ad2, Ad3, Ad4, and Ad9 by virtue of their different electrophoretic mobilities in SDS-PAGE (Fig. 3, lane 4, Ad5 *ts147*, versus lanes 5 to 8, Ad2, Ad3, Ad4, and Ad9, respectively). In coinfections of Ad5 *ts147* with Ad4 and Ad9, the hexon proteins found in virus particles correspond to the hexon produced by the wild-type virus. Lanes 1 to 4 show a dilution of Ad5 *ts147* particles and the resulting hexon protein signal detected by silver staining. In a coinfection with Ad4 (Ad5 *ts147* at 10% packaging efficiency), we would anticipate an Ad5 *ts147* hexon signal similar to the intensity observed for 1.4×10^8 particles in the Ad5 dilution if the Ad5 *ts147* hexon was present in the purified virus particles. Similarly, in a coinfection with Ad9 (Ad5 *ts147* at 4.4% packaging efficiency), we would anticipate an Ad5 *ts147* hexon signal similar to the intensity observed for 6×10^7 particles if the Ad5 *ts147* hexon was present in the purified virus particles. Clearly, no Ad5 *ts147* hexon monomers were observed in the particles isolated from the cells coinfecting with Ad5 *ts147* and either Ad4 or Ad9. Unexpectedly, the hexons from the viral particles obtained from coinfection with Ad5 *ts147* and Ad2 were mixtures of Ad2 and Ad5 *ts147* hexon proteins (lanes 9 and 13). Hexon protein bands of almost equivalent intensity for Ad2 and Ad5 *ts147* were observed, suggesting that the defect in the nuclear transport of the Ad5

FIG. 3. Analysis of hexon proteins in mixed virus particles obtained from coinfection of A549 cells with Ad5 *ts147* and different wild-type Ad serotypes. Hexon protein present in purified virus particles was analyzed by silver staining of viral proteins separated by SDS-PAGE. Lanes 1 to 4, hexon protein from a dilution of Ad5 *ts147* particles (lane 1, 6×10^7 particles; lane 2, 2×10^8 particles; lane 3, 6×10^8 particles; lane 4, 2×10^9 particles); *ts147* particles were produced in cells infected at the permissive temperature of 32°C; lanes 5 to 8, hexon proteins of different wild-type Ad serotypes; lanes 9 to 16, the results from two experiments (Expt. 1 and Expt. 2) examining the hexon content of mixed virus particles obtained from coinfection of A549 cells with Ad5 *ts147* and Ad2 (lanes 9 and 13), Ad3 (lanes 10 and 14), Ad4 (lanes 11 and 15), or Ad9 (lanes 12 and 16).

ts147 hexon was rescued by coinfection with Ad2. In coinfections of Ad5 *ts147* with Ad3, a band at the apparent mobility of Ad5 *ts147* hexon was apparent in purified virus particles (lanes 10 and 14); however, a band of identical mobility was present in Ad3 particles isolated from a single-virus infection as well (lane 6). In addition, there was no apparent increase in the relative intensity of the faster-migrating band seen in the particles from the coinfection with Ad3 compared to the single infection (lane 6 versus lanes 10 and 14). In fact, the relative intensity of this band appeared lower in the particles from the coinfection with Ad3. Thus, we believe the presence of the Ad5 *ts147* hexon in particles from coinfections with wild-type Ad3 to be minimal or nonexistent. We conclude that Ad5 *ts147* DNA is packaged into virus particles utilizing solely the hexon protein of Ad3, Ad4, and Ad9.

To determine if the Ad5 *ts147* DNA-containing particles that were pseudopackaged with the hexons of other Ad serotypes were infectious, A549 cells were infected with equal numbers of the mixed particles obtained from each of the coinfection experiments. The infectivity of the Ad5 *ts147* pseudopackaged virions was measured by Southern blot analysis of total nuclear DNA isolated at 6 and 48 h after infection (Fig. 4). A 6-h time point was used to attempt to detect input viral DNAs before the onset of replication, but the signals were below the level of detection (Fig. 4, lanes 1 to 4). In contrast, replicated viral DNA was readily detected at the 48-h time point, indicative of virus infection and genome amplification (lanes 5 to 8). The significant increase in viral DNAs observed at the 48-h time point in comparison to the 6-h time point clearly demonstrates that the coinfecting viruses were infectious and replicated. Quantification of these results is shown in Table 2. In each case, the relative replication ratio of Ad5 *ts147* in comparison to the coinfecting wild-type viruses was very similar to those found in the initial coinfection experiments (Table 1). These results demonstrate that the pseudopackaged Ad5 *ts147* viruses were infectious. In summary, we conclude that Ad5 DNA can be packaged into virus particles that con-

FIG. 4. Pseudopackaged particles of Ad5 *ts147* DNA are infectious. Southern blot analysis was used to quantify the replication of viruses recovered in the coinfections of A549 cells. Lanes 1 to 4, DNAs isolated 6 h postinfection from cells infected with mixed particles from the Ad5 *ts147* plus Ad2 (lane 1), Ad4 (lane 2), Ad3 (lane 3), or Ad9 (lane 4); lanes 5 to 8, DNAs isolated 48 h postinfection from cells infected with mixed particles from Ad5 *ts147* plus Ad2 (lane 5), Ad4 (lane 6), Ad3 (lane 7), or Ad9 (lane 8). The asterisks indicate the Ad5 *ts147* DNA fragments.

tain the hexon proteins of other Ad serotypes and that these particles are infectious.

DISCUSSION

A limitation of using Ad as a therapeutic vector for gene therapy is the host immunological responses to the virus. One way of circumventing a preexisting humoral immunity to a particular serotype of Ad is to deliver the therapeutic viral genome in the capsid of a different serotype that would not be neutralized by the antibodies present. It has been shown previously that the genome of Ad5 can be packaged in the capsid of Ad2 (15, 35). Ad5 and Ad2 are evolutionarily very closely related viruses (1); both are members of subgroup C. The aim of the experiments presented here was to determine whether virus particles containing the hexons of Ads from other subgroups are capable of packaging the Ad5 genome. Utilizing representatives of subgenera B (Ad3) D (Ad9), and E (Ad4), our results showed that, indeed, the Ad5 genome could be

pseudopackaged into capsids containing the hexons derived from viruses that are not members of subgroup C, albeit at a lower efficiency. These results demonstrated that there is no subgroup specificity for packaging of the Ad5 genome, at least with regard to hexons from subgroups B, D, and E. That the hexons can be switched may not be surprising, given the conservation of the amino acid sequence across subtypes—79% (for hexon from a subgroup D virus, Ad48) to 88% (for the subgroup C virus Ad5) of the amino acids are identical to those of the Ad2 hexon. More than 99% of the variability between the hexons of different Ad serotypes is accounted for in seven hypervariable regions (12) that map to the exterior of the protein and include the serotype-specific epitopes (36).

There are at least 12 other proteins that contribute to the capsid structure of the virion and several additional proteins that participate temporarily in the assembly process (41). In the coinfections, all of these proteins were synthesized by both the Ad5 *ts147* and wild-type viruses of different serotypes, except for hexon and possibly pVI. In addition to the mutation in the hexon gene of Ad5 *ts147*, growth of this virus at the nonpermissive temperature results in reduced levels of pVI compared to the levels seen with wild-type Ad (24). We do not know if this reduction occurred in the coinfections as well, but if so, pVI, which interacts with hexons, would be replaced by the pVI of the coinfecting wild-type virus in these experiments. It has been shown previously that pVI from different subtypes of Ad, including nonprimate Ads, can bind the hexon trimers of Ad2 (30). It would be interesting to know if any of the other structural and nonstructural proteins segregate in a subtype-specific fashion or if the subtype source for the proteins in the capsids from these mixed infections is random. We examined the population of pentons from viruses obtained in the coinfections; Ad3, Ad4, and Ad9 penton monomers can be distinguished from that of Ad5 penton by their different mobilities using SDS-PAGE. We observed pentons from both wild-type viruses of different serotypes and Ad5 with the viruses obtained in the coinfection experiments (data not shown), demonstrating that the pentons of Ad5 can interact with the hexons of subgroups other than subgroup C. Interestingly, Gall et al. (15) were unable to generate an Ad5/Ad7 virus chimera that had the Ad5 hexon gene replaced by the Ad7 hexon gene, although they could generate an Ad5 virus with an Ad2 hexon replacement. Ad7 is a member of subgenus B. They surmised that there may be an incompatibility of the Ad7 hexon in its interaction with other Ad5 proteins, for example, the 100K protein. The 100K protein binds hexon and is involved in the trimerization and nuclear translocation of hexons (7). The fact that the Ad5 genome was packaged into virus particles containing subtype B (Ad3) hexons, when all other proteins of the subtype B were present, supports their interpretation.

The subtypes used to provide the helper function in these experiments are closer evolutionary relatives to subtype C than to subtypes A and F (1). B and E are more closely related to each other than to C, and D probably diverged in its evolution between the C and B viruses (1). Not only are these viruses related by their DNA sequences, but also viruses of subtypes B, C, D, and E all cause ocular and respiratory infections (18). We were also interested in determining if Ad5 genomes could be packaged in capsids with the hexons from Ad subtypes less closely related to subtype C, that is, from subgroups A and F.

TABLE 2. Pseudopackaged Ad5 particles are infectious

Virus used with Ad5 <i>ts147</i>	Relative replication ratio ^a
Ad2	0.24, 0.38
Ad3	0.19, 0.22
Ad4	0.69, 0.5
Ad9	0.55

^a The relative replication ratio of Ad5 *ts147* was calculated by dividing the replication ratio of Ad5 *ts147* from the infection of A549 cells with mixed virus particles by the original packaging efficiency shown in Table 1. The data reflect the results of two independent experiments for Ad2, Ad3, and Ad4 and one experiment for Ad9.

FIG. 5. Comparison of the nucleotide sequences of the packaging regions of the different Ad serotypes. Shown are the nucleotides of the packaging region of Ad5 (nt 242 to 386) and the equivalent regions of Ad2, Ad3, Ad4, and Ad9. A1, A2, A5, and A6 refer to the A repeats identified in Ad5 that are required for efficient packaging of the viral DNA, with the conserved TTTGN₈CG consensus indicated in the dark boxes (where N is any nucleotide). Similarly conserved sequences are also indicated in the nucleotide sequences of the other serotypes.

Consistent with the evolutionary distance from Ad5, these viruses also have a different tropism, since they infect cells of the gastrointestinal tract (18). We attempted to use Ad12, a member of subgroup A, in pseudopackaging experiments. Results from these experiments, however, were inconclusive because Ad12 did not grow reproducibly in mixed infections of A549 cells with Ad5 *ts147*. We could not use Ad40 and Ad41 of subgroup F in coinfection experiments in A549 cells since these viruses do not grow well in established cell culture lines (42).

Packaging of the Ad genome occurs by recognition of packaging sequences at the left end of the viral genome (38) and, presumably, by the incorporation of the DNA into a preformed viral capsid. At present, no specific viral protein(s) has been conclusively identified that directs the packaging event. However, many DNA viruses do require a viral protein for initiating packaging (13), and it seems likely that Ad is no exception. Furthermore, there may be a cellular protein(s) involved in packaging, referred to as P complex (39). This complex binds the packaging sequences of Ad5 in a manner consistent with playing a role in the packaging process. It is likely that the complex would bind the packaging sequences of Ad2, which are virtually identical to those of Ad5 (Fig. 5). However, it is not known whether this complex binds the equivalent packaging regions of Ad3, Ad4, and Ad9. The fact that the Ad5 genome was pseudopackaged into capsids containing hexons

from viruses of subgenera B, D, and E demonstrates that the communication, whether direct or indirect, between the packaging factor(s) of Ad5 and the hexons from the different Ad serotypes was not impaired. An alignment of the nucleotide sequences of the packaging regions of the Ad genomes used in these experiments is shown in Fig. 5. The A repeat elements identified in the genome of Ad5 that are crucial for packaging include a conserved TTTG followed by 8 nt and a CG, as indicated in the figure (38). There is strong conservation of these sequences, particularly within the equivalent A5 and A6 repeats among the different subtypes, suggesting that these viruses may use the same packaging factors.

The pseudopackaged Ad5 virions that were obtained in the coinfections were infectious. These pseudopackaged Ad5 virions were infectious at a level similar to the Ad5 *ts147* genome packaged in virus particles containing its own hexons. Therefore, switching hexons, and possibly other structural proteins, does not impair the infectivity or uncoating process of the pseudopackaged virion. These results demonstrate that the Ad5 genome can be packaged not only within the hexon coat of a virus from the same subgenera but also in a broad range of viruses containing hexons from other subgenera as well. This expands the possibilities for methods of evading the preexisting immunity of the host when using Ad as a therapeutic vector. For this approach to be feasible for the production of gene therapy vectors, inhibition of the packaging of viral genomes of the wild-type viruses of different Ad serotypes will be required. The Cre-Lox system (reviewed in reference 26) could be utilized to inhibit the packaging of the genomes of different Ad serotypes while allowing the packaging of an Ad5-based vector. This approach could be used to scale up the production of pseudopackaged Ad vectors.

ACKNOWLEDGMENTS

We thank Ece Erturk, Jared Evans, Amy Ostrom, and Ziv Sandalon for many helpful discussions and critical reviews of the manuscript. We thank Gia Feeney for excellent technical assistance and Hamish Young for generously providing Ad5 *ts147*.

This research was supported by Public Health Service grant AI41636.

REFERENCES

- Bailey, A., and V. Mautner. 1994. Phylogenetic relationships among adenovirus serotypes. *Virology* **205**:438–452.
- Beer, S. J., C. B. Matthews, C. S. Stein, B. D. Ross, J. M. Hilfinger, and B. L. Davidson. 1998. Poly(lactic-glycolic) acid copolymer encapsulation of recombinant adenovirus reduces immunogenicity in vivo. *Gene Ther.* **5**:740–746.
- Benihoud, K., P. Yeh, and M. Perricaudet. 1999. Adenovirus vectors for gene delivery. *Curr. Opin. Biotechnol.* **10**:440–447.
- Bouvet, M., B. Fang, S. Ekmekcioglu, L. Ji, C. D. Bucana, K. Hamada, E. A. Grimm, and J. A. Roth. 1998. Suppression of the immune response to an adenovirus vector and enhancement of intratumoral transgene expression by low-dose etoposide. *Gene Ther.* **5**:189–195.
- Brenner, M. 1999. Gene transfer by adenovectors. *Blood* **94**:3965–3967.
- Bromberg, J. S., L. A. Debruyne, and L. Qin. 1998. Interactions between the immune system and gene therapy vectors: bidirectional regulation of response and expression. *Adv. Immunol.* **69**:353–409.
- Cepko, C. L., and P. A. Sharp. 1982. Assembly of adenovirus major capsid protein is mediated by a nonviral protein. *Cell* **31**:407–415.
- Chirmule, N., K. Propert, S. Magosin, Y. Qian, R. Qian, and J. Wilson. 1999. Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther.* **6**:1574–1583.
- Chirmule, N., A. Truneh, S. E. Haecker, J. Tazelaar, G. Gao, S. E. Raper, J. V. Hughes, and J. M. Wilson. 1999. Repeated administration of adenoviral vectors in lungs of human CD4 transgenic mice treated with a nondepleting CD4 antibody. *J. Immunol.* **163**:448–455.
- Christ, M., M. Lusk, F. Stoeckel, D. Dreyer, A. Dieterle, A. I. Michou, A. Pavirani, and M. Mehtali. 1997. Gene therapy with recombinant adenovirus

- vectors: evaluation of the host immune response. *Immunol. Lett.* **57**:19–25.
11. Cichon, G., and M. Strauss. 1998. Transient immunosuppression with 15-deoxyspergualin prolongs reporter gene expression and reduces humoral immune response after adenoviral gene transfer. *Gene Ther.* **5**:85–90.
 12. Crawford-Miksza, L., and D. P. Schnurr. 1996. Analysis of 15 adenovirus hexon proteins reveals the location and structure of seven hypervariable regions containing serotype-specific residues. *J. Virol.* **70**:1836–1844.
 13. Fujisawa, H., and P. Hearing. 1994. Structure, function and specificity of the DNA packaging signals in double-stranded DNA viruses. *Semin. Virol.* **5**:5–13.
 14. Gall, J., A. Kass-Eisler, L. Leinwand, and E. Falck-Pedersen. 1996. Adenovirus type 5 and 7 capsid chimera: fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. *J. Virol.* **70**:2116–2123.
 15. Gall, J. G. D., R. Crystal, and E. Falck-Pedersen. 1998. Construction and characterization of hexon-chimeric adenoviruses: specification of adenovirus serotype. *J. Virol.* **72**:10260–10264.
 16. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairu. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**:59–72.
 17. Harvey, B.-G., R. Hackett, T. El-Sawy, T. K. Rosengart, E. A. Hirschowitz, M. D. Lieberman, M. L. Lesser, and R. G. Crystal. 1999. Variability of human systemic humoral immune responses to adenovirus gene transfer vectors administered to different organs. *J. Virol.* **73**:6729–6742.
 18. Horwitz, M. S. 1996. Adenoviruses, p. 2149–2171. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fields virology*, 3rd ed. Raven Press, Philadelphia, Pa.
 19. Ilan, Y., R. Prakash, A. Davidson, Jona, G. Drogue, M. S. Horwitz, N. R. Chowdhury, and J. R. Chowdhury. 1997. Oral tolerization to adenoviral antigens permits long-term gene expression using recombinant adenoviral vectors. *J. Clin. Invest.* **99**:1098–1106.
 20. Jooss, K., L. A. Turka, and J. M. Wilson. 1998. Blunting of immune responses to adenoviral vectors in mouse liver and lung with CTLA4Ig. *Gene Ther.* **5**:309–319.
 21. Kaplan, J. M., and A. E. Smith. 1997. Transient immunosuppression with deoxyspergualin improves longevity of transgene expression and ability to readminister adenoviral vector to the mouse lung. *Hum. Gene Ther.* **8**:1095–1104.
 22. Kaplan, J. M., J. A. St. George, S. E. Pennington, L. D. Keyes, R. P. Johnson, S. C. Wadsworth, and A. E. Smith. 1996. Humoral and cellular immune responses of nonhuman primates to long-term repeated lung exposure to Ad2/CFTR-2. *Gene Ther.* **3**:117–127.
 23. Kass-Eisler, A., L. Leinwand, J. Gall, B. Bloom and E. Falck-Pedersen. 1996. Circumventing the immune response to adenovirus-mediated gene therapy. *Gene Ther.* **3**:154–162.
 24. Kauffman, R. S., and H. S. Ginsberg. 1976. Characterization of a temperature-sensitive, hexon transport mutant of type 5 adenovirus. *J. Virol.* **19**:643–658.
 25. Kay, M. A., A. X. Holterman, L. Meuse, A. Gown, H. D. Ochs, P. S. Linsley, and C. B. Wilson. 1995. Long-term hepatic adenovirus-mediated gene expression in mice following CTLA4Ig administration. *Nat. Genet.* **11**:191–197.
 26. Kochanek, S. 1999. High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum. Gene Ther.* **10**:2451–2459.
 27. Lochmuller, H., B. J. Petrof, G. Pari, N. Larochelle, V. Dodelet, Q. Wang, C. Allen, S. Prescott, B. Massie, J. Nalbantoglu, and G. Karpati. 1996. Transient immunosuppression by FK506 permits a sustained high-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic (mdx) mice. *Gene Ther.* **3**:706–716.
 28. Mack, C. A., W. R. Song, H. Carpenter, T. J. Wickham, I. Kovesdi, B. G. Harvey, C. J. Magovern, O. W. Isom, T. Rosengart, E. Falck-Pedersen, N. R. Hackett, R. G. Crystal, and A. Mastrangeli. 1997. Circumvention of anti-adenovirus neutralizing immunity by administration of an adenoviral vector of an alternate serotype. *Hum. Gene Ther.* **8**:99–109.
 29. Mastrangeli, A., B. G. Harvey, J. Yao, G. Wolff, I. Kovesdi, R. G. Crystal, and E. Falck-Pedersen. 1996. “Sero-switch” adenovirus-mediated in vivo gene transfer: circumvention of anti-adenovirus humoral immune defenses against repeat adenovirus vector administration by changing the adenovirus serotype. *Hum. Gene Ther.* **7**:79–87.
 30. Matthews, D. A., and W. C. Russell. 1994. Adenovirus protein-protein interactions: hexon and protein VI. *J. Gen. Virol.* **75**:3365–3374.
 31. Merrill, C. R., M. E. Bisher, M. Harrington, and A. C. Steven. 1988. Coloration of silver-stained protein bands in polyacrylamide gels is caused by light scattering from silver grains of characteristic sizes. *Proc. Natl. Acad. Sci. USA* **85**:453–457.
 32. Moffatt, S., J. Hays, H. HogenEsch, and S. K. Mittal. 2000. Circumvention of vector-specific neutralizing antibody response by alternating use of human and non-human adenoviruses: implications in gene therapy. *Virology* **272**:159–167.
 33. Norby, E. 1969. Structural and functional diversity of adenovirus capsid components. *J. Gen. Virol.* **5**:221–256.
 34. O’Riordan, C. R., A. Lachapelle, C. Delgado, V. Parkes, S. E. Wadsworth, A. E. Smith, and G. E. Francis. 1999. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum. Gene Ther.* **10**:1349–1358.
 35. Parks, R., C. Eveleigh, and F. Graham. 1999. Use of helper-dependent adenoviral vectors of alternative serotypes permits repeat vector administration. *Gene Ther.* **6**:1565–1573.
 36. Rux, J. J., and R. M. Burnett. 2000. Type-specific epitope locations revealed by X-ray crystallographic study of adenovirus type 5 hexon. *Mol. Ther.* **1**:18–30.
 37. Sarnow, P., P. Hearing, C. Anderson, N. Reich, and A. J. Levine. 1982. Identification and characterization of an immunologically conserved adenovirus early region 11,000 Mr protein and its association with the nuclear matrix. *J. Mol. Biol.* **162**:565–583.
 38. Schmid, S. I., and P. Hearing. 1999. Adenovirus assembly, p. 85–89. In P. Seth (ed.), *Adenoviruses: basic biology to gene therapy*. R. G. Landes, Austin, Tex.
 39. Schmid, S. I., and P. Hearing. 1998. Cellular components interact with adenovirus type 5 minimal DNA packaging domains. *J. Virol.* **72**:6339–6347.
 40. Schmitz, H., R. Wigand, and W. Henrich. 1983. Worldwide epidemiology of human adenovirus infections. *Am. J. Epidemiol.* **117**:455–466.
 41. Shenk, T. 1996. Adenoviridae: the viruses and their replication, p. 2111–2148. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fields virology*, 3rd ed. Raven Press, Philadelphia, Pa.
 42. Tiemessen, C. T., and A. H. Kidd. 1994. Adenovirus type 40 and 41 growth in vitro: host range diversity reflected by differences in patterns of DNA replication. *J. Virol.* **68**:1239–1244.
 43. Tripathy, S. K., H. B. Black, E. Goldwasser, and J. M. Leiden. 1996. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nat. Med.* **2**:545–550.
 44. Yang, Y., Q. Li, H. C. Ertl, and J. M. Wilson. 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* **69**:2004–2015.
 45. Yang, Y., F. A. Nunes, K. Berencsi, E. E. Furth, E. Gonczol, and J. M. Wilson. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* **91**:4407–4411.
 46. Yang, Y., Q. Su, I. S. Grewal, R. Schilz, R. A. Flavell, and J. M. Wilson. 1996. Transient subversion of CD40 ligand function diminishes immune responses to adenovirus vectors in mouse liver and lung tissues. *J. Virol.* **70**:6370–6377.
 47. Yang, Y., Q. Su, and J. M. Wilson. 1996. Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. *J. Virol.* **70**:7209–7212.
 48. Ye, S., S. Wittereder, K. Tang, C. O’Sullivan, and B. C. Trapnell. 1994. Adenovirus-mediated gene transfer for cystic fibrosis: quantitative evaluation of repeated in vivo administration to the lung. *Gene Ther.* **1**:192–200.