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Selective Targeting of Human Cells by a Chimeric Adenovirus Vector Containing a Modified Fiber Protein

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The adenovirus fiber protein is responsible for attachment of the virion to unidentified cell surface receptors. There are at least two distinct adenovirus fiber receptors which interact with the group B (Ad3) and group C (Ad5) adenoviruses. We have previously shown by using expressed adenovirus fiber proteins that it is possible to change the specificity of the fiber protein by exchanging the head domain with another serotype which recognizes a different receptor (S. C. Stevenson et al., J. Virol. 69:2850–2857, 1995). A chimeric fiber cDNA containing the Ad3 fiber head domain fused to the Ad5 fiber tail and shaft was incorporated into the genome of an adenovirus vector with E1 and E3 deleted encoding β-galactosidase to generate Av9LacZ4, an adenovirus particle which contains a chimeric fiber protein. Western blot analysis of the chimeric fiber vector confirmed expression of the chimeric fiber protein and its association with the adenovirus capsid. Transduction experiments with fiber protein competitors demonstrated the altered receptor tropism of the chimeric fiber vector compared to that of the parental Av1LacZ4 vector. Transduction of a panel of human cell lines with the chimeric and parental vectors provided evidence for a different cellular distribution of the Ad5 and Ad3 receptors. Three cell lines (THP-1, MRC-5, and FaDu) were more efficiently transduced by the vector containing the Ad3 fiber head than by the Ad5 fiber vector. In contrast, human coronary artery endothelial cells were transduced more readily with the vector containing the Ad5 fiber than with the chimeric fiber vector. HeLa and human umbilical vein endothelial cells were transduced at equivalent levels compared with human diploid fibroblasts, which were refractory to transduction with both vectors. These results provide evidence for the differential expression of the Ad5 and Ad3 receptors on human cell lines derived from clinically relevant target tissues. Furthermore, we show that exchange of the fiber head domain is a viable approach to the production of adenovirus vectors with cell-type-selective transduction properties. It may be possible to extend this approach to the use of ligands for a range of different cellular receptors in order to target gene transfer to specific cell types at the level of transduction.

In susceptible cells, the adenovirus cellular entry pathway is an efficient process which involves two separate cell surface events (22). First, a high-affinity interaction between the adenovirus capsid fiber protein and an unidentified cell surface receptor mediates the attachment of the adenovirus particle to the cell surface. A subsequent association of the penton with the cell surface integrins αvβ3 and αvβ5, which act as coreceptors, potentiates virus internalization (22). Competition binding experiments with intact adenovirus particles and expressed fiber proteins have provided evidence for the existence of at least two distinct adenovirus fiber receptors which interact with the group B (Ad3) and group C (Ad5) adenoviruses (1, 14, 18). Although Ad5 and Ad3 utilize different fiber binding receptors, αv integrins enhance entry of both serotypes into cells (14). This suggests that the binding and entry steps are unlinked events and that fiber attachment to various cell surface molecules may permit productive entry. It is likely that additional receptors exist for other adenovirus serotypes, although this remains to be demonstrated.

Adenovirus vectors derived from the human group C Ad5 serotype are efficient gene delivery vehicles which readily transduce many nondividing cells. Adenovirus infects a broad range of cells and tissues, including lung, liver, endothelium, and muscle (19). High-titer stocks of purified adenovirus vectors can be prepared, a factor which makes the vector suitable for in vivo administration. Various routes of in vivo administration have been investigated, including intravenous delivery for liver transduction and intratracheal instillation for gene transfer to the lung. As the adenovirus vector system is being more widely applied, it is becoming apparent that some cell types may be refractory to recombinant adenovirus infection. Both the fiber binding receptor and αvβ3 or αvβ5 integrins are important for high-efficiency infection of target cells. Efficient transduction requires fiber-mediated attachment as demonstrated by the effectiveness of recombinant soluble fiber in blocking gene transfer (3). Transduction of cells which lack fiber receptors occurs with much lower efficiency and requires high multiplicities of input vector (2, 11). Fiber-independent transduction likely occurs through direct binding of the penton base RGD sequences to cell surface integrins. Blockade of the RGD-integrin pathway reduces gene transfer efficiencies by several fold (2, 11), but the effect is less complete than blockade of the fiber-receptor interaction, suggesting that the latter is more critical.

Low-level gene transfer may result from a deficiency in one of the components of the entry process in the target cell. For example, inefficient gene transfer to human pulmonary epithelia has been attributed to a deficiency in αvβ5 integrins (3). Other cell types, such as vascular endothelial and smooth muscle cells, have been identified as being deficient in fiber-dependent transduction because of a low level of the Ad5 receptor (24). Several approaches have been undertaken to target adenovirus vectors to improve or enable efficient transduction of

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target cells. These strategies include alteration of the penton base to selectively target specific cell surface integrins (21, 24) and modification of the fiber protein with an appropriate ligand to redirect binding (15, 18). The approach we have undertaken involves engineering the fiber head domain to include novel receptor specificities. We previously examined the binding of recombinant Ad5 and Ad3 fiber proteins to cellular receptors and demonstrated that the receptor specificity of the fiber protein can be altered by exchanging the head domains between these two fiber proteins (18). This report describes the incorporation of a modified fiber gene into the Ad5 adenovirus genome via homologous recombination to generate a recombinant adenovirus particle which contains a chimeric fiber protein. This modified fiber redirects binding of the vector to the Ad3 receptor. In addition, we present evidence for the differential expression of the Ad5 and Ad3 receptors on various human cell types. The Av9LacZ4 vector described here may be useful for the transduction of cell types or tissues which are difficult to transduce with the wild-type Ad5 fiber vectors. These studies provide a basis for targeting adenovirus vectors to specific cell types and tissues by engineering the fiber protein to contain novel receptor specificities.

MATERIALS AND METHODS

Recombinant fiber plasmid. A shuttle plasmid was constructed for homologous recombination at the right-hand end of Ad5-based adenovirus vectors. This shuttle plasmid, referred to as "prepac," contains the last 8,886 bp from bp 25,171 to 34,057 of the Ad5dl327 genome cloned into pBluescript SK II(+) (Stratagene) and was kindly supplied by Soumitra Roy. The wild-type Ad5 fiber cDNA contained within prepac was replaced with the 5TS3Ha cDNA by PCR gene overlap extension (9). The 5TS3H gene construct contains the Ad5 fiber tail and shaft domains (5TS; amino acids 1 to 403) fused with the Ad3 fiber head region (3H; amino acids 136 to 319) as described previously (18). The 5TS3Ha cDNA was modified to contain native 3′ downstream sequences of the wild-type 5F cDNA. In addition, the last two codons of the Ad3 fiber head domain, GAC TGA, were mutated to correspond to the wild-type 5F codon sequence, GAA TAA, to maintain the Ad5 fiber stop codon and polyadenylation signal. The Ad5 fiber 3′ downstream sequences were added to the 5TS3Ha cDNA with the pgem5TS3H plasmid (18) used as the template and with the following primers: P1, 5′-CATCTGCAGCATGAAGCGCGCAAGACCGTCTGAAGATA-3′ (scs4); and P2, 5′-CGTTGAAACATAACACAAACGATTCTTTATTCATCTTCTCTA ATATAGGAAAAGGTAA-3′ (scs80). Overlappinghomologous sequences were added to prepac with the following primers: P3, 5′-TTACCTTTTCCTATATTAG AGAAGATGAATAAAGAATCGTTTGTGTTATGTTTCAACG-3′ (scs79); and P4, 5′-AGACAAGCTTGCATGCCTGCAGGACGGAGC-3′ (scs81). Amplified products of the expected size were obtained and were gel purified. A second PCR was carried out with the end primers P1 and P4 to join the two fragments together. The DNA fragment generated in the second PCR contained the 5TS3Ha cDNA with the last two codons mutated to the wild-type 5F sequence and the appropriate 3′ downstream prepac sequences. The 5TS3Ha PCR fragment was digested with *Nde*I and *Sse* 8387 and was cloned directly into prepac to create the fiber shuttle plasmid prep5TS3Ha.

Generation of recombinant adenoviruses. The modified 5TS3Ha fiber cDNA was incorporated into the genome of Av1LacZ4, an adenovirus vector with E1 and E3 deleted encoding β-galactosidase, by homologous recombination between Av1LacZ4 and the prep5TS3Ha fiber shuttle plasmid to generate the chimeric fiber adenovirus vector referred to as Av9LacZ4. Human embryonic kidney 293 cells (ATCC CCL-1573) were obtained from the American Type Culture Collection (Rockville, Md.) and cultured in Iscove's modified Eagle's medium (MEM) containing 10% heat-inactivated fetal bovine serum (HIFBS). Cotransfections of 293 cells were carried out with 10 μg of *Not*I-digested prep5TS3Ha and 1.5 μg of *Srf*I-digested Av1LacZ4 genomic DNA with a calcium phosphate mammalian transfection system (Promega Corporation, Madison, Wis.). The 293 cells were incubated with the calcium phosphate-DNA precipitate at 37°C for 24 h. The precipitate was removed, and the monolayers were washed once with phosphate-buffered saline (PBS). The transfected 293 cell monolayers were overlayered with 1% SeaPlaque agarose in MEM supplemented with 7.5% HIFBS, 2 mM glutamine, 50 U of penicillin per ml, 50 μg of streptomycin sulfate per ml, and 1% amphotericin B. Adenovirus plaques were isolated after approximately 14 days. Individual plaques were expanded, and genomic DNA was isolated and screened for the presence of the chimeric fiber 5TS3Ha cDNA by *Sca*I restriction enzyme digestion and confirmed by Southern blot analysis with the Ad3 fiber head as a probe. Positive plaques were subjected to two rounds of plaque purification to remove parental Av1LacZ4 contamination. The Av9LacZ4 vector after two rounds of plaque purification was expanded and purified by conventional techniques by CsCl ultracentrifugation. The adenovirus titers (particles per milliliter) were determined spectrophotometrically (6, 20) and compared with the biological titer (PFU per milliliter) determined with 293 cell monolayers as described previously (16). The ratio of total particles to infectious particles (particles per PFU) was calculated. DNA was isolated from each vector and digested with *Dra*I, *Sca*I, or *Eco*RI and *Bam*HI to confirm the identity of each. The schematic diagrams of the Av9LacZ4 and parental Av1LacZ4 vectors are shown in Fig. 1.

Expression of fiber constructs in baculovirus. As described previously (18), the baculovirus expression system (Clontech, Palo Alto, Calif.) was used to generate fiber proteins for receptor binding studies. Recombinant baculovirus vectors were used which expressed either the Ad5 fiber or Ad3 fiber proteins. *Spodoptera frugiperda* cells (Sf21) were cultured as monolayers at 27°C in Grace's supplemented insect cell medium containing 10% HIFBS, 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, and 2.5 μg of amphotericin B per ml. Large-scale infections of Sf21 cells with each recombinant fiber baculovirus were carried out, and fiber-containing cell lysates were prepared as described previously (18).

The Ad5 fiber protein was purified from the Sf21 cell lysates as described previously (18). Briefly, the Ad5 fiber trimer was purified to homogeneity by a two-step purification procedure utilizing a DEAE-sepharose column and then a Superose 6 gel filtration column equilibrated in PBS with a fast-performance liquid chromatography system (Pharmacia). Protein concentrations of the purified Ad5 fiber trimer and the insect cell lysates containing the Ad3 fiber (3F/CL) were determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as the assay standard.

The expression of fiber proteins was verified by sodium dodecyl sulfate (SDS)–4 to 15% polyacrylamide gradient gel electrophoresis (PAGE) under denaturing conditions and Western immunoblot analysis. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by use of a small Transblot apparatus (Bio-Rad, Hercules, Calif.) for 30 min at 100 V. After the transfer was completed, the PVDF membrane was transiently stained with Ponceau red, and the molecular mass standards were marked directly on the membrane. The molecular mass standards used ranged from 200 to 14 kDa (Bio-Rad). Nonspecific protein binding sites on the PVDF membrane were blocked with a 5% dried milk solution in 10 mM Tris (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, and 0.04% Tween 20 for 1 h at room temperature or overnight at 4°C. The membrane was then incubated for 1 h at room temperature with a 1:10,000 dilution of the primary anti-Ad2 fiber monoclonal antibody, 4D2-5 (ascites kindly provided by J. Engler, University of Alabama), or with a 20-μg/ml concentration of a partially purified anti-Ad3 fiber specific rabbit polyclonal antibody generated against the baculovirus-expressed Ad3 fiber head domain (18). The membrane was developed either with a 1:10,000 dilution of the secondary sheep anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRPO)-conjugated antibody (Amersham Lifesciences, Arlington, Ill.) or with a 1:2,000 dilution of donkey anti-rabbit IgG-HRPO with an enhanced chemiluminescence system (Amersham Lifesciences). The membrane was exposed to film for approximately 3 to 60 s.

Production of an anti-Ad3 fiber-specific antiserum. The fiber head region of the Ad3 fiber was expressed with the baculoviral expression system as described previously (18). The insect cell lysate containing the Ad3 fiber head was used for immunizations of New Zealand White rabbits according to standard protocols (Lofstrand Labs, Ltd., Gaithersburg, Md.). The IgG fraction was isolated and was applied to an affinity column containing covalently bound insect cell lysate proteins. The unbound fraction from this affinity column was obtained and tested for immunoreactivity against the Ad5, Ad3, and chimeric 5TS3H fiber proteins by Western blot analysis.

Competitive virus transduction assay. The receptor tropism of the recombinant adenoviruses was evaluated with a virus transduction assay in the presence of fiber protein competitors. Monolayers of HeLa cells (ATCC CCL 2) cultured in Dulbecco's MEM (DMEM) with 10% HIFBS, 100 U of penicillin per ml, and 100 μg of streptomycin sulfate per ml contained in 12-well dishes were incubated with various dilutions of either purified Ad5 fiber trimer protein (0.05 to 100 μg/ml) or with an insect cell lysate containing the Ad3 fiber (100 to 2,000 μg/ml) for 1 h at 37°C in a total volume of 0.2 ml of DMEM–2% HIFBS. The chimeric fiber Av9LacZ4 or parental Av1LacZ4 adenovirus vector was then added in a total volume of 5 μl to achieve a total particle/cell ratio of 100 by dilution of the virus into DMEM–2% HIFBS. Virus transductions were carried out for 1 h at 37°C. The monolayers were washed once with PBS, 1 ml of DMEM–10% HIFBS was added per well, and the cells were incubated for an additional 24 h to allow for β-galactosidase expression. The cell monolayers then were fixed with 0.5% glutaraldehyde in PBS, and then were incubated with a mixture of 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in 0.5 ml of PBS. The cells were stained for approximately 24 h at 37°C. The monolayers were washed with PBS, and the average number of blue cells per high-power field was quantitated by light microscopy with a Zeiss ID03 microscope; three to five fields were counted per well. The average number of blue cells per high-power field was expressed as a percentage of the control which did not contain competitor fiber protein. Each concentration of competitor was carried out in triplicate, and the average percentage ± standard deviation was expressed as a function of added competitor fiber protein. Each experiment was carried out three to four times, and data from a representative experiment are shown.

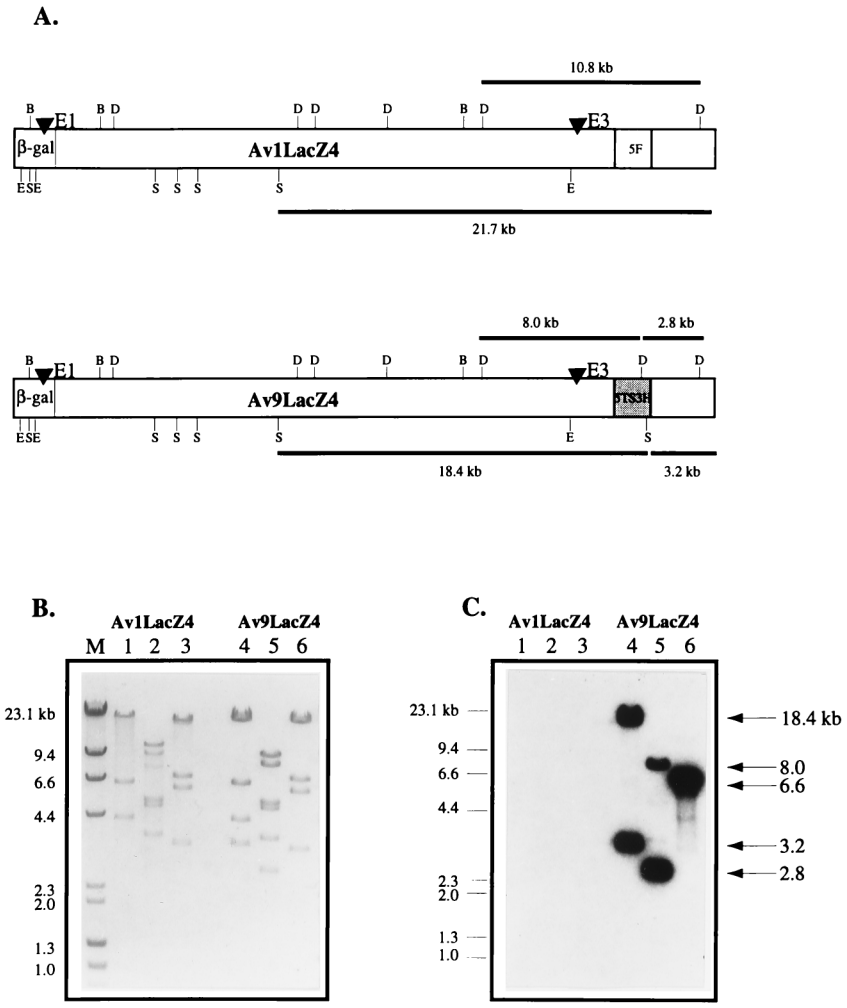

FIG. 1. Genomic analysis of the wild-type Av1LacZ4 and chimeric fiber Av9LacZ4 adenovirus vectors. (A) *Sca*I (S), *Dra*I (D), *Eco*RI (E), and *Bam*HI (B) restriction endonuclease sites are indicated on the schematic diagram for each vector. The predicted *Dra*I and *Sca*I restriction fragments and the expected sizes for Av1LacZ4 and Av9LacZ4 are highlighted. DNA was isolated from each vector and digested with the indicated restriction endonucleases, and Southern blot analysis was carried out by standard procedures. (B) The digested DNA samples (0.4 μg) were applied to a 0.8% agarose gel and stained with ethidium bromide to visualize the individual DNA fragments. The combined λDNA/*Hin*dIII and ϕX174 RF DNA/*Hae*III DNA size markers (M) are indicated. The Av1LacZ4 wild-type vector was digested with *Sca*I (lane 1), *Dra*I (lane 2), and *Eco*RI and *Bam*HI (lane 3). The Av9LacZ4 chimeric fiber vector was digested with *Sca*I (lane 4), *Dra*I (lane 5), and *Eco*RI and *Bam*HI (lane 6). (C) The digested DNA fragments as shown in panel B were transferred to a Zetaprobe membrane and hybridized with the ^{32}P-labeled 500-bp Ad3 fiber head domain probe at approximately 10^6 cpm/ml and exposed to film for 12 h. The expected fragments derived from Av9LacZ4 which hybridized with the Ad3 fiber head probe are indicated.

Cell culture. The transduction efficiency of Av9LacZ4 and Av1LacZ4 was surveyed with a panel of human cell lines. HeLa (human cervical carcinoma), MRC-5 (human embryonic lung diploid fibroblast; ATCC CCL-171), FaDu (human squamous carcinoma; ATCC HTB 43), and THP-1 (human monocyte; ATCC TIB-202) cells were obtained from the American Type Culture Collection and cultured in the recommended medium. Human umbilical vein endothelial cells (HUVEC; CC-2517) and human coronary artery endothelial cells (HCAEC; CC-2585) were obtained from the Clonetics Corporation (San Diego, Calif.) and cultured in the recommended medium. Each cell line was transduced with the chimeric fiber Av9LacZ4 or the wild-type Av1LacZ4 adenovirus vector at 0, 10, 100, and 1,000 total particles per cell for 1 h at 37°C in a total volume of 0.2 ml of culture medium containing 2% HIFBS. The cell monolayers were then washed once with PBS, and 1 ml of the appropriate culture medium containing 10% HIFBS was added. THP-1 cells were incubated with the indicated concentration of vector for 1 h at 37°C in a total volume of 0.2 ml of culture medium containing 2% HIFBS, and then 1 ml of complete medium containing 10% HIFBS was added. The cells were incubated for 24 h to allow for β-galactosidase expression. The cell monolayers were then fixed and stained with X-Gal as described above. The incubation of each cell line in the X-Gal solution varied from 1.5 h up to 24 h, depending on the amount of background staining found in the mock-infected wells. The percentage of transduction was determined by light microscopy by counting of the number of transduced, blue cells per total cells in a high-power field with a Zeiss ID03 microscope; three to five fields were counted per well. Each vector dose was carried out in triplicate, and the average percentage of transduction per high-power field (mean ± standard deviation; $n = 3$ wells) was determined and expressed as a function of added vector. Each cell line was transduced at least three times, and the data represent the mean percentage of transduction ± standard deviation from three independent experiments.

RESULTS

Construction of an adenovirus vector containing a chimeric fiber gene. It was previously shown by using chimeric fiber proteins expressed in vitro and in insect cells that the receptor specificity of the adenovirus fiber protein can be altered by an exchange of the head domain with that of another serotype which recognizes a different receptor (18). To generate an adenovirus vector particle with an altered receptor specificity, the chimeric fiber gene construct containing the Ad3 fiber head domain fused to the Ad5 fiber tail and shaft, 5TS3H, was incorporated into the adenovirus genome of Av1LacZ4. For the precise replacement of the wild-type Ad5 fiber gene, a

shuttle plasmid was constructed which contained the last 8,886 bp of the Ad5dl327 genome from 73.9 to 100 map units, including the Ad5 fiber gene, E4, and the right inverted terminal repeat (ITR). This shuttle plasmid was used for incorporation of modified fiber genes into the backbone of an adenovirus vector with E1 and E3 deleted, Av1LacZ4, via homologous recombination. This strategy replaces the native Ad5 fiber with the chimeric 5TS3H fiber sequences cloned within the prep5TS3Ha shuttle plasmid. The resulting vector, Av9LacZ4, contains the nucleus-targeted β-galactosidase cDNA and the Ad3 fiber head domain. This approach will allow for any modification to the native fiber sequence to be incorporated within the adenovirus genome.

Both the parental Av1LacZ4 and chimeric fiber Av9LacZ4 vectors are shown schematically in Fig. 1. The Ad3 fiber head region introduces additional *Dra*I and *Sca*I restriction enzyme sites within the Av1LacZ4 genome, which were used to identify the recombinant virus. Plaques which yielded the predicted *Dra*I and *Sca*I diagnostic fragments as indicated in Fig. 1A were selected and expanded. Genomic DNA isolated from the purified chimeric fiber Av9LacZ4 and the parental Av1LacZ4 viruses was analyzed by restriction enzyme digestion and agarose gel electrophoresis (Fig. 1B). The expected DNA fragments were obtained for both the Av9LacZ4 and wild-type Av1LacZ4 viruses. Diagnostic 18.4- and 3.2-kb fragments were found after *Sca*I digestion of the Av9LacZ4 genomic DNA (Fig. 1B, lane 4), indicating the presence of the Ad3 fiber head domain. *Dra*I restriction endonuclease digestion of Av9LacZ4 also confirmed the presence of the Ad3 fiber head domain as indicated by the 8.0- and 2.8-kb diagnostic fragments (Fig. 1B, lane 5). *Eco*RI and *Bam*HI digestion produced identical restriction patterns for both vectors as expected (Fig. 1B, lanes 3 and 6). Southern blot analysis with the Ad3 fiber head probe demonstrated the expected hybridization pattern for all restriction endonuclease digestions for both vectors (Fig. 1C). The 18.4- and 3.2-kb *Sca*I and 8.0- and 2.8-kb *Dra*I diagnostic fragments of Av9LacZ4 hybridized with the Ad3 fiber head probe (Fig. 1C, lanes 4 and 5). The 6.6-kb *Eco*RI/*Bam*HI fragment which contains the full-length 5TS3H fiber gene was also detected (Fig. 1C, lane 6). Southern blot analysis with the Ad5 fiber head probe (data not shown) demonstrated the expected hybridization pattern for Av1LacZ4 and confirmed that the chimeric fiber Av9LacZ4 virus preparation was free of parental Av1LacZ4 virus.

Characterization of adenovirus particles containing the chimeric fiber. Expression and assembly of the chimeric 5TS3H fiber protein into the adenovirus capsid were examined by Western blot analysis of CsCl-purified virus stocks. Equivalent numbers of the parental (Av1LacZ4) and chimeric (Av9LacZ4) particles were subjected to SDS-PAGE (4 to 15% polyacrylamide gradient gel) under denaturing conditions. A control virus containing a full-length Ad3 fiber was also analyzed. Western immunoblot analysis was carried out with an antifiber monoclonal antibody, 4D2-5 (Fig. 2A), and a rabbit polyclonal antibody specific for the Ad3 fiber head domain (Fig. 2B). The 4D2-5 antibody recognizes a conserved epitope located within the N-terminal tail domain of the fiber protein (8) and reacts with both the Ad5 (5F) and the Ad3 (3F) fiber proteins (18). As shown in Fig. 2A, the Av1LacZ4 (lane 1) and Av9LacZ4 (lane 2) viruses contain fiber proteins with sizes of approximately 62 to 63 kDa which react with the 4D2-5 antibody, while the Ad3 fiber virus contains a fiber protein with a size of approximately 35 kDa (Fig. 2A, lane 3). The presence of the Ad3 fiber head (3FH) domain within the 5TS3H chimeric fiber was confirmed by Western blot analysis with a rabbit polyclonal antibody specific for the Ad3 fiber. The rabbit anti-3FH polyclonal antibody did not bind to the Ad5 fiber protein in Av1LacZ4 and was specific for the 35-kDa Ad3 fiber protein in the control virus (Fig. 2B, lane 6) and the Ad3 fiber head domain contained within the chimeric 5TS3H fiber protein in Av9LacZ4 (Fig. 2B, lane 5).

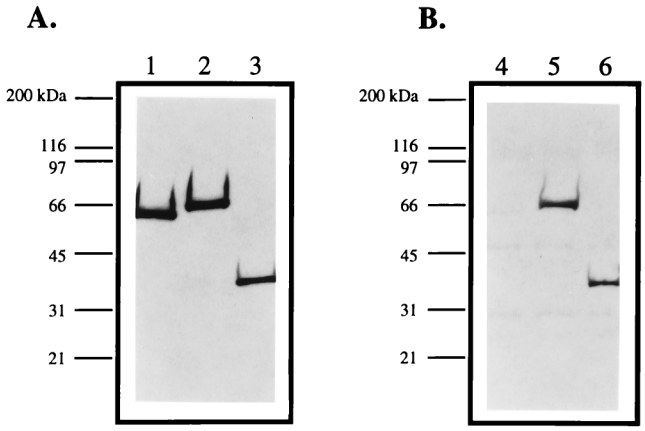

FIG. 2. Western immunoblot analysis of adenovirus capsid proteins. Equivalent numbers of adenovirus particles for the Av1LacZ4 (lanes 1 and 4) and Av9LacZ4 (lanes 2 and 5) vectors or a control virus containing the full-length Ad3 fiber protein (lanes 3 and 6) were subjected to SDS-PAGE (4 to 15% polyacrylamide gradient gel) and Western blot analysis under denaturing conditions. (A) A total of 2×10^{10} adenovirus particles were applied per lane, and the membrane was developed with the antifiber monoclonal antibody 4D2-5 and an anti-mouse IgG-HRPO-conjugated secondary antibody by chemiluminescence. (B) A total of 6×10^{10} particles were applied per lane, and the membrane was developed with a rabbit anti-Ad3 fiber-specific polyclonal antibody and donkey anti-rabbit IgG-HRPO-conjugated secondary antibody by chemiluminescence. The positions of molecular mass markers are indicated.

The biological titers and particle numbers of the chimeric fiber (Av9LacZ4) and parental (Av1LacZ4) adenoviruses were compared. Biological titers determined with 293 cell monolayers indicated plaque-forming titers of 2.6×10^{10} and 4.5×10^{10} PFU/ml for the Av1LacZ4 and Av9LacZ4 virus preparations, respectively. The total particle concentrations were determined spectrophotometrically and were 1.45×10^{12} and 1.25×10^{12} particles/ml for Av1LacZ4 and Av9LacZ4, respectively. Thus, the ratios of particle number to PFU titer were similar for both viruses, 55.8 versus 27.8 total particles/PFU, respectively. An increased ratio of particle number to infectious titer has previously been reported for Ad3 compared to Ad2 (1). However, the replacement of the Ad5 fiber head domain with the Ad3 fiber head domain did not adversely affect the cellular production of the adenovirus containing the chimeric fiber protein or significantly change the ratio of total physical to infectious particles.

Receptor binding specificity of the modified fiber adenovirus. To evaluate the receptor binding properties of the chimeric fiber vector compared to those of the native Ad5 fiber vector, transduction experiments were carried out in the presence of recombinant fiber protein competitors. Cells were preincubated with purified Ad5 fiber protein or with an insect cell lysate containing the Ad3 fiber protein prior to transduction with the chimeric fiber or native Ad5 fiber vector. Figure 3 shows the results of transduction experiments in which HeLa cels were incubated with increasing amounts of Ad5 fiber protein (Fig. 3A) or with the Ad3 fiber competitor (Fig. 3B) prior to transduction with the Av9LacZ4 or Av1LacZ4 vector. Transduction of HeLa cells with Av1LacZ4 decreased with increasing amounts of Ad5 fiber trimer protein, with maximal competition occurring between 0.1 and 1.0 μg/ml. In contrast, the purified Ad5 fiber trimer did not block the transduction of the Av9LacZ4 chimeric fiber adenovirus. These results confirm

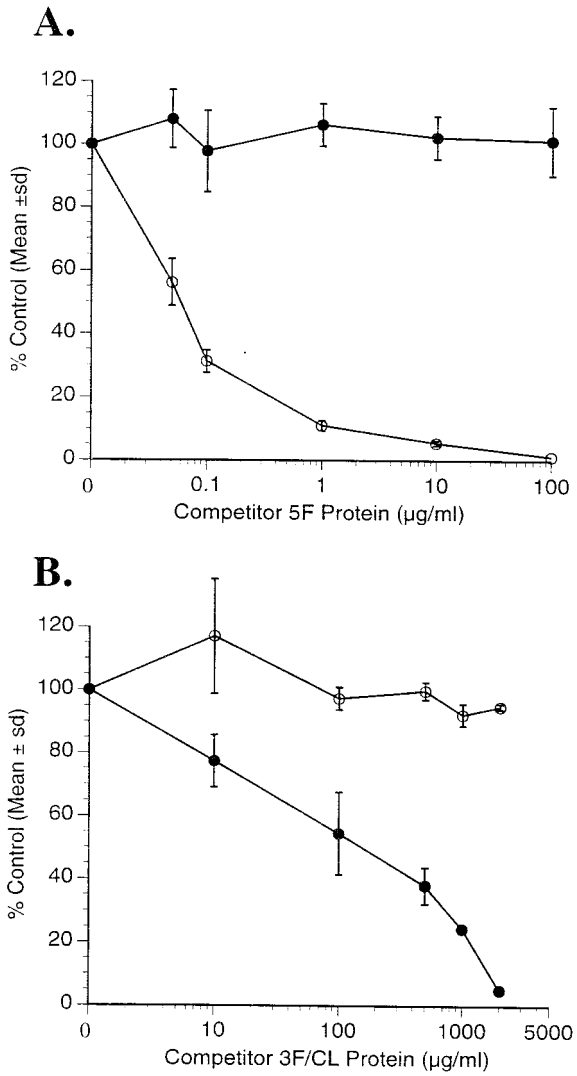

FIG. 3. Competition virus transduction assay. HeLa cell monolayers were incubated with increasing concentrations of purified Ad5 fiber trimer protein (5F [A]) or with an insect cell lysate containing the Ad3 fiber protein (3F/CL [B]) prior to transduction with 100 total particles per cell of either the Av1LacZ4 (open circles) or Av9LacZ4 (closed circles) adenovirus vector. After 24 h, the cells were analyzed for β-galactosidase expression as described in Materials and Methods. The percentage of adenovirus transduction at each concentration of competitor is plotted. Each point is the average ± standard deviation (sd) of three independent determinations for a representative experiment.

that the wild-type Av1LacZ4 and chimeric fiber Av9LacZ4 vectors bind to different cell surface receptors. This conclusion was supported by the reciprocal experiment shown in Fig. 3B. Increasing concentrations of the Ad3 fiber competitor decreased the Av9LacZ4 transduction of HeLa cells but did not influence transduction with the wild-type Av1LacZ4 vector. The competition between the Ad3 fiber competitor and Av9LacZ4 was specific, since control experiments carried out with insect cell lysates which did not contain the Ad3 fiber protein did not result in competition (data not shown). These results indicate that transduction of HeLa cells by Av9LacZ4 is mediated by the chimeric fiber protein which interacts with the Ad3 receptor. Thus, the modification of the Ad5 fiber head domain has resulted in a change in receptor tropism of an adenovirus vector.

Transduction of human cell lines by the chimeric fiber vector. Since the identity of the Ad5 and Ad3 receptors is unknown, there is relatively little information available concerning their cellular distribution. We hypothesized that differential expression of the Ad5 and Ad3 receptors on different human cells might be reflected in the differential transduction by the parental Av1LacZ4 and chimeric fiber Av9LacZ4 vectors. We investigated the transduction properties of a number of human cell lines by the two vectors. Several cell lines were included which had been identified as negative for Ad5 fiber adenovirus receptor binding (11, 18) and/or refractory to Av1LacZ4 infection (unpublished data). Cells were infected with the chimeric fiber Av9LacZ4 or the wild-type Av1LacZ4 adenovirus at particle/cell ratios of 0, 10, 100, and 1,000 in a total volume of 0.2 ml of culture medium. Twenty-four hours later, the cells were stained with X-Gal as described in Materials and Methods. Shown in Fig. 4 are representative photographs of the Av1LacZ4 and Av9LacZ4 transduction of HeLa cells (Fig. 4A and B); MRC-5, a human embryonic lung fibroblast cell line (Fig. 4C and 4D); and FaDu, a human squamous cell carcinoma line (Fig. 4E and 4F) monolayers at a dose of 1,000 virus particles per cell. Both vectors transduced HeLa cell monolayers with similar efficiencies. In contrast, differential transduction of the MRC-5 and FaDu cell lines was found. Both the MRC-5 and FaDu cells were relatively refractory to Av1LacZ4 transduction but were readily transduced with Av9LacZ4.

The percentage of transduction of each cell line was quantitated, and the fraction of HeLa, MRC-5, and FaDu cells transduced as a function of dose is shown in Fig. 5. HeLa cells (Fig. 5A) were equally susceptible to transduction with both vectors, indicating that both the Ad5 and Ad3 receptors are present on the cell surface. The MRC-5 (Fig. 5B) human embryonic lung cell line was efficiently transduced with the chimeric fiber Av9LacZ4 vector. The percentage of transduction with Av9LacZ4 was dose dependent, with approximately 80% transduction at the vector dose of 1,000. Less-efficient transduction of MRC-5 cells with Av1LacZ4 was observed, suggesting that these cells either lack or express low levels of the Ad5 receptor. In contrast, the Ad3 receptor appears to be abundant on this cell type. The FaDu cell monolayers (Fig. 5C) were also transduced more efficiently with Av9LacZ4, with 75% of the cells transduced at the vector dose of 1,000 compared to only 7% transduction achieved with Av1LacZ4 at the same vector dose.

Av1LacZ4 and Av9LacZ4 were used to compare the levels of transduction of a number of additional human cell lines. Figure 6 summarizes data for each of the cell lines examined at the virus particle/cell ratios of 100 (Fig. 6A) and 1,000 (Fig. 6B). The cell lines assessed in addition to the HeLa, MRC-5, and FaDu cell lines included HDF (human diploid fibroblasts), THP-1 (human monocytes), HUVEC, and HCAEC. Cells were infected with Av9LacZ4 or Av1LacZ4 adenovirus vectors at particle/cell ratios of 100 and 1,000, and 24 h later were stained with X-Gal as described in Materials and Methods. The fraction of transduced cells for each cell line at the indicated vector dose was determined. As shown previously, HeLa cells were transduced at equivalent levels with both adenovirus vectors, while HDF cells were refractory to Av1LacZ4 as well as Av9LacZ4 transduction. HDF cells are negative for Ad5 fiber binding, indicating that these cells lack or express low levels of the Ad5 receptor (18). The transduction data presented in Fig. 6 for HDF cells suggest that these cells lack or express low levels of the Ad3 receptor as well.

This analysis identified several human cell lines which were differentially transduced by the parental Av1LacZ4 and chimeric fiber Av9LacZ4 vectors. MRC-5, FaDu, and THP-1 cells

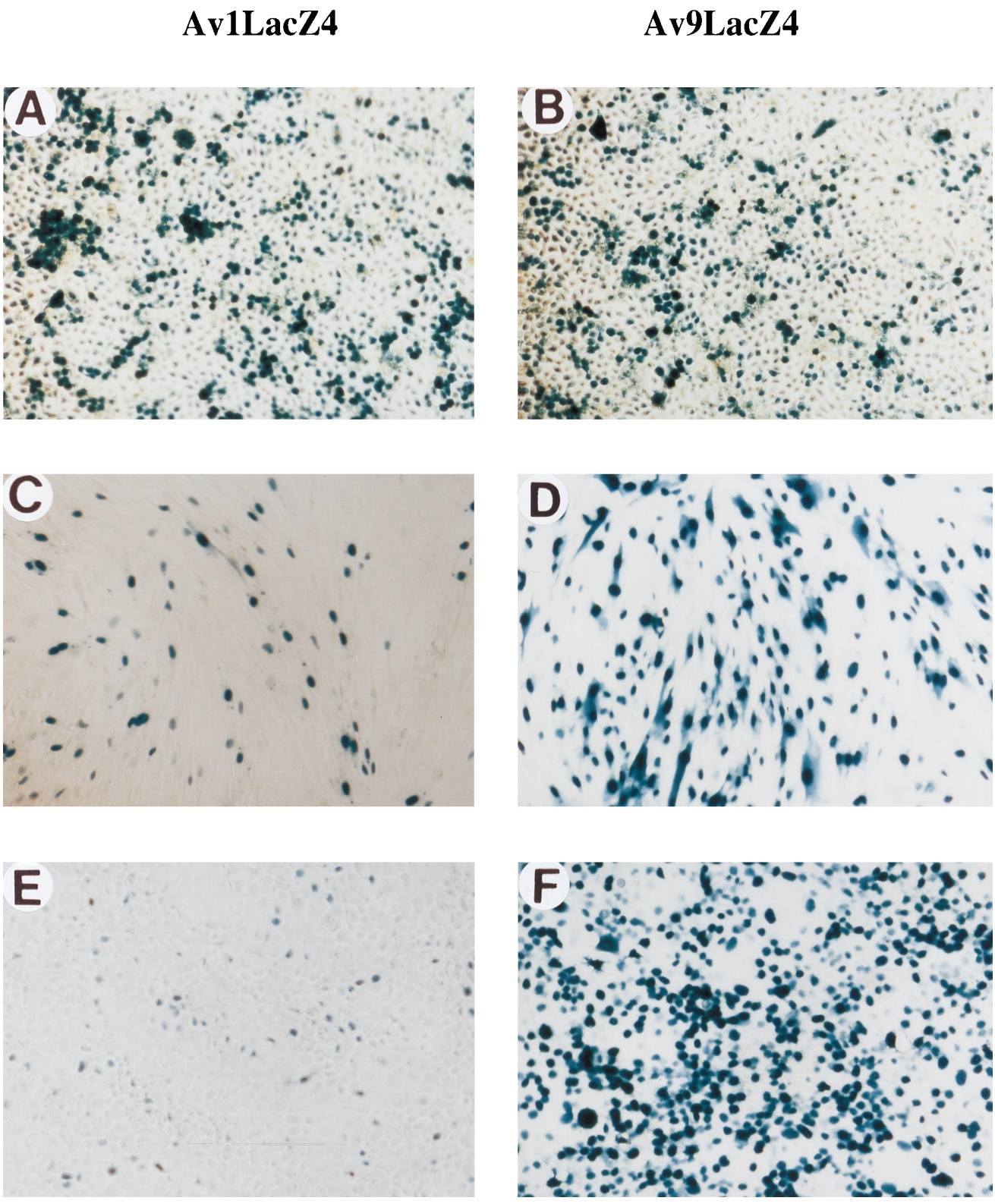

FIG. 4. Differential adenovirus-mediated transduction properties of human cell lines. HeLa (A and B), MRC-5 (C and D), and FaDu (E and F) cells were transduced with the Av1LacZ4 (A, C, and E) or Av9LacZ4 (B, D, and F) vector at 1,000 total particles per cell. After 24 h, the cells were analyzed for β-galactosidase expression as described in Materials and Methods. Representative photomicrographs are shown.

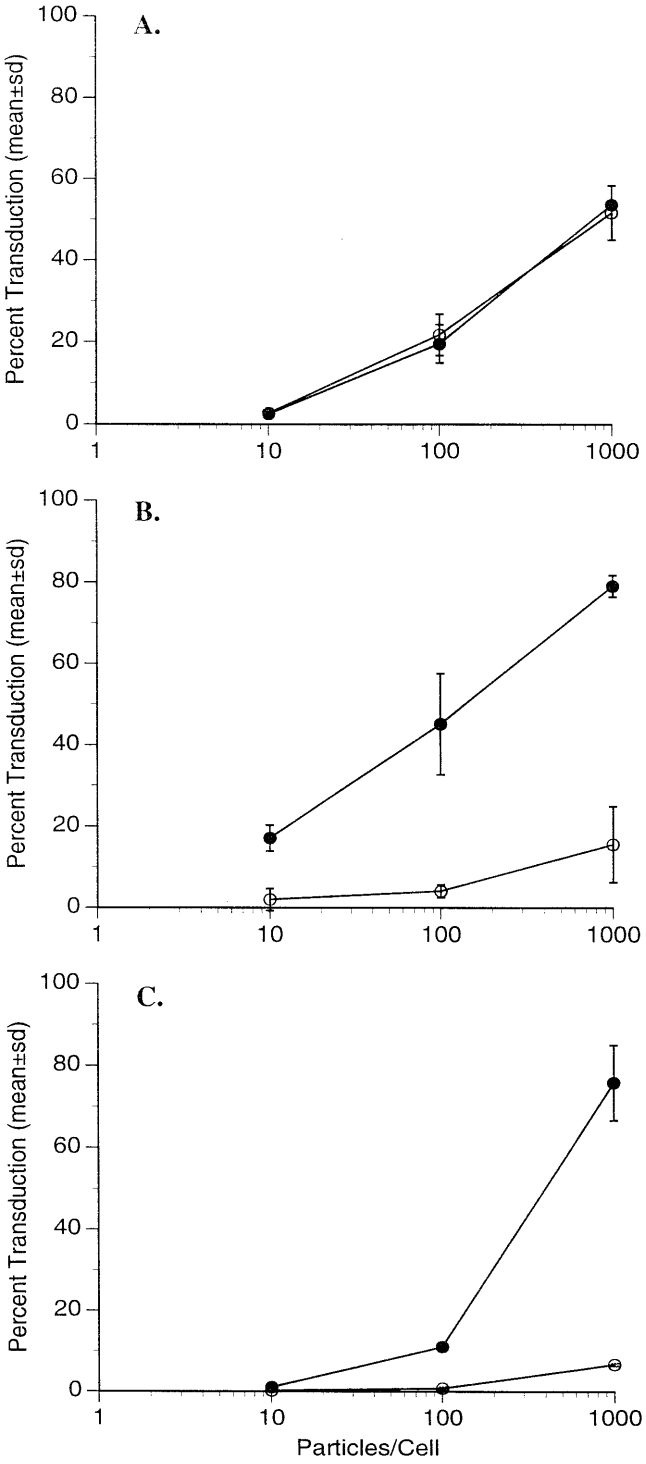

FIG. 5. Adenovirus-mediated transduction properties of HeLa (A), MRC-5 (B), and FaDu (C) human cell lines. The indicated cells were transduced with 0, 10, 100, and 1,000 total particles per cell of the Av1LacZ4 (open circles) or Av9LacZ4 (closed circles) vector for 1 h at 37°C in a total volume of 0.2 ml of culture medium. After 24 h, the cells were fixed and stained with X-Gal as described in Materials and Methods. The percentage of transduced cells per high-power field was determined for each vector dose. The data represent the average percentage of transduction ± standard deviation (sd) for three independent experiments, and each vector dose was carried out in triplicate. The percentage of transduction of HeLa, MRC-5, and FaDu cells at each vector dose is displayed.

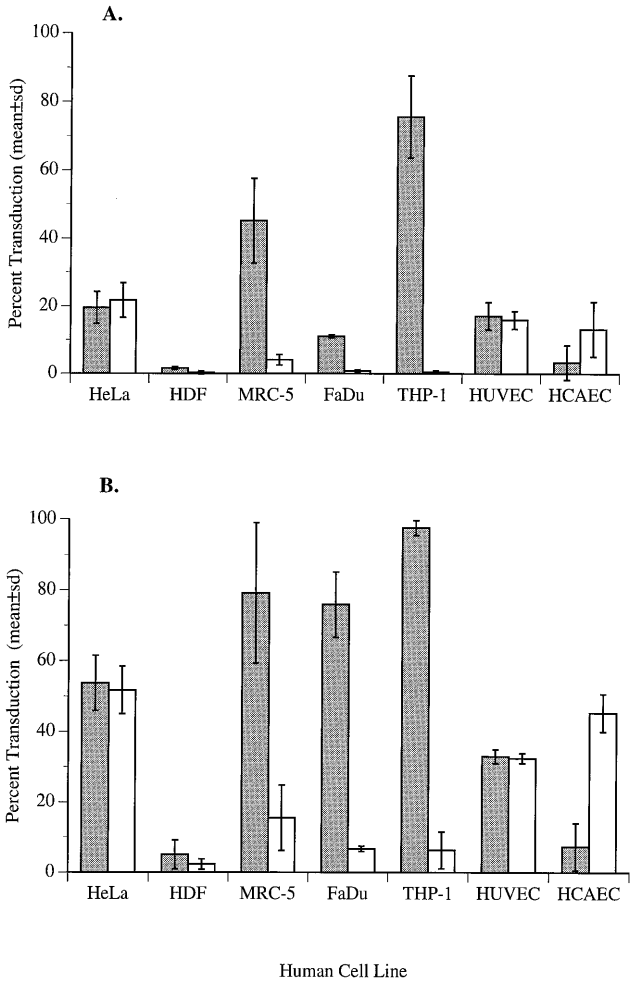

FIG. 6. Differential adenovirus-mediated transduction properties of human cell lines. The percentage of transduction efficiency for each cell line infected with the Av1LacZ4 (open bars) or Av9LacZ4 (shaded bars) vector is displayed for vector doses of 100 (A) and 1,000 (B) particles per cell. The data represent the mean ± standard deviation (sd) from three independent experiments.

were efficiently infected with the recombinant vector containing the Ad3 fiber head in a dose-dependent manner (Fig. 6A and B), suggesting that the Ad3 receptor is more abundant than the Ad5 receptor on these cell types. At the vector dose of 1,000 particles per cell, approximately 45% of the HCAEC were transduced with the wild-type fiber Av1LacZ4 vector, while only 7.3% were transduced with the chimeric fiber Av9LacZ4 vector. Venous endothelial cells (HUVEC) were equivalently transduced with both vectors. Differences in the transduction of arterial and venous endothelial cells with Av1LacZ4 and Av9LacZ4 reveal the differential expression of the Ad3 and Ad5 receptors on cells derived from different regions of the vasculature. These data taken together demonstrate the differential expression of the Ad5 and Ad3 receptors on human cell lines derived from target tissues which are of potential clinical relevance.

DISCUSSION

A major goal in gene therapy research is the development of vectors and delivery systems which can achieve efficiently targeted in vivo gene transfer and expression. Vectors are needed

which maximize the efficiency and selectivity of gene transfer to the appropriate cell type for expression of the therapeutic gene and which minimize gene transfer to other cells or sites in the body which could result in toxicity or unwanted side effects. Of the virus vectors under investigation for in vivo gene transfer applications, the adenovirus system has shown considerable promise and has undergone extensive evaluation in animal models as well as early clinical evaluation in lung disease and cancer. Key features of adenovirus vectors are the efficiency of transduction and the resulting high levels of gene expression which can be achieved in vivo. This property is derived from the ability to prepare high-titer stocks of purified vector and from the remarkable efficiency of each of the steps in the adenovirus entry process leading to gene expression (4). Attachment of adenovirus particles to the cell is mediated by a high-affinity interaction between the fiber protein and the cellular receptor (17). Following binding, virion entry into many cell types is facilitated by an interaction between RGD peptide sequences in the penton base and the αvβ3 and αvβ5 integrins which act as coreceptors (22). In the absence of the high-affinity interaction of the fiber protein with its receptor, virus binding and transduction can still occur but with reduced efficiency. This process of fiber-independent binding and transduction is believed to occur via a direct association between the penton base and cellular integrins (11). As the first step in the cellular transduction process, the interaction between the fiber protein and the cell is an attractive and logical target for controlling the cell specificity of transduction by adenovirus vectors. We and others have previously shown that the receptor binding domain of the fiber protein resides within the trimeric globular head domain (7, 13, 18). The interaction of the fiber head domain with its receptor thus determines the binding specificity of adenoviruses. Consequently, manipulation of the fiber head domain represents an opportunity for control of the cell specificity of transduction by adenovirus vectors.

In order to test this concept experimentally, we took advantage of the fact that adenoviruses of the group B and group C serotypes bind to different cellular receptors (1, 14, 18). Chimeric fiber proteins were prepared which exchanged the head domains of the Ad3 and Ad5 fiber proteins. Cell binding and competition studies with the recombinant chimeric fiber proteins confirmed the role of the fiber head domain in receptor binding and showed that an exchange of head domains resulted in a corresponding change of receptor specificity between the Ad3 and Ad5 receptors (18). In the present study, we have extended this analysis by the construction of an Ad5-based adenovirus vector, Av9LacZ4, which contains the fiber head domain from Ad3. The fiber-modified vector was prepared by a gene replacement strategy with the β-galactosidase-expressing vector Av1LacZ4 used as a starting point. A plasmid cassette containing the Ad5/Ad3 chimeric fiber gene construct 5TS3H was used for homologous recombination with the Av1LacZ4 genome, resulting in the precise substitution of the Ad5 fiber gene with the chimeric fiber gene containing the Ad3 fiber head to generate Av9LacZ4. Following plaque purification, molecular analysis of the recombinant vector genome provided confirmation of the fiber gene replacement in the vector. Western blot analysis of purified vector particles with an antiserum specific for the Ad3 fiber verified the expression and assembly of the chimeric 5TS3H fiber protein into functional adenovirus particles. The changed receptor specificity of the Av9LacZ4 chimeric fiber vector was confirmed by competition with recombinant fiber proteins which showed that transduction of 293 cells was effectively blocked by soluble Ad3 fiber but not by Ad5 fiber. These data confirm previous results obtained from binding experiments with recombinant fiber proteins and extend the analysis to intact adenovirus particles. Furthermore, the changed receptor specificity of the Av9LacZ4 vector establishes experimentally that the tropism of adenovirus vectors can be altered by manipulation of the head domain.

While the manuscript for this article was in preparation, a similar fiber-modified adenovirus vector was described by Krasnykh et al. (12). Cell binding and transduction experiments with fiber protein competitors provided evidence for the alteration of the receptor tropism of an Ad5 luciferase vector to that of Ad3. In the present study, we have analyzed the recombinant vector particles by Western blotting and provide evidence that the particles contain exclusively the chimeric 5TS3H fiber protein (Fig. 2). In addition, the results obtained with the Av9LacZ4 vector extend these studies by demonstrating that the chimeric fiber adenovirus vector differentially transduces several human cell lines.

The titer, yield, and ratio of physical to infectious particles of the fiber chimeric vector Av9LacZ4 and the parental Ad5 vector Av1LacZ4 were similar, indicating that the fiber head exchange did not significantly alter the growth properties of the vector on 293 cells. It has been reported that the infectivity of Ad3 is significantly less than that of Ad5, with Ad3 having a particle/PFU ratio approximately 20 times that of Ad5 (1). The similar infectivity of the Av9LacZ4 vector to the parental Av1LacZ4 vector shows that the efficiency of entry of an Ad5-based vector via either the Ad5 or Ad3 receptor is similar. This suggests that the differences in the infectivity between Ad5 and Ad3 are not due to the use of a different receptor for binding and must reflect other differences between the two serotypes. The finding that the infectivities of the Av1LacZ4 and Av9LacZ4 vectors in 293 cells are similar leads to the important conclusion that the binding specificity of adenovirus vectors can be completely changed without adversely affecting the subsequent steps in entry and disassembly of the vector particles leading to nuclear gene delivery and expression. The implication of this result is that the function of the fiber receptor is primarily to promote efficient cellular attachment and that cell entry is an independent event which is not necessarily dependent on the molecule used for attachment. Therefore, it should be possible to modify the fiber protein to promote vector attachment to a range of different cell surface molecules without compromising the ability of the vector to enter the cell. This conclusion is supported by a recent report of a fiber-modified adenovirus which binds to ubiquitously expressed cell surface proteoglycans and as a result has an extended cell tropism (23). It should therefore be possible to construct other adenovirus vectors containing fiber proteins modified to contain ligands for cellular receptors which are expressed in a cell-specific manner and as a result to achieve cell-selective transduction.

The importance of the interaction between the fiber protein and the cellular fiber receptor for adenovirus infectivity is underscored by the fact that blockade of this interaction by soluble fiber protein results in the efficient inhibition of transduction (Fig. 3). Furthermore, cells which lack or express low levels of the cellular fiber receptor are inefficiently transduced, and high levels of input vector are needed to achieve gene transfer (11). Recent clinical experience with adenovirus vectors in the treatment of cystic fibrosis lung disease has revealed a previously unsuspected resistance of human airway cells to transduction by Ad5-based vectors (5, 26). It has been proposed that patterns of expression of both the αv integrins and the fiber attachment receptors may be involved in limiting transduction of human airway cells in vivo (3, 26). Evidence for a correlation between the level of αv integrin expression on human pulmonary epithelial cells and the efficiency of adenovirus vector transduction supports this hypothesis (3).

The distribution of the Ad5 fiber attachment receptor on primary human cells is poorly characterized, largely due to the fact that its identity is unknown. However, it is increasingly clear that many human cell lines and a number of primary cells are refractory to transduction by Ad5-based vectors because of low levels or absence of the Ad5 fiber receptor. As noted previously, the Ad3 fiber receptor, while also as yet unknown, is clearly distinct from the Ad5 fiber receptor. Consequently, if differences in the patterns of expression of the two receptors exist, they should be reflected in a differential transduction efficiency by vectors which attach to either the Ad5 or Ad3 fiber receptors. In support of this hypothesis, we have identified several human cell lines which were inefficiently transduced by the Ad5 vector Av1LacZ4 and which could be transduced more efficiently by the chimeric fiber vector Av9LacZ4. These include a human head and neck tumor line, FaDu; a human lung epithelial cell line, MRC-5; and a human monocytic cell line, THP-1. Transduction of HeLa cells and HUVEC was equally efficient with both vectors. In contrast, HCAEC were more efficiently transduced by Av1LacZ4 than by Av9LacZ4. Since the only difference between the two vectors is the identity of the fiber head domain, the differences observed in transduction are fiber dependent and must be a result of the differential expression of the two fiber receptors. The overlapping but distinct cellular distribution of the fiber receptors for Ad5 and Ad3 which is revealed by these results will likely be of practical value in designing vectors for transduction of specific human target cells. For example, the results obtained with the THP-1 cell line suggest that gene transfer to the monocyte/macrophage lineage will be more efficient with vectors having the Ad3 receptor tropism than that of Ad5. Previous studies have demonstrated that human hematopoietic cells, monocytes, T lymphocytes, and THP-1 cells were refractory to adenovirus vector transduction because of an apparent lack of Ad5 fiber receptors and were transduced only at high doses of input Ad5 vector (10, 11). The efficient transduction of monocytes with the Av9 vector may be useful in designing strategies for the treatment of cardiovascular disease and atherosclerosis by targeting macrophage cells in vessel wall lesions. Similarly, the FaDu cell data indicate that certain tumor cells will be more effectively transduced with the Av9LacZ4 vector than with the Av1LacZ4 vector. Further analysis of the relative efficiency of the Av9LacZ4 and Av1LacZ4 vectors in transducing different primary human cell types and tissues will establish the specific applications for which the Av9-based vectors will be advantageous. It will also be of considerable interest to compare the tropism of the two vectors in vivo, particularly under circumstances in which transduction with Ad5-based vectors is inefficient.

The ability to modify adenovirus vectors to improve or enable transduction will increase the efficiency of adenovirus-mediated gene transfer. Modifications to the adenovirus fiber protein such as the head replacement strategy described in the present study are an approach which can lead to highly selective transduction of target cells. Head domains from other fiber proteins can be used to construct chimeric fibers which target vectors to alternative adenovirus receptors exploiting natural differences in the tropism of different adenovirus serotypes. Novel fiber proteins can also be constructed by replacement of the fiber head domain with other trimeric proteins, fusion of peptide sequences to the Ad5 fiber C terminus (15), or addition of peptide ligands within exposed loop regions of the fiber head domain (25). These strategies will lead to the development of customized adenovirus vectors which selectively target specific cell types.

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