

Generation of Recombinant Adenovirus Vectors with Modified Fibers for Altering Viral Tropism

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To expand the utility of recombinant adenovirus vectors for gene therapy applications, methods to alter native viral tropism to achieve cell-specific transduction would be beneficial. To this end, we are pursuing genetic methods to alter the cell recognition domain of the adenovirus fiber. To incorporate these modified fibers into mature virions, we have developed a method based on homologous DNA recombination between two plasmids. A fiber-deleted, propagation-defective rescue plasmid has been designed for recombination with a shuttle plasmid encoding a variant fiber gene. Recombination between the two plasmids results in the derivation of recombinant viruses containing the variant fiber gene. To establish the utility of this method, we constructed a recombinant adenovirus containing a fiber gene with a silent mutation. In addition, we generated an adenovirus vector containing chimeric fibers composed of the tail and shaft domains of adenovirus serotype 5 and the knob domain of serotype 3. This modification was shown to alter the receptor recognition profile of the virus containing the fiber chimera. Thus, this two-plasmid system allows for the generation of adenovirus vectors containing variant fibers. This method provides a rapid and facile means of generating fiber-modified recombinant adenoviruses. In addition, it should be possible to use this system in the development of adenovirus vectors with modified tropism to allow cell-specific targeting.

Recombinant adenoviruses have demonstrated great utility in the context of a variety of strategies to accomplish gene therapy (32, 51, 57). One of the principal features of recombinant adenoviruses which has recommended their use relates to the unique ability of these vectors to accomplish direct in vivo gene delivery. In this regard, recombinant adenovirus vectors have been shown to be capable of efficient in situ gene transfer to parenchymal cells of various organs, including the lung, brain, pancreas, gallbladder, and liver (7, 15, 18, 31, 34, 37, 38, 43, 45). This has allowed the use of these agents in approaches aimed at treating inherited genetic diseases, such as cystic fibrosis, whereby the delivered vector may be contained within the target organ (7, 14, 15, 18, 31, 34, 37, 38, 43, 45). In addition, the ability of the adenovirus vector to accomplish in situ tumor transduction has allowed the development of a variety of anticancer gene therapy approaches for compartmentalized disease (1, 12, 23, 35, 52). Again, these approaches have been directed toward nondisseminated disease, whereby vector containment favors tumor cell-specific transduction.

Despite the versatility of adenovirus vectors in these contexts, the full potential of the recombinant virions for in vivo gene transfer applications is not currently exploitable because the promiscuous tropism of the virus allows widespread, unrestricted tissue transduction after systemic in vivo vector delivery (30, 55). Thus, approaches based on vascular vector delivery to specific organ sites would be undermined by ectopic, nontargeted cellular transduction. This biologic feature of the virion has therefore limited gene therapy approaches to the aforementioned compartmentalized disease models whereby anatomic containment favors some level of selective target cell transduction.

A strategy to overcome this limitation would be the modifi-

cation of the cell binding domains of the adenovirus to allow interaction with cellular receptors in a specific manner. Adenovirus binds to eukaryotic cells by virtue of specific receptor recognition by domains in the knob portion of the fiber protein (29, 36, 54) which protrude from each of the 12 vertices of the icosahedral capsid. Thus, modifications of this region of the adenoviral capsid might be predicted to alter the native tropism of the virus (29, 36, 54). To this end, we have previously developed methods to introduce physiologic ligands into the structure of the adenovirus fiber protein as a means to redirect the tropism of the virion in a targeted manner (41). As a further step toward the development of a tropism-modified virus, we report here a novel genetic method to introduce modified fiber genes into adenovirus particles. In addition, we show that incorporation of a chimeric fiber can alter the tropism profile of the derived virus. The described method provides a rapid and facile means to produce recombinant adenovirus vectors with modified fibers. This methodology may thus be useful in the derivation of additional fiber modifications for purposes of cell-specific targeting via tropism-modified adenovirus vectors.

MATERIALS AND METHODS

Cells. 293 cells (26) were obtained from Microbix (Toronto, Ontario, Canada) and maintained in Dulbecco's modified Eagle's medium-Ham's F12 (DMEM-F12) supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO₂.

Construction of recombinant plasmids. A fiber rescue plasmid was constructed for recombination with shuttle plasmids containing fiber variants. For this construction, plasmid pBR322 was modified to provide restriction sites of utility. First, the *Cla*I site was destroyed by linearization with *Cla*I, the termini were blunted by a Klenow enzyme fill-in reaction, and the plasmid was recircularized. The resultant plasmid, pBRΔ*Cla*, was then digested with *Pvu*II and ligated with the *Mun*I linker, 5'-CCCAATTGGGG-3', resulting in plasmid pBR.MUN, which served as the cloning vector for subsequent constructions. Three distinct segments comprising the adenovirus genome were then cloned into pBR.MUN. First, a 1.9-kb *Nde*I-*Mun*I fragment was excised from the genome of the recombinant serotype 5 adenovirus (Ad5)-Luc 3. This recombinant adenovirus is a replication-competent vector containing the firefly luciferase gene in place of the deleted E3 region (44) and was generously provided by F. Graham (McMaster University, Hamilton, Ontario, Canada). The Ad5-Luc 3 1.9-kb DNA fragment was cloned into the corresponding sites of pBR.MUN to

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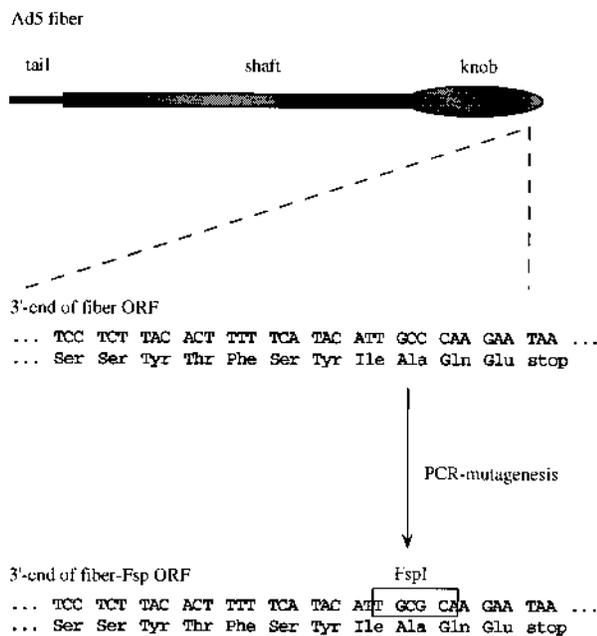


FIG. 1. Construction of a mutant fiber gene containing a functionally silent mutation. The 3' portion of the fiber gene encodes the knob domain of the fiber protein. PCR mutagenesis was used to generate a single-base substitution (C to G) which creates a silent mutation resulting in a novel recognition site for the restriction endonuclease *FspI*.

create plasmid pBR.MN2. This plasmid contains a unique *ClaI* site within the luciferase segment of the cloned Ad5-Luc 3 *NdeI-MunI* fragment. This *ClaI* site and a *MunI* site were then used to clone a 4.0-kb *ClaI-MunI* DNA fragment from plasmid pJM17 (40), containing the joined adenovirus inverted terminal repeats. Plasmid pJM17 contains a full-size, circular adenovirus genome and was generously provided by F. Graham. Since the *ClaI* site of interest in pJM17 overlaps Dam methylation sites, to provide accessibility for *ClaI* digestion, the plasmid was isolated from the Dam⁻ *Escherichia coli* strain JM110. The resultant plasmid, pAR, contains the pBR.MUN backbone flanked by two segments of adenovirus genomic DNA which normally flank the fiber gene in the Ad5-Luc 3 genome. To complete the construction of the rescue plasmid, a 30-kb *ClaI* fragment from Ad5-Luc 3 genomic DNA was cloned into the unique *ClaI* site of pAR. After electroporation of the ligated DNA into *E. coli* SURE cells (Stratagene, La Jolla, Calif.), ampicillin-resistant clones were isolated for restriction analysis. The complete rescue plasmid was designated pVK5.

A fiber shuttle plasmid was generated for incorporation of fiber variants into the adenovirus genome by recombination with the adenovirus fiber rescue plasmid pVK5. To construct the shuttle plasmid, a 3.6-kb *PacI-KpnI* DNA fragment of Ad5-Luc 3 DNA was cloned into the corresponding sites of the commercial vector pNEB193 (New England Biolabs, Cambridge, Mass.). The resulting plasmid, pNEB.PK3.6, contains a complete copy of the Ad5 fiber gene flanked by two segments of Ad5-Luc 3 DNA approximately 1.1 and 0.8 kb in length.

To create a silent mutation in the adenovirus fiber gene, a PCR-based mutagenesis method was used to modify codon Ala-579 of the fiber open reading frame (ORF) from GCC to GCG (Fig. 1). This substitution at position 1737 of the fiber ORF creates a novel recognition site for the restriction endonuclease *FspI*. Two pairs of primers were designed for this mutagenesis: primer F1 (5'-AAC AAA ATG TGG CAG TCA AAT AC-3'), primer F2 (5'-CAT ACA TTG CGC AAG AAT AAA G-3'), primer R1 (5'-CTT TAT TCT TGC GCA ATG TAT G-3'), and primer R2 (5'-TGA TGC ACG ATT ATG ACT CTA CC-3'). Primers F2 and R1 are complementary to the site of the mutation; primers F1 and R2 are complementary to DNA sequences outside the mutation site and designed as partners for R1 and F2, respectively. Generation of the mutation was accomplished via two sequential PCRs. First, primer pairs F1-R1 and F2-R2 were used with pNEB.PK3.6 to generate two DNA fragments overlapping at the mutation site. These two fragments were then used as the template for a second PCR with primer pair F1-R2. The DNA fragment generated via the second PCR contained the mutated alanine codon. To transfer the mutated segment of the fiber gene into pNEB.PK3.6, the PCR product was digested with *BstXI* and *MunI*. The 0.36-kb fragment generated was used to replace the analogous segment in pNEB.PK3.6. The DNA of the new plasmid, pNEB.PK.FSP, was partially sequenced to confirm the presence of the mutation.

To facilitate genetic manipulation of the Ad5 fiber gene, a master plasmid, pBS.F5wt, was made as follows. Plasmid pTF5F (41) was digested with *Acc65I*,

treated with Klenow enzyme, digested with *MunI*, and ligated with *EcoICRI-EcoRI*-digested pBluescript KS II (Stratagene). The resulting plasmid, pBS.F5wt, contains the full-length fiber ORF followed by part of the 3' untranslated region of the gene. To engineer a site suitable for the construction of fiber fusions, a unique *EcoICRI* restriction site was introduced at the 3' end of the fiber ORF by PCR-based mutagenesis. Primers F5.F1 (5'-ATG AAG CGC GCC AGA CCG TCT GAA G-3') and F5.R1 (5'-TTA GAG CTC TTG GGC AAT GTA TGA AAA AGT G-3') were used with pTF5F as a template to amplify the modified fiber gene. The PCR product was then digested with *BglII*, and a 0.3-kb DNA fragment was cloned into *BglII-EcoRV*-digested pBS.F5wt, resulting in pBS.F5.LEU. As a result of these modifications, the last GAA codon of the fiber ORF was mutated to GAG, and CTC was added to the sequence. This resulted in a unique *EcoICRI* restriction site at the 3' end of the fiber gene. To facilitate the subcloning of the chimeric fiber gene constructed in pBS.F5.LEU into the fiber shuttle vector pNEB.PK3.6, a segment of the 3' untranslated region of the fiber gene was synthesized as two oligonucleotides (5'-CTC TAA AGA ATC GTT TGT GTT ATG TTT CAA CGT GTT TAT TTT TCA ATT GAA GCT TAT-3' and 5'-CGA TAA GCT TCA ATT GAA AAA TAA ACA CGT TGA AAC ATA ACA CAA ACG ATT CTT TAG AG-3') and cloned into *EcoICRI-ClaI*-digested pBS.F5.LEU. The resulting plasmid, pBS.F5.UTR, was then used for all subsequent modifications of the fiber gene.

To generate recombinant fiber genes encoding chimeric fibers consisting of the Ad5 fiber tail and shaft domains with knob domains derived from other adenoviruses, plasmid pSHAFT was made as follows. Two PCR primers (5'-ATG CAC CAA ACA CAA ATC CCC TCA A-3' and 5'-CTC TTT CCC GGG TTA GCT TAT CAT TAT TTT TG-3') were used to modify the sequence of the Ad5 fiber gene coding for the TLWT motif which is highly conserved in most characterized mammalian adenovirus fiber genes (9). The DNA fragment generated with these primers from Ad5-Luc 3 genomic DNA was then digested with *NcoI* and cloned into *NcoI-EcoICRI*-digested pBS.F5.UTR. Plasmid pSHAFT contains a truncated sequence of the Ad5 fiber gene with a unique *SmaI* site located next to a Leu codon preceding the TLWT coding sequence. This plasmid was then used to construct a chimeric Ad5/Ad3 fiber gene. For this construction, a portion of the Ad3 fiber gene coding for the knob domain was PCR amplified by using plasmid pBR.Ad3Fib (generously provided by J. Chrobozcek, Grenoble, France) and a pair of primers: 5'-TAT GGA CAG GTC CAA AAC CAG AAG C-3' and 5'-TTT ATT AGT CAT CTT CTC TAA TAT AGG AAA AGG-3'. The PCR product was then cloned into *SmaI-EcoICRI*-digested pSHAFT, resulting in pBS.F5/3, which contains a chimeric fiber gene coding for the tail and shaft domains of Ad5 and the knob domain of Ad3 fiber.

To subclone the recombinant fiber gene into the fiber shuttle vector, a 0.73-kb *NcoI-MunI* DNA fragment of pBS.F5/3 was cloned into *NcoI-MunI*-digested pNEB.PK3.6, resulting in pNEB.PK.F5/3.

Generation of recombinant adenoviruses. To construct a recombinant adenovirus with a fiber gene containing the silent mutation, plasmids pNEB.PK.FSP and pVK5 were used for cotransfection of 293 cells as previously described (24). Two weeks posttransfection, the cells from tissue culture dishes showing cytopathic effects were lysed by freeze-thawing, and the lysate was then used for infection of monolayers of 293 cells. Virus isolated from infected cells 2 days later was used for purification of viral DNA as described previously (25). DNA isolated from the new virus, as well as DNA isolated from Ad5-Luc 3, was digested with *FspI*, and the products were analyzed by agarose gel electrophoresis.

Recombinant adenovirus containing chimeric Ad5/Ad3 fibers was generated by *in vitro* recombination between pVK5 and pNEB.PK.F5/3, using methods described above. To confirm the identity of the rescued virus, its DNA was characterized by restriction digestion with *DraI* and *ScaI*.

Expression of Ad5 and Ad3 knobs in *E. coli*. The knob domains of Ad5 and Ad3 fibers were expressed in *E. coli* with N-terminal His₆ tags, using the pQE30 expression vector (Qiagen, Hilden, Germany). Ad5-Luc 3 DNA and plasmid pBR.Ad3Fib were used as templates for PCR to amplify the knob domains of the respective fiber genes. Primers for these reactions were F5.F (5'-TTT AAG GAT TCC GGT GCC ATT ACA GTA GGA A-3'), F5.R (5'-TAT ATA AGC TTA TTC TTG GGC AAT GTA TGA-3'), F3.F (5'-CTC GGA TCC AAT TCT ATT GCA CTG AAA AAT AAC-3'), and F3.R (5'-GGG AAG CTT AGT CAT CTT CTC TAA TAT AGG AAA AGG-3'). Each pair of primers amplified a DNA sequence coding for the knob domain plus the last repeat of the shaft domain of the corresponding fiber polypeptide. Both PCR products were then digested with *BamHI* and *HindIII* and cloned into *BamHI-HindIII*-digested pQE30, resulting in plasmids pQE.KNOB₅ and pQE.KNOB₃. Recombinant proteins isolated from *E. coli* M15(pREP4) cells harboring pQE.KNOB₅ and pQE.KNOB₃ were purified on Ni-nitrilotriacetic acid agarose columns (Qiagen). The ability of both proteins to form homotrimers was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of boiled and unboiled samples as described before (41, 46). The concentrations of the purified knobs were determined by the Bradford protein assay (Bio-Rad, Hercules, Calif.), using bovine immunoglobulin G as the standard.

***In vitro* gene transfer mediated by the recombinant adenovirus containing chimeric fiber protein.** The tropism of the recombinant adenovirus vectors containing the type 5 fiber (Ad5-Luc 3) or the chimeric fiber consisting of the tail and shaft of Ad5 and the knob of Ad3 (Ad5/3-Luc 3) was evaluated by using the purified knob proteins to inhibit viral infection of target cells. The recombinant

adenovirus vectors used in this analysis carried the firefly luciferase reporter gene to facilitate this assay, which was performed as described previously (29). Monolayers of 293 cells in a 24-well tray were washed once with phosphate-buffered saline (PBS) and incubated at room temperature with 0.25 ml of various dilutions of knob in PBS. After receptor binding had been allowed to proceed for 10 min, cells were infected at a multiplicity of infection of 10 PFU per cell by addition of the recombinant adenovirus diluted in 0.25 ml of DMEM-F12 plus 4% FCS and incubation for 30 min at room temperature. The cells were then washed with DMEM-F12 plus 2% FCS and incubated in DMEM-F12 plus 2% FCS for 30 h at 37°C to allow luciferase expression. The cells were then lysed and assayed for luciferase activity by using a luciferase assay system (Promega, Madison, Wis.) according to the manufacturer's protocol.

Virus binding assay. The virus binding assay was performed in essentially the same way as the gene transfer assay described above. The viruses Ad5-Luc 3 and Ad5/3-Luc 3 were labeled with ^{125}I by using Bolton-Hunter reagent (Du Pont NEN, Boston, Mass.) according to the manufacturer's recommendations and purified by gel filtration on NAP-5 columns (Pharmacia, Piscataway, N.J.). 293 cells preincubated with PBS or with serial dilutions of the knobs in PBS were incubated with 0.25 ml of radiolabeled virus with a specific activity of 3.6×10^6 cpm/ml. After 30 min at room temperature, the cells were washed once with DMEM-F12 plus 2% FCS and collected. Radioactivity of the samples was determined with an Auto-Gamma 5000 γ counter (Packard Instruments, Downers Grove, Ill.).

RESULTS

Construction of a two-plasmid rescue system for derivation of adenovirus fiber recombinants. Methods have been developed to produce recombinant adenovirus vectors by using *in vivo* homologous recombination. These methods are based on noninfectious adenovirus genome constructs undergoing recombination in target cells to yield an infectious viral genome capable of propagating progeny virions. Techniques reported to date have included the use of overlapping linear DNA constructs (3, 4, 16) as well as the use of plasmid-based systems (40). In the latter instance, a two-plasmid strategy involving recombination between a shuttle plasmid, containing foreign gene sequences, and a rescue plasmid, providing the required viral functions, has been widely used (40).

For our strategy to generate adenovirus fiber variants, we have used the latter methodology. As a first step toward this goal, we constructed a fiber rescue plasmid for recombination with fiber variant-containing constructs. This plasmid, pVK5, was designed to possess the key attributes of described rescue plasmid vectors. The fiber rescue plasmid contains a viral genome joined at the inverted terminal repeats within a prokaryotic vector backbone. The fiber gene was deleted from the adenovirus genome via substitution with a segment of a bacterial plasmid. In addition, this prokaryotic vector backbone segment results in an oversized (40-kb), unpackagable adenovirus genome (5, 24). Thus, plasmid pVK5 would not be capable of generating progeny virions after transfection into eukaryotic cells because of its size plus the fact that viral fiber functions are essential for lateral infection and thus progeny plaque generation (6, 22).

The derived rescue plasmid differs in several additional respects from previously described adenovirus plasmid recombination systems (3, 4, 16, 25, 26). In this construction, we have retained the adenoviral E1A and E1B regions to allow replication of derived recombinant adenoviruses in a variety of target cells. Deletion of these regions could be accomplished, however, provided that viral rescue procedures are carried out in the context of an E1-transcomplementing cell line, such as 293 (26). In addition, we have incorporated a luciferase reporter gene within the viral genome of the rescue plasmid. To accomplish this, we have used DNA segments derived from the recombinant adenovirus Ad5-Luc 3 (44), which contains a firefly luciferase reporter gene in place of the deleted E3 region. The luciferase gene was included to provide an additional

means of monitoring viral progeny in our recombination system.

For use with the fiber rescue plasmid pVK5, we also derived a fiber shuttle plasmid. This plasmid was designed to provide a complete copy of the fiber gene for the generation of a recombinant viral genome. To achieve efficient recombination with the rescue plasmid, the shuttle vector must contain flanking regions of viral DNA homologous to corresponding regions in the rescue plasmid. The lengths of these flanks require sufficient overlap to provide efficient homologous recombination between the two plasmids. The fiber shuttle vector, pNEB.PK3.6, was thus designed to provide these functional requirements. In addition, pNEB.PK3.6 contains several unique restriction sites convenient for making modifications of the fiber gene.

Generation of a modified fiber gene for incorporation into the fiber rescue system. The design of the fiber rescue system was undertaken to allow construction of adenovirus vectors possessing modified fiber genes. The ultimate goal of this strategy is the introduction of ligands into the cell binding domains of the adenovirus fiber to modify viral tropism. To this end, we have previously created fiber-ligand fusion genes capable of expressing fiber-ligand chimeras in eukaryotic systems (41). These fusion molecules have been shown to possess key attributes predicting incorporation into viral particles, including a pattern of native fiber biosynthesis and assumption of authentic trimeric quaternary protein conformation (41). Thus, as a next step in developing tropism-modified virions, a system for incorporation of fiber variants into mature particles was required.

In this regard, several factors dictated that the efficiency of the fiber rescue system be validated prior to use in the production of fiber-ligand chimeras with modified tropism. Primarily, it was not clear if the fiber-ligand chimeras could mediate viral infection via the internalization pathway of the heterologous ligand. Furthermore, it was unclear if the addition of the ligand would adversely affect entry through the native adenovirus pathway via its intrinsic receptor. Thus, for initial proof of concept, we required a fiber variant which could be validated in the context of a new virion but which we knew would not impair plaquing efficacy after a recombinational event. The lack of any defective phenotype associated with this mutation would predict its authentic cellular processing. To this end, a fiber mutation which is silent with respect to viral infectivity was derived for our initial validation of the fiber rescue system.

To derive this variant fiber gene, PCR-based mutagenesis was used. This methodology was designed to introduce a silent mutation in the native fiber gene by means of a single-base substitution which created an additional recognition site for the restriction endonuclease *FspI* (Fig. 1). This new recognition site provided a convenient means to validate the introduction of the designed mutation into the 3' end of the fiber ORF by simple restriction analysis. We confirmed that this mutagenesis procedure introduced an additional *FspI* site into the fiber gene, as evidenced by the predicted restriction endonuclease digestion pattern and by DNA sequencing (data not shown).

Generation of recombinant adenovirus with a modified fiber gene. After validation of the presence of the silent fiber mutation, the mutated segment of the fiber gene was incorporated into the pNEB.PK3.6 fiber shuttle vector. To construct a recombinant adenovirus containing the mutated fiber gene, the newly generated fiber shuttle plasmid, pNEB.PK.FSP, and the rescue plasmid, pVK5, were cotransfected into 293 cells. The schema for the predicted recombination event is shown in Fig. 2. In this strategy, recombination between homologous regions

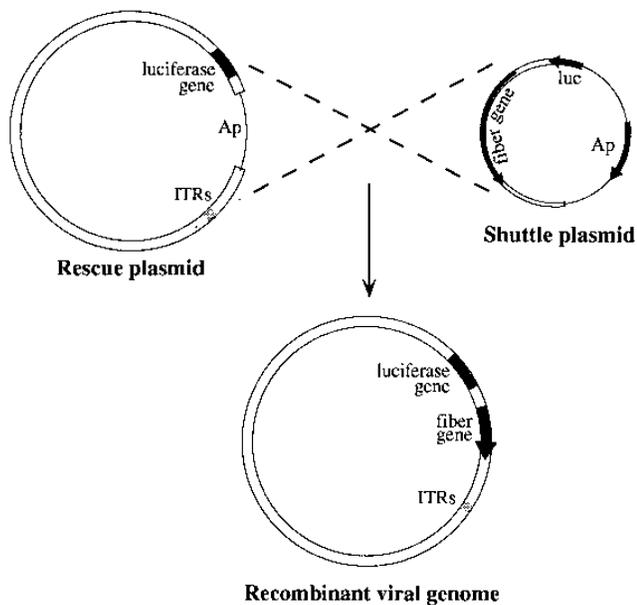


FIG. 2. Schema of in vivo homologous recombination between the fiber rescue and shuttle plasmids to generate an adenovirus vector containing fiber variants. The recombinant genome contains the fiber variant gene originating from the shuttle plasmid.

of the two plasmids would be predicted to yield an intact viral genome in which the fiber defect in the rescue plasmid was rectified. Such a genome would be capable of generating progeny virions, as evidenced by plaque formation. To be successful in generating progeny virus, the recombination event would thus require excision of the oversized stuffer segment in the deleted fiber gene region, as well as incorporation of an intact, functional fiber gene into the rescue plasmid.

After cotransfection of 293 cells with the fiber shuttle and rescue plasmids, a cytopathic effect was noted, indicating the presence of infectious viral progeny. Control transfections with pVK5 alone or pNEB.PK.FSP alone did not result in a cytopathic effect, confirming that the component plasmids were not individually capable of deriving viral progeny. Cells from cotransfected plates with evidence of viral propagation were lysed to release virus, which was then expanded to permit genomic DNA analysis. Viral DNA was subjected to restriction endonuclease analysis with *FspI* to confirm the presence of the silent mutation. The pattern of this digestion was as predicted by virtue of incorporation of the mutated fiber gene containing a novel *FspI* recognition site (Fig. 3A). In this regard, an 11-kb fragment was noted in Ad5-Luc 3 genomic DNA digested by *FspI*, corresponding to the right terminus of the viral genome (Fig. 3B). In contrast, in the DNA isolated from the virus rescued from the cotransfected cells, *FspI* digestion yielded DNA fragments of 8 and 3 kb. These findings were consistent with the concept that a recombinational event between the fiber shuttle and rescue plasmids had resulted in the derivation of a recombinant adenovirus incorporating the modified fiber gene.

The design of the two-plasmid rescue system incorporated a luciferase reporter gene for monitoring the homologous recombination event. The firefly luciferase reporter gene was originally placed in the context of an E3-deleted region in the replication-competent recombinant adenovirus Ad5-Luc 3 (44). In the two-plasmid rescue system, the location of part of the luciferase ORF in the 5' fiber flanking region would predict

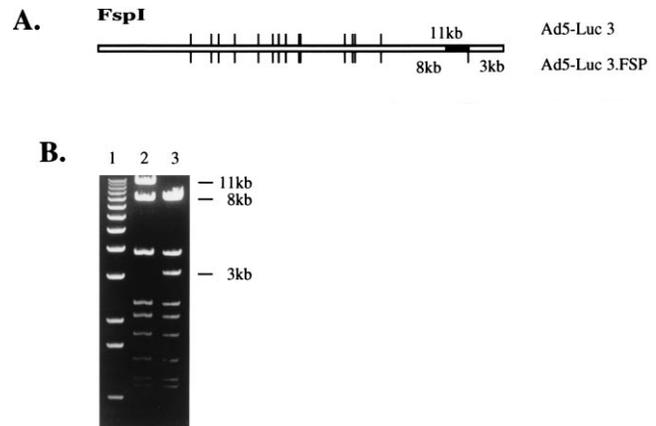


FIG. 3. Analysis of recombinant adenovirus containing fiber variant generated by the two-plasmid system. (A) Predicted map of restriction endonuclease recognition sites for Ad5-Luc 3 and the fiber variant derivative, Ad5-Luc 3.FSP. The filled box represents the fiber gene. (B) Analysis of genomic DNA derived from Ad5-Luc 3 and Ad5-Luc 3.FSP by restriction endonuclease *FspI* digestion. Lane 1, 1-kb marker; lane 2, Ad5-Luc 3 *FspI* digest; lane 3, Ad5-Luc 3.FSP *FspI* digest. Dashes indicate *FspI* restriction fragments of 11, 8, and 3 kb. The 11-kb fragment in the Ad5-Luc 3 *FspI* digest is replaced by 8- and 3-kb fragments in the Ad5-Luc 3.FSP digest, indicating the presence of a novel restriction site in the recombinant genome.

its involvement in at least a subset of productive recombinational events. As expected, progeny virions have demonstrated the capacity to accomplish efficient transfer of the luciferase gene to heterologous cells, as has been noted with the parent vector (data not shown). Thus, the incorporation of this reporter gene provided an additional method of validating the fidelity of the recombinational events allowing progeny virus derivation.

Generation of a recombinant adenovirus with chimeric fibers. Having demonstrated the utility of the two-plasmid fiber rescue system, our next step was to use this methodology to derive an adenovirus containing modified fibers. This analysis would serve to validate that a given fiber variant was structurally compatible with incorporation into a capsid structure. In addition, such a chimeric virus would demonstrate the feasibility of this approach for tropism modification based on altering the adenovirus cell recognition domain localized within the fiber knob. As a first step, we engineered a chimeric fiber gene encoding the tail and shaft domains from Ad5 and the knob domain from Ad3. After construction, the chimeric Ad5/Ad3 fiber gene was incorporated into the fiber shuttle vector, and homologous recombination was accomplished by using the fiber rescue plasmid as previously described.

In this procedure, viral plaques were derived and expanded so that the identity of the purified virus could be validated by genetic analysis. The introduction of the DNA encoding the Ad3 fiber knob domain into the chimeric fiber gene created several restriction endonuclease sites which are absent in the sequence encoding the Ad5 knob (Fig. 4A). In this regard, restriction polymorphisms would be predicted with respect to the restriction endonucleases *DraI* and *ScaI*. These differences thus provided a convenient means to validate the presence of the sequence encoding the Ad3 knob domain within the Ad5/Ad3 chimeric fiber gene. For this analysis, genomic DNA was derived from both the parent virus, Ad5-Luc 3, and the chimeric virus, Ad5/3-Luc 3. This viral genomic DNA was then subjected to restriction digestion with *DraI* and *ScaI* followed by agarose gel electrophoresis (Fig. 4B). In this analysis, it was observed that the predicted restriction pattern dictated by the

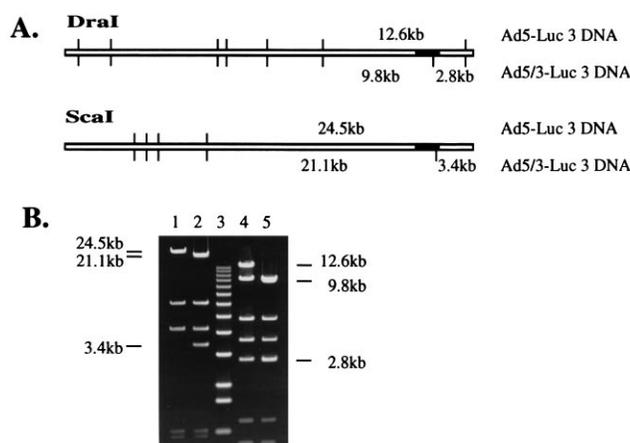


FIG. 4. Analysis of recombinant adenovirus containing chimeric fibers. (A) Predicted maps of *DraI* and *ScaI* restriction endonuclease recognition sites for Ad5-Luc 3 and Ad5/3-Luc 3. The filled box represents the fiber gene. (B) Analysis of genomic DNA derived from Ad5-Luc 3 and Ad5/3-Luc 3. Lane 1, Ad5-Luc 3 *ScaI* digest; lane 2, Ad5/3-Luc 3 *ScaI* digest; lane 3, 1-kb marker; lane 4, Ad5-Luc 3 *DraI* digest; lane 5, Ad5/3-Luc 3 *DraI* digest. Dashes indicate restriction fragments of 24.5, 12.6, 9.8, 3.4, and 2.8 kb.

introduction of the Ad3 fiber knob gene segment was indeed observed in the genome of the Ad5/3-Luc 3 adenovirus vector. Thus, analysis of genomic DNA derived from the fiber chimeric virus confirmed the presence of the fiber Ad5/Ad3 chimera gene. The identity of the chimeric fiber gene within the adenovirus genome was also validated by direct DNA sequence analysis (data not shown).

Analysis of the receptor recognition profile of the chimeric adenovirus vector. The construction of the adenovirus vector containing the chimeric fiber protein was undertaken to achieve altered receptor tropism for purposes of targeted gene delivery. In this regard, Ad3 and Ad5 achieve cellular entry via distinct cell surface receptors (17, 54). Thus, to validate the functional utility of constructing the chimeric fiber, we sought to demonstrate that entry of Ad5/3-Luc 3 occurred via the pathway dictated by the knob domain of the fiber. As the receptors for Ad3 and Ad5 coexist on many types of cells, including 293 cells, it was necessary to be able to validate specific entry via each pathway. Since previous studies have demonstrated the ability of recombinant trimeric knobs to block binding of the corresponding adenovirus or recombinant fiber protein to its receptor (29, 36, 54), we expressed recombinant Ad5/Ad3 knobs in *E. coli* and used SDS-PAGE to confirm that the purified proteins were trimeric (data not shown). Various concentrations of each recombinant knob were preincubated with 293 cell monolayers prior to infection with the parent adenovirus vector Ad5-Luc 3 or the modified adenovirus Ad5/3-Luc 3. Since both viruses carry the gene encoding firefly luciferase, viral infectivity was measured indirectly by determination of luciferase activity in the infected cells. Thus, entry via the Ad5 receptor was confirmed by competition with the recombinant type 5 knob, and entry via the Ad3 receptor was confirmed by competition with the type 3 knob.

This analysis confirmed that competition with the recombinant type 5 knob inhibited the infectivity of the parent virus Ad5-Luc 3 in a dose-dependent manner. When used at a concentration of 100 $\mu\text{g/ml}$, the type 5 knob inhibited 97% of the maximal luciferase activity, confirming that specific entry was via the Ad5 cellular receptor (Fig. 5A). The specificity of this interaction was confirmed in that type 3 fiber knob was not

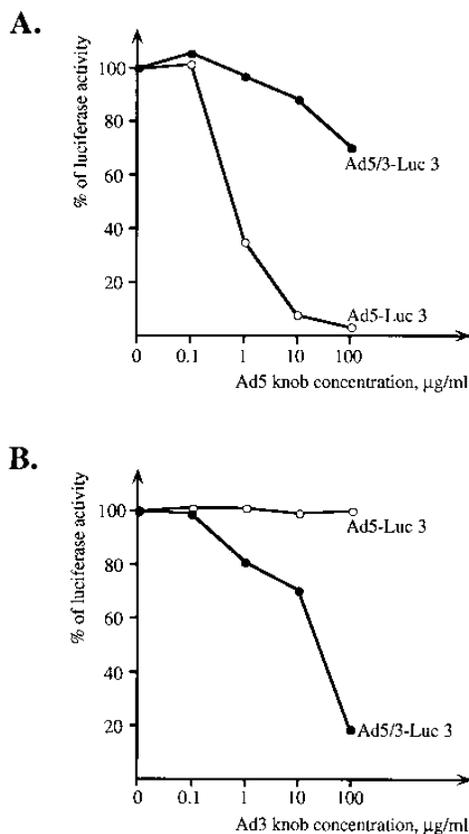


FIG. 5. Type-specific inhibition of adenovirus infectivity by recombinant knobs. 293 cells were preincubated with either type 5 (A) or type 3 (B) knob at the indicated concentrations for 10 min at room temperature to allow receptor binding. Ad5-Luc 3 or Ad5/3-Luc 3 was then added at a multiplicity of infection of 10, and incubation was continued for another 30 min at room temperature. The viruses were aspirated, and complete medium was added before transfer of the cells to 37°C. After 30 h, the cells were lysed and luciferase activity was determined. Luciferase activity is given as a percentage of the activity in the absence of blocking by recombinant knob. Each point represents the mean of two determinations.

capable of blocking the gene transfer by Ad5-Luc 3 in competition experiments (Fig. 5B). A similar analysis was then carried out with the Ad5/3-Luc 3 chimeric virus. In competition experiments using the type 3 knob, it was observed that the Ad5/3-Luc 3-mediated gene transfer could be blocked in a dose-dependent manner. At a concentration of 100 $\mu\text{g/ml}$, the type 3 knob inhibited 80% of the maximal luciferase activity of Ad5/3-Luc 3 (Fig. 5B). Conversely, gene transfer by this modified virus was only minimally inhibited by high concentrations of type 5 knob (Fig. 5A). These findings thus confirm that Ad5/3-Luc 3, which contains a chimeric fiber protein with the knob domain derived from Ad3, achieved cellular entry via the Ad3 pathway. Thus, the overall specificity of viral entry was dictated exclusively by the knob domain of the chimeric fiber.

The data obtained in the gene transfer experiment were corroborated by the results generated by the radiolabeled virus binding assay (Fig. 6).

DISCUSSION

In this report, we have described methods for the derivation of adenovirus vectors containing modified fibers. Using a two-plasmid rescue system, we have shown that adenovirus particles containing variant fiber gene constructs can be derived.

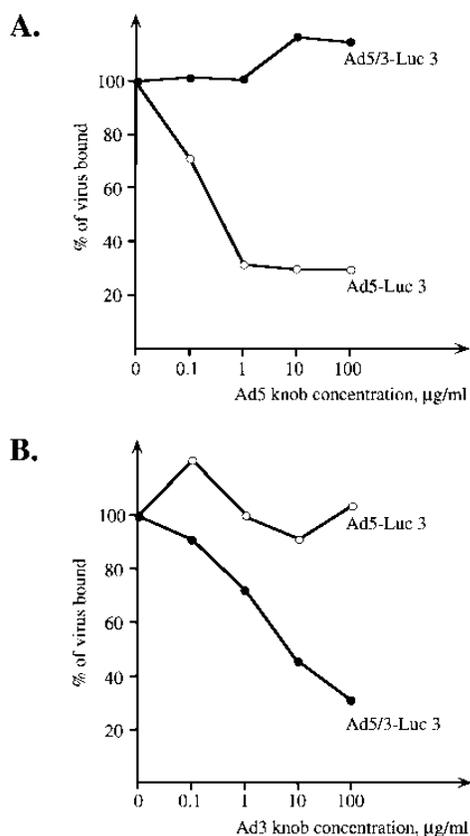


FIG. 6. Type-specific inhibition of the cell binding activity of radiolabeled adenovirus by recombinant knobs. 293 cells were preincubated with either type 5 (A) or type 3 (B) knob at the indicated concentrations for 10 min at room temperature. ^{125}I -labeled Ad5-Luc 3 or Ad5/3-Luc 3 was then added (8×10^5 cpm per well), and incubation was continued for another 30 min at room temperature. The viruses were aspirated, and the cells were collected for radioactivity determination. The amount of bound virus is given as a percentage of the labeled virus bound to the monolayer in the absence of blocking by recombinant knob. Each point represents the mean of three determinations.

Furthermore, we have used this method to produce an adenovirus vector containing a fiber chimera. It was shown that this modification alters the cellular receptor-binding profile of the chimeric adenovirus. The dose-dependent inhibition of Ad5/3-Luc 3 infectivity by Ad3 knob is similar to that observed previously by Henry et al., who used Ad5 knob to block Ad5 infection (29). Moreover, the nonspecific inhibition of Ad5/3-Luc 3 by Ad5 knob is similar to that reported by the same authors when they used an irrelevant ligand to block Ad5 infection. These findings thus support the concept that altering the cell recognition domains of the adenovirus represents an approach to achieve cell-specific targeting of gene delivery in the context of gene therapy strategies.

It has been recognized that the potential utility of recombinant adenovirus vectors for gene therapy has been limited by the promiscuous tropism of the parent virus. Consequently, attempts have been made to alter the tropism of the virion by modifying the viral domains involved in specific recognition of target cell receptors. These proposed modifications are based on presently characterized aspects of the viral entry pathway. In this context, it has been shown that the globular carboxy-terminal knob domain of the adenovirus fiber protein is the ligand for attachment to the adenovirus primary cellular receptor. This understanding is based on studies which demon-

strated that adenovirus infection can be blocked by recombinant adenovirus trimeric knob and by an antiknob antibody (29, 36); thus, the knob is both necessary and sufficient for virion binding to host cells.

Given these findings, modification of the knob domain of the fiber is a logical means to achieve alterations in adenoviral tropism. In this regard, Stevenson et al. have constructed recombinant chimeric fiber molecules consisting of the shaft domain of Ad5 and the knob domain of Ad3 (54). They demonstrated that the fiber-binding profile of the chimera was determined by its knob domain, thus confirming the exclusive role of the knob domain in dictating binding to the primary adenovirus cellular receptor. Furthermore, these chimeric fibers exhibited parameters consistent with their proposed use in viral tropism modification: they could assume the native trimeric conformation, which is necessary for association of the mature fiber protein with the penton base in the formation of the adenovirus capsid (47).

Our study extends these findings in that we have shown that the chimeric Ad5/Ad3 fiber is also capable of being incorporated into mature viral particles. Fiber associates with penton base by virtue of noncovalent interactions between the amino terminus of the fiber trimer and a conserved domain within the penton base (6). Alterations which perturb the ability of fiber monomers to achieve their native trimeric confirmation or alter the amino terminus of the molecule interfere with the ability of fiber to associate with penton base in the context of the mature capsid (47). It was thus not apparent from this earlier study that the chimeric fiber proteins would also be capable of incorporation into mature particles. Using our two-plasmid rescue system, we were able to demonstrate that the fiber chimeras were indeed capable of capsomeric association. Thus, this system may be more generally useful in determining which structural modifications of the fiber protein are compatible with the ability of fiber to assume its native configuration and, more importantly, allow assembly of fiber into mature particles on the basis of association with penton base capsomers.

Following viral attachment via the fiber knob domain, the next step in adenovirus infection is internalization of the virus by receptor-mediated endocytosis (27, 48, 50). This process is mediated by the interaction of Arg-Gly-Asp (RGD) sequences in the penton base with secondary host cell receptors represented by the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (2, 39, 61, 62). These secondary receptors are responsible for the major aspects of internalization after fiber binding. Recombinant fiber binding to its receptor does not trigger internalization (62). However, association of the recombinant fiber with recombinant penton base does trigger internalization (62). On the basis of this recognition, strategies have been proposed to alter viral tropism by modulating the penton-integrin component of the adenovirus entry pathway. To this end, Wickham et al. have derived recombinant penton bases which exhibit distinct cellular internalization profiles based on specific peptide sequences replacing the RGD region of the penton base (60). These alterations would be of utility for tropism modification in situations in which lack of cognate integrins on target cells was the limiting factor of adenovirus infection.

Several lines of evidence, however, suggest that the secondary receptor restrictions may not be relevant in the context of our retargeting strategy. In this regard, we have previously used adenoviruses for their endosomal lysis properties in the context of receptor-mediated gene delivery in molecular conjugate vectors (42). In this schema, we achieved incorporation of binding-incompetent adenoviruses into the molecular conjugate configuration while using antifiber antibody to block the

primary cell attachment of the adenovirus. These studies demonstrated that adenovirus receptor binding was not necessary for virus-mediated facilitation of conjugate internalization. Thus, in this model, conjugate-DNA complex binding was achieved via a nonviral receptor and internalization was accomplished by viral mechanisms. This established that adenovirus binding and internalization can be functionally uncoupled. The findings of the present study are in accord with this concept, in that the chimeric adenovirus achieved primary binding via the Ad3 receptor with subsequent internalization steps achieved via domains of the penton base of Ad5. In this regard, the efficient reporter gene delivery accomplished via the recombinant adenovirus incorporating the chimeric Ad5/Ad3 fiber suggests the ability of the type 3 knob to function in a coordinated and effective manner with other relevant type 5 capsid components to achieve viral entry. This may suggest a more general concept that binding of modified fibers to specific cellular receptors might be sufficient to achieve the retargeting of adenovirus vectors. After binding, the native adenovirus internalization mechanism mediated by the penton base would then permit the infection of the target cells.

The development of the described fiber rescue system will permit the derivation of adenovirus vectors containing a variety of modified fibers. On the basis of these findings, it should be feasible to incorporate fiber variants with targeting potential into mature viral particles by using these same methods. This capacity will be of key utility in the context of strategies to develop tropism-modified adenoviruses capable of targeted, cell-specific gene delivery (20, 41). In this regard, genetic methods have been successfully used to alter the tropism of retrovirus vectors toward the goal of cell-specific targeting. This has been accomplished both by pseudotyping (19, 21, 59, 63) and by direct genetic modifications of the envelope glycoprotein of the retrovirus particle (10, 11, 13, 28, 33, 49, 53, 58). In the latter instance, cell-specific targeting has been achieved by using ligands (13, 28, 33, 58) or single-chain antibodies (10, 11, 49, 53) in fusion with the envelope glycoprotein as targeting moieties. Thus, a substantial body of work has validated the concept of tropism modification of retrovirus vectors as a means to achieve targeted, cell-specific gene delivery (reviewed in reference 20).

Despite these advancements in retrovirus vector development, the direct utility of these maneuvers with regard to practical gene therapy approaches is not immediately apparent, since retroviruses are highly labile *in vivo* (8, 56). This phenomenon is understood to reflect effective humoral system-mediated clearance subsequent to intravascular delivery. It must therefore be recognized that the various retrovirus targeting maneuvers are not of a high level of utility in the context of strategies designed to accomplish direct, *in vivo* transduction subsequent to systemic administration. Thus, despite the acquisition of a targeting capacity, these modifications have not allowed a more generalized use of retrovirus vectors for transduction of nonlocalized targets. In contrast, adenoviruses are highly competent in achieving direct, *in vivo* gene delivery (7, 55). Thus, modifications to adenovirus vectors allowing cell-specific targeting would appear to be of direct utility in gene therapy approaches.

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