

Unique Biologic Properties of Recombinant AAV1 Transduction in Polarized Human Airway Epithelia*

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The choice of adeno-associated virus serotypes for clinical applications is influenced by the animal model and model system used to evaluate various serotypes. In the present study, we sought to compare the biologic properties of rAAV2/1, rAAV2/2, and rAAV2/5 transduction in polarized human airway epithelia using viruses purified by a newly developed common column chromatography method. Results demonstrated that apical transduction of human airway epithelia with rAAV2/1 was 100-fold more efficient than rAAV2/2 and rAAV2/5. This transduction profile in human airway epithelia (rAAV2/1 \gg rAAV2/2 = rAAV2/5) was significantly different from that seen following nasal administration of these vectors to mouse lung (rAAV2/5 > rAAV2/1 \gg rAAV2/2), emphasizing differences in transduction of these serotypes between these two species. In stark contrast to rAAV2/2 and rAAV2/5, rAAV2/1 transduced both the apical and basolateral membrane of human airway epithelia with similar efficiency. However, the overall level of transduction across serotypes did not correlate with vector internalization. We hypothesized that differences in post-entry processing of these serotypes might influence the efficiency of apical transduction. To this end, we tested the effectiveness of proteasome inhibitors to augment nuclear translocation and gene expression from the three serotypes. Augmentation of rAAV2/1 apical transduction of human polarized airway epithelia was 10-fold lower than that for rAAV2/2 and rAAV2/5. Cellular fractionation studies demonstrated that proteasome inhibitors more significantly enhanced rAAV2/2 and rAAV2/5 translocation to the nucleus than rAAV2/1. These results demonstrate that AAV1 transduction biology in human airway epithelia differs from that of AAV2 and AAV5 by virtue of altered ubiquitin/proteasome sensitivities that influence nuclear translocation.

Adeno-associated virus (AAV)² is a nonpathogenic parvovirus with a 4.7-kb single-stranded DNA genome (1). At least

eight serotypes of the AAV family have been identified and evaluated as recombinant vectors (2), and many more have been isolated and cloned from non-human primates (3) and other species. While these various serotypes have very similar genomic organizations, their infectious tropisms vary greatly as a result of evolutionary diversity among capsid proteins. The identification of optimal AAV serotypes for a given tissue target has been an area of intense interest in gene therapy research (4, 5). AAV type 2 is the first primate AAV to be cloned and the most extensively studied (6, 7).

Recombinant AAV2 (rAAV2) has been effectively developed into a highly promising vector system for gene therapy of various human genetic diseases including cystic fibrosis (CF) (8, 9). CF is a recessive genetic disease caused by a gene defect in the cystic fibrosis transmembrane regulator (CFTR). The CFTR defect most strongly affects the lung and leads to progressive and life-threatening bacterial lung infection. rAAV2-mediated CFTR gene transfer to the human CF lung has been extensively studied in clinical trials. Phase I and II trials for CF lung disease with rAAV2 have demonstrated a promising safety profile and persistence of viral DNA genomes in airway epithelia. However, CFTR gene expression in these trials was sufficiently low to preclude the detection of transgene-derived CFTR mRNA (10–13). Studies of rAAV2 transduction *in vitro* using polarized human airway epithelia models has revealed that the post-entry intracellular trafficking barriers and impaired processing of rAAV2 appear to be primarily responsible for the observed reduced transgene expression following apical infection of human airway epithelia (14, 15). Similar findings demonstrating that rAAV2 has impaired nuclear transport and uncoating have also been described for primary murine hematopoietic cells and fibroblasts (16–18).

Our previous studies demonstrated that co-administration of proteasome inhibitors, such as tripeptidyl aldehyde or/and anthracycline compounds, resulted in a dramatic enhancement of transgene expression following rAAV2 apical infection of human polarized airway epithelia *in vitro* and of mouse lungs *in vivo* (14, 15). Modulating proteasome activity under these conditions did not result in increased virion uptake or reduced virion degradation. Furthermore, proteasome modulation did not appear to directly affect the efficiency of second strand synthesis in airway epithelia as judged by a comparison of self-complementary and full-length vector transduction (19). Rather, augmentation of transgene expression by proteasome inhibitor treatment was accompanied by an increase in capsid ubiquitination and enhanced nuclear uptake of virions in the

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² The abbreviations used are: AAV, adeno-associated virus; rAAV, recombinant AAV; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; HPLC, high performance liquid chromatography; CHO, Chinese hamster ovary; LLnL, N-acetyl-L-leucyl-L-leucyl-L-norleucine; PBS, phosphate-buffered saline.

infected cells (14, 15, 20). Treatment of human CF airway epithelia with proteasome inhibitors at the time of rAAV2 or rAAV5 infection also dramatically enhances CFTR gene delivery and the correction of CFTR-mediated short circuit currents (21). Although proteasome inhibitor treatment may eventually be an effective adjunct method to enhance rAAV-mediated gene delivery in clinical trials, the identification of alternative rAAV serotypes less susceptible to ubiquitin/proteasome-mediated blocks would be a preferred route to improving current clinical trials.

It has been reported that rAAV type 1, 5, and 6 vectors transduce mouse lungs much more efficiently than type 2 vectors (22–25). However, a recent report has demonstrated that rAAV2 and rAAV5 transduction biology in human and mouse polarized airway epithelia significantly differs, questioning the validity of using mouse models for testing the preclinical efficacy of various AAV serotypes (26). Given the species-specific differences in transduction with various AAV serotypes and the fact that rAAV1 biology of transduction has yet to be evaluated in human airway epithelia, we sought to directly compare the biological properties of rAAV1, rAAV2, and rAAV5 transduction in polarized human airway epithelia. These studies revealed that rAAV1 was 100-fold more effective at transducing human polarized airway epithelia from the apical membrane in comparison to the other serotypes. Interesting differences in the polarity of transduction from the apical and basolateral membranes between these three serotypes also exist. In contrast to rAAV2 and rAAV5, rAAV1 equally transduced both the apical and basolateral membranes of polarized human airway epithelia without polarity bias of virion entry. Studies comparing the extent of ubiquitin/proteasome-sensitive nuclear transport and transduction between these serotypes also suggest that higher levels of rAAV1 transduction in human airway epithelia might be the result of more efficient intracellular processing of this serotype.

EXPERIMENTAL PROCEDURES

Recombinant AAV Vector Production—Since similar vector purity is paramount for studies comparing the biology of different AAV serotypes, we developed a uniform procedure for purification of rAAV2/2, rAAV2/1, and rAAV2/5 vectors using an adenovirus-free packaging system and HPLC (high performance liquid chromatography). Adenovirus helper plasmid pAD Helper 4.1, AAV2 helper plasmid pBS-HSP-RC2.3, and AAV5 helper pBS-HSP-R2C5 were provided by Targeted Genetics Inc. (Seattle, WA). AAV1 helper plasmid p5E18RXC1 was a kind gift from the Vector Core at the University of Pennsylvania. All the viral stocks were generated from the same AAV2 proviral plasmid pAV2-CMVluc-flag, which was also provided by Targeted Genetics Inc. The identical rAAV2 genome was encapsidated into different AAV serotype capsids to generate rAAV2 virus (AV2/2.Luc) and pseudotyped rAAV1 (AV2/1.Luc) and rAAV5 (AV2/5.Luc) viruses. Viruses were purified with a similar procedure by iodixanol discontinuous gradient ultracentrifugation as described previously (27), followed with HPLC on a PI-Porous ion-exchange column as described below. Viral titers were determined as DNase-resistant particles using a TaqMan PCR approach and were confirmed using

slot blot hybridization with a ^{32}P -labeled probe against the luciferase gene.

pAD Helper 4.1 and pAV2-CMVluc-flag plasmids were cotransfected with one of the AAV helper plasmids (pBS-HSP-RC2.3, pBS-HSP-R2C5, or p5E18RXC1) into 293 cells. For a forty 150-mm plate preparation, the amount of each plasmid used in the transfection mixture was 1.5 mg of pAD Helper 4.1, 0.5 mg of pAV2-CMVluc-flag and 1.0 mg of AAV helper. The cells were harvested at 72 h post-transfection and lysed by three rounds of freezing and thawing followed by sonication. The crude lysates were further incubated with DNase I for 30 min followed by 0.05% trypsin, 1% deoxycholate for another 30 min. Before being loaded on iodixanol discontinuous gradients, cell lysates were clarified by centrifugation at $7000 \times g$ for 10 min. The clarified supernatant was diluted with 10 mM Tris-HCl buffer, pH 8.0, to a final volume of 24 ml, and NaCl was also added to a final concentration of 1 M during the dilution. Using Beckman 36.2 ml OptiSeal centrifuge tubes, the preparation of iodixanol discontinuous gradients was adopted from methods described by Zolotukhin *et al.* (27) by underlaying 12 ml of clarified lysate with 9 ml of 15% iodixanol, 6 ml of 25% iodixanol, 5 ml of 40% iodixanol, and 4.2 ml of 54% iodixanol. For a forty 150-mm plate scale preparation, two tubes were needed. The loaded tubes were centrifuged in a Beckman VTi50 rotor at 45,000 rpm for 2 h at 18 °C. The rAAV band of ~4–5 ml was located in the 40% iodixanol layer and was collected with a syringe inserted at the 40%/54% interface. The AAV-containing fractions were combined and diluted to 200 ml with loading buffer A (20 mM Tris-HCl, pH 8.0, 20 mM NaCl) and directly loaded on a 4.6×100 -mm (column volume: 1.7 ml) anion-exchange Poros PI column (Applied Biosystem, Boston, MA) using a Beckman 2000 BioSysTM HPLC work station at the flow rate of 4 ml/min. After a brief wash with the loading buffer (A buffer), the column was eluted in a 15-column volume linear NaCl gradient from 20 mM to 1 M with high salt buffer B (20 mM Tris-HCl, pH 8.0, 1 M NaCl) at the flow rate of 4 ml/min. AV2/1.Luc eluted from the column at the gradient of 50% B buffer; AV2/2.Luc at 45%; and AV2/5.Luc at 40%, as shown in Fig. 1A. The purity of viral preparations was assessed using a 10% SDS-PAGE stained with Coomassie Blue.

Cell Culture—293 cells were cultured as monolayers in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and penicillin-streptomycin, and were maintained in a 37 °C incubator at 5% CO₂. CHO-K1 (ATCC catalog number CCL-61) cells were propagated in Kaighn's modification of Ham's F-12 medium (F12K) containing 2 mM L-glutamine and 1.5 g/liter sodium bicarbonate, supplemented with 10% fetal bovine serum and penicillin-streptomycin, and maintained in a 37 °C incubator at 5% CO₂. Polarized human airway epithelia were generated as described previously from lung transplant airway tissue by growing airway epithelia on 12 mm Millicell membrane inserts (Millipore Corp., Bedford, MA) followed by differentiation at an air-liquid interface (28).

Chemicals—Tripeptidyl aldehyde inhibitor LLnL (*N*-acetyl-L-leucyl-L-leucyl-L-norleucine, also called MG101) was purchased from Boston Biochem (Boston, MA) and dissolved in Me₂SO as a 40 mM stock solution. Doxorubicin (also called adriamycin) was purchased from Calbiochem and dissolved in

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phosphate-buffered saline (PBS) as a 500 μM stock solution. Iodixanol was purchased as 60% aqueous solution "Opti-PrepTM" (Axis-Shield PoC AS, Oslo, Norway) and further diluted with phosphate-buffered saline containing 1 mM MgCl_2 and 2.5 mM KCl to 15, 20, 40, and 55% (w/v) for use in gradient cushions during viral isolation.

Virus Infections—293 cells and CHO cells were grown in 24-well plates to 70% confluence prior to infection with rAAV at a multiplicity of infection equal to 100 particles/cell. Viral infection was performed in 0.25 ml of serum-free medium. At 2 h post-infection, 0.25 ml of medium with 20% FBS was added to bring the final serum level to 10%. Viruses were left in the medium for an additional 22 h. At 24 h post-infection, luciferase assays were performed to quantify gene expression. rAAV infections of fully differentiated polarized human airway epithelia were performed as described previously (14, 15). In brief, 2×10^9 particles of rAAV were diluted in culture medium to 50- μl volume to infect each Millicell insert from either the apical or basolateral compartment for a 16-h period. Following infection, the medium and virus were removed, and cultures were returned to an air-liquid interface with fresh medium. When proteasome inhibitors were applied, chemicals were added directly to the viral inoculum when infection was initiated and left in the medium only during the infection period. Control wells not treated with proteasome inhibitors received an equivalent amount of vehicle using the same protocol. Luciferase assays were performed 3 days post-infection. *In vivo* gene delivery studies utilized 4-week-old C57BL6 mice infected with AV2/1.Luc, AV2/2.Luc, and AV2/5.Luc virus by nasal instillation as described previously (15). rAAV vectors were diluted with PBS, and 30 μl of virus containing 6×10^{10} particles of rAAV was intranasally instilled in each mouse. Mice were sacrificed 2 weeks after infection, and the lungs were collected for luciferase expression assays.

Assays for Internalization of rAAV—Fully differentiated human polarized airway epithelia were infected from the apical or basal membrane with 10^{10} particles of rAAV vector as described above. At 2 h post-infection, virus was removed from the epithelia by washing thoroughly with PBS. Each Millicell insert was put into a 50-ml conical tube containing 40 ml of PBS and washed by gently inverting the tube. This was repeated three times to ensure that all the non-specifically bound viruses were removed. After washing, the Millicell inserts were fed with fresh medium and put back into a 37 °C incubator for 5 min or 22 h. Low molecular weight Hirt DNA was then extracted from the rAAV infected epithelia as described previously (14, 15). $\frac{1}{10}$ of the Hirt DNA preparation was used for viral genome quantification by PCR.

Quantitative Analysis of rAAV Genome by TaqMan PCR—TaqMan PCR was used to quantify the physical titers of the viral stocks and the copies of viral genome in the Hirt DNA from rAAV-infected cells (29). PCR primers and the TaqMan probe were designed with *Primer Express* V1.5 (PE Applied Biosystems, Foster City, CA). The forward primer, P1 (5'-TTTT-TGAAGCGAAGGTTGTGG-3'), and the reverse primer, P2 (5'-CACACACAGTTCGCCTCTTTG-3'), were designed to amplify a 73-bp fragment of the AV2Luc DNA. The TaqMan probe (5'-ATCTGGATACCGGGAAAACGCTGGGCGTT-

AAT-3') was designed following the general principle outlined by the manufacturer. The TaqMan probe was synthesized by IDT (Coralville, IA) and tagged at the 5' end with 6-carboxyl-fluorescein as the reporter, and with Dark Hole Quencher 1 (BHQ1) at the 3' end as the quencher. The 25- μl PCR mixture consisted of 5 μl of Hirt DNA sample ($\frac{1}{10}$ of the preparation), primers P1 and P2 (final concentration 300 nM), TaqMan probe (final concentration 100 nM), and 12.5 μl TaqMan Universal Master Mix (Bio-Rad). The PCR reaction was performed and analyzed using a Bio-Rad MyIQTM PCR detection system and software. The reaction condition was set as one cycle at 95 °C for 10 min followed by a two-step PCR procedure consisting of 15 s at 95 °C and 1 min at 60 °C for 40 cycles. All standard dilutions, controls, and unknown samples were run in triplicate. The average value of the copy number was calculated according to a standard AAV2 vector dilution series. A standard curve for AV2Luc was accepted as linear when the slope fell between -3.0 and -3.4 , and the coefficient of correlation was above 0.99.

Subcellular Fractionation of rAAV Genomes—Human polarized airway epithelia were infected from the apical membrane with 2×10^{10} particles of rAAV vector as described above. The infections were performed in the presence or absence of proteasome inhibitors (40 μM LLnL and 5 μM doxorubicin) for 16 h. The Millicell inserts were then washed twice with medium to remove the viruses and were re-fed with proteasome inhibitor-free fresh medium and incubated for two more days. At 3 days post-infection, airway cells were trypsinized from the Millicell membrane, washed twice with 5 ml of PBS, and then transferred to a microcentrifuge tube in 1 ml of PBS. Cytoplasmic and nuclear fractions were then isolated from the cell pellet as described previously (15, 30). The subcellular fractions were then digested with 0.25 mg/ml proteinase K at 56 °C for 60 min followed by heating at 95 °C for 15 min to inactivate proteinase K. TaqMan PCR was performed to quantify the copies of viral genome in each fraction.

RESULTS

Purification of rAAV1, -2, and -5 Serotypes—With the goal of comparing transduction efficiencies (*i.e.* transgene expression) between AAV2/1, AAV2/2, and AAV2/5 vectors, it was critical to develop a similar strategy of purification for these three serotypes to avoid potential differences in transduction caused by variation in virus purity. Although CsCl fractionation has been adopted previously for this purpose, we have found this method insufficient to purify virus from cellular impurities. To this end, we developed a rapid and uniform purification procedure to produce high purity rAAV from these three serotypes. As shown in Fig. 1A, the three serotypes eluted from the HPLC ion-exchange column in the range of 40–50% NaCl with slight variations for each serotype. Coomassie Blue staining of 10% SDS-PAGE demonstrated three major capsid bands with no detectable impurities (Fig. 1B). The total recovery of rAAV DNase-resistant particles from the crude lysate was $\sim 60\%$ (80% recovery from iodixanol fractionation and 75% recovery from HPLC ion exchange). The infectivity of AV2/1.Luc, AV2/2.Luc, and AV2/5 was tested on two different cell lines. The target cells were infected with rAAV at 100 viral DNase-resistant particles/

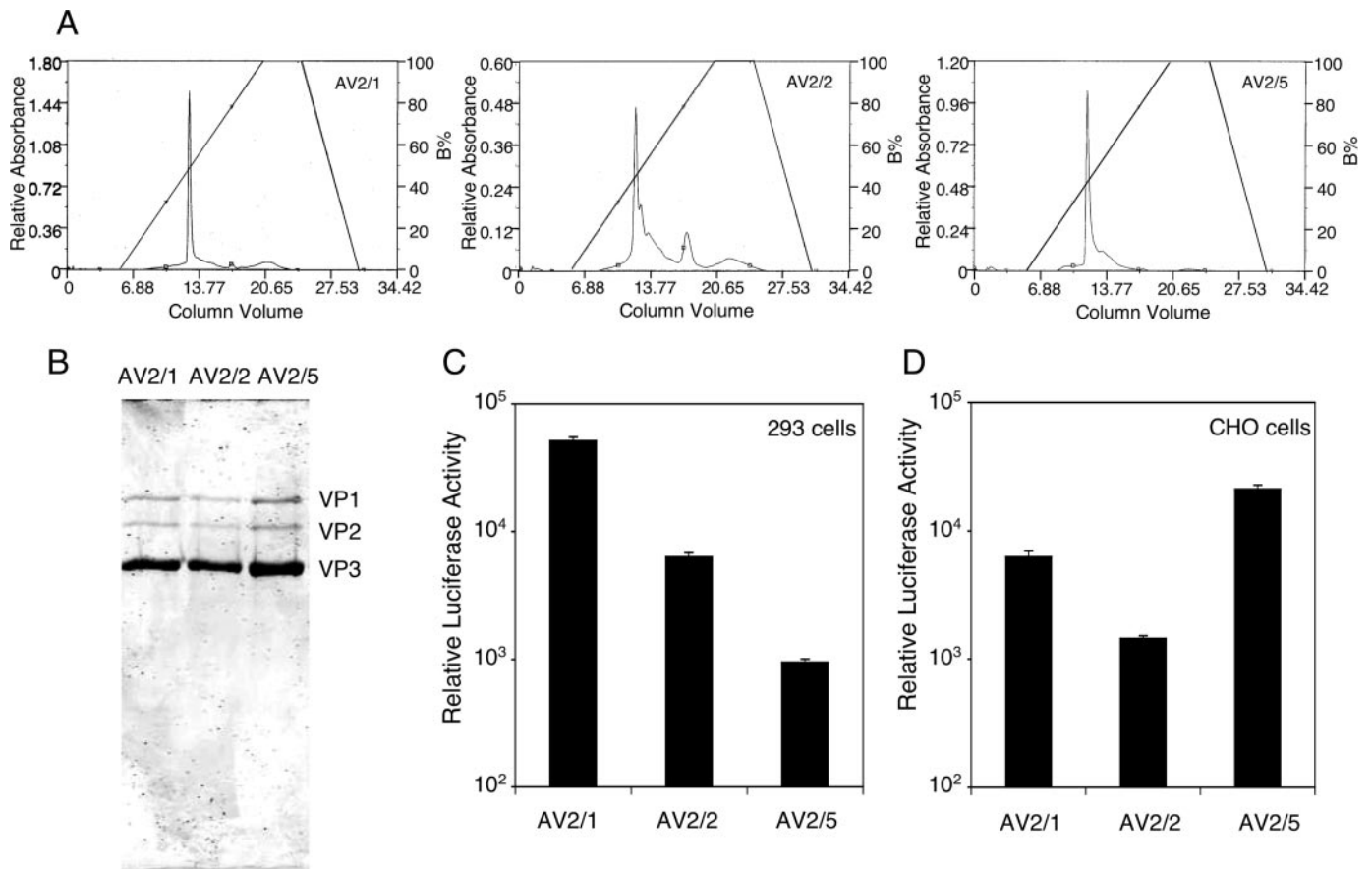


FIGURE 1. HPLC purification of rAAV type 1, 2, and 5 vectors by ion-change HPLC. *A*, HPLC chromatographic profiles for rAAV2/1, rAAV2/2, and rAAV2/5 on a 4.6×100 -mm Poros PI column at a flow rate of 4 ml/min. The chromatograph was monitored by absorption at 280 nm on the left y axis. The buffer set consisted of low salt loading buffer (A buffer: 0.02 M NaCl, 20 mM Tris-HCl, pH 8.0) and high salt buffer (B buffer: 1 M NaCl, 20 mM Tris-HCl, pH 8.0). The same buffer set was used for isolation of all three AAV serotypes. A linear 0.02–1 M NaCl gradient (from 0 to 100% B buffer) with 15 column volumes was adopted in the elution. The right y axis denotes percent Buffer B in the effluent. rAAV2/1, rAAV2/2, and rAAV2/5 were eluted from the column at the gradient at 0.51, 0.46, and 0.41 M NaCl, respectively. *B*, 5×10^{10} particles of each HPLC-purified virus were resolved on a SDS-PAGE, and the capsid proteins (VP1, VP2, and VP3) were visualized with Coomassie Brilliant Blue staining. No obvious impurities were detected under these conditions. The infectivity of purified rAAV serotypes was compared on 293 cells (*C*) and CHO-K1 cells (*D*). Cells were cultured in 24-well plates to 70% confluence and infected with AV2/1.Luc, AV2/2.Luc, or AV2/5.Luc at a multiplicity of infection of 100 particles/cell. Transduction efficiencies, as assessed by luciferase expression, were evaluated at 24 h post-infection. Data represents the mean (\pm S.E.) relative luciferase activity (per well) for four independent experiments.

cell, and transgene expression was assessed at 24 h post-infection as shown in Fig. 1, *C* and *D*. Not surprisingly, the various serotype of rAAV demonstrated different transduction efficiencies in 293 cells and CHO-K1 cells. rAAV2/1 and rAAV2/2 efficiently infected both human and rodent cell lines. In contrast, rAAV2/5 transduced CHO cells more efficiently than 293 cells.

rAAV2/1 Transduces Human Polarized Airway Epithelia from the Apical Basolateral Membranes Much More Efficiently than rAAV2/2 and rAAV2/5—It has been reported that gene delivery to mouse lung using rAAV1, rAAV5, and rAAV6 is significantly higher than with rAAV2 (15, 22–25). However, recently the validity of predicting rAAV serotype specificity for transduction of human airway epithelia using *in vivo* mouse models has been questioned (26). This study uncovered species-specific differences in the efficiency of rAAV5 and rAAV2 transduction between human and mouse polarized airway epithelia that explain the greater efficiency of *in vivo* rAAV5 transduction in mouse lung; rAAV5 transduction of mouse lung and polarized airway epithelia is ~ 75 –100-fold greater than that with rAAV2, whereas human polarized airway epithelia are

transduced with rAAV5 and rAAV2 with near equivalent efficiencies (15, 26). These studies suggested that inherent differences in tropism for these two serotypes exist in mouse and human airway epithelia. As part of the present study aimed at evaluating rAAV1 transduction in human airway epithelia, we sought to extend this type of interspecies comparison to all three serotypes tested. Consistent with a previous report comparing AAV1, -2, and -5 gene delivery in mouse lung (23), we observed that our methods of generating and purifying rAAV from these three serotypes also demonstrated that AV2/2 virus was the least efficient at expressing luciferase transgene in mouse lung, with 30- and 100-fold higher levels of transduction observed with AV2/1 and AV2/5 vectors, respectively (Fig. 2*A*). When these same viruses were tested on human airway epithelium (Fig. 2*B*), apical transduction with AV2/1 was 100-fold higher than with AV2/2 or AV2/5. As observed previously, AV2/2 and AV2/5 vectors transduced the apical surface of human airway epithelia with similar efficiency (15); this was clearly different from the transduction efficiency observed following infection of mouse lung with these two serotypes. Interestingly, AAV1 capsid was the only serotype with the ability to

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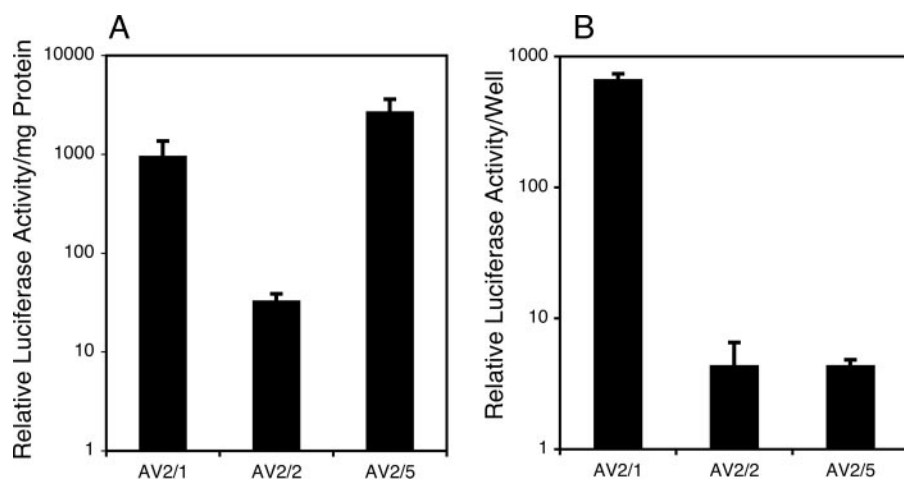


FIGURE 2. **Species-specific differences in airway transduction with AAV1, -2, and -5 serotypes.** A, comparison of rAAV vector serotypes in mouse lung. C57/BL6 male mice (4-week-old) were infected with 6×10^{10} particles of AV2/1.Luc, AV2/2.Luc, or AV2/5.Luc by nasal inhalation. At 2 weeks post-infection, relative luciferase activity of whole lung homogenates was assessed and normalized per mg of total protein. The value represents the mean (\pm S.E.) relative luciferase activity per mg protein of lung homogenate ($n = 3$). B, a similar serotype comparison of transduction was performed on differentiated human airway epithelia grown at an air-liquid interface in Millicell inserts. 2×10^9 particles of AV2/1.Luc, AV2/2.Luc, or AV2/5.Luc were applied to the apical surface of polarized human airway epithelial cultures for 24 h. Luciferase expression was assayed at 3 days post-infection. Data represents the mean (\pm S.E.) relative luciferase activity (per well) from 10 independent infections ($n = 10$).

efficiently transduce both mouse and human airway epithelia. This finding led us to hypothesize that AAV1 capsid has unique biologic properties for transduction of human airway epithelia that are different from those of AAV2 and AAV5 serotypes.

To further investigate differences in the biology of AAV1 transduction of human airway epithelia, as compared with AAV2 and AAV5, we assessed the polarity of transduction with these three serotypes. Assessment of rAAV infection from the apical and basolateral membranes of three independent donor-derived human airway epithelia cultures demonstrated that AV2/1 virus lacked polarity bias of transduction (*i.e.* transduced the apical and basolateral membrane with equal efficiency) (Fig. 3). This observation was significantly different from that for AV2/2 and AV2/5, in which transduction following basolateral membrane infection of human airway epithelia was significantly greater than that following apical infection (Fig. 3). These findings supported the original hypothesis that enhanced transduction of human airway epithelia with rAAV1, as compared with rAAV2 and rAAV5, may be the result of divergent biologic properties.

Differences in Endocytosis of rAAV1, -2, and -5 Cannot Account for Enhanced Transduction of Human Airway Epithelia with rAAV1—We hypothesized that the enhanced rAAV1 transduction observed from the apical membrane of human airway epithelia might be due to increased receptor-mediated uptake of this serotype. Since the receptor for AAV1 has yet to be elucidated, we chose to directly compare the extent of endocytosis of the various AAV serotypes by analyzing viral genomes using TaqMan PCR of Hirt DNA. Cells were infected with equal amounts of virus from either the apical or basal membrane for 2 h followed by extensive washing of the transwell. Hirt DNA was then prepared at both 2 and 24 h post-infection time points. TaqMan PCR analysis of total viral genomes present in the Hirt DNA at 2 h following apical and

basolateral infection revealed a significantly different pattern than that for AAV2 and AAV5 serotypes (Fig. 4A). While AAV2 and AAV5 demonstrated preferred entry from the basolateral surface (the efficiency of rAAV2 and rAAV5 entry from basolateral membrane was 100- and 10-fold higher than from apical membrane, respectively), AAV1 entered both the apical and basolateral membranes with equal efficiency. Interestingly, despite the 100-fold higher ability of AAV1 to transduce polarized airway epithelia from the apical membrane in comparison to the other two serotypes (*i.e.* express transgene), there was only a 2–3-fold variation in the number of viral genome copies present in cells with all three serotypes at 2 h post-infection from the apical membrane (Fig. 4A). Another interesting difference was that

AAV2 genomes appeared to be slightly less stable following apical infection, decreasing 3-fold by 24 h; however, the abundance of AAV1 and AAV5 genomes were not significantly changed over this period (Fig. 4B). These findings strongly suggest that difference in viral entry and stability of viral genomes do not account for the 100-fold higher level of apical transduction seen with rAAV1 vectors as compared with the other serotypes.

In contrast to apical infection, results correlating the level of basolateral transduction with the abundance of viral genomes in Hirt DNA between the various serotypes were much more complex. Although near equivalent levels of viral genomes were seen at 2 h post-apical and basolateral infection with rAAV1, the stability of rAAV1 viral genomes following basolateral infection at 24 h was significantly decreased (Fig. 4). Given that the level of transduction (*i.e.* transgene expression) following apical and basolateral infection with rAAV1 was equivalent, this finding suggests that the viral genomes that remain at 24 h post-infection are more competent for transgene expression following basolateral infection. Alternatively, processing of the AAV1 virion following basolateral infection may be more rapid than following apical infection. In contrast to AAV1, enhanced AAV2 uptake from the basolateral as compared with the apical membrane appeared to correlate well with enhanced transduction from the basolateral membrane (Fig. 4). Although rAAV5 also demonstrated on average enhanced transduction from the basolateral as compared with apical membrane, there was considerable variability (10-fold) in the degree of this difference between the three independent donor samples (Fig. 3). There were no major differences in the number of viral genomes in Hirt DNA following apical rAAV5 infection at the 2- and 24-h time point (Fig. 4). However, a 10-fold decline in the abundance of AAV5 viral genomes in Hirt DNA between 2 and 24 h following basolateral infection was observed. This observation

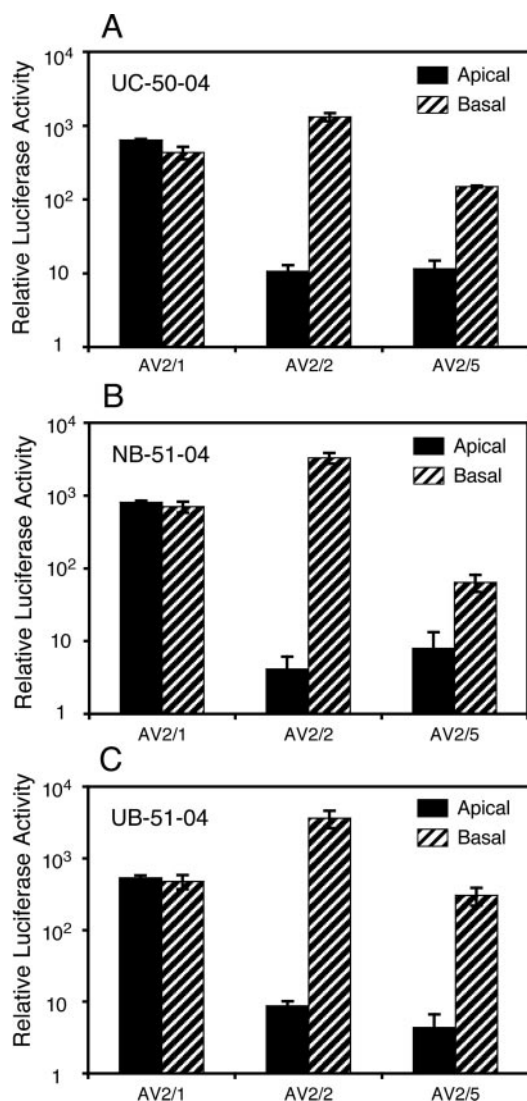


FIGURE 3. Comparisons of transduction efficiencies for different rAAV vector serotypes following infection of the basolateral and apical membrane of human polarized airway epithelia. 2×10^9 particles of AV2/1.Luc, AV2/2.Luc, or AV2/5.Luc were applied to either the apical surface or the basolateral side of human polarized airway epithelia for 24 h. At 3 days post-infection, relative luciferase activities in each well were assessed. Data represent the mean (\pm S.E.) relative luciferase activity (per well) in airway epithelia from three independent tissue donor samples: donor 1 ($n = 3$) (A), donor 2 ($n = 4$) (B), and donor 3 ($n = 3$) (C).

was similar to that seen following basolateral infection with rAAV1 and may represent a conserved more rapid processing of these two serotypes through basolateral infection pathways.

Serotype-specific Differences Exist in Proteasome-dependent Transduction of Polarized Airway Epithelia—Findings demonstrating near equivalent levels of rAAV1, -2, and -5 uptake from the apical membrane of polarized human airway epithelia (Fig. 4A) suggest that more efficient intracellular processing of rAAV1 likely accounts for its 100-fold higher level of transduction as compared with rAAV2 and rAAV5. We have previously demonstrated that apical transduction of human polarized airway epithelia with rAAV2 or rAAV5 is enhanced >1000 -fold by the combined co-administration of proteasome inhibitors LLnL and doxorubicin (15). The ubiquitin/proteasome pathway has been thought to play a major role in rAAV2 and rAAV5

transduction of human airway epithelia and other cultured cell lines (14, 31). These previous studies have demonstrated that transient exposure of cells to proteasome inhibitors at the time of rAAV infection enhances ubiquitination of rAAV capsids and nuclear uptake of virions (14, 15). We hypothesized that AAV1 virions might be less susceptible to the ubiquitin/proteasome pathway as compared with AAV2 and AAV5 virions. To this end, we compared the effect of proteasome inhibitors on apical transduction of human airway epithelia with the various serotype vectors. As shown in Fig. 5, A and B, treatment with proteasome inhibitors more significantly enhanced transduction with rAAV2 and rAAV5 vectors (~ 700 -fold) as compared with rAAV1 (~ 70 -fold). When we compared the effect of proteasome inhibitors on transduction following basolateral infections, the induction on rAAV1 was still 3–4-fold lower than rAAV2 and rAAV5 (100-fold for rAAV1 versus 300- and 380-fold for rAAV2 and rAAV5, respectively), although differences were not as large as what we observed following apical infection (data not shown). These findings support the concept that intracellular processing of rAAV1 is less affected by the ubiquitin/proteasome pathway than the other serotypes.

Given that proteasome inhibitors have been previously shown to enhance nuclear accumulation of rAAV2 in human airway cell lines (15), we sought to determine whether the differences in apical transduction between these three serotypes were due to differential sensitivity of viral nuclear transport to the ubiquitin/proteasome pathway. To test this hypothesis, we compared nuclear uptake of the three vector serotypes in human polarized airway epithelia in the presence or absence of proteasome inhibitors (LLnL and doxorubicin). 3 days following apical infection, polarized airway epithelial cells were harvested from support membranes and subjected to nuclear and cytoplasmic fractionation. TaqMan PCR was then used to assess proteasome inhibitor-dependent changes in the distribution of viral DNA. The results from these experiments are summarized in Fig. 5C. As hypothesized, proteasome inhibitors significantly enhanced nuclear accumulation of viral DNA for only rAAV2 and rAAV5 serotypes. The net proteasome inhibitor-mediated increase in nuclear viral DNA was 15% for rAAV2 and 20.6% for rAAV5, both of which were statistically significant ($p < 0.05$). Proteasome inhibitors also slightly increased rAAV1 nuclear viral DNA by 8.7%; however, this increase was not statistically significant. Although proteasome inhibitors did not enhance nuclear uptake of rAAV as effectively as previously observed in IB3 cells (15), the trends are consistent with the hypothesis that nuclear transport of rAAV2 and rAAV5 are more susceptible to the ubiquitin/proteasome pathway than rAAV1.

Although proteasome inhibitors appear to more greatly enhance nuclear uptake of rAAV2 and rAAV5 than rAAV1, one as of yet unexplained observation was the finding that a similar percentage of viral genomes were contained within the nuclear fraction for each serotype in the absence of proteasome inhibitors. Given that rAAV1 expresses its transgene 100-fold more effectively than rAAV2 and rAAV5 following apical infection (Fig. 2B), factors other than nuclear transport may also contribute to the higher level of transduction observed with rAAV1. Two potential mechanistic and/or technical issues may explain

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this observation. First, to achieve adequate TagMan PCR signals following subcellular fractionation of the small sample size, it was necessary to use 10-fold higher titers for infection than are used in gene expression studies. Given recent observations that higher titers of rAAV2 infection result in more efficient intracellular processing in HeLa cells (29), this may have led to an enhanced movement of rAAV2 and rAAV5 to the nucleus in the subcellular fractionation studies. Second, a plausible biologic explanation of the data may be that rAAV1 can more rapidly uncoat following entry into the nucleus, and that proteasome inhibitors may enhance this process for rAAV2 and rAAV5.

DISCUSSION

Intracellular processing of rAAV vectors has been increasingly recognized as an important feature that influences the efficiency of transduction in a given cell type (7, 15, 17, 29). Impaired intracellular trafficking of rAAV2 to the nucleus has also been linked to reduced efficiency of uncoating in primary murine hematopoietic cells (16). Furthermore, the rate of nuclear uncoating of AAV virions appears to be significantly more efficient for serotypes such as AAV6 and AAV8 following liver transduction (32). Our findings that rAAV1 transduces the apical surface of human airway epithelia 100-fold more efficiently than rAAV2 and rAAV5 vectors, despite near equivalent levels of viral uptake, suggested that rAAV1 intracellular processing may also fall into a unique category of serotypes best suited for airway transduction in humans.

Several salient features of rAAV1 transduction biology in human airway epithelia support the claim that this serotype has unique properties that are different from those of rAAV2 and rAAV5. First, unlike AAV2 and AAV5, the AAV1 capsid is capable of transducing both apical and basolateral membranes of polarized airway epithelia with similar efficiency. Second, unlike rAAV5, rAAV1 transduced mouse and human airway epithelia with high efficiency. The lack of a significant difference in the efficiency of rAAV1 between species also supports the notion that AAV1 transduction in airway epithelia may be

quite different from that of AAV5. Third, apical transduction with rAAV1 was more refractory to induction by proteasome inhibitors than the other two serotypes. This finding suggests that the ubiquitin/proteasome system plays less of a functional role in modulating rAAV1 transduction of human airway epithelia.

It is widely accepted that efficient AAV uptake by cell surface receptors does not always result in efficient transgene expression. This has been well documented not only in polarized airway epithelia but also in primary murine embryo fibroblast, hepatocytes, and other cell types (16–18,33). This feature of AAV transduction is highlighted in our findings of apical transduction of the three serotypes studied; comparative analysis between serotypes demonstrated near equivalent levels of viral uptake from the apical membrane for the various serotypes, despite 100-fold differences in transduction. These findings support post-entry intracellular barriers including intracellular trafficking, nuclei transport, virion uncoating, and viral genome conversion as critical potential pathways in limiting apical gene transfer with certain serotypes. In contrast, our studies comparing apical and basolateral transduction with the extent of viral uptake for a given serotype correlated quite well; the level of viral uptake at 2 h post-infection demonstrated similar polarity differences as seen in transduction. A potential explanation for the differences in polarity of transduction with the three serotypes likely involves receptor entry pathways. In the context of AAV1, the same receptor may reside on the apical and basolateral membrane. This AAV1 receptor may be processed from the apical and basolateral membrane of airway epithelial cells in a similar fashion, giving rise to equivalent levels of transduction. However, our findings demonstrating that the abundance of rAAV1 genomes declines more rapidly following basolateral as compared with apical infection, despite equivalent levels of transduction from both membranes, argues that this simplistic view may not be totally correct. In contrast, AAV2 and AAV5 may utilize different receptor entry pathways at the apical and basolateral membranes. Differences in abundance

and/or processing of primary binding receptors and/or co-receptors at the apical and basolateral membrane could account for the observations in the current studies demonstrating enhanced basolateral transduction by rAAV2 and rAAV5.

Differences in AAV serotype transduction of polarized human airway epithelia likely initiate at the cell surface via interactions with receptors that determine the intracellular fate of a given serotype. Although several receptors and co-receptors have been identified for rAAV2 (heparan sulfate proteoglycan, FGFR1, α V β 5 integrin) (34–36), none of these proteins can be detected on the apical membrane of polarized human airway epithelia (37). Hence, in the context of

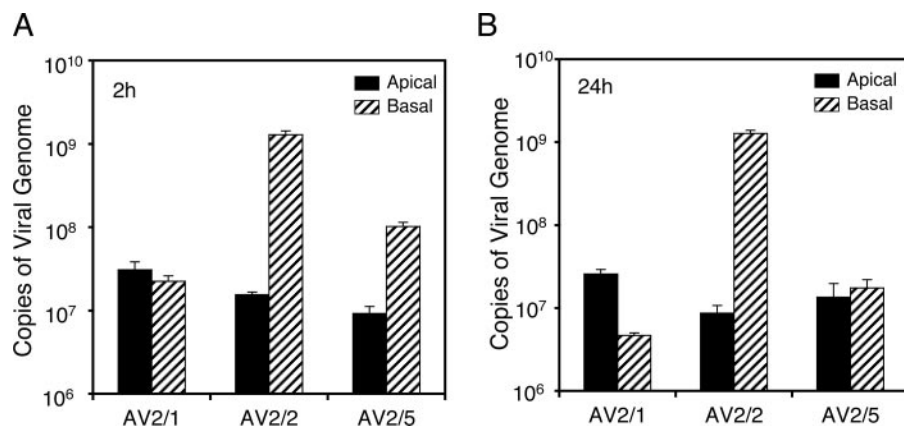


FIGURE 4. Serotype differences in rAAV internalization from the apical and basolateral membrane. Polarized human airway epithelia were infected from the apical or basolateral membrane with 10^{10} particles of each rAAV serotype, and the abundance of vector genomes in Hirt DNA was assessed at 2 and 24 h post-infection. Viral infections were performed for 2 h by applying virus to the apical or basolateral membrane followed by extensive washing to remove unbound virus. Hirt DNA was extracted from infected cells immediately after washing at 2 h post-infection (A) or after 22 h of an additional incubation period (24 h post-infection) (B). TaqMan PCR was then performed to quantify viral genomes in the Hirt DNA. Data represent the mean (\pm S.E.) viral genome copies from five independent infections ($n = 5$).

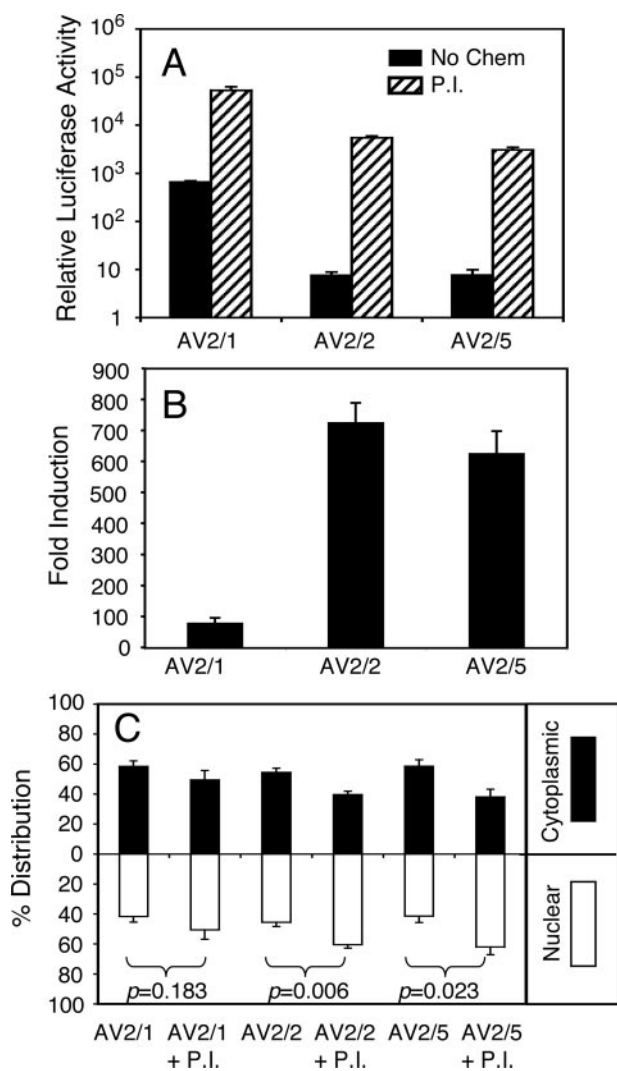


FIGURE 5. Co-administration of proteasome-modulating agents LLnL and doxorubicin induced rAAV2 and rAAV5 transduction from the apical membrane more effectively than rAAV1. 2×10^9 particles of AV2/1.Luc, AV2/2.Luc, or AV2/5.Luc were applied to the apical membrane of polarized human airway epithelial cultures for 24 h in the absence or presence of LLnL (40 μ M) and doxorubicin (5 μ M). *A*, luciferase expression was assayed at 3 days post-infection. Data represent the mean (\pm S.E.) relative luciferase activity (per well) for $n = 10$ independent infections from three independent tissue samples. *B*, the fold induction in luciferase reporter expression shown in *A* following the addition of proteasome inhibitors for each serotype (mean \pm S.E.). *C*, cellular fractionation was performed to evaluate the cellular distribution of viral genomes in the absence or presence of proteasome inhibitors. Cells were lysed, and the cytoplasmic and nuclear fractions were isolated at 3 days post-infection for quantification viral genome copy number using TaqMan PCR. Data represent the mean (\pm S.E., $n = 4$) percentage of viral genomes in the cytoplasmic (solid bars) and nuclear (open bars) fractions for each serotype in the presence or absence of proteasome inhibitors (P.I.).

rAAV2, the apical entry pathway has yet to be determined. In contrast, it has been reported that the apical membrane of airway epithelia contains abundant 2,3-linked sialic acid, a binding receptor for rAAV5 (38). The receptor for AAV1 has not yet been identified and hence it is difficult to predict how this entry pathway may enhance transduction from the apical membrane with this serotype.

It is clear that increased transgene expression following apical infection of human airway epithelia with rAAV1 was not due to enhanced virus uptake in comparison to rAAV2 and

rAAV5. Hence, we conclude that post-entry processing of rAAV1 at the level of intracellular trafficking, nuclear transport and/or virion uncoating are likely more efficient for this serotype. The ubiquitin/proteasome pathway has been associated with limiting rAAV2 and rAAV5 transduction from the apical surface of airway epithelia. Modulating proteasome activity with proteasome inhibitor appears to accelerate nuclear uptake of these two serotypes to a greater extent in airway epithelia as compared with rAAV1. However, proteasome-dependent enhancement of nuclear uptake for rAAV2 and -5 (although greater than that for rAAV1) cannot fully explain the 10-fold greater ability of proteasome inhibitors to augment rAAV2- and rAAV5-mediated transgene expression as compared with rAAV1. Hence, the mechanistic difference responsible for enhanced rAAV1 transduction appears to involve both enhanced efficiency of nuclear uptake and nuclear processing of virions.

In summary, our studies demonstrate that rAAV1 transduces polarized human airway epithelia from the apical membrane significantly more effectively than rAAV2 and 5. This difference in biology appears to be an evolutionary advantage of the AAV1 virion that imparts more efficient intracellular and nuclear processing without interference from the ubiquitin/proteasome system. Hence the use of the AAV1 serotype for gene therapy of diseases such as cystic fibrosis may circumvent the need for chemical augmentation with proteasome inhibitor to achieve functional correction of disease.

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