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Sites in the AAV5 capsid tolerant to deletions and tandem duplications

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ABSTRACT

Gene therapy vectors based on adeno-associated virus (AAV) have shown much promise in clinical trials for the treatment of a variety of diseases. However, the ability to manipulate and engineer the viral surface for enhanced efficiency is necessary to overcome such barriers as pre-existing immunity and transduction of non-target cells that currently limit AAV applications. Although single amino acid changes and peptide insertions at select sites have been explored previously, the tolerance of AAV to small deletions and tandem duplications of sequence has not been globally addressed. Here, we have generated a large, diverse library of $>10^5$ members containing deletions and tandem duplications throughout the viral capsid of AAV5. Four unique mutants were identified that maintain the ability to form viral particles, with one showing improved transduction on both 293T and BEAS-2B cells. This approach may find potential use for the generation of novel variants with improved and altered properties or in the identification of sites that are tolerant to insertions of targeting ligands.

1. Introduction

Adeno-associated virus (AAV)[1] is considered one of the most promising vectors for gene therapy due to its non-pathogenic, non-toxic nature and its ability to achieve long term gene expression in a variety of cell lines, tissues and animal models [1–5]. However, despite early promise in clinical trials with recombinant AAV serotype 2 (rAAV2) vectors [6,7], there still remain a number of barriers that limit the efficacy of AAV vectors. For example, the heparin sulfates, which are the primary receptors for AAV2, are widespread, leading to difficulties in achieving good tissue dispersion or specific cell targeting [8]. Alternatively many therapeutic target cells express very low levels of receptors, necessitating high vector doses. The apical surfaces of airway epithelial cells, a common target for many lung diseases, typically have limited expression of viral receptors [9–11]. Finally, the pre-existence of neutralizing antibodies against AAV in the general population, ~32% of individuals in one study [12], may limit initial efficacy and hinder repeated administrations. Thus, there is a need to be able to engineer viruses to specific target cells, enhance infection, reduce non-specific transduction, and overcome pre-existing neutralizing antibodies and other extracellular barriers.

Earlier attempts to understand and engineer the AAV surface have predominantly focused on serotype 2. Mutagenesis studies have identified sites amenable to insertion of foreign peptides [13–16] as well as sites critical for AAV2 receptor [16,17] and antibody binding [18–20]. Single amino acid changes on the AAV surface can dramatically change such phenotypes as viral titer, receptor binding, and tissue tropism exhibited by closely related AAV serotypes [21]. These early studies often involved the individual construction of many capsid mutants and subsequent identification of new phenotypes. For example, 38 virus capsid mutants containing peptide insertions at 25 unique sites within the AAV2 capsid were constructed by Shi et al. to identify insertion sites [14] while Wu et al. [16] constructed 93 mutants at 59 different positions in the AAV2 capsid gene by site directed mutations. More recently, directed evolution approaches have been utilized to introduce point mutations randomly throughout the capsid and subsequently selected for enhanced properties such as neutralizing antibody evasion [22,23]. However, the tolerance of AAV to randomly introduced deletions or insertions has not been addressed.

Serotypes other than AAV2 have been shown to have specific and superior transduction efficiencies for a variety of tissues [24–26]. For example, AAV5 may be the vector of choice for gene therapy targeted for the lungs; it transduces the lungs more efficiently than serotype 2 [27,28] and recognizes PDGFR [29], a receptor expressed on the apical surface of the airways. AAV5 is the most

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[1] *Abbreviations used:* AAV, adeno-associated virus; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; rAAV2, recombinant AAV serotype 2 vectors.

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distant serotype within the parvovirus family, with only a 54–56% sequence homology to AAV2 [30,31], but like all the other AAV serotypes, it is composed of three structural proteins designated VP1, VP2, and VP3 present at a ratio of 1:1:10. These capsid genes are all encoded in the same open reading frame and expressed from the same viral p40 promoter. The amino acid sequence of the major capsid protein VP3, contained within the two larger capsid proteins, is the primary protein that constitutes the surface topology of the virus. Here we have randomly introduced deletions and tandem duplications (rather than point mutations) on the AAV5 capsid to examine how tolerant AAV5 is to such mutations and to identify sites on the capsid that may be amenable to the insertion of targeting peptides.

2. Materials and Methods

2.1. Cell culture

Low passage 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (Pen/Strep). BEAS-2B cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep. All cells were cultured at 37 °C in a 5% CO_2 environment. For screening, cells were seeded at a density of 3.5×10^6 cells in 15 cm plates and for flow cytometry studies cells were seeded at 10,000 cells/well in 24-well plates and allowed to grow for 24 h before manipulation.

2.2. Plasmids

The pDIM-N2 plasmid [32] served as the backbone plasmid for most of the library manipulation. SpeI and MluI restriction enzyme sites were introduced outside of the BstEII and RsrII sites within the AAV5 cap gene by PCR on pACYC184-AAV5 plasmid which contains the full sequence of wild-type AAV5 (1–4642 nt). This fragment was subsequently moved into the pDIM-N2 plasmid to yield pDIM-N2-AAV5. The BtgzI restriction enzyme site within the AAV5 cap gene was silently removed using the QuikChange Site-Directed Mutageneis Kit (Stratagene) per manufacturer instructions using the primers, forward 5′-CACCATAGCCAACAACCTCACCTCC-3′ and reverse 5′-TGTTGGCTATGGTGGTGGTGGAGTC-3′.

A plasmid containing the 'marker' sequence was created such that PvuII digestion would yield the desired marker fragment shown in Fig. 1B. The marker sequence was derived from a fragment of the gene encoding YFP. Primers were used such that a PvuII restriction enzyme site would start 7 bp away from the end of the BtgzI site (forward 5′-TCATCGCAGCTGCAGCTTACATCGCCCACCTACGGCAAGC TG-3′ and reverse 5′-TCATCGCAGCTGCAGCTTACATCGCCTTGTA CAGCTCGTCCATG-3′). The primers were used to amplify a portion of the YFP gene, and transferred into the pDIM-N2 plasmid. After the plasmid was transformed into DH5α cells, amplified and purified, digestion with PvuII yielded the blunt ended 'marker' sequence.

For the final step of the library, all mutations made on the pDIM-N2-AAV5 plasmid needed to be transferred back into the pACYC184-AAV5 plasmid with the rest of the cap and rep genes. To minimize any transfer of un-digested wild-type capsids into the final library, an internal stop codon and unique restriction enzyme site (AflII) were introduced into the cap gene, following the instructions on the Phusion site-directed mutagenesis kit (New England BioLabs) using the primers, forward 5′-CAAAATCTTCAACACTTAAGTCAAA GAG-3′, and reverse 5′-ACTCTGAGGGACCGGGGTCTG-3′ primers. The library in the pDIM-N2-AAV5 plasmid was transferred into this modified pACYC184-AAV5 plasmid instead of the wild-type. This final library was digested with AflII to further ensure there were no wild-type plasmids arising from incomplete digestions before the transfection/screening steps.

2.3. Construction of the plasmid library

For the initial step in the library process, the pDIM-N2-AAV5 plasmid was treated with serial dilutions of diluted DNase I (1.6–0.0016 mU/ug DNA) in a buffer containing 1 mM $MnCl_2$, 0.05 M Tris–HCl and 0.05 mg/mL BSA. The reaction was allowed to take place at 22 °C for 10 min until the reaction was stopped by the addition of 1.2 μL of 1 M EDTA to 100 μl of the reaction mixture and heating to 75 °C for 10 min. The reactions were analyzed by agarose gel electrophoresis to determine the concentration of DNase I that yielded the largest amount of linear fragments but also with a fair amount of supercoiled DNA, indicating that the DNA had not been over-digested. After gel purification (Qiagen) of the linear fragment, the ends were then repaired with 1 U T4 DNA polymerase and 160 U T4 DNA ligase/μg of DNA in ligase buffer with 200 μM dNTPs. The reaction proceeded for 20 min at 12 °C until it was terminated by the addition of EDTA to a final concentration of 10 mM. The 'marker' sequence was ligated into the linearized and repaired library with T4 DNA ligase overnight at 16 °C. Ligations were done in 10 μL reactions with 2000 U T4 DNA ligase, 100 ng of the repaired library and 30 ng of the 'marker' sequence (~1:3 mole ratio) in T4 DNA ligase buffer and 5% w/v of 8000 MW PEG. The plasmids were amplified by transformation into electrocompetent DH5α *Escherichia coli* cells and the resulting plasmid library purified (Qiagen). This initial library containing the 'marker' sequence was then digested with BstEII, RsrII, AseI and AhdI. The largest fragment was gel purified (Qiagen) and ligated into another pDIM-N2 plasmid in the same fashion as described above. Another transformation amplification and purification yielded a plasmid library containing the AAV5 capsid with the 'marker' sequence contained within. This 'marker' sequence was removed by enzymatic digestion with BtgzI. The linear fragment was gel purified and repaired as described above. Overnight ligations were done in 10 μl reactions with 100 ng of the repaired library to recircularize the plasmid.

2.4. AAV5 library Screening/production and titration

For the production and screening of the AAV5 library, 293T cells in a 15 cm diameter plate were transfected, by Polyfect Transfection Reagent (Qiagen) according to manufacturer instructions, with 13 μg of an adenovirus helper plasmid (pAd12) containing the VA RNA, E2, and E4, and 6, 60, 600 ng or 6 μg of the plasmid library. The DNA was pre-mixed and added to polyfect reagent at a ratio of 10 μl:1 μg. After 48 h, the cells were harvested, lysed via freeze/thaw cycles and addition of 0.5% w/w deoxycholic acid, and free nucleic acids were digested with benzonase. After the cell debris was removed by centrifugation, the recovered virus was purified using a CsCl gradient. The nuclease resistant genomic particle titer was determined by quantitative real-time PCR using the TaqMan system (BioRad) with probes specific to the AAV5 wt genome (forward 5′-ACCAACATCGCGGAGGC-3′ and reverse 5′-CCCTCCTCCCACCAAAT GA-3′). Viruses in CsCl were dialyzed 24 h using 0.5 ml Slide-A-Lyzer (Pierce) in 500 ml of PBS with changing of the buffer three or four times.

2.5. Sequencing of AAV library clones

The dialyzed viral library was collected and the AAV served as a template for PCR. PCR products were analyzed by gel electrophoresis, digested with RsrII/BstEII, cloned into Topo- AAV5 cap (AAV5 nucleotides 1700–4500 nt) which contains cap ORF and its endogenous promoter. After transformation into *E. coli*, random colonies were selected for sequencing. All sequencing was done at Genewiz, Inc.

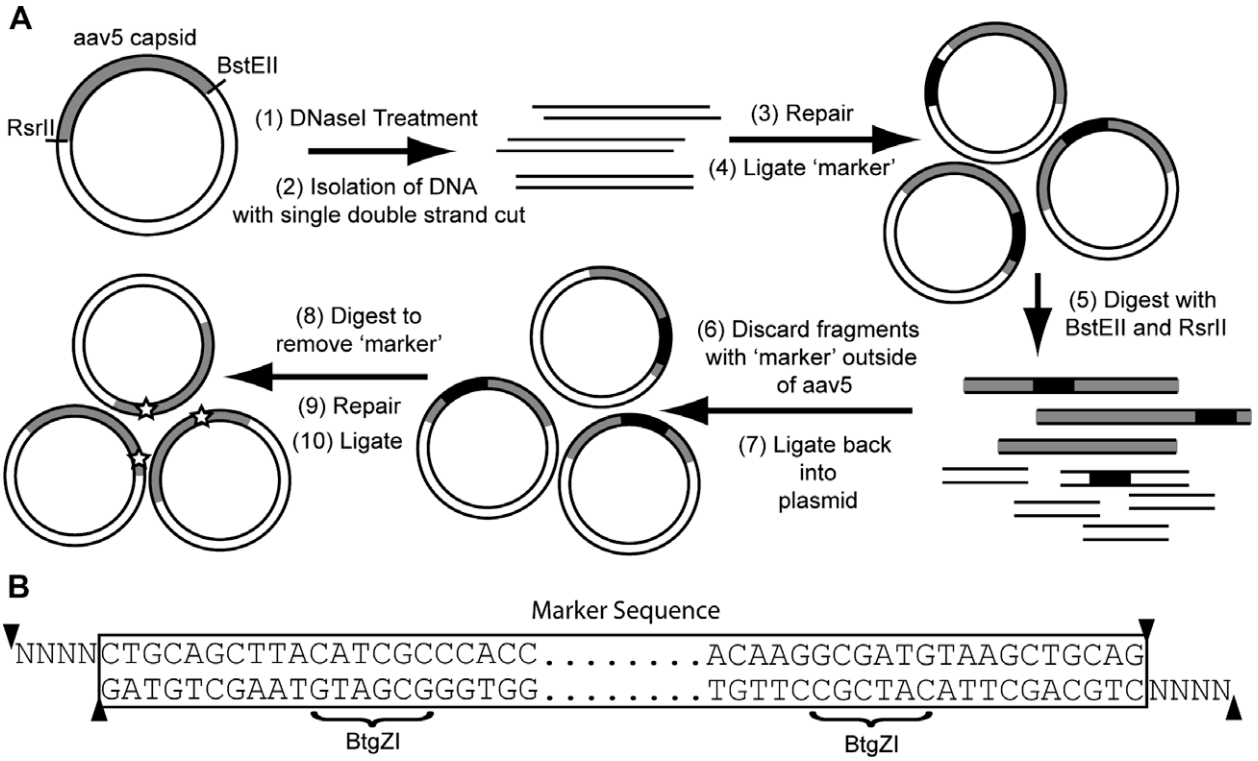

Fig. 1. Construction of the deletion/duplication library. (A) A plasmid containing the adeno-associated virus serotype 5 (AAV5) capsid gene between unique restriction enzyme sites BstEII and RsrII was digested with dilute concentrations of DNaseI to create on average, one random double-stranded break per plasmid molecule. After purification of DNA with a single double strand cut, ends were repaired to form blunt ends. Repair of 5′ overhangs results in tandem duplications and repair of 3′ overhangs results in deletions. Recircularization of the vector occurred such that a 'marker' sequence was inserted at the site of the single double-stranded break. Digestion with BstEII and RsrII yields two different fragment sizes containing the AAV5 gene, the larger one contained the 'marker' sequence. Purification of such fragments significantly decreases the number of wild-type sequences in the final library. The 'marker' sequence was subsequently removed using BtgzI digestion, repaired and ligated to create a library with random deletions and tandem duplications. (B) The 'marker' sequence contains two BtgZI restriction enzyme sites ten nucleotides away from either end. The enzyme cuts one strand at the end of the marker and the other strand four nucleotides outside of the marker sequence, as indicated by the dark triangles. Upon digestion and repair by a polymerase to form blunt ends, the marker sequence is completely removed without leaving any non-AAV5 sequences behind.

2.6. Recombinant AAV5β−actin-GFP production and titration

Recombinant (r) AAV5, and library clone viruses expressing β-actin-GFP were produced using a four-plasmid procedure as previously described [33]. Briefly, semiconfluent 293T cells were transfected by calcium phosphate with four plasmids: an adenovirus helper plasmid (pAd12) containing the VA RNA and coding for the E2 and E4 proteins; two AAV helper plasmids containing the AAV2 *rep* and Topo-AAV5 with wt or library cap, respectively, and a vector plasmid containing AAV2 inverted terminal repeats flanking an CMV β-actin-GFP expression cassette. Forty-eight hours post-transfection the cells were harvested by scraping into TD buffer (140 mM NaCl, 5 mM KCl, 0.7 mM K_2HPO_4, 25 mM Tris–HCl {pH 7.4}) and the cell pellet was collected by low-speed centrifugation. Cells were lysed in TD buffer by three cycles of freeze–thaw. The clarified lysate (obtained by further low-speed centrifugation) was treated with 0.5% w/w deoxycolic acid and 100 U/ml Benzonase for 30 min at 37 °C. The virus was then purified using CsCl gradients. Particle titers were determined by qPCR. Amplification was detected using an ABI 7700 sequence detector (ABI). Specific primers for CMV were designed by using the Primer Express program (ABI): CMV forward 5′-CATCTACGTATTAGTCATC GCTATTACCAT-3′, CMV reverse 5′-TGGAAATCCCCGTGAGTCA-3′. Following denaturation at 96 °C for 10 min, cycling conditions were 96 °C for 15 s, 60 °C for 1 min for 40 cycles. The viral DNA in each sample was quantified by comparing the fluorescence profiles with a set of DNA standards. The rAAV particle titers were $1–5 \times 10^{12}$ DNAse resistant particles per ml.

2.7. Gene transfer assay in vitro

293T and BEAS-2B were incubated with purified wt or library clones of AAV5 β-actin-GFP at an MOI (genomic) of 3000. Four days later, cells were harvested with trypsin/EDTA and re-suspended in PBS. Reporter gene activity (5000–10,000 cells per sample) was detected using FACSCaliber flow cytometer (Becton Dickinson).

3. Results

3.1. Construction of the Library

To create our library of random tandem deletions and duplications, we targeted the region within the AAV5 genome between two unique restriction enzyme sites, BstEII and RsrII, which codes for amino acids 156–666 (VP1 protein numbering). The overall library construction scheme is depicted in Fig. 1A. The DNA fragment between BstEII and RsrII was initially transferred into a smaller plasmid, which was subsequently treated with dilute DNase I to generate, on average, one random double strand break per plasmid molecule (steps 1 and 2). Double strand breaks created by DNase I can have blunt ends, 5′ overhangs or 3′ overhangs. T4 DNA polymerase, which has 5′ → 3′ polymerase and 3′ → 5′ exonuclease activity, was used to make both types of overhangs blunt (step 3), resulting in tandem duplication of sequences from 5′ overhangs and deletions from 3′ overhangs.

Recircularization of the plasmid at this juncture would result in a library containing deletions and tandem duplications in the capsid gene fragment. However, since DNase I does not specifically target the capsid gene fragment, a large fraction of our library would contain deletions and duplications elsewhere in the plasmid and an unaltered capsid gene. This prevalence of wild-type capsid in our library would hamper our ability to identify functional capsids with deletions and tandem duplications. We minimized this possibility by designing a 'marker' sequence, which was used to select for those library members in which DNase I digested within our target sequence (Fig. 1B). This marker sequence was inserted into the plasmid after the repair step during cyclization of the plasmid (step 4, Fig. 1A). After subsequent digestion with BstEII and RsrII (step 5), capsid gene fragments with a marker sequence were isolated by agarose gel electrophoresis based on size (~2150 bp) and ligated into a new plasmid (steps 6 and 7). The marker sequence was excised (step 8) using the restriction enzyme BtgzI. The marker sequence was designed to contain BtgzI sites near both ends. BtgzI digests 10 and 14 nucleotides outside its recognition sequence; thus, the marker sequence could be cleanly removed leaving only DNA originating from the capsid gene (Fig. 1B). Once the marker was excised, a final repair step made blunt ends and DNA was re-circularized (steps 9 and 10) to make our final plasmid library, which was comprised of 1.4×10^5 transformants.

Nine naïve library members were sequenced to verify successful library construction and examine the variety and the lengths of deletions and tandem duplications (Fig. 2). The mutations appeared randomly distributed throughout the targeted gene (Fig. 2A) and both deletions and tandem duplications were found (Fig. 2B). More deletions than duplications were identified, with the largest deletion being 119 bp and all others being much smaller, averaging about 18 bp. This bias towards deletions is typical of random digestion libraries made with DNase I [34]. Since wild-type sequences would result when DNAse I digestion produced blunt ends, we expected wild-type sequences to exist in the library. However, the fact that no wild-type sequences were found amongst the nine naïve library members indicates that they are not overly prevalent in the library and that our strategy to minimize wild-type sequences using the 'marker' sequence was successful. The majority of naïve library members contain frame shift mutations resulting in non-functional capsid proteins.

3.2. Screening for mutants able to assemble AAV5 particles

We evaluated our library on 293T cells to identify modified capsids that were capable of assembling into a viral particle. We expected to isolate wild-type sequences despite our efforts to eliminate them from the naïve library. Based on previous work on libraries of this type [34], we expected that approximately 1–10% of naïve library members will contain neither deletions nor duplications (i.e. they will be wild-type). The presence of non-mutated sequences is unavoidable in many types of mutagenesis libraries. We were unsure whether functional mutant library members could be identified under this selection scheme, in which wild-type sequences will be strongly selected—both because wild-type infects these cells efficiently and because of the prevalence of wild-type sequences in the library. Functional mutants would have to appear with a significant frequency for our selection process to isolate them.

The library was first subcloned into the original plasmid (pACYC184-AAV5 plasmid) with the remainder of the cap and the wild-type rep genes. 293T cells were then co-transfected with varying concentrations of the library plasmid (6–6000 ng) and adenovirus helper plasmid to determine the lowest concentration of library plasmid needed for detectable viral production. Minimizing library plasmid DNA during transfection helped ensure that the viral particles produced contained the DNA that coded for their capsid protein thereby maintaining the genotype–phenotype linkage necessary for directed evolution. Virus production was determined by real-time PCR. Transfection with 600 ng of library plasmid was the lowest concentration that yielded viruses.

Forty-eight hours after transfection with 600 ng of library plasmid, viruses were harvested, treated with benzonase and purified via CsCl centrifugation. After isolation of peak viral fractions, the single stranded DNA genome was amplified, cloned and individual transformants sequenced. Not surprisingly, the most frequently identified sequence was wild-type, representing 13 out of 20 clones sequenced. These were members of the library and not clon-

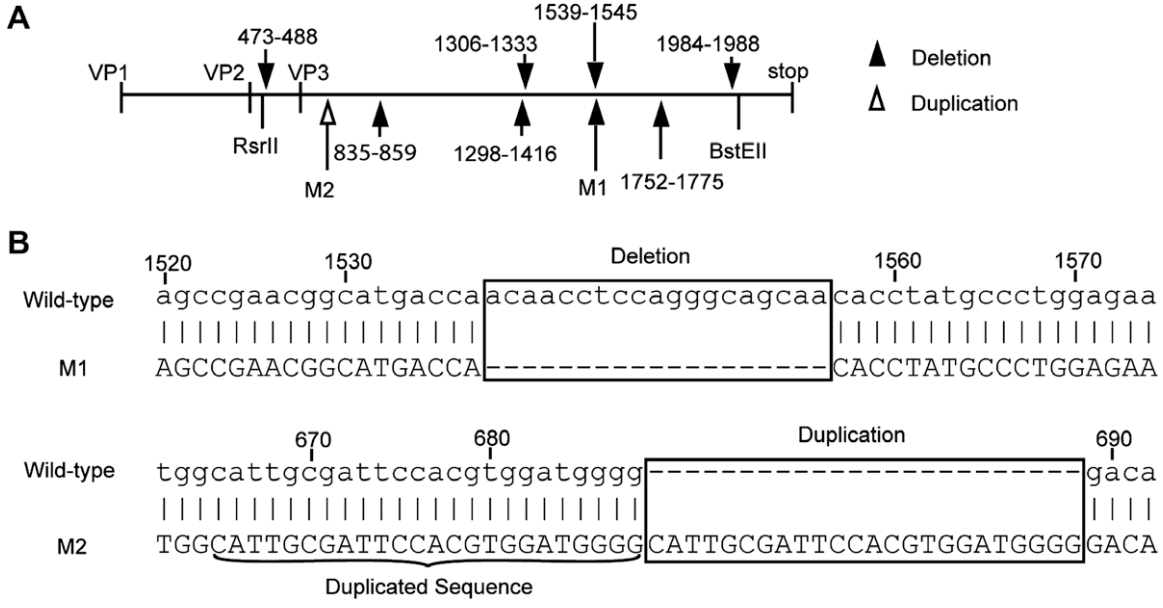

Fig. 2. Naïve Library Members. (A) Mutations were distributed randomly throughout the targeted region, with more deletions (filled arrows) than duplications (open arrows) identified. The numbers indicate the number of the deleted nucleotides (nucleotide 1 is the first nucleotide of the gene encoding VP1). (B) Examples of the types of mutations in the library are shown here. Clone M1 has a 19 bp deletion and clone M2 a 24 bp duplicated sequence.

ing artifacts or contamination because our library capsid gene contained a unique silent mutation by design, which was present in all 20 clones. Recovery of wild-type capsids validates that our experimental procedure can be used to isolate functional capsids from a library of mostly non-functional capsids.

Most interestingly, four novel sequences comprised the remaining seven clones—two with duplications and two with deletions (Table 1). Unlike typical naïve library members, these library members were in-frame and all contained short deletions (6 or 9 bp) or tandem duplications (3 or 6 bp).

3.3. Testing of transduction efficiencies in vitro

To compare the ability of our four newly isolated mutants to produce viral particles compare with wild-type AAV5, recombinant particles were produced that encoded the GFP-β-actin gene and then purified by isopycnic CsCl centrifugation. All mutants produced comparable genomic particle titers to the wild-type vector except for R12, which had no detectable viral titer (Table 1). It was not apparent whether R12 failed to package genetic material or whether the particles did not assemble, as unlike AAV2 there is no capsid ELISA available to look for assembled AAV5 particles. We next tested the infectivity of these viral particles (R9, R31 and R39) compared with wild-type. Viral transduction efficiencies were determined in vitro on two different cell lines: 293T and BEAS-2B (immortalized bronchial epithelial cells). Viruses were added to cells at an MOI of 3000 (calculated as DNase I resistant particles) and after 96 h, expression of the GFP transgene was assessed via flow cytometry. R31 and R39 showed some transduction (Table 1). However, R9 showed a moderate improvement in the transduction of BEAS-2B cells. This was unexpected since selective pressure was not applied for improved transduction during the process for identifying functional capsid variants.

4. Discussion

Recent studies have demonstrated the feasibility of using directed evolution approaches for the creation of large numbers of mutants and subsequent screening to identify new AAV vectors. However, the mutations have been either limited to single amino acid changes dispersed throughout the capsid protein [22,23] or a mixture of different serotype capsids as in the case of gene shuffling [35,36]. Here we have demonstrated a new approach to identifying sites throughout the viral capsid that are tolerant to deletions and duplications (i.e. insertions).

Our approach takes advantage of the nature of the ends of double-stranded DNA cuts produced by DNaseI digestion of plasmid DNA, which upon subsequent blunting of the ends using DNA polymerase creates deletions and tandem duplications. We were able to create a large and diverse library with $>1 \times 10^6$ transformants after step 4 in the library construction scheme (Fig. 1A) and $\sim 1 \times 10^5$ transformants after each subsequent transformation step (steps 7 and 10). Mutations of variable lengths were distributed throughout our target gene, with the majority of the mutations (deletions or duplications) being relatively small in length. Except for the largest deletion (119 bp), all mutations ranged from 4 to 27 bases, which would correspond to roughly 1–9 amino acids, a size comparable to that of foreign peptides inserted into AAV capsids in previous studies [14–16].

Due to the inherent nature of the mutations we made, few viruses were expected to be still infectious. We identified four unique mutants after selecting for packaging in 293T cells and sequencing 20 clones. Sequencing of more clones will likely result in the identification of additional functional sequences; however, since two clones were found more than once, this suggests that the number of additional functional sequences may not be extensive. The sequences identified all have small changes (1–3 amino acids) relative to those found in a random sequencing of the naïve library. Even with the use of the 'marker' sequence, wild-type constituted almost 65% of isolated virions. These sequences presumably resulted from DNAse I digestions that produced blunt ends and are unavoidable. If precautions had not been taken to minimize wild-type sequences in our final library, it is unlikely that we would have been able to isolate any mutants as they would have been overwhelmed by the number of wild-type sequences. Thus, minimization of wild-type sequences using the marker sequence was critical in the identification of new capsid sequences. A selection scheme that does not favor wild-type AAV (i.e. the use of cells that are poorly infected by AAV5) will also likely reduce the number of wild-type sequences identified as well as favor novel sequences with improved transduction properties on such cells.

Although this library was subjected to selection for the ability to assemble into viruses and not for any improved properties it is encouraging that we were able to identify a clone with improved transduction abilities. The two most commonly identified mutations, R12 and R39 occurs at position 492, possibly suggesting that this site is highly tolerant to changes. However, recombinant GFP-R39 had negligible transduction of both 293T and BEAS-2B cells and GFP-R12 did not produce a detectable viral titer. This may indicate that mutations near this area may interrupt a critical capsid interaction necessary for both transduction and particle assembly or genome packaging.

The sequence alignment of five different AAV serotypes (1, 2, 4, 5, and 6) shows that three of the four mutations identified here (R12, R31, R39) occur in regions of low sequence conservation (Fig. 3). The R31 deletion continues a large deletion that occurs

Table 1
Selected library members and their corresponding particle titers and transduction efficiencies of recombinant AAV-GFP-actin.

	Mutation[a]	Occurences[b]	rAAV-GFP titer[c]	Transduction efficiency[e]	
				BEAS-2B	293T
Wild-type	–	13	3.9E10	1200 ± 120	200 ± 18
R9	Dup 574–575	1	2.8E10	3400 ± 340	240 ± 10
R12	Dup 492	2	–[d]	N/A	N/A
R31	Δ 176–178	1	3.2E10	245 ± 50	30 ± 20
R39	Δ 492–493	3	3.5E10	50 ± 12	–[f]

[a] Dup indicates a duplication and Δ indicates a deletion of the amino acids indicated by the numbers. Numbering is based on the amino acid sequence of VP1.
[b] Number of occurrences out of 20.
[c] DNase resistant genomic particle titer (viral particles/ml) of recombinant AAV with GFP-actin was assessed by real-time PCR.
[d] No viral titer was detected for this mutant.
[e] Transduction efficiencies of BEAS-2B and 293T cells, expressed as number of transduced cells/10^8 viral particles. Viruses were added to cells at a genomic MOI of 3000 and fluorescence was quantified 96 h later by FACS. Transduced cells were defined as cells with fluorescence greater than 99% of untransduced cells. Data represents $n = 3$ independent experiments with each condition done in triplicates with >5000 cells counted. N/A indicates that the experiment was not performed.
[f] No transduction was detected.

in the AAV5 sequence. In contrast, the most promising mutant, R9, contains an insertion between glutamine and serine, two residues conserved in at least four of the five serotypes. This site is also one amino acid away from a site previously shown to be amenable to peptide insertion on the AAV2 surface [14]. This supports the idea that this method can successfully identify sites that are tolerant to larger peptide insertions. Although the R9 mutation lies close to the heparin binding site, the primary receptor interaction for AAV2, AAV5 does not bind to heparin sulfates at this location [29]. Although atomic resolution models of AAV5 are not currently available, by comparing the sequence alignment with AAV2 and superimposing onto the solved structure of AAV2, it appears that R9, R12, and R39 are all on the surface (Fig. 4). Both locations appear close to sites other researchers have identified in AAV2 that are tolerant to insertions (red) [13–16]. The Δ 176–178 deletion of R31 is not depicted in this figure as it lies outside the solved

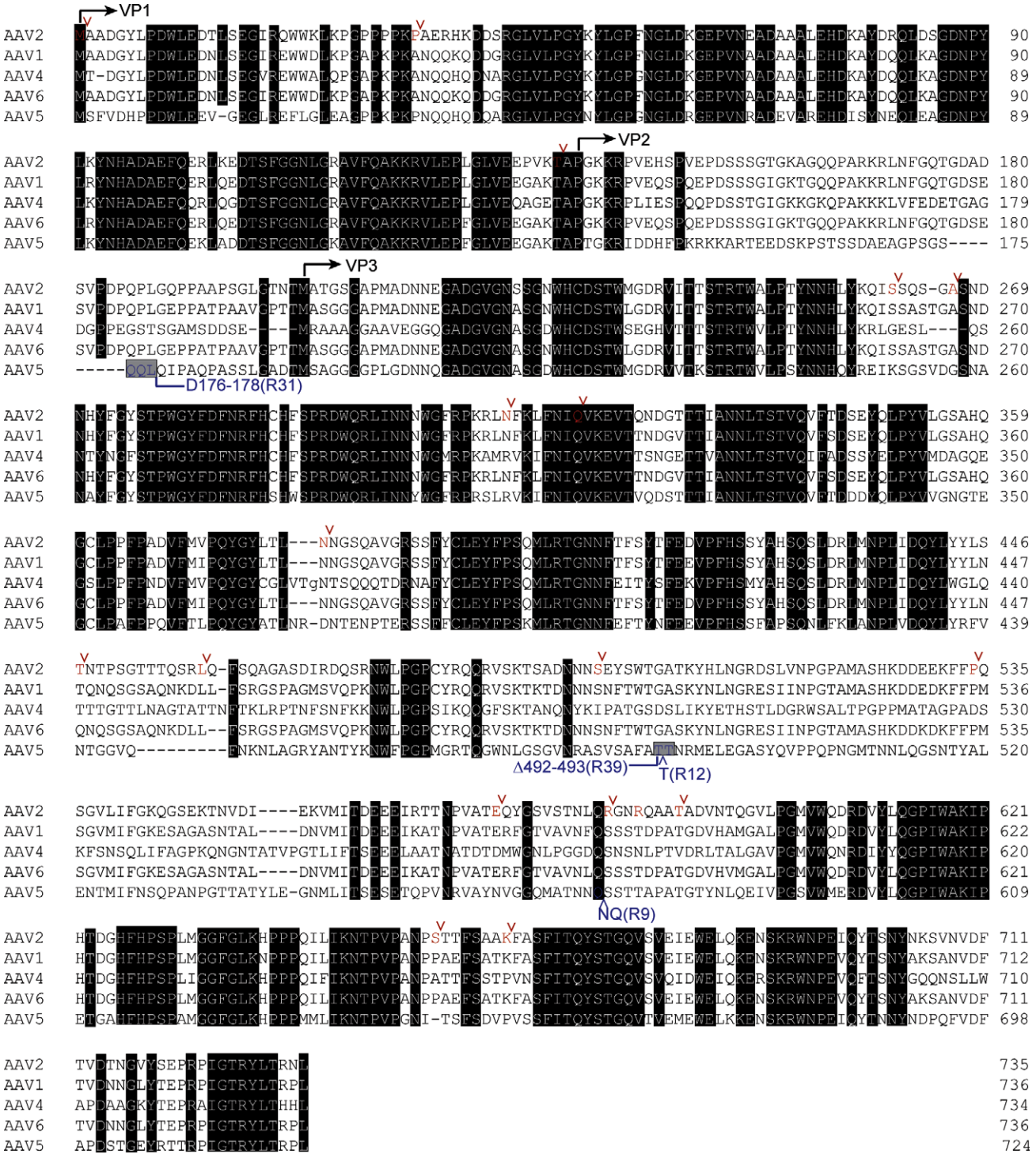

Fig. 3. Sequence alignment of Cap genes of AAV 1, 2, 4, 5, and 6. Alignments were made using the MACAW software. Residues that are highly conserved across all serotypes are highlighted in black. Sites within the AAV2 capsid previously shown to be amenable to insertions [13–16] are shown in red, and sites in AAV5 identified here are shown in blue. All insertions occur immediately after the colored residue and deletions are colored in grey. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

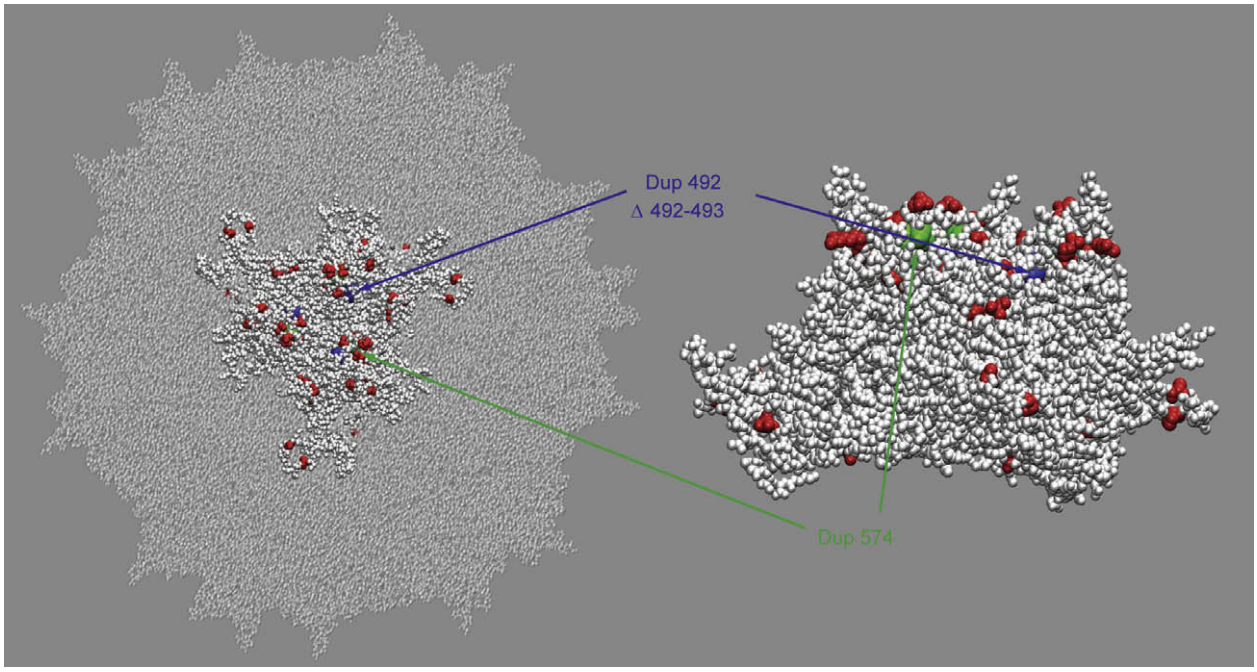

Fig. 4. Molecular model of the AAV2 capsid trimer. The surface of the trimeric unit of AAV2 is highlighted from a top view and from a side view. Locations of the mutations identified on the AAV5 surface (Δ 492–493, Dup 492 and Dup 574) are approximated by sequence alignment with the AAV2 genome and are shown in blue and green. Identified mutations are exaggerated in size for clarity. Previously identified sites in AAV2 that are tolerant to insertions [13–16] are shown in red. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

region within AAV2. These exact mutations have not been previously identified; however Δ 492–493 and Dup 492, occur in a region with low sequence homology, such that it is difficult to pinpoint exactly where on the AAV2 structure this mutation in AAV5 corresponds.

Although the mutations that led to a functional virus were all small mutations, it is reasonable to expect that these sites may be tolerant to insertions of larger peptides. Large tandem duplications may be more difficult to produce, as evidenced by the predominance of deletions in our naïve library. Further screening of this library with a selective pressure that does not favor wild-type may identify novel mutants and/or identify larger scale changes in the capsid. Although R9, the most successful mutant identified here, lies close to a previously identified site on AAV2 and may have been identifiable via rational design, the random mutagenesis method introduced here should be broadly applicable to any virus, including those with unsolved structures not amenable to rational design approaches. Viruses more tolerant to changes in the capsid may yield more variants, leading to further understanding of the viral surface for future engineering.

Acknowledgments

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