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Directed evolution of adeno-associated virus yields enhanced gene delivery vectors

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Adeno-associated viral vectors are highly safe and efficient gene delivery vehicles. However, numerous challenges in vector design remain, including neutralizing antibody responses, tissue transport and infection of resistant cell types. Changes must be made to the viral capsid to overcome these problems; however, very often insufficient information is available for rational design of improvements. We therefore applied a directed evolution approach involving the generation of large mutant capsid libraries and selection of adeno-associated virus (AAV) 2 variants with enhanced properties. High-throughput selection processes were designed to isolate mutants within the library with altered affinities for heparin or the ability to evade antibody neutralization and deliver genes more efficiently than wild-type capsid in the presence of anti-AAV serum. This approach, which can be extended to additional gene delivery challenges and serotypes, directs viral evolution to generate 'designer' gene delivery vectors with specified, enhanced properties.

AAV is a 4.7-kilobase, single-stranded DNA virus that contains two genes[1]. *Rep* encodes four proteins necessary for genome replication (Rep78, Rep68, Rep52 and Rep40), whereas *cap* expresses three structural proteins (VP1–3) that assemble to form the viral capsid. AAV depends upon a helper virus such as an adenovirus for active replication and in the absence of a helper establishes a latent state in which its genome is maintained episomally or integrated into the host genome. A number of homologous human and nonhuman primate AAV serotypes have been identified[2–5]; of these, AAV2 is the best characterized as a gene delivery vehicle and the only one that has been used in clinical trials[6–8].

Recombinant gene delivery vector systems (rAAV), first generated based on AAV serotype 2 in the 1980s[9], have been shown to offer numerous major advantages. First, rAAV vectors are safe, as wild-type AAV is nonpathogenic[10]. In addition, rAAV offers the capability for efficient gene delivery and sustained transgene expression in numerous tissues, including muscle[11], lung[12], liver[13,14] and central nervous system[15,16]. Furthermore, rAAV has enjoyed some success in human clinical trials[6–8].

Despite these successes, several problems remain. For example, the majority of the human population has already been exposed to various AAV serotypes, and as a result a significant fraction of any patient population harbors neutralizing antibodies that block gene delivery[17–21]. Additional problems with rAAV vectors include limited tissue dispersion for serotypes that bind heparan sulfate (for example, AAV2 and AAV3)[3,22]; poor infection of refractory cell types such as stem cells[23,24]; challenges with high-efficiency, targeted gene delivery to specific cell populations; and a finite transgene carrying capacity[25].

Because these problems arise from the capsid proteins that mediate gene delivery, the capsid must be reengineered to overcome them. Site-directed point and insertional mutagenesis of the AAV2 *cap* genes indicate that the viral capsid can tolerate some limited modifications and maintain its infectious properties[26,27]. Furthermore, peptide insertion into discrete capsid locations has been successfully applied to generate rAAV2 vectors with some cell-selective gene transfer capabilities[28–31]. However, the molecular basis of some viral properties, such as antibody neutralization[18,19] and virus-cell interactions[2,4,32], is distributed throughout the primary sequence of the capsid; these properties are unlikely to be efficiently modulated by serial site-directed capsid modifications even if the AAV structures are available[33,34]. Therefore, a higher-throughput approach would aid the design of vectors with functional enhancements.

Nature's approach to functional diversification has been the evolution of numerous AAV serotypes[2,4,5], and detailed examination of these types will undoubtedly yield both a wealth of basic AAV virology information and delivery vectors with new properties. However, it would be advantageous to develop a complementary vector-engineering approach that can create novel vehicles with a desired set of specified properties. Directed evolution has been used to generate enzymes with novel catalytic features[35,36], antibodies with enhanced binding affinities[37,38] and retroviruses with new properties[39]. We have built upon this work to develop an efficient and high-throughput method to generate rAAV vectors with improved capabilities. Furthermore, we have applied this method to create rAAV2-based vectors that evade neutralizing antibodies *in vitro* and *in vivo* and that have altered receptor-binding properties.

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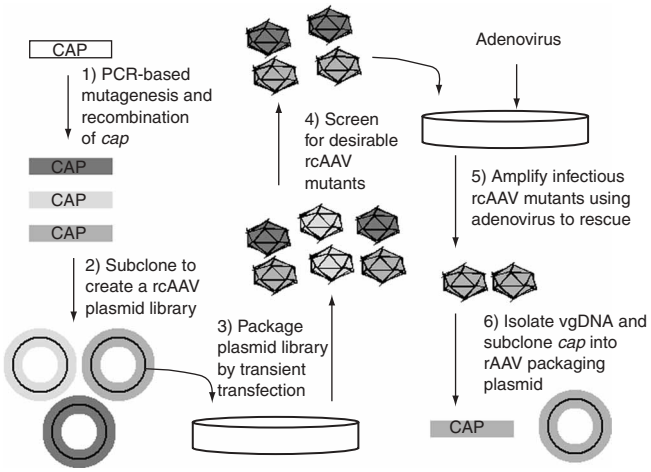

Figure 1 A forward genetics approach to creating 'designer' rAAV vectors. The entire *cap* gene was subjected to (1) mutagenesis and recombination using PCR-based methods. The mutant DNA was then (2) inserted into a rcAAV packaging plasmid to created a viral plasmid library. A viral particle library was created by (3) AAV helper-free transfection of the viral plasmid library into HEK 293 cells. This library was then (4) selected to isolate mutants with a desired set of properties. The resulting pools were (5) added to HEK 293 cells and rescued by the addition of adenovirus to select for only infectious particles and to amplify the small number of choice mutants. Several rounds of selection and amplification can be conducted to enrich the pool of choice mutants. Finally, (6) vector genome (vgDNA) from these mutants was extracted, amplified via PCR, and cloned into a rAAV helper plasmid to package 'designer' rAAV vectors. This enriched mutant pool can also be subjected to additional rounds of mutagenesis and selection, if desired.

RESULTS

Library generation

To generate a library of *cap* mutants with point mutations randomly distributed throughout VP1–3, we performed error-prone PCR followed by the staggered extension process[40]. Cloning the fragments yielded a viral plasmid library, pSub2Cap2*, with $>10^6$ independent clones (that is, bacterial colonies) after transformation. This plasmid diversity is large, although the resulting viral library diversity could potentially be smaller due to some nonsense or missense mutations yielding abnormal VP1–3 production. The viral plasmid was significantly diluted and packaged into AAV virions essentially as previously described[16]. The resulting AAV library can be selected for any variety of new properties or functions, and because the genotype of these variants is physically linked to their phenotype, the novel capsid sequence can readily be recovered by DNA sequence analysis of the encapsidated AAV genome (**Fig. 1**).

Heparin-binding mutants

As an initial gauge of how the library's sequence diversity translated into capsid functional diversity, we subjected CsCl-purified library particles to heparin affinity chromatography. Wild-type AAV elutes between 450 and 550 mM NaCl (**Fig. 2a**)[41]. In stark contrast, the AAV mutant library elutes at a wide range of salt concentrations, from the 150 mM load to the final 750 mM fraction, demonstrating that the library encompasses significant functional diversity. To generate mutants with either low or high heparin affinity, the 150 mM and 600–650 mM NaCl elution fractions were separately amplified by low multiplicity of infection (MOI) of human embryonic kidney epithelial (HEK) 293 cells, followed by adenovirus serotype 5 (Ad5) infection to

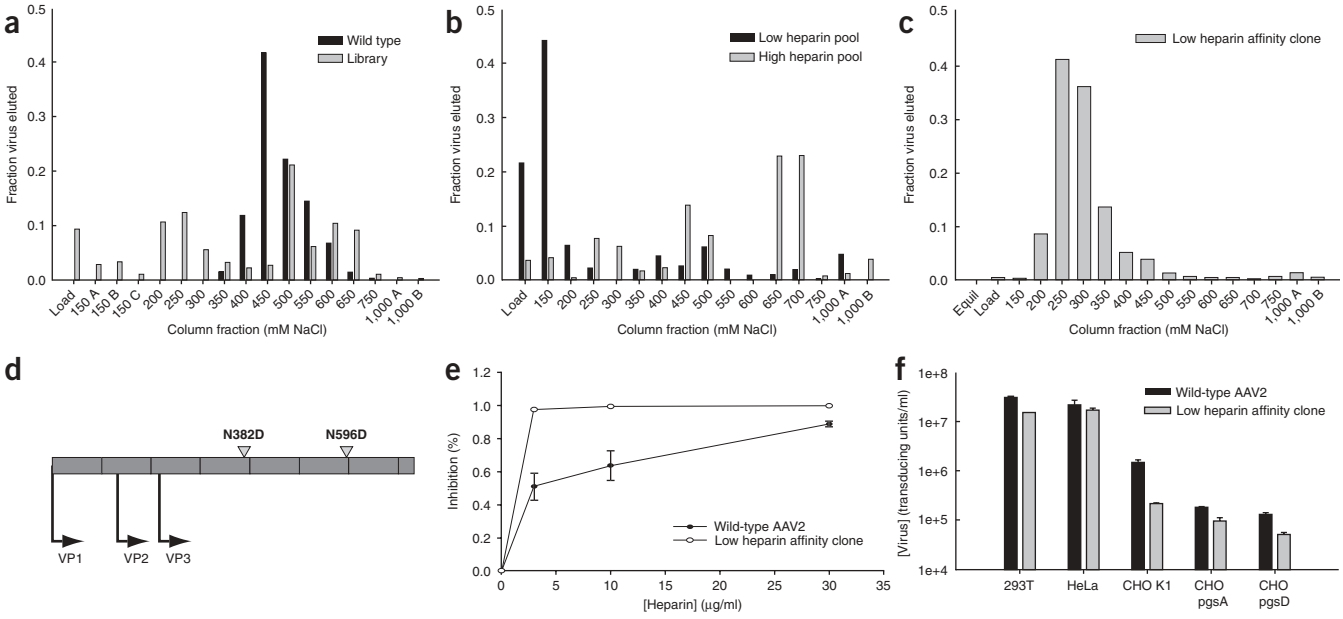

Figure 2 Heparin-binding characteristics of wild-type AAV versus the viral library. (**a**) The heparin affinity column chromatogram of wild-type AAV and the mutant library is shown, where virus gradually eluted from the column as the NaCl concentration was increased. Pools of virus from the load fraction and the 600-650 mM fractions were amplified by infection of HEK 293 cells followed by addition of adenovirus. (**b**) Chromatograms of the resulting pools from the mutant library are shown, and individual clones were subsequently isolated from the load and 700 mM fractions. (**c**) One clone exhibited reduced heparin affinity, and its (**d**) sequence data revealed numerous novel mutations. (**e**) Infection of HEK 293 cells by rAAV-GFP packaged in mutant or wild-type capsid revealed that the mutant is significantly more susceptible to inhibition by soluble heparin. (**f**) Infection of wild-type CHO cells and two variant lines expressing reduced heparan sulfate levels with rAAV-GFP packaged with wild-type or mutant capsid revealed that the low-affinity heparin-binding mutant was more sensitive to lower heparan sulfate levels.

Table 1 Neutralizing antibody titer of antibody-evading mutants

Clone	Neutralizing antibody titer reciprocal serum dilution
(+)	1,520
r1.2	482
r1.3	556
r1.4	583
r1.5	743
r2.1	606
r2.3	545
r2.4	536
r2.5	625
r2.15	15.8

The neutralizing antibody (NAB) titer for each clone was determined by fitting data in **Figures 4a–b** to a simple exponential decay. NAB titers are reported as the reciprocal of the volume fraction of serum necessary to reduce infectivity to 37% of the value measured in the absence of serum.

induce AAV replication and refractionation on the heparin column. After two such rounds of enrichment, the two resulting viral pools eluted from the column predominantly at 150 mM or 750 mM (**Fig. 2**). Notably, because each enrichment round involved viral infection and replication, these pools were composed of infectious virus.

Individual capsid clones were isolated from the two pools, inserted into a rAAV helper plasmid for recombinant vector production[42] and sequenced (**Fig. 2d** and **Supplementary Fig. 1** online). rAAV-green fluorescent protein (GFP) produced from the clones was fractionated on a heparin column to verify their modified heparin affinity, and one high-titer clone eluted at 250 mM NaCl (**Fig. 2c**), significantly lower than the wild-type capsid. This mutant's transduction properties were further analyzed by infection of both HEK 293 cells in the presence of soluble heparin, as well as wild-type Chinese hamster ovary (CHO) cells and two mutant lines expressing reduced heparan sulfate levels[43]. When heparan sulfate binding became limiting, transduction with the low-heparin-affinity mutant was reduced (**Figs. 2e,f**). Other low-and high-affinity clones were obtained, but some failed to package high-titer recombinant virus (**Supplementary Fig. 4** online). The ability to isolate diverse heparin-binding mutants from the AAV mutant library after only one round of mutagenesis demonstrates the utility of this approach for creating vectors with novel properties.

Creation of antibody-evading mutants after one and two rounds of evolution

We next applied this approach to a significant clinical problem: vector inactivation by neutralizing antibodies. Rabbit anti-AAV2 neutralizing serum with a neutralizing antibody titer (defined as the amount of serum necessary to reduce infectivity to 37%, or 1/e) for wild-type AAV of 1:1,500 was produced (**Table 1**), comparable to neutralizing antibody titers in human serum[17,18,20] (**Supplementary Note** online). Preimmune sera collected from two animals before rAAV inoculations, as well as serum depleted of IgG protein through incubation with protein A beads, had no neutralizing effect (data not shown), indicating IgG-dependent neutralization.

To isolate antibody-evading mutants, we selected the library against neutralizing serum. Wild-type, library or selected virus from the previous step was preincubated with varying antiserum amounts, added to HEK 293 cells and rescued by adenovirus (**Fig. 3**). The serum level was escalated after each step. After three selection steps, the virus pool maintained infectivity even after preincubation with a 1:30 dilution of serum. Viral genomic DNA was extracted from the selected antibody-evading mutant pool, and the *cap* sequence was PCR-amplified and inserted into both pSub2, to produce replication-competent AAV (rcAAV), and pXR2, to produce recombinant vectors. Five clones were sequenced and packaged. Four were packaged successfully and were tested for their ability to evade neutralization. In the absence of serum, infectious titers for these clones were equivalent to normal rAAV-GFP (**Supplementary Table 1** online). However, in the presence of serum, all four clones had neutralizing antibody titers that were ~threefold improved over wild-type capsid (**Fig. 4a** and **Table 1**). Moreover, a substantial (>1%) fraction of cells were still transduced at a 1:37.5 dilution of serum. Although each clone had distinct mutations, they all shared a T716A mutation near the C terminus of VP3. In fact, the r1.3 clone had only the T716A mutation, indicating that this mutation was likely responsible for most, if not all, of the antibody-resistant phenotype. The packaged rcAAV mutants also showed enhanced ability to evade neutralization (data not shown). Therefore, one mutagenesis and three selection steps generated mutant capsids with a threefold improved neutralizing antibody titer as compared to wild-type capsid and a ~7.5% infectivity at serum levels that completely neutralized wild-type infectivity.

To further improve the antibody-evading property of the mutants, we subjected the previous pool to a second round of evolution, that is, mutagenesis followed by three selection steps. In contrast to the first evolution round (**Fig. 3a**), we were able in the second round to rapidly escalate the levels of serum used (**Fig. 3b**). After amplification of *cap* from the selected library and insertion into pXR2, seven mutants isolated from a pool generated rAAV-GFP, which exhibited only partial neutralization at a 1:15 serum dilution. Infectious titers of five clones were very similar to that of wild-type capsid, whereas two mutants had titers 100- to 1,000-fold lower than wild-type capsid and were thus not pursued (**Supplementary Table 1** online). Antibody neutralization analysis revealed that four clones had neutralizing antibody titers similar to those isolated in the first round (**Fig. 4b**). However, clone r2.15 was only mildly neutralized, and when we repeated the neutralization assay using up to a 1:2.35 serum dilution (**Fig. 4c**), the neutralizing antibody titer of r2.15 was 96-fold improved compared to wild type (**Table 1**). Over 10% of virus remained

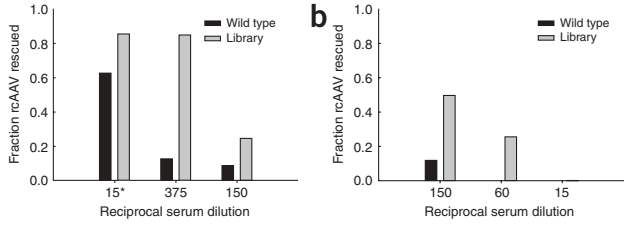

Figure 3 Selection of antibody-escape mutants from generated viral capsid libraries. AAV viral particles were incubated with various amounts of anti-AAV2 antiserum before addition to HEK 293T cells. Viral particles that productively infected cells were rescued by addition of Ad5 and quantified by QPCR. The fraction rescued was calculated at each serum dilution (that is, selection stringency) by normalizing the rescued AAV titer in the presence of serum by the rescued AAV titer in the absence of serum. (**a**) The first selection step in the first round of evolution indicated the viral capsid library is more infectious even in aggregate as compared to wild type, at a 1:375 serum dilution. The asterisk denotes that the first serum sample was primary antiserum, collected after only the first inoculation of rabbits with AAV. (**b**) In the second round of evolution, over 50% of the library was rescued at the 1:150 serum dilution as compared to the 10% in the first round, indicating progress over the first round. In both panels **a** and **b**, the viral pools rescued at the highest stringencies were amplified and subjected to two additional selection steps.

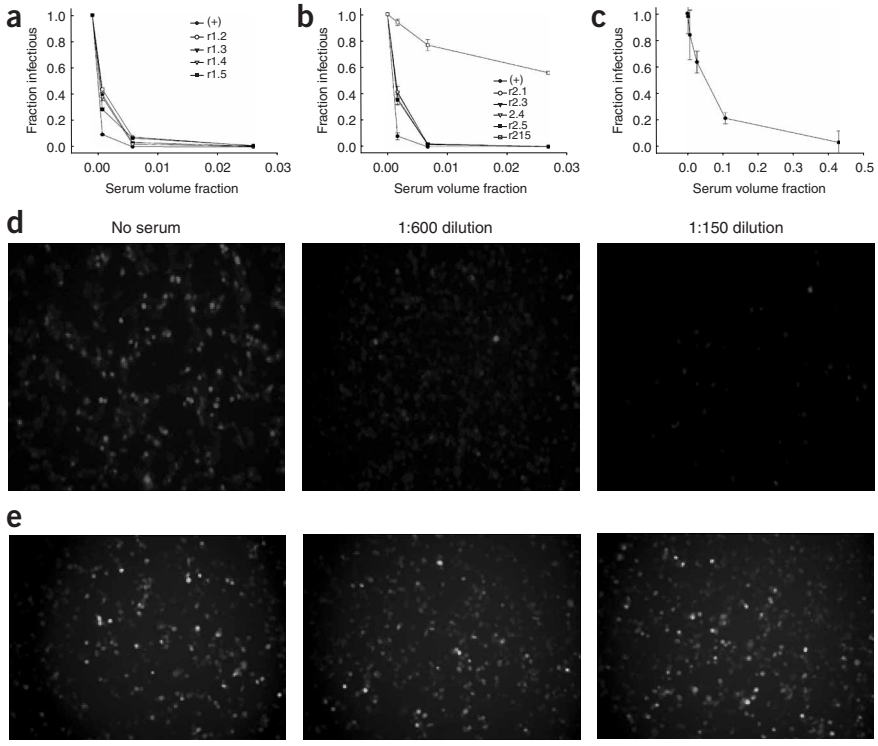

Figure 4 Neutralization profiles of antibody-evading mutants. (**a**) Antibody-evading mutants isolated after one round of mutagenesis were used to package high-titer rAAV-GFP and incubated with anti-AAV2 rabbit polyclonal serum before addition to HEK 293 cells at an MOI of 1. The fraction of infectious particles remaining was determined by fluorescence flow cytometry and normalized by the infectious titer in the absence of serum. The neutralizing antibody titer was determined by fitting these points to an exponential curve. (**b**) Of the five antibody-evading mutants isolated after a further round of mutagenesis, r2.15 exhibited a significant further improvement in antiserum evasion. Error bars indicate the standard deviation of triplicate samples, and trend lines are shown for clarity. (**c**) Further characterization of the r2.15 mutant neutralization profile was conducted using larger amounts of serum to determine a 96-fold improved antiserum evasion (that is, neutralizing antibody). (**d**) Representative panels of HEK 293 cells showed reduced GFP expression when infected with wild-type rAAV GFP in the presence of serum. (**e**) In contrast, delivery of r2.15 mutant AAV GFP yielded no reduction in GFP expression at the same serum levels.

infectious at a serum dilution of 1:7, and 3% persisted at a 1:2.35 serum dilution.

In vivo analysis of antibody evasion

We next conducted an *in vivo* assay in which high-titer rAAV-Epo vector based on the r2.4 and r2.15 mutants was produced, preincubated with antiserum and analyzed for its ability to mediate erythropoietin gene delivery after injection in the mouse hind limb. This represented a rigorous assay where high concentrations of antibody were allowed to fully bind and opsonize virus before injection. Notably, with no serum, the two mutants yielded a higher hematocrit than wild-type capsid, a result that may be further investigated (**Fig. 5**). However, as serum was increased, r2.4 yielded increased hematocrits at 1–2 orders of magnitude higher serum concentrations, and r2.15 at 2–3 orders of magnitude higher serum concentrations, compared to wild-type capsid. A serum-only control did not increase hematocrits (data not shown).

Sequencing revealed that the mutations in r2.4 were identical to r2.15, except for two additional substitutions at T567S and N587I (**Fig. 6a**). Because N587 lies in the heparin-binding domain, we analyzed the heparin affinity of r2.15, along with r1.2, r1.5 and r2.3. The three latter clones had a wild-type binding phenotype, whereas r2.15 showed a slight reduction in heparin affinity (**Supplementary Fig. 2** online). To ensure that the selected mutants could evade the neutralizing responses of more than one animal, vector was tested against the serum of the second rabbit immunized against AAV2 and evaded the neutralizing responses of this serum to a similar extent (**Supplementary Fig. 3** online).

DISCUSSION

There has been major progress in the development of safe, efficient and therapeutically relevant adeno-associated virus vectors[6,9,11,12,15,16]. However, many challenges remain, including widespread preexisting immunity against human serotypes, targeted and efficient delivery, limited packaging capacity[25] and infection of nonpermissive cell types[23,24]. These problems are not surprising, since the parent AAVs are products of natural evolutionary pressures that select for survival rather than for performance in human gene therapy. To overcome these and other problems, the viral capsid must be modified. We have accordingly developed a high-throughput approach to engineer AAV variants with improved properties. Specifically, large ($>10^6$) AAV2 libraries with randomly distributed capsid mutations were

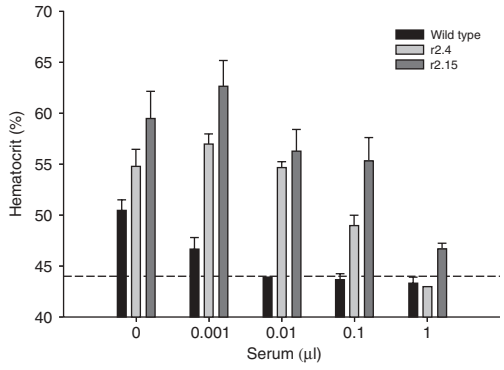

Figure 5 rAAV-Epo was produced with wild-type, r2.4 and r2.15 capsids. Vector was preincubated with varying levels of potent rabbit antiserum (1:6,000 neutralizing antibody), which allowed anti-AAV2 antibodies to opsonize vector particles before vector injection into the hind-limb muscle of adult mice. Two weeks after injection, hematocrit levels were measured and revealed that gene delivery mediated by the two antibody-evading mutants persists at serum levels that completely neutralize wild-type rAAV-Epo ($P < 0.005$). Error bars indicate the standard deviation of $n = 4$ samples, and the dotted line represents the basal hematocrit value. Results 3 weeks after injection were quantitatively similar (data not shown).

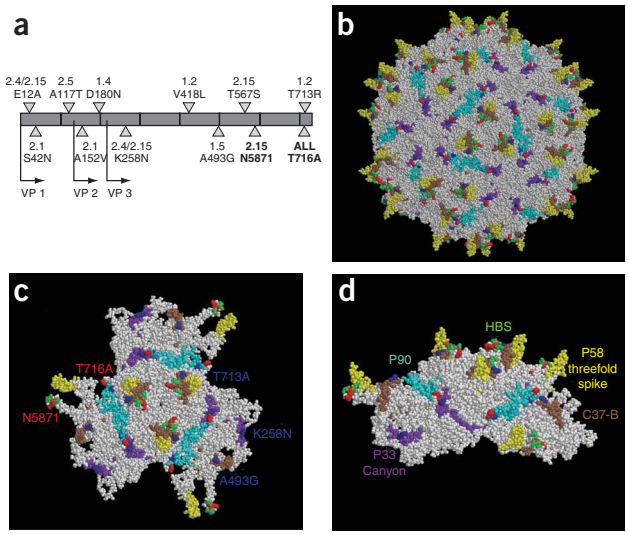

Figure 6 Mutation profile of neutralizing antibody-evading mutants. (**a**) Sequence data indicate that all nine mutants that had neutralizing titers threefold better than wild type contain the T716A mutation. The r2.15 mutant, which had a neutralizing titer 96-fold better than wild type, has two mutations absent from any other mutant. T567S is a conservative mutation that lies in a minor epitope associated with the A20 monoclonal antibody[19], whereas N587I lies in the heparin-binding domain[50], whose disruption has been shown to confer limited escape from neutralization by serum from certain human patients[44]. Therefore, the N587I mutation may be responsible for improvement from the first to the second round. (**b**) A molecular model of the full AAV2 capsid, based on the solved structure[34], is shown using Raswin. (**c**) Surface mutations found in the r2.15 mutant are labeled on a VP2 hexamer, with the phenotypically key N587I and T716A mutations shown in red and the other mutations in blue (see text for details). Mutated residues have been slightly exaggerated for clarity. (**d**) Several previously identified antigenic regions[18,19] as well as the heparin-binding site (HBS)[50] are labeled using various colors on a side view of the VP2 hexamer.

generated and selected for altered receptor binding and evasion of antibody neutralization.

Affinity chromatography selection yielded mutants with altered heparin affinity. Lower-affinity mutants may be desirable as gene delivery vectors when wide dissemination through a tissue or region is needed. AAV variants with reduced affinity for heparin have previously been generated[27,32], but none of our mutations overlap with any previously identified, and none of the resulting amino acid changes correspond to the sequences of orthologous positions in other AAV serotypes (**Fig. 2d** and **Supplementary Fig. 4** online). Two mutations that occurred on surface-accessible regions of VP3—N382D on the inner shoulder of the threefold spike and N596D on the outer shoulder of the threefold spike—resulted in a gain of negative charge. The low-heparin-affinity mutant's efficiency was reduced when heparin binding became limiting (**Figs. 2e,f**). A high-affinity mutant yielded low-titer vector but could enable insights into receptor-binding mechanisms.

The heparin-selection results confirm two key attributes of the method. First, the heparin chromatograms (**Fig. 2a**) demonstrate that the viral library contains considerable functional diversity. Second, the library was packaged by substantial dilution of mutant viral genome DNA, but a fraction of cells could have received multiple plasmids and therefore generated undesired chimeric mutant particles. A previous study that generated AAV peptide-display libraries used an additional packaging step to avoid chimeric capsids[30]. However, our ability to enrich low-and high-heparin-binding pools (**Figs. 2b,c**) as well as to isolate clonal variants from these pools indicates that the method does effectively link *cap* genotype with capsid phenotype and that the conceivable presence of chimeras does not substantially affect the evolution process.

We next applied the directed evolution approach to an important clinical problem: antibody neutralization. Several epidemiological studies have found that from 35–96% of the human population is seropositive for AAV1–5, and 18–67% harbors antibodies capable of significantly neutralizing rAAV1–5 gene delivery[12,17–20]. Therefore, preexisting immunity from 'accidental vaccination' by the parent virus could render AAV2 gene therapy ineffective in as much as two-thirds of the population. Mapping of dominant neutralizing epitopes has revealed the presence of both linear and complex conformation epitopes[18,19], rendering the rational design of variants that can escape antibody neutralization difficult. One study found that variants with peptides inserted at the heparin-binding site, originally generated for targeted gene delivery[28], reduced sensitivity to neutralizing antibodies[44]. However, success varied with the particular serum sample used, and infectious titer was reduced 10- to 1,000-fold, depending on the inserted peptide. Thus, disrupting one epitope can reduce antibody neutralization, but several mutations may be necessary to create a general neutralization-free vector. In addition, our more modest point mutations could achieve antibody-evasion without significantly altering vector tropism for situations in which maintaining heparin binding is desired.

Selecting the AAV2 library for efficient gene delivery in the presence of neutralizing antiserum yielded numerous successful variants, including one with a 96-fold improvement and the ability to mediate moderate gene delivery even at a ~1:2 serum dilution. This reduced neutralization is similar to the neutralization of other AAV serotypes by anti-AAV2 serum[4,45]. We further demonstrated that two mutants, r2.4 and r2.15, evaded antibody neutralization *in vivo* at serum levels several orders of magnitude greater than those required to neutralize delivery with the wild-type capsid. Surprisingly, both mutants also mediated enhanced gene delivery compared with wild type in the absence of serum (**Fig. 5**). Analysis of mutations present in 12 different clones suggests two key mutations in VP3, N587I and T716A, are largely responsible for the antibody-evasion phenotype. The N587I mutation lies on the surface of the threefold spike within the heparin-binding site[32] (**Figs. 6c,d**) but maintains high infectivity and only slightly reduces heparin affinity (**Supplementary Fig. 2** online). Interestingly, antibody-escape mutants in a number of picornaviruses also occur in receptor-binding sites with no change in receptor binding[46]. The T716A mutation found in all antibody-evading mutants lies on the surface of the twofold dimple region (**Figs. 6c,d**), previously identified as an immunogenic region[18]. Interestingly, AAV4 (only weakly neutralized by anti-AAV2 serum[45]) also has an alanine at position 716, raising the possibility that directed evolution could mirror some changes generated by natural evolution.

Our directed evolution approach can readily be extended to additional AAV gene delivery challenges. Future efforts may evolve AAV variants to escape neutralization by pooled human sera representative of a potential patient population. Furthermore, the mutant library may contain variants capable of more efficient delivery to problematic cells, such as stem cells resistant to all AAV serotypes attempted[23,24], provided that the problems reside in the capsid structure. In addition, variants could be selected to overcome specific blocks in the intracellular AAV gene delivery pathway[47]. Moreover, because the gene transfer characteristics of alternate AAV serotypes

vary in properties such as their cell surface receptors[43,48], tissue tropism[3] and overall delivery efficiency[3], they offer rich starting material for additional evolution.

In summary, we present a high-throughput approach to evolve AAV viral variants with advantageous gene delivery properties, and we have applied this approach to generate AAV2 mutants with altered receptor binding and neutralizing antibody-evasion properties. This method allows one to stipulate the criteria a designer vector must satisfy rather than attempting to identify a natural variant that likely meets only some of those needs. Furthermore, no initial mechanistic knowledge of the capsid properties is needed, and analysis of the results enhances our understanding of the capsid's structure-function relationships. For example, as more useful variants are isolated, pooling their sequence information with natural AAV serotypes should lead to the identification of adaptable regions in the capsid that can be tailored without compromising transduction. This approach therefore has potential to enhance knowledge of both AAV basic virology and vector engineering.

METHODS

Library generation and vector packaging. An AAV2 *cap* ORF genetic library was generated by error-prone PCR followed by the staggered extension process as described[40], using 5′-GCGGAAGCTTCGATCAACTACGC-3′ and 5′-GG GGCGGCCGCAATTACAGATTACGAGTCAGGTATCTGGTG-3′ as forward and reverse primers, respectively. To construct pSub2, a 1.8 kilobase linear fragment was generated by PCR using 5′-GCGGAAGCTTCGATCAACTACGC-3′ and 5′-GATGCCGGGAGCAGACAAGCCCGTCAGGGC-3′ with pSub201 (ref. 9) as template. Both this fragment and pSub201 were digested with *Hin*dIII and *Cla* I, and the products were ligated to create the 5.7-kb rcAAV packaging plasmid, pSub2, into which the resulting *cap* product could be inserted after *Hin*dIII/*Not* I digestion. AAV was produced and purified by CsCl centrifugation essentially as previously described[16]. Briefly, in a ~75% confluent 15-cm dish of HEK 293 (ATCC) cells, 25 μg of pHelper (Stratagene) or pXX6, 25 μg of pBluescript (Stratagene), and 7 or 70 ng of the pSub2Cap2* plasmid library were transfected by the calcium phosphate method. This 1:2 × 10^{-4} molar ratio was calculated such that >90% of cells received approximately one member of the pSub2Cap2* plasmid library, assuming each cell receives ~50,000 total plasmids[49]. Viral libraries were harvested as described[16], and virus was purified using CsCl density centrifugation. In the case of selection for antibody evasion, the above procedure was repeated using viral genomic DNA from a selected pool of mutant rcAAV as the template for error-prone PCR.

For all experiments, the AAV genomic titer was determined by extracting vector DNA as previously described[16] followed by quantification using real time PCR on a Bio-Rad iCycler using SYBR Green dye (Molecular Probes).

Heparin column chromatography. Approximately 10^{12} AAV library particles were loaded onto a 1-ml HiTrap heparin column (Amersham) previously equilibrated with 0.15 M NaCl and 50 mM Tris at pH 7.5. Washes were performed using 0.75 ml volumes of Tris buffer with increasing increments of 50 mM NaCl up to 750 mM, followed by a 1 M wash. As a control, rAAV-GFP was also subjected to this chromatography. Mutant virus from both a combined pool of the load and 150-mM fraction (low heparin affinity) and the 650-mM fraction (high heparin affinity) were added to 10^7 HEK 293T cells at a genomic MOI of 1–10. Cells were superinfected with adenovirus serotype 5 (Ad5) 2 d after infection, and virus was harvested 3 d later. Both crude lysates and viral particles precipitated by ammonium sulfate were characterized by heparin column chromatography as above. To isolate individual viral clones from library fractions that eluted at different salt concentrations, viral DNA was extracted from the fractions, amplified by PCR, and inserted into pSub2Cap2. These single clone rcAAV mutants were then packaged as previously described, subjected to heparin column chromatography to verify their phenotype, and then sequenced at the UC Berkeley DNA Sequencing Facility.

***In vitro* transduction assays.** Either wild-type or mutant rAAV-GFP particles were added to HEK 293T, HeLa, CHO K1, CHO pgsA and CHO pgsD cell lines at a genomic MOI of 3,000. After 48 h, the fraction of green cells was quantified by flow cytometry at the UC Berkeley Cancer Center (Beckman-Coulter EPICS). Heparin inhibition studies were performed identically, except that viral particles were preincubated with varying amounts of heparin (0–30 μg/ml) in 75 μl of PBS (pH 7.4) for 1 h at 37 °C before addition to HEK 293T cells. The medium was changed 1 h later. After 48 h, the fraction of green cells was quantified by flow cytometry.

Antiserum generation and library antibody neutralization screen. Polyclonal sera containing neutralizing antibodies against AAV2 were generated in two New Zealand White rabbits in accordance with the UC Berkeley Animal Care and Use Committee and National Institutes of Health (NIH) standards for laboratory animal care. Briefly, 5 × 10^{10} CsCl-purified rAAV2 particles carrying β-galactosidase cDNA were mixed with 0.5 ml TiterMax adjuvant (CytRx) and injected into the anterior hind-limb muscle. Two boosts were performed at 3-week intervals using the same AAV dosage, followed by antiserum collection. Adjuvant was not used in the last boost.

One round of evolution consisted of *cap* mutagenesis followed by three selection steps against neutralizing serum. Both wild-type and a mutant rcAAV library were incubated with varying amounts of serum (0–7.5 μl) in 75 μl of PBS (pH 7.4) for 30 min at 25 °C, followed by addition to 2.5 × 10^5 HEK 293 cells in a 6-well format. After 48 h, AAV was rescued from infected cells by addition of Ad5, and cells were harvested 24 h later. The rescued rcAAV was subjected to two additional infection-and-rescue steps in the presence of increasing amounts of serum.

Clonal antibody neutralization screen. Individual viral clones from the library fraction that successfully infected cells even in the presence of neutralizing antibody were inserted into the pXR2 rAAV packaging plasmid[42], and rAAV-GFP was produced as above. To assess the extent of neutralization of these antibody-evading rAAV variants, they were incubated with varying amounts of rabbit sera as above, followed by addition to 2.5 × 10^5 HEK 293 cells in 12 well format at an MOI of 1. At 48 h after infection, the fraction of green cells was quantified by flow cytometry. Iodixanol gradient purified rAAV mutants[41] were subjected to heparin column chromatography as above.

***In vivo* neutralization assay.** pXR2 containing wild-type, 2.4 or 2.15 *cap* were used to package pAAV-CB-mEpo, a vector expressing murine erythropoietin under the control of the chicken β-actin enhancer/cytomegalovirus promoter (a kind gift of J. Wilson, University of Pennsylvania). High-titer rAAV-Epo was produced and purified as described[16]. 2.0 × 10^{10} rAAV-Epo particles were incubated with varying amounts of high-titer rabbit sera (**Supplementary Fig. 3** online) for 30 min, then injected into the hind-limb muscle of 8-week-old female BALB/c mice (Jackson Laboratories, $n = 4$). Beginning 14 d after infection, animals were retroorbitally bled and hematocrits determined every 7 d. Animal studies were approved by the UC Berkeley Animal Care and Use Committee and conducted in accordance with NIH guidelines on laboratory animal care.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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