From Virus Evolution to Vector Revolution: Use of Naturally Occurring Serotypes of Adeno-associated Virus (AAV) as Novel Vectors for Human Gene Therapy

D. Grimm and M.A. Kay*

Stanford University, School of Medicine, Departments of Pediatrics and Genetics, 300 Pasteur Drive, Stanford, California, 94305, USA

Abstract: Gene transfer vectors based on the human adeno-associated virus serotype 2 (AAV-2) have been developed and tested in pre-clinical studies for almost 20 years, and are currently being evaluated in clinical trials. So far, all these studies have provided evidence that AAV-2 vectors possess many properties making them very attractive for therapeutic gene delivery to humans, such as a lack of pathogenicity or toxicity, and the ability to confer long-term gene expression. However, there is concern that two restrictions of AAV-2 vectors might limit their clinical use in humans. First, these vectors are rather inefficient at transducing some cells of therapeutic interest, such as liver and muscle cells. Second, gene transfer might be hampered by neutralizing anti-AAV-2 antibodies, which are highly prevalent in the human population. In efforts to overcome both limitations, an increasing number of researchers are now focusing on the seven other naturally occurring serotypes of AAV (AAV-1 and AAV-3 to -8), which are structurally and functionally different from AAV-2. To this end, several strategies have been devised to cross-package an AAV-2 vector genome into the capsids of the other AAV serotypes, resulting in a new generation of "pseudotyped" AAV vectors. *In vitro* and *in vivo*, these novel vectors were shown to have a host range different from AAV-2, and to escape the anti-AAV-2 immune response, thus underscoring the great potential of this approach. Here the biology of the eight AAV serotypes is summarized, existing technology for pseudotyped AAV vector production is described, initial results from pre-clinical evaluation of the vectors are reviewed, and finally, the prospects of these promising novel tools for human gene therapy are discussed.

Keywords: Adeno-associated virus, serotype, viral vector, pseudotype, cross-packaging

INTRODUCTION

Among the large variety of mammalian and non-mammalian viruses currently being developed as vectors for human gene transfer, some of the most promising candidates are represented by the smallest of all viruses under investigation, the adeno-associated viruses (AAV). The prototype of this human virus genus, AAV serotype 2 (AAV-2), has been at the center of intense studies since its discovery in the late 1960's, and has continuously been engineered and improved as a vector. To date, a wealth of data from pre-clinical and recent clinical evaluation shows that the virus and vectors derived thereof provide a combination of properties that are highly advantegous for application in humans, and unique amongst all viral vector systems [Grimm and Kleinschmidt, 1999]. These properties include the lack of pathogenicity and toxicity, ability to infect dividing and quiescent cells of various tissue origin, and the potential for site-specific integration into the host chromosome or formation of stable episomal DNA forms, either of which results in long-term gene expression from the recombinant AAV-2 genome. Moreover, technology for vector production and purification has steadily been improved, leading to current state-of-the-art methods that allow simple generation of high-titer, high-purity AAV-2 vector stocks in a short amount of time [Grimm and Kleinschmidt, 2000].

The enthusiasm initially associated with AAV-2 vectors diminished, when it became apparent that the virus is rather inefficient at infecting a number of cell types of particular clinical interest, such as hematopoietic cells, or liver and muscle cells. Transduction of the latter two is feasible with AAV-2, and well tolerated in humans according to recent phase I clinical trials [Kay *et al*., 2000], but the vector dose required to achieve therapeutic gene transfer in these tissues might be high. Moreover, a second long overlooked parameter prone to limiting the use of AAV-2 in humans is the high prevalence of antibodies against the virus, with estimates of up to 80% of all humans being seropositive. A high proportion of these individuals carry antibodies that are able to neutralize infection of cells with the virus *in vitro*, and although never proven, it is widely assumed that this might also be relevant *in vivo*. Consequently, these individuals might be resistant to transduction with AAV-2, which renders the vector essentially useless for treatment of these subjects. In addition to the cases where it was naturally acquired, neutralizing anti-AAV-2 immunity might also result from one-time treatment with AAV-2 vectors. This would leave the patients impervious to a repeated administration of the same vector, which might be needed to replenish or increase the population of transgene-expressing cells.

*Address correspondence to this author at the Stanford University, School of Medicine, Departments of Pediatrics and Genetics, 300 Pasteur Drive, Stanford, California, 94305, USA; Tel: 650-498-6531; Fax: 650-498-6540; E-mail: markay@stanford.edu

1566-5232/03 $41.00+.00

Once aware of the potential drawbacks of AAV-2, efforts were undertaken to overcome these hurdles. For instance, bi-specific antibodies were coupled to the AAV-2 capsid to broaden the vector's host range [Bartlett *et al*., 1999], or the recipient's immune system was transiently suppressed to allow vector re-administration [Halbert *et al*., 1998]. While it remained unclear whether these approaches could ever be translated into the human patient, a much more elegant solution was provided by the naturally occurring serotypes of AAV. There are seven primate AAVs in addition to AAV-2 which to date have been isolated, cloned, sequenced and accordingly named AAV-1 and AAV-3 to -8 [Muramatsu *et al*., 1996; Chiorini *et al*., 1997,1999b; Rutledge *et al*., 1998; Xiao *et al*., 1999; Gao *et al*., 2002]. All these viruses were initially found in different laboratories as contaminants in adenovirus preparations, with the exceptions of AAV-5, which was directly obtained from a human clinical specimen [Bantel-Schaal and zur Hausen, 1984], and the latest two members, AAV-7 and -8, which thus far only exist as partial molecular clones derived from rhesus monkey DNA [Gao *et al*., 2002]. Simian origins were also suggested for AAV-1, due to the fact that reactive antibodies against AAV-1 exist in non-human primates [Xiao *et al*., 1999], and for AAV-4, which also primarily infects non-human primates (African green monkey) [Chiorini *et al*., 1997].

Independent of their putative reservoir, all seven non-type-2 AAVs are able to transduce human cells in culture, and are thus essentially interesting as vectors for human gene therapy. Importantly, their engineering as vectors is readily achievable, since the genomes of all AAVs are available as easy-to-manipulate plasmids, and since technology developed for AAV-2 vector production can be applied to the alternative serotypes in a straight-forward manner. From the limited, but progressively growing literature on the generation and pre-clinical evaluation of these vectors, it is now becoming clear that they might inded fulfill the initial hopes : *in vitro* and *in vivo*, they can infect cells which are difficult to transduce with conventional AAV-2 vectors. Moreover, they can evade immune responses in animals having received an initial AAV-2 vector, or a vector based on another AAV serotype. Thus, while it is still too early to take the vectors into the clinic, the sum of data accumulated over the last 6 years is encouraging, and suggests the start of an exciting revolution in the field of AAV vectorology.

In this article, we will provide a comprehensive overview of current knowledge of AAV structure and biology (Part I), and will then critically review existing reports on the pre-clinical testing of novel gene transfer vectors based on AAV serotypes (Part II).

PART I: FROM VIRUS EVOLUTION…

In the following two chapters, we will use the AAV-2 prototype as a model to describe the unique structure (1.) and biology (2.) of the seven other AAV types. Since AAV-2 itself has been extensively reviewed over the years, we will limit our description to aspects relating to the other serotypes, and refer the reader to earlier literature for further basic information about AAV-2 [e.g., Muzyczka, 1992; Berns and Linden, 1995]. A summary of some of the information provided in the next two chapters can be found in Table **1**.

Table 1. Characterization of AAV Serotypes 1 to 8

Serotype	Natural host	Genome size (nt)	ITR size (nt)	Homology to AAV-2[e]	Receptor known	Cell binding inhibited by			HA activity	Ubiquitinated
						trypsin	mucin	heparin		
1	Monkey	4718	143	80%						
2	Human	4681	145	100%	yes	yes		yes	no	yes
3[a]	Human	4722	145	82%				yes	no	
4	Monkey	4767	144	75%	yes	no	yes	no	yes	
5	Human	4642	167	55%	yes		yes	no	yes	yes
6[b]	Human	4683	145/143[d]	82%				no		
7	Monkey	4721[c]		84%						
8	Monkey	4393[c]		84%						

[a] Two variants of AAV-3 were described and denoted AAV-3A [Muramatsu *et al*., 1996] and AAV-3B [Rutledge *et al*., 1998]. Both were derived from an ATCC stock of AAV-3 and are thus assumed to be naturally occurring sequence variants. AAV-3B is predominantly used and in the text will thus be referred to as AAV-3; it differs from AAV-3A in 16 nucleotides and 11 amino acids [Rutledge *et al*., 1998].

[b] Whether or not AAV-6 is a genuine serotype, or only a hybrid of AAV-1 and -2 with immunological identity to AAV-1, is a matter of intense controversy (see text). Since this issue has not been conclusively resolved yet, AAV-6 will be referred to as "serotype" in the text.

[c] Thus far, only the *rep* and *cap* genes of AAV-7 and -8 were cloned and sequenced; the full-length genomes will be larger by the size of the ITRs.

[d] The ITRs of AAV-6 are identical to those of AAV-2 (left, 145 nt) and AAV-1 (right, 143 nt).

[e] Percentages represent sequence homology on the DNA level; they are averages from often differing reports, and do not always reflect sequence homology over the full-length genome.

Blank fields indicate that this aspect has not been reported yet. For details and references, see text. HA, hemagglutination activity; nt, nucleotides.

1. Structure of AAV Serotypes

The AAV-2 particle consists of an icosahedral, non-enveloped capsid of about 20 nm in diameter, composed of three different capsid proteins, VP1, VP2, and VP3, in a ratio of 1:1:10, and containing a single stranded DNA genome of 4681 nucleotides. Very similar capsid morphologies and genome lengths were also found for the other AAV types, with AAV-5 carrying the smallest genome (4642 nucleotides) and AAV-4 the largest (4767 nucleotides). The full-length genome of AAV-7 is probably even larger than that of AAV-4, since the 4721 nucleotides long sequence cloned so far excludes the ends of the genome [Gao *et al*., 2002]. Typically, the genome ends are 143-146 nucleotides each (only the AAV-5 ITR is extended to 167 nucleotides) and are self-complementary, thus forming inverted terminal repeats (ITRs). In the AAV life cycle, they serve as the origin of viral DNA replication and encapsidation, as well as play roles in gene expression and genome persistence in the infected cell [Berns and Linden, 1995]. Two elements within the ITRs are crucial to these functions and are indeed found in all ITRs cloned thus far, i.e., a binding site (*rbs*) for the AAV Rep proteins (the AAV non-structural proteins, see below), and a "terminal resolution site" (*trs*), which is nicked by the endonuclease function of the Rep proteins. Between AAV-1 to -4 and AAV-6, the ITRs display greater than 95% DNA sequence homology, while the ITR of AAV-5 is distinct from the other AAV types with only about 60% homology. Consequently, as shown by Chiorini *et al*. [1999a,b] and confirmed by our group [Grimm *et al*., in press], the AAV-5 ITR is not a template for nicking by Rep proteins from any AAV type other than 5. This is not due to a lack of Rep binding to the ITR, since AAV-2 and AAV-5 Rep proteins are able to bind the ITR of the other serotype [Chiorini *et al*., 1999a], but rather related to the unique sequence of the AAV-5 *trs* and its positioning relative to the *rbs*. As will be described later in Part II, this affects the development of AAV vector production strategies involving Rep or ITR elements from AAV-5.

Within the AAV-2 genome, two large open reading frames (orf, *rep* and *cap*), three promoters and one polyadenylation site were identified (Fig. **1**, for further information on AAV-2 genome and gene products see Muzyczka, 1992). This general organization is conserved throughout the eight AAV types, in particular between AAV types 1 to 3 and 6, which exhibit 80-90% sequence homology between *rep* and *cap* orfs, as well as between the ITRs [Rutledge *et al*., 1998]. The almost 99% DNA sequence homology between AAV-1 and AAV-6, and the identity in sequence of the first 508 nucleotides of AAV-6 to those of AAV-2, led to the hypothesis that AAV-6 represents a naturally occurring hybrid of AAV types 1 and 2 [Xiao *et al*., 1999]. Nevertheless, as will be described later, AAV-6 is functionally different from both AAV-1 and -2 and offers some interesting properties as a vector. Between 80 and 90% homology in nucleic and amino acid sequence of VP1 were also found when AAV-7 and -8 were recently compared to AAV types 1, 2, 3 and 6 [Gao *et al*., 2002].

Some marked differences in genome sequence and organization were noted for AAV-4 and -5, when they were aligned with the other AAV serotypes. For AAV-4, an approximate 90% degree of homology to AAV-2 was found for the ITRs and the *rep* orf, although there were some minor changes, such as the positioning of the first promoter (p7, equivalent to p5 in AAV-2) within the genome [Chiorini *et al*., 1997]. However, a dramatic drop in DNA and protein sequence homology to about 60-70% was noted, when the *cap* orf and the VP1 protein were aligned with the other serotypes. Importantly, most of the differences lie in regions which are presumably on the exterior surface of the assembled viral capsid, providing a reasonable explanation for the distinct tropism and serology of AAV-4 (see Part II).

Even greater differences were found for AAV-5, which is therefore often classified as the most divergent member of the AAV family. At the nucleotide level, the overall homology to the other serotypes is only about 55% for both orfs, and similar low degrees of homology were reported for the ITR [Bantel-Schaal *et al*., 1999; Chiorini *et al*., 1999b]. Likewise, AAV-5 proteins are only 50-60% homologous to their counterparts of the seven other AAV types. Although the AAV-5 Rep proteins share about 90% homology with Rep of other AAVs in their central part, the degree of homology drops to 10-25% for the C-terminal part of these proteins. This results in slight alterations of a zinc finger motif located in this region and probably affects secondary protein structure [Bantel-Schaal *et al*., 1999]. Large differences are also found for the VP proteins, which are less than 45% homologous within AAV types 1 to 6, compared to greater than 80% homology when AAV-4 and -5 are excluded from the alignment [Bantel-Schaal *et al*., 1999]. This is because the AAV-4 and -5 *cap* orfs differ from the other AAVs as well as from each other, resulting in AAV-5 capsid proteins which are only 50-55% homologous to those of AAV-1 to -3 and AAV-6, or to those of AAV-4, respectively. Similar to AAV-4, the great diversity in amino acid sequence of AAV-5 capsid proteins provides the virus with a unique host range and serology (see Part II).

Interestingly, despite their overall divergence, the Rep and capsid proteins of AAV-1 to -8 share epitopes which are recognized by mono- or polyclonal antibodies previously raised against the respective proteins of AAV-2 [Grimm *et al*., in press]. These antibodies thus represent useful tools for future studies of wildtype and recombinant AAV serotypes. Thus far, the use of these antibodies to detect VP proteins of AAV-1 to -6 in Western Blot analyses has already shown that the proteins differ markedly in their migration pattern in SDS polyacrylamide gels [Grimm *et al*., in press; Rabinowitz *et al*., 2002]. Considering that they are composed of almost identical numbers of amino acids, one explanation might be that AAV serotype capsid proteins undergo specific posttranslational modifications in the cells, which should be worth studying further.

The initial reports on the cloning of AAV-5 indicated that despite the differences in sequence, the virus is identical to the other AAVs in terms of genome organization, i.e., structure and location of orfs, promoters, intron and polyadenylation site, with an exception being the ITRs. The only changes noted were the absence of a binding site for the transcription factor YY1 in the p5 promoter, and a shift of another transcription factor (EivF) binding site from p5 to a locus upstream of p40 [Bantel-Schaal *et al*., 1999], suggesting that AAV-5 gene expression is regulated differently from AAV-2. Surprisingly, a recent report by Qiu

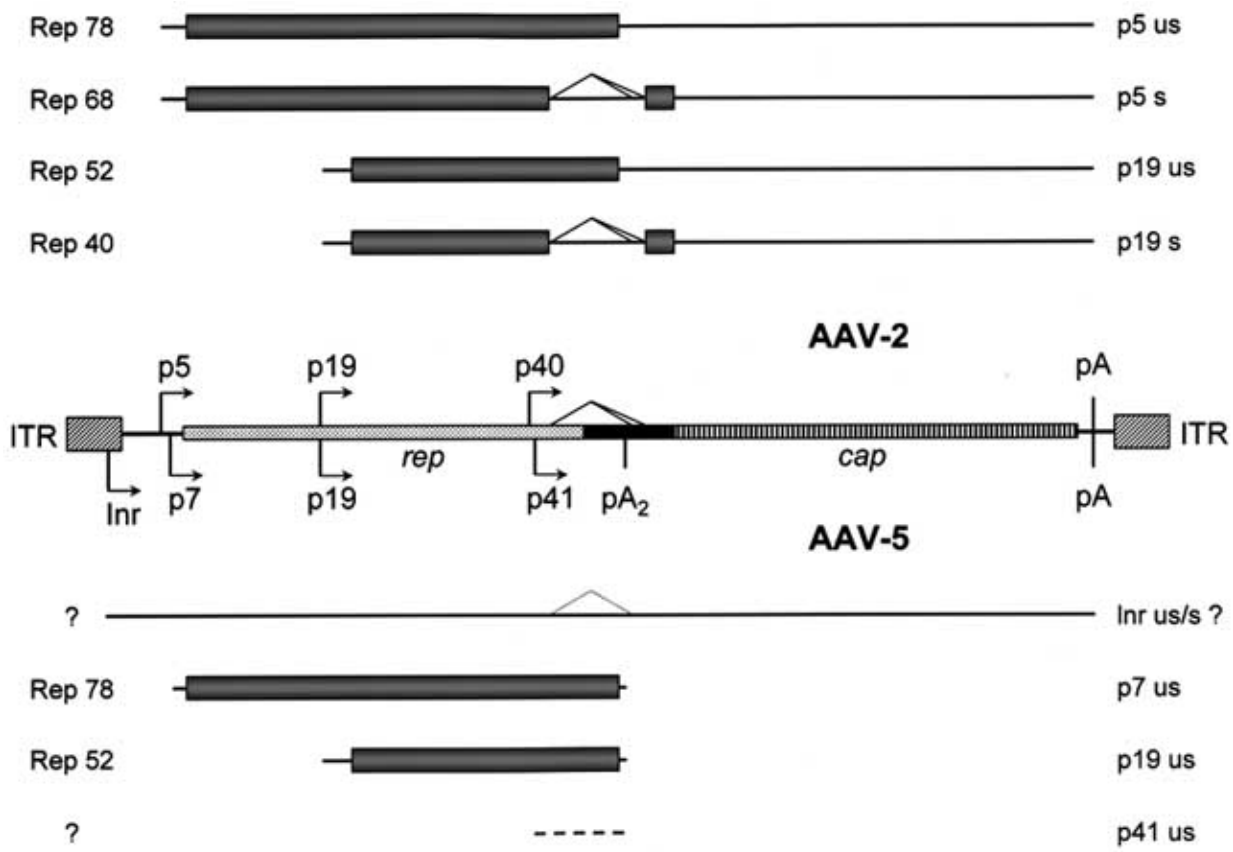

Fig. (1). Structure of wildtype AAV-2 and -5 genomes and transcription products. Depicted in the center is an AAV genome with structures common to AAV-2 and -5, i.e., ITRs (inverted terminal repeats) at both ends, as well as *rep* and *cap* genes (the black box indicates overlap). Shown above the genome are the three AAV-2 promoters (arrows), and the polyadenylation signal (pA) and the central intron (depicted as caret, with one donor and two acceptor sites). Shown below are corresponding elements in AAV-5, along with the additional Inr transcription initiation site in the left ITR, and the second polyadenylation site (pA_2) within the intron. Not shown is the AAV-5 intron, which is in the same spot as in AAV-2, but 81 nucleotides shorter. The grey boxes with lines (above and below the schematic genome) symbolize the translation products (names on left) and transcripts (names on right; us, unspliced; s, spliced), respectively, of the AAV-2/-5 *rep* genes (*cap* products are similar for both viruses and thus not shown). The nature and translation of the AAV-5 Inr and p41 transcripts is unclear [Qiu *et al*., 2002].

et al. [2002] now showed that AAV-5 is in fact much more divergent in structure and function from the other AAVs than previously assumed. First, AAV-5 generates a unique and abundant transcript from the left ITR, using an initiation site that maps to the *trs* site and extends to the right end of the genome (Fig. **1**). Whether this transcript is translated, and its function for the virus is unclear. A second interesting find was an additional polyadenylation site located within the AAV-5 intron, which is preferentially used by the RNAs generated by the p7 (p5 in AAV-2) and p19 promoters, but not by transcripts originating from the ITR or the p41 (p40 in AAV-2) promoter. This likely explains why unspliced Rep proteins (Rep78 and Rep52) were predominant in AAV-5/adenovirus coinfected cells, while the spliced versions (Rep68 and Rep40) seemed absent. Third, the AAV-5 intron was found to be 81 nucleotides smaller than that of AAV-2.

2. Biology of AAV Serotypes

Characteristic for all AAV serotypes is their dependence on a helpervirus for productive infection, which led to their classification as dependoviruses [Berns and Linden, 1995]. This helpervirus is typically adenovirus, but others such as herpes simplex virus (HSV) can also exert helper function. Interestingly, while seroconversion for AAV-2 (and AAV-3) occurs early in childhood and thus closely follows adenovirus, for AAV-5 it does not occur until 15 to 20 years of age, which is similar to HSV. This suggests that instead of adenovirus, HSV may be the natural helper for AAV-5 [Georg-Fries *et al*., 1984], and further emphasizes that AAV-5 is a more distantly related member of the AAV family.

A second helpervirus-related difference between AAV-5 and AAV-2 was noted by Qiu *et al*. [2002]. In contrast to

AAV-2, for which adenovirus dramatically stimulates gene expression and RNA splicing [Muzyczka, 1992], these events occur efficiently from AAV-5 independent of a helpervirus. This may translate into the strong expression of AAV-5 capsid and Rep52 proteins previously found by us and others [Grimm *et al*., in press; Brument *et al*., 2002]. Although this suggests that the requirement of the AAV-5 life cycle for a helper is more limited, a helpervirus is still required for a productive AAV-5 infection. Nevertheless, it is tempting to speculate that the strong expression of AAV-5 proteins provides advantages for the development of AAV-5-based vectors. Results from vector production indeed indicate that particle titers from helper plasmids expressing AAV-5 capsid proteins are higher than those from helpers based on other serotypes [Grimm *et al*., in press; Chiorini *et al*., 1999b] (see also Part II).

Another biological property common to all AAV types, next to the dependence on a helpervirus, is the requirement for binding to specific receptors for infection of cells. For AAV-2, heparan sulfate proteoglycans (HSPG) were suggested as primary receptors [Summerford and Samulski, 1998]. Moreover, human fibroblast growth factor (hFGF) receptor 1, as well as $\alpha_v\beta_5$ integrin are said to function as coreceptors, although this is controversial [Qiu *et al*., 1999]. A possible role of HSPG was also discussed for AAV-3, but evidence is inconsistent. Infection of cultured cells with AAV-3 was inhibited by soluble heparin, which indicated binding to HSPG, but the heparin dose required was 10-fold higher than for AAV-2. This led the authors to suggest that HSPG are not the physiological AAV-3 receptor [Handa *et al*., 2000]. This was further supported by the fact that AAV-2 and -3 vectors differ in the subtypes of hematopoietic cells they infect, and they also do not compete with each other for transduction [Muramatsu *et al*., 1996; Handa *et al*., 2000]. Instead of HSPG, a 42 kD protein was proposed as the AAV-3 receptor, but the nature of this protein was never elucidated [Handa *et al*., 2000]. On the other hand, Rabinowitz *et al*. [2002] saw that unlike serotypes 1, 4 and 5, but similar to AAV-2, AAV-3 vectors are dependent on heparan sulfate for infection of various cells. Moreover, they found purification of AAV-3 feasible using heparin sulfate affinity chromatography. Taken together, these data yet again support the idea that AAV-3 interacts with HSPG. Perhaps this inconsistency is related to differences in binding affinity and specificity between AAV serotypes 2 and 3, but this idea requires further validation.

In a series of three reports, one group has provided compelling evidence that 2,3-linked sialic acid (SA) is a key component of the receptor complex for AAV serotypes 4 and 5 [Walters *et al*., 2001,2002; Kaludov *et al*., 2001]. Sialic acid is the most common terminal glycosyl residue on proteins, with the 2,3-linkage being most abundant, which provides an explanation for the broad host range of AAV-5 noted thus far. Binding to SA also explains why AAV-4 and -5, unlike AAV-2 and -3, are able to hemagglutinate erythrocytes (Table **1**). Interestingly, AAV-4 hemagglutinates erythrocytes from several species, while for AAV-5 this reaction is limited to cells from rhesus mokeys [Kaludov *et al*., 2001]. This indicates that the agglutinin is different, and in fact, the exact nature of the sialic acid linkage was found to be crucial : AAV-4 specifically binds to 2,3 O-linked SA, while AAV-5 binds to 2,3 N-linked SA. The fact that AAV-5 infection of cells can also be inhibited by soluble 2,6-linked SA suggests that next to the 2,3-linked form, 2,6-linked SA might also serve as part of an AAV-5 receptor complex [Kaludov *et al*., 2001]. It is currently unclear whether SA is the only receptor for AAV-4 and -5, or if SA must be present on particular proteins on the cell surface.

An interesting hypothesis by Walters *et al*. [2002] suggests that AAV-5 has evolved to escape the mucin barrier present in human airways, which might serve as a soluble receptor complex due to its richness in O-linked carbohydrates. *In vitro*, mucin indeed bound AAV-4 specifically and inhibited virus uptake, but had no effect on AAV-5. This would explain why AAV-5 can infect human airway epithelial cells from the apical surface, despite its protection by a mucin layer. It should be pointed out, however, that AAV-5 has never been isolated from the human lung. Notable at this point is also that although the binding of AAV-5 to mucin appears to be unspecific and not physiologically relevant, an affinity chromatography protocol based on immobilized mucin was recently developed for purification of AAV-5 vectors [Auricchio *et al*., 2001b] (see Part II).

To date, the receptors for AAV types 1 and 6 to 8 remain to be identified. Virus competition experiments [e.g., Halbert *et al*., 2001] suggest the receptors to be different from those of AAV-2. A weak interaction of AAV-6 with heparin was reported [Halbert *et al*., 2001], but soluble heparin did not inhibit infection of cultured cells, making HSPG unlikely receptor candidates for AAV-6. Likewise, for the closely related AAV-1, Rabinowitz *et al*. [2002] demonstrated that the virus cannot be purified using heparin affinity chromatography, and that infection of cultured cells is independent of heparan sulfate. Together this rules out a role for HSPG in the AAV-1 life cycle. It is not unlikely that the AAV-1 receptor is similar to that of AAV-7, which is also unknown, since AAV-1 and -7 are equally efficient at transducing muscle, and both serotypes are highly homologous in DNA and amino acid sequences of the capsid genes and proteins [Gao *et al*., 2002]. The AAV-8 receptor appears highly prevalent on murine liver cells [Gao *et al*., 2002], which should aid in its identification.

Two studies by Bantel-Schaal *et al*. [2002] and Yan *et al*. [2002] have shed light on the intracellular fate of AAV serotype 5. The first group investigated the entry pathway of AAV-5 in HeLa cells in the absence of a helpervirus, and proposed a model in which the virus first binds to the apical cell surface, especially at microvilli. It is then taken up predominantly via coated pits and vesicles, although virus particles were also observed occassionally in non-coated pits and vesicles, suggesting that AAV-5 uses multiple entry pathways. Later, the virus was detected in the Golgi network, indicating that AAV-5 uses cellular trafficking routes not described as part of the endocytotic process of any other AAV serotype, or of viruses in general. Although the final fate of AAV-5 in the cell remained unclear, there was no evidence for intact AAV-5 particles in the nucleus. This is in contrast to AAV-2 [Bartlett *et al*., 2000] and thus again highlights the diversity amongst AAV serotypes. In the second study, Yan and coworkers [2002] demonstrated that similar to AAV-2 [Duan *et al*., 2000], AAV-5 capsids

become ubiquitinated in cells and subsequently degraded by the proteasome machinery. Proof was provided by adding proteasome-inhibitors to cells infected with AAV-2 or -5 vectors, resulting in augmented transduction with both vectors, although the extent of induction varied with the cell type tested. In fact, in differentiated myoblast cells, the same group had previously reported that addition of proteasome inhibitors led to a decrease of transgene expression in AAV-5-infected cells, while expression from AAV-2 increased [Duan *et al*., 2001]. Addition of proteasome inhibitors had no effect on the stability of the viral genomes, which was unexpected since increasing the percentage of intact capsids should protect more internalized genomes from enzymatic degradation. The fact that heat-denatured AAV-2 or -5 particles were better substrates for *in vitro* ubiquitination suggests that in the cell, endosomal processing of viral particles preludes capsid ubiquitination. This would be consistent with a model where ubiquitination of AAV capsids serves as a positive signal for viral particle disassembly, enhancing completion of the AAV life cycle. It is unclear how the viral capsid is rendered receptive to ubiquitination in the endosomes, but the process itself may be a crucial step in the life cycle of all AAV serotypes.

A final observation relating to AAV serotype biology was reported by Duverger *et al*. [2002], who found that full or empty capsids of AAV-2 are capable of enhancing cell apoptosis following its induction by chemotherapeutic agents, whereas capsids of AAV-5 are not. While basically interesting, it remains to be determined which part of the viral capsid is responsible, and why the effect was not observed with AAV-5 or another parvovirus tested (H-1).

PART II : … TO VECTOR REVOLUTION

Following the description of the basic biology of AAV serotypes, the next two chapters will provide an overview of the state-of-the-art technology for production of vectors based on these viruses (1.), with particular focus on the different strategies for vector generation (1.1.) and purification (1.2.). The information given will also be summarized in Fig. **2**. Subsequently (2.), we will critically review published results from the pre-clinical evaluation of AAV serotype vectors, exemplifying their unique and interesting tissue tropisms (2.1.) and immunological aspects (2.2.).

1. Production of AAV Serotype Vectors

The development of technology for generation and purification of vectors based on serotypes of AAV has gained from the experience made over the last 20 years with AAV-2. Fortunately, all the basic improvements and tools developed for AAV-2 [Grimm and Kleinschmidt, 1999,2000] have also proven useful for the production of vectors based on other AAV serotypes. It is thus not surprising that as compared to two decades of continuous progress on AAV-2 vector production, it took only 6 years to establish equally advanced technology for the other AAVs, as reviewed in the following two chapters.

1.1. Protocols for Vector Generation

In general, production of vectors based on any AAV serotype follows the principles established for AAV-2, requiring similar components and using protocols largely identical to those developed for AAV-2. AAV-2 vector production has been widely reviewed, and the strategies to adapt the protocols to generation of vectors based on other serotypes were also the topic of a recent review. We will therefore limit the information provided here to basic steps and refer the reader to the literature for further details [Grimm and Kleinschmidt, 1999,2000; Grimm, 2000,2002].

There are four components that are required for generation of vectors based on any AAV serotype (Fig. **2**): first, an AAV vector plasmid, consisting of a transgene expression cassette flanked by AAV packaging signals (ITRs), traditionally derived from AAV-2. Since the capsids of AAV types 1 to 6 are similar in diameter (this is likely also true for AAV-7 and -8), the size of the foreign DNA to be encapsidated, including the ITRs, must not exceed the approximate 5 kb packaging limit of AAV. The second components are the AAV *rep* and *cap* genes, encoding the Rep proteins which replicate and encapsidate the recombinant vector genome, or the capsid proteins which form the viral shell and thus determine the serotype of the vector particles, respectively. Needed third is a set of adenoviral genes (E2A, E4orf6, VA RNA), whose products provide helper functions in the various steps of AAV vector particle generation. Formerly delivered through wildtype adenovirus, these genes are now available as non-infectious plasmid clones. All three DNAs must be introduced into cultured cells, which represent the fourth component. This can be achieved by transient transfection of the cells with the three plasmids, i.e., the AAV vector, the AAV helper, and the adenovirus helper plasmid (AAV *rep* and *cap* can be combined with the adenoviral genes in one plasmid, reducing the number of plasmids to two [Grimm *et al*., 1998]). Alternatively, the DNAs can be delivered through recombinant heterologous viruses used to infect the cells, which is more efficient than transfection, but associated with a higher safety concern. Finally, they can be stably introduced into cell lines, but this approach is less versatile and more difficult to accomplish.

Of these three different strategies, the first two (transfection or infection of cells) have been adapted to generation of vectors from serotypes other than 2, with the main change being the origin of the AAV *cap* gene in the AAV helper (plasmid or heterologous virus). In this respect, it was a crucial discovery that expression of AAV-2 Rep proteins along with capsid proteins of another serotype results in formation of particles that package AAV-2 vector DNA. This made it feasible to simply replace the AAV-2 *cap* gene within existing optimized AAV-2 helper plasmids with *cap* of alternative AAV serotypes, resulting in novel hybrid helpers allowing the cross-packaging of AAV-2 vector genomes into capsids of any serotype (as determined by the *cap* gene). Importantly, the efficiency of this process, which is also being referred to as pseudotyping, is not reduced as compared to traditional AAV-2 vector generation, at least from vector production protocols relying upon transient plasmid transfection [Grimm *et al*., in press; Rabinowitz *et al*., 2002].

This could not be predicted, as it was unclear whether AAV-2 Rep proteins would interact with capsid proteins from another serotype, as is required for AAV DNA

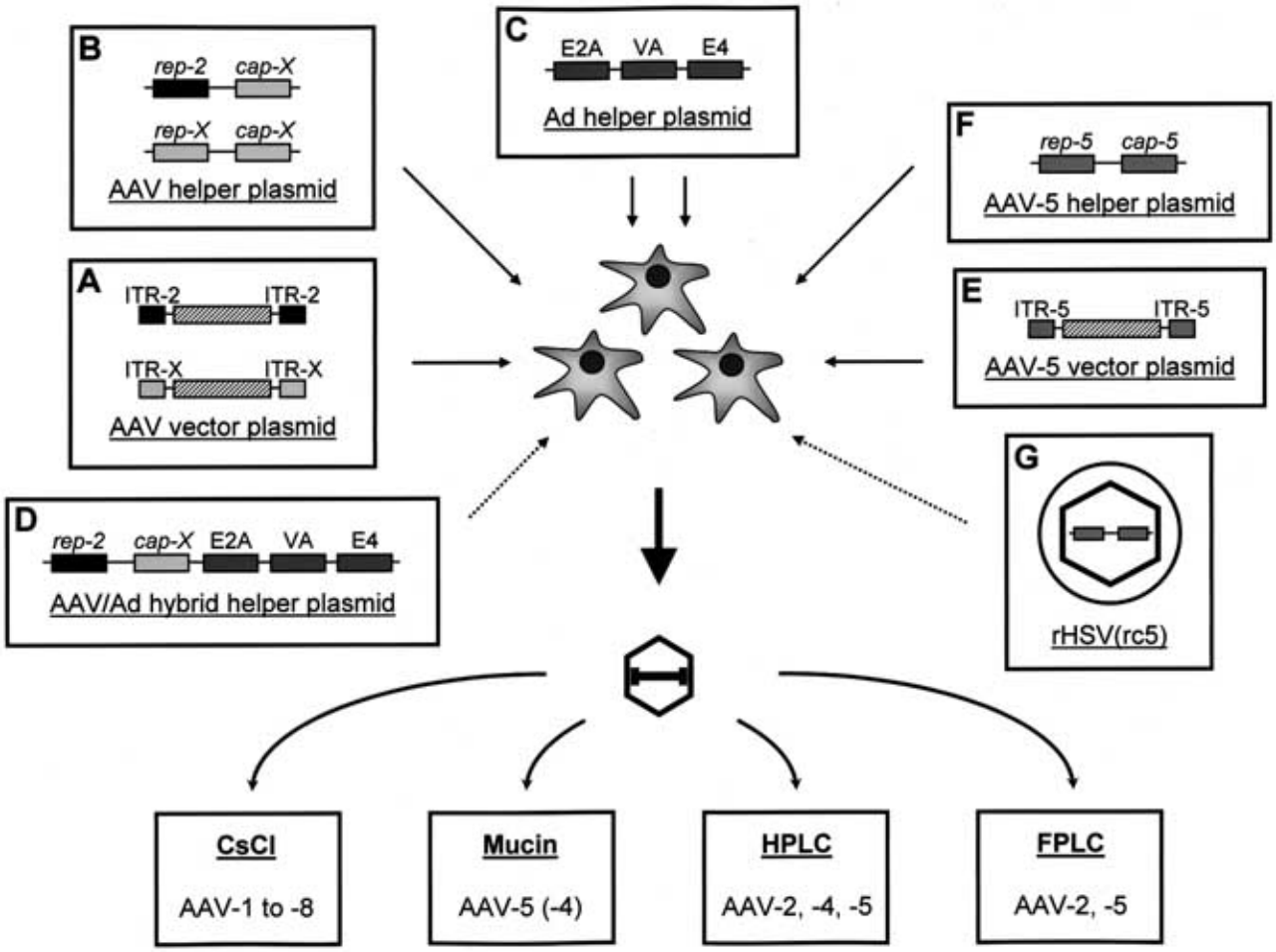

Fig. (2). Strategies for AAV serotype vector production. Summarized are approaches and components for generation and purification of vectors from all eight AAV isolates. Packaging of vector DNA carrying ITRs from any non-type-5 AAV is feasible by triple transfection of cells, with an AAV vector plasmid (typically with AAV-2 ITRs, or other ITRs, **A**), an AAV helper plasmid (expressing *cap* of any serotype typically next to *rep* of AAV-2, or of another serotype, **B**), and an adenoviral (Ad) helper plasmid (**C**). AAV and Ad helper genes can also be provided from a single hybrid construct (**D**). Packaging of AAV-5 ITR-containing vector DNA is achieved by triple transfection with the AAV-5 vector plasmid (**E**), as well as an AAV-5 helper plasmid (expressing AAV-5 *rep* and *cap*, **F**) and the Ad helper plasmid (**C**). Like for non-type-5 vectors, AAV-5 and Ad helper genes can be provided from a common plasmid (not shown) [Smith *et al*., 2002]. Alternatively, AAV-5 vector-transfected cells can be infected with a recombinant herpesvirus carrying AAV-5 genes, and delivering helper functions (rHSV(rc5), **G**). Resulting particles are purified using one of four different methods (see text for details).

encapsidation [King *et al*., 2002]. There is indeed evidence that combining Rep and capsid proteins from two different serotypes is not necessarily optimal for vector production. For instance, cross-packaging of AAV-2 vector DNA into AAV-4 capsids was 5-fold more efficient, when AAV-4 instead of AAV-2 Rep proteins were expressed from the helper plasmid [Grimm *et al*., in press]. Likewise, Rabinowitz *et al*. [2002] obtained higher vector titers from expressing hybrid Rep proteins, which had the AAV-2 Rep N-terminus fused to the C-terminus of Rep proteins from the serotype that also provided *cap*, than from full-length AAV-2 Rep. These reports indicate an optimal interaction between Rep and capsid proteins when both are derived from the same serotype, with the domain for interation residing in Rep's C-terminal portion. On the other hand, co-expression of Rep and capsid proteins from AAV-6 was less efficient than expressing AAV-2 *rep* together with AAV-6 *cap* from the helper plasmid [Grimm *et al*., in press; Halbert *et al*., 2001]. This suggests that Rep and capsid protein requirements may vary between the serotypes, and optimal combinations must be elucidated experimentally.

An interesting challenge in this respect is presented by AAV-5. The problem posed by this virus is that as mentioned earlier, AAV-5 Rep proteins cannot nick AAV-2 ITRs, which is required for excision and replication of the viral genome. It is therefore not feasible to express Rep and capsid proteins of AAV-5 for packaging of AAV-2 vector DNA into AAV-5 capsids, although this setting might provide strongest protein interactions. Instead, probably less optimal helpers are used for pseudotyping of AAV-2 vectors

with AAV-5 capsids, which express AAV-2 Rep together with AAV-5 capsid proteins. Interestingly, particle titers from these helpers are still higher than those of other serotype particles prepared under identical conditions [Grimm *et al*., in press; Wustner *et al*., 2002], which may be due to the stronger expression of AAV-5 capsid proteins. This would underscore the general idea that AAV vector titers are mainly determined by expression of capsid proteins from the helper [Grimm *et al*., 1998,1999].

Helper plasmids expressing AAV-5 *rep* and *cap* are not useful for encapsidation of AAV-2 vector DNA, but they allow packaging of vector genomes carrying ITRs of AAV-5 into capsids from AAV-5 (Fig. **2**). Such AAV-5 vector DNAs might exhibit unique properties in mammalian cells and thus represent interesting objects of study. Noteworthy in this respect is that vector DNAs carrying ITRs from two other serotypes, AAV-3 or -6, were also reported [Rutledge *et al*., 1998]. In contrast to the AAV-5 DNAs, encapsidation of these two alternative vector constructs was readily achieved with helpers expressing AAV-2 or -4 Rep proteins, albeit the AAV-6 vector gave lower titers than an AAV-2 vector, correlating with reduced vector DNA replication [Grimm *et al*., in press; Halbert *et al*., 2001].

The only AAV serotype packaging approach not based on transient plasmid transfection was reported by Wustner *et al*. [2002], who engineered a recombinant herpesvirus to express AAV-5 Rep and capsid proteins. The virus is consequently useful only for encapsidation of AAV-5 vector DNAs. This was indeed achieved by infection of cells after transfection with the AAV-5 vector, or by co-infection with the herpesvirus and AAV-5 particles, which circumvents a transfection step. However, the efficiency of either approach was at least 10-fold lower as compared to transient transfection of vector and helper plasmids, probably related to cytopathic effects caused by the herpesvirus. As a further drawback, the herpesvirus-based approach involves major safety concerns.

1.2. Protocols for Vector Purification

A prerequisite for the successful transition of AAV-2 vectors to human clinical trials was an improvement in methods for vector purification. CsCl density gradient centrifugation, which long provided the only means of purifying recombinant AAV-2, was time consuming, could not easily be scaled up, and yielded particle preparations that were contaminated with cellular proteins and had poor infectivity. Therefore, along with increasing knowledge of the interactions of AAV-2 with the HSPG receptor, conventional CsCl-based technology was replaced with novel affinity chromatographies based on heparin or sulfate ions [e.g., Clark *et al*., 1999]. These methods are significantly faster and more convenient, but unfortunately, they are useless for AAV serotypes which do not depend on HSPG for infection, such as AAV-4 and -5. Accordingly, when Rabinowitz *et al*. [2002] evaluated heparin columns for purification of vectors based on AAV serotypes 1 to 5, only AAV-3 displayed specific binding and elution profiles similar to AAV-2, while recovery of AAV types 1, 4 and 5 was poor.

In view of the need for alternative methods to purify AAV serotype vectors, Auricchio *et al*. [2001b] developed a single-step affinity column for purification of vectors derived from AAV-5. The method is based on the observation that 2,3-linked sialic acid is required for infection of cells with AAV-5 (see above). Thus, when mucin, a sialic acid-rich protein, was coupled to CnBr-activated Sepharose, AAV-5 bound to the resulting column and could be eluted with high salt (0.4 M NaCl). Comparison of the particles to vectors purified by conventional CsCl sedimentation showed similar yields, transduction efficiencies and infectivity characteristics *in vitro* and *in vivo*. However, the mucin-treated particles were cleaner by Coomassie-stain. While this suggests the method is advantegeous over CsCl purification, in addition to being faster and more amenable for scale-up, it has two drawbacks. First, the mucin column is only useful for vectors based on AAV-5 (probably also for AAV-4, which also binds 2,3-linked sialic acid, albeit this was not demonstrated), but not for the other serotypes. Second, in contrast to CsCl sedimentation, allowing for enrichment of genome-containing particles, the mucin method concentrated both full and empty particles, which is highly unwanted.

The latter drawback is overcome by an improved method reported from Kaludov *et al*. [2002]. This group developed a two-step process, involving anion-exchange high-performance liquid chromatography (HPLC), followed by particle concentration by use of high molecular weight retention filters. Although successful use of the technology was only demonstrated for AAV-2, -4 and -5 vectors, it should be suitable for all AAV serotypes, since it does not rely on affinity for specific molecules. The method is again faster and easier than CsCl sedimentation, and yields particles of superior purity (judged by electron microscopy), with greater than 90% of them full. Moreover, for reasons not understood, the HPLC method allows removal of non-transducing particles from the vector preparation. This resulted in better than 100% recovery of transducing material for all three serotypes tested, although only 10% of the starting viral genomes were retained. Together, this represents a 100-fold decrease of the particle-to-infectivity ratio for the final vector stock compared to the crude cell lysate. These data are encouraging, but some issues require further investigation and validation. In particular, although likely, it remains to be demonstrated that the protocol is useful for serotypes other than the three tested. It is reasonable to assume that adaption will not be straight-forward, considering that a large variety of parameters had to be tested to separately optimize the method for AAV-2, -4 and -5, such as type of ion-exchange resins, pH and salt conditions.

A second disadvantage is the protocol's requirement for extensive detergent and exonuclease treatment of crude cell lysates prior to HPLC, since these agents are difficult to eliminate from, or detect in final preparations. Therefore, Brument *et al*. [2002] presented yet another method which does not require pre-purification of cell lysates. The method is a two-step fast performance liquid chromatography (FPLC), using a cation-exchanger, followed by an anion-exchanger resin. For AAV-2 and -5 vectors, the method gave relatively poor recoveries of transducing particles of 33 to 44%, which is not unusual for multi-step purification procedures. Such yields are similar to those from CsCl gradients, and the method is thus clearly inferior to the

previously described HPLC method. There were no differences in biological characteristics between vectors purified by FPLC or by CsCl, although the FPLC-treated particles were slightly purer in electron microscopy analyses. However, in contrast to CsCl or HPLC, FPLC purification does not enrich full particles, resulting in vector stocks with the same homogeneous ratio of empty and full particles as crude cell extracts. Thus, the only benefit provided by the FPLC method is the lack of need for pre-treatment of crude cell lysates, and the hope that like HPLC, it can also be applied to other serotypes of AAV.

In conclusion, there is unfortunately no current alternative to conventional CsCl sedimentation to purify vectors based on all known AAV serotypes under identical conditions, as is required for reasonable side-by-side comparison of those vectors. There is however good reason to hope that novel methods based on liquid chromatography (HPLC or FPLC), such as the two reviewed here and a similar one most recently reported by Zolotukhin *et al*. [2002], will be further developed and streamlined for all serotypes, to eventually replace CsCl-based protocols. Together with improved methods for vector generation, this should allow scale-up of AAV serotype vector production under conditions of good manufacturing practice, which would pave the way for future clinical analyses of the vectors.

2. Pre-Clinical Evaluation of AAV Serotype Vectors

The rationale for developing alternative AAV serotypes as vectors is the hope that the divergence of capsid proteins of the wildtype viruses will translate into vector particles that differ in tissue tropism and serology. In a large number of *in vitro* experiments (not reviewed here), unique tropisms were indeed confirmed for vectors from all AAV serotypes, and partially related to binding of the particles to specific receptors. Importantly, the results were confirmed and extended under physiologically more relevant conditions *in vivo*, as reviewed in the following chapter (2.1.). The subsequent chapter (2.2.) summarizes data from the serological analyses of AAV serotype vectors, showing that as hoped for, *in vitro* and *in vivo* the particles can at least partially escape humoral immune responses directed against the viral capsids of particular serotypes.

2.1. Tropism of AAV Serotypes for…

This chapter will describe recently published analyses of tropism and transduction characteristics of AAV serotype vectors in various small animals (rat, mouse and rabbit), and in six different clinically relevant tissues, eye, pancreas, CNS, muscle, lung and liver. All findings will be summarized in Fig. **3**.

… The Eye

The eye is an interesting target for somatic gene therapy, since a number of inherited diseases affecting the eye have known gene defects. A typical example is Leber's congenital amaurosis (LCA), the earliest and most severe form of retinal degeneration. This disease, which arises from specific mutations in cells in the retinal pigmented epithelium (RPE), causes up to 10% of all congenital blindness in children [Harris, 2001]. The eye is further attractive for gene therapy because it is an enclosed organ being divided into small compartments, which is ideal for precise vector delivery. Moreover, animal models are available for many of the diseases affecting cells in the eye. There is extensive literature describing successful *in vivo* gene transfer to the eye with AAV-2 vectors, with one of the most impressive reports demonstrating restoration of vision in a canine model of LCA [Acland *et al*., 2001]. AAV-2 vectors efficiently transduce photoreceptors and RPE cells, which both are interesting targets for gene delivery, since mutations in either of them can lead to retinal degeneration development.

Four recent reports now demonstrate that these cells, as well as additional cells in the eye, are also transduced by alternative AAV serotype vectors, and that transduction occurs faster and more efficiently. In the first study, Auricchio *et al*. [2001a] pseudotyped *gfp*-expressing AAV-2 vector genomes with capsids from AAV-1, -2 or -5, and injected equal doses of the resulting particles into the murine eye, either subretinally or intravitreally. Ophthalmoscopic examination of Gfp expression from day 2 after injection up to 4 months later showed two things. First, the viruses differed in onset of gene expression : the AAV-1 vector expressed already at day 4 after subretinal injection, whereas Gfp expression from AAV-2 or -5 was detected no earlier than at day 14 (AAV-5) or day 28 (AAV-2). Once detectable, expression was stable for the duration of the experiment in all three cases. Second, the viruses also differed in the cell type transduced : following subretinal administration, the AAV-1 vector specifically and efficiently transduced the RPE layer, but AAV-2 and -5 targeted both photoreceptors and RPE cells. While AAV-2 primarily infected photoreceptors and to a smaller extent RPE cells, the opposite was observed for AAV-5. The latter moreover expressed stronger than AAV-2. Following intravitreal injection, AAV-2 transduced the inner retina efficiently, whereas the other two vectors expressed poorly in this region. The important conclusion was that exchanging capsids of AAV vectors allowed efficient targeting of specific retinal cell types, and influenced onset and level of gene expression. It should be noted however, that only one vector dose was evaluated, and the method used to quantify Gfp expression (fluorescence microscopy) was not very sensitive. In fact, using confocal microscopy, Sarra *et al.* [2002] saw Gfp expression from AAV-2 vectors applied under identical conditions as early as three days after injection.

In a subsequent study, the authors exploited the AAV-1 vector's capability to efficiently transduce the RPE for expression of genes encoding anti-angiogenic factors (AAF) in a mouse model of retinopathy of prematurity (ROP) [Auricchio *et al*., 2002a]. Anti-angiogenic therapies may be useful in the eye, where angiogenesis is involved in ischemic retinopathies and choroidal neovascularization, two of the most common causes of blindness at all ages. The AAF-expressing AAV-1 vectors were injected subretinally into one eye, and the other eye received a non-therapeutic vector expressing a reporter gene. Only the AAF-treated eyes showed decreased neovascular pathology, and inner retinal neovascularization was significantly reduced, indicating success of the approach. This encouraging result now requires confirmation in a long-term study, in which, as was

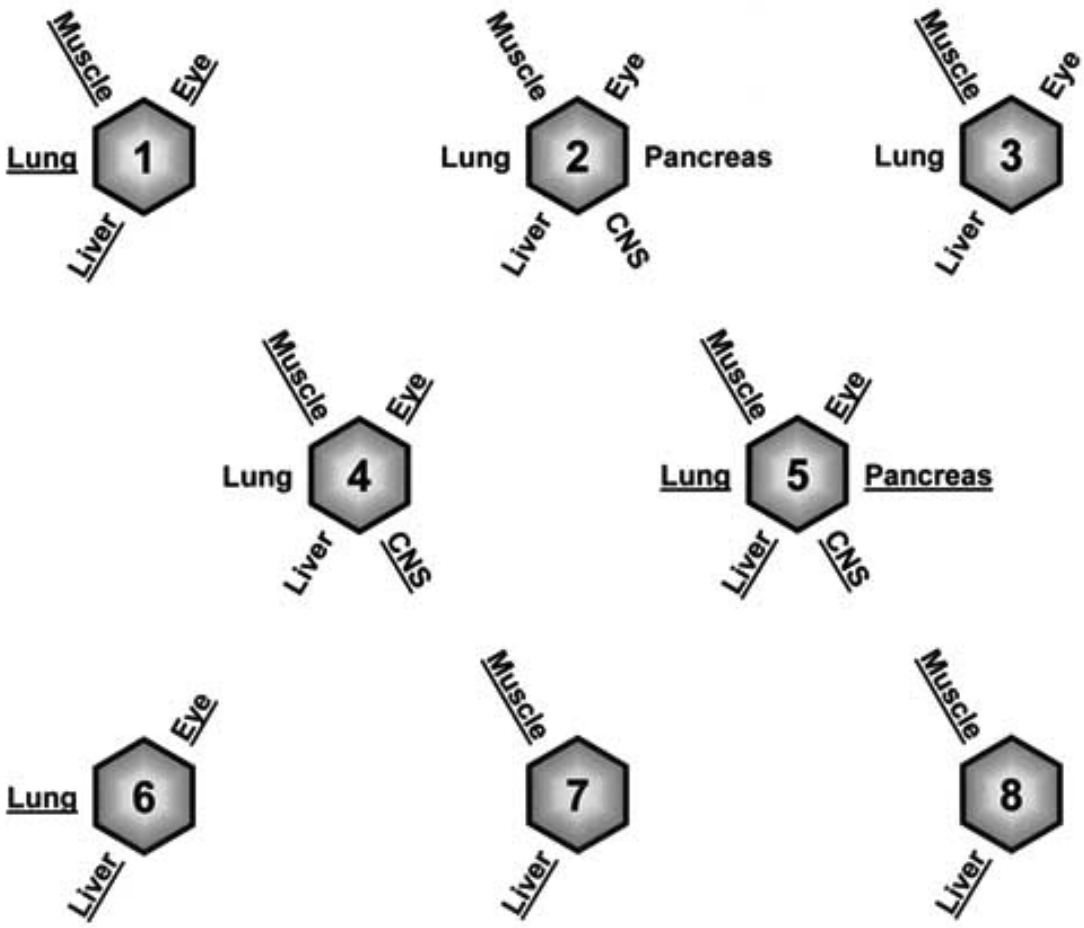

Fig. (3). AAV serotype vector tropism. Shown are the eight known AAV isolates together with the tissues each has been studied in *in vivo* thus far. In underlined tissues, the particular AAV type was found to prove beneficial over AAV-2, usually through stronger or faster transgene expression. For details and references, see text.

suggested by the authors, it may be interesting to test combinatorial therapies using different AAFs.

Superior transduction in the eye by alternative AAV serotypes was independently confirmed by Yang *et al*. [2002]. Basically, their study supports the initial report from Auricchio *et al*. [2001a], showing faster and stronger expression from AAV-5 as compared to AAV-2, and transduction of both photoreceptors and RPE cells by either vector. Importantly, this study also highlights the impact of vector dose on strength and onset of gene expression from AAV serotype vectors. At a low dose, the AAV-2 vector did not express even 5 weeks after injection, whereas AAV-5 already gave strong expression. However, a 100-fold increase in vector dose in a second experiment resulted in expression from both vectors at this timepoint. Moreover, from the increased dose, an up to 400-fold higher number of transgene-expressing cells was noted for the AAV-5 vector, as compared to AAV-2. This was corroborated by an up to 30-fold higher number of vector genome copies per retina. Two other vector serotypes gave either no transduction in the retina (AAV-3), or like AAV-5 showed fast and specific expression in the RPE (AAV-6). The latter is in line with the findings reported from Auricchio *et al*. [2001a] for the closely related AAV type 1.

Importantly, the results from the mouse studies were confirmed in another animal model by Rabinowitz *et al*. [2002]. These authors injected *gfp*-expressing AAV-2 vector genomes, pseudotyped with capsids from AAV-1 to -5, subretinally into rat eyes. Basically consistent with the other reports, fastest and strongest expression was obtained from AAV-5 and -4, followed by AAV-1. The AAV-2 and -3 pseudotype vectors only expressed upon prolongued incubation of the animals, and only to a minor extent. Although the cell types transduced were not evaluated, this

study further supports the notion that in particular AAV-5 might be a most promising candidate for gene transfer to the eye.

In sum, results from four studies in murine and rat retina consistently indicate that vectors based on AAV types 1, 5 and 6 hold promise for targeting photoreceptors and RPE cells, by providing levels and kinetics of gene expression that differ from AAV-2. It must be noted however, that conclusive interpretation of the results was hampered by the fact that in the majority of experiments, particle doses used differed significantly (up to 100-fold, [Yang *et al*., 2002]) between the serotypes, or were obviously too low to expect transduction. Or in some cases, serotype vectors which were compared side-by-side expressed different transgenes, which together with the varying vector doses makes it almost impossible to evaluate the quantitative data reported. Therefore, these parameters should be considered carefully when attempting the next exciting step, to translate the comparison of AAV serotype vectors into larger animals, such as the canine LCA model [Acland *et al*., 2001].

… The Pancreas

Gene transfer to the pancreas, in particular the pancreatic islet beta cells, is a desirable goal to fight insulin-dependent diabetes mellitus (type 1 diabetes). This autoimmune disease is characterized by invasion of immune cells into islets and subsequent inflammation, resulting in eventual destruction of the insulin-producing beta cells. Although relief can be provided by islet allografts, these are frequently rejected and autoimmunity recurs. With gene therapy, genes encoding immunoregulatory, cytoprotective or antiapoptotic proteins could be transferred to islet allografts, to help the islets survive and resist the host immune response, and thus provide a long-term solution. AAV-2 vectors were reported to transduce pancreatic islet cells from various species, e.g. pork or rat [Prasad *et al*., 2000], without altering glucose-induced insulin secretion from these cells, but the efficiency of gene transfer was too low for clinical applications.

In the only study thus far investigating alternative AAV serotypes for islet gene transfer, Flotte *et al*. [2001] suggested that vectors based on AAV-5 might be more efficient than AAV-2. This was shown by infecting cultured murine islet cells with equal doses of *lacZ*-expressing vectors of both serotypes, and only the AAV-5 vector resulting in abundant LacZ-positive cells. This study was rather simple in design and the result must therefore be considered preliminary, in particular since only one (not exactly defined) vector dose was used. Nevertheless, the finding of increased efficacy from AAV-5 as compared to AAV-2 is promising and should be interesting to confirm in islets from different species, using potentially therapeutic genes, and including more serotypes of AAV.

… The CNS

Gene therapy in the CNS is a promising approach to achieve focal or global delivery of therapeutic genes. The specific aim is to treat a variety of CNS disorders such as autosomal dominant spinocerebellar ataxias (SCA), a degenerative disease of the cerebellum. This disease, which results from a loss Purkinje cells and particular neurons, has well defined underlying genetic defects, making it ideal for treatment by gene therapy to prevent or slow the degenerative process. Successful gene transfer with AAV-2 vectors in the CNS is documented in a large number or reports, showing efficient, non-toxic and long-term transgene expression from the vectors, preferentially in neurons [e.g., Kaspar *et al*., 2002]. Two limitations however warrant the investigation of other AAV serotypes in the CNS : first, the inability of AAV-2 to infect clinically interesting cell types other than neurons, such as astrocytes, and second, its often limited spread beyond the immediate injection sites, which opposes its use for treatment of diseases with global pathology. Three encouraging recent reports suggest that these two hurdles can indeed be overcome by the use of vectors based on alternative AAV serotypes.

In the first of these studies, Davidson *et al*. [2000] injected vectors derived from AAV-2, -4 and -5, expressing a *lacZ* reporter gene, into the lateral ventricel or the striatum of mice. They found that the AAV-4 and -5 vectors transduced on average 10-fold more cells after intra-ventricular injection than the AAV-2 vector, with all vectors mainly targeting ependymal cells. Interestingly, the AAV-4 vector also provided fastest onset of gene expression : early (3 weeks) after injection, it transduced 100-fold more cells than AAV-2, and 10-fold more than AAV-5, which later (15 weeks) was equally efficient. The results were strikingly different after intra-striatal injection, where AAV-5 was most efficient at 3 and 15 weeks. Moreover, expression from AAV-4 and -5 remained stable, but was completely lost from AAV-2 after 15 weeks. In addition, the vectors differed in the types of cells and cerebral regions transduced : AAV-2 targeted mainly cells in the striatum, whereas AAV-4 selectively transduced the ependyma. AAV-5 resulted in diffuse transduction in multiple regions, including the striatum and neocortex, where it infected a mix of neurons and astrocytes. This findings is important, since the latter are not accessible with AAV-2. A final intriguing result was that only the AAV-5 vector led to an extensive spread of transduced cells beyond the injection site.

These results were confirmed in a follow-up report [Alisky *et al*., 2000], showing that administration of AAV-5 to murine cerebellar cortex led to extensive distribution of the virus beyond the site of injection. This was not due to retrograde transport, in contrast to an FIV (feline immunodeficiency) vector also analysed, but rather resulted from physical spread of AAV-5. Both vectors transduced neurons in the molecular and Purkinje cell layer, whereas transduction of Golgi neurons was limited, and almost absent for granule neurons. This suggests a selectivity of AAV-5 (and FIV) vectors among potential target neurons, which might be exploitable for diseases caused by degeneration of Purkinje cells, such as the initially mentioned SCA.

Mastakov *et al*. [2002a] presented a further independent report comparing vectors based on AAV-2 and -5 in the rat CNS. Their goal was to investigate whether co-infusion of heparin with *gfp*-expressing vectors of either serotype into the striatum would lead to higher distribution of transduced cells. The experiment's rationale was that soluble heparin should block interaction of AAV-2 with the HSPG receptor and thus facilitate particle diffusion. Indeed, the heparin/AAV-2 group showed dramatic increase in vector spread and number of transgene expressing cells, as compared to a saline control group. In contrast, consistent

with the fact that AAV-5 neither binds HSPG nor heparin, there were no differences between both groups for this vector. In good agreement with the two above described studies, the AAV-5 vector led to extensive spread from the injection site, although distribution appeared less homogeneous and more patchy than for AAV-2. Vector spread occurred mainly around the major blood vessels in Virchow's spaces, which could explain the wider distribution of AAV-5 in the mammalian brain.

Last but not least, Hughes *et al*. [2002] investigated the use of vectors based on AAV serotypes 2, 4 and 5 to transduce neural progenitor cells, which may provide cell replacement for neurodegenerative disease therapy. However, in contrast to adenoviral and FIV vectors also evaluated, none of the three AAV serotypes infected the cells *in vitro*, which for AAV-4 and -5 is related to a lack of receptors on these cells.

In conclusion, three of four studies provide compelling evidence that vectors based on serotypes of AAV, in particular AAV-4 and -5, are promising candidates for further evaluation in the brain, providing unique tropisms, strengths and kinetics of expression, as well as the potential for wider spread (AAV-5). Unfortunately, the same issues affecting the earlier reviewed eye studies also apply here and require further validation of the CNS data, and particular consideration when the approaches are translated into larger animal models. First, it appears highly questionable to compare serotype vectors in doses that either largely differ, such as those used by Davidson *et al*. [2000] with up to 30-fold differences within one experiment. Likewise, doses were in some cases extremely low, such as the multiplicity of infection of two used by Hughes *et al*. [2002]. Second, it is crucial to only compare particles that carry identical vector genomes, regarding the serotype origin of the ITRs. This is because these genetic elements are central to long-term persistence of the vector sequences in the cell, and might also impact short-term expression. It is thus impossible to quantitatively evaluate data obtained weeks or months after injection with vectors carrying ITRs from either AAV-2 or AAV-5, which unfortunately applies to all studies reviewed above. Along the same lines, Mastakov *et al*. [2002a] used two very different methods to determine titers of their AAV-2 or -5 preparations, which is a third unnecessary source of error that is easy to avoid.

Considering these parameters in future work, it will be interesting to evaluate other AAV serotypes in the CNS, in small and large animal models. This is particularly the case for AAV types for which the receptors are still unknown and for which results are thus not predictable, i.e., AAV-1 and -6 to -8. For example, an exciting comparison would be that of AAV-1 and -6, for which fast kinetics and strong gene expression were obtained in the eye (see above), with AAV-4 and -5, which thus far appear most efficient in the CNS.

… The Muscle

Skeletal muscle, like liver and lung (see below), is a potential target for AAV-based gene therapy currently being intensively investigated, due to three advantegeous inherent properties of this tissue : first, a variety of inherited muscle diseases have well defined genetic causes, such as Duchenne muscular dystrophy, making them amenable to gene therapy. Second, muscle might be exploited as a platform to produce secreted therapeutic proteins that are absent or under-produced in patients, such as human blood clotting factors, whose deficiency causes hemophilia. Third, muscle is easily accessible without the need for surgical procedures, as opposed to most other potential target tissues. Thus far, a large number of studies demonstrated successful gene transfer to skeletal muscle with AAV-2 vectors, resulting in stable long-term transgene expression [e.g., Kessler *et al*., 1996; Duan *et al*., 1998a]. However, the efficiency of gene transfer is usually rather poor, suggesting a need for high AAV-2 vector doses to achieve therapeutic goals in humans. First reports now indicate that particular AAV serotypes may be more efficient than AAV-2, and thus provide better candidates for targeting the muscle.

The first to evaluate vectors based on serotypes other than 2 was Xiao *et al*. [1999], who intramuscularly injected mice with AAV-1 or -2 particles, carrying an AAV-2 vector genome encoding a secreted reporter protein. Analysis of blood protein expressed from the vectors showed that the AAV-1 vector outperformed AAV-2, although the difference in expression was less than two-fold.

Superior efficiency of AAV-1 was further confirmed by two groups specifically developing AAV serotypes for muscle-based gene therapy for hemophilia B. First, Chao *et al*. [2000] injected equal doses of AAV vector genomes encoding the canine factor IX gene pseudotyped with capsids from AAV serotypes 1 to 5, into murine skeletal muscle (hind limbs). While all vectors expressed biologically active canine Factor IX (cFIX) protein, the levels of expression varied dramatically 12 weeks after injection : AAV-1 was most efficient with levels of greater than 100 μg/ml cFIX, followed by AAV types 5, 3, 4 and finally 2, which only expressed 90 ng/ml. Thus, the difference between AAV-1 and -2 was greater than 1000-fold, in contrast to the report by Xiao *et al*. [1999], which might be due to the different mice and reporters used.

In a detailed study a year later, the same vectors were compared in a clinically more relevant FIX-mutant murine model of hemophilia B [Chao *et al*., 2001]. The results basically support the previous report, again, the AAV-1 vector expressed 300-fold more biologically active FIX than AAV-2, following injection of equal vector doses into the gastrocnemius. It was moreover found that in contrast to AAV-2, administration of the AAV-1 vector did not elicit a humoral immune response ("FIX inhibitor") to circulating FIX protein. Consequently, it was speculated that the supranormal levels of FIX expressed from AAV-1 had induced tolerance in the animals. This was an interesting hypothesis pointing to yet another potential advantage of AAV serotypes, and thus clearly warrants further investigation.

The muscle hierarchy of AAV serotype vectors initially proposed by Chao *et al*. [2000] was recently confirmed in three different strains of mice, and with two different vector cassettes [Rabinowitz *et al*., 2002]. In all cases, the AAV-1 vector was most efficient at transducing muscle, followed by AAV-5, although exact levels of expression were not provided. Surprising differences were observed for the three other serotypes, where the efficiency of transduction appeared to depend on the transgene. Thus, for one

transgene, the AAV-4 vector was consistently least efficient, while the AAV-2 vector was outperformed by all others with a different gene. This interesting novel observation points to the importance of considering DNA sequences in the vector genome, in addition to the ITRs, when comparing serotype-specific transduction.

The results from Fraites Jr. *et al*. [2002], who independently studied AAV-1 and -2 vectors in a murine model of Pompe disease (deficiency of acid α-glucosidase, GAA), further support the usefulness of AAV-1 for muscle transduction. Injection of GAA-expressing AAV-1 vectors into the tibialis interior muscle of GAA-mutant mice led to complete amelioration of glycogen accumulation, corroborated by increased GAA enzymatic activity. The levels were supposedly high enough in patients with Pompe disease to achieve systemic restoration of GAA activity in non-muscle tissues. In contrast, the AAV-2 vector resulted in only normal levels of GAA below the therapeutic systemic threshhold, albeit it should be noted that a 50-fold lower dose was given for this vector.

Two further reports by Hildinger *et al*. [2001] and Duan *et al*. [2001] focused on another alternative AAV serotype, AAV-5. The groups provided data on the potential usefulness of AAV-5, as well as delivered a possible model for AAV-5 transduction in muscle. In the first study, *lacZ*-expressing AAV-2 or -5 vectors were injected in equal doses into murine muscle (tibialis anterior), which resulted in two-fold higher β-galactosidase enzymatic activity for AAV-5 over AAV-2. This supports the previous notion that AAV-5 is another serotype superior to AAV-2 in muscle, albeit probably not to the extent reported for AAV-1. A second interesting result of this study was that *lacZ* expression from the AAV-5 vector was further increased when the vector genome contained ITRs from AAV-5, instead of from AAV-2 as in the initial experiment. Together with the dependence of transduction on transgene sequences noted by Rabinowitz *et al*. [2002], this indicates that it might be necessary not only to exchange vector capsids, but also to modify the DNA sequences packaged into the viral particle, in order to optimize gene transfer to a particular tissue.

In the second study, Duan *et al*. [2001] analysed potential mechanisms for the more efficient transduction of murine muscle with AAV-5. First, superiority of AAV-5 was confirmed by injecting luciferase-expressing AAV-2 or -5 vectors into the anterior tibialis muscle of normal mice, or mdx mice (a model for Duchenne muscular dystrophy). For both normal and dystrophic muscle, the AAV-5 vector resulted in greater than 200-fold higher luciferase activity at 1 and 4 weeks after injection, than AAV-2. Interestingly, expression from the dystrophic muscle was reduced with both vectors when *gfp* was used as a transgene, which was related to different immunogenicities of *gfp* and luciferase in the setting of Duchenne muscular dystrophy. It yet again indicates that in muscle, and in general, the nature of the transgene influences expression from AAV serotype vectors. Second, to unravel the molecular reasons for AAV-5's increased efficiency, AAV-2 and -5 vectors were compared in undifferentiated myoblasts and differentiated myofibers. The intriguing finding was that despite similar levels of transduction by the two vectors in undifferentiated cells, efficiency from the AAV-5 vector increased greater than 500-fold, and dropped an order of magnitude for AAV-2, with the process of cell differentiation. This was not due to differences in viral binding to the cells, since the AAV-2 vector bound even more efficiently than AAV-5 to undifferentiated and differentiated cells. Moreover, although cell differentiation led to an upregulation of the AAV-5 SA receptor and an eight-fold stronger AAV-5 binding, this alone could not explain the dramatic increase in gene expression. Thus, it was eventually suggested that intracellular processing of the two viruses was different. In fact, significantly higher levels of single-stranded viral genomes were found in differentiated cells following infection with the AAV-5 vector, when compared to AAV-2, indicating a higher stability of input genomes delivered by AAV-5. However, it remains to be determined if this also affects double-stranded genomes, which represent the transcriptionally active form of the viral genome. The important main conclusion was that AAV-2 transduction of muscle is limited by intracellular barriers, which can be overcome by switching to alternative AAV serotypes, in particular AAV-5.

Last but not least, Gao *et al*. [2002] compared the newest AAV types, AAV-7 and -8, to AAV-1 and -2 vectors in murine muscle (tibialis anterior). When two different transgenes were delivered by the vectors, AAV-1 was most efficient in one case, followed by AAV-7. The opposite was observed for the other transgene, which once again emphasizes the central role of the vector sequences. The authors concluded that AAV-7 is equally efficient as AAV-1 in muscle, and thus yet another serotype worth developing further. The AAV-2 vector expressed poorest from both transgenes (although the AAV-2 preparation probably had impacted activity, as it also performed surprisingly poorly in a separate approach to transduce liver), and AAV-8 gave intermediate results.

In conclusion, results from the analyses of AAV serotype vectors in muscle thus far are extremely promising, considering the compelling evidence that at least three of them, AAV-1, -5 and -7, might be more efficient than AAV-2. It is unfortunately hard to evaluate the extent to which they differ, for the same reasons outlined above for other tissues, i.e., major inconsistencies in the design of the experiments. For instance, Fraites Jr. *et al*. [2002] injected significantly (50-fold) more AAV-1 than AAV-2 vector in their study on Pompe disease, and Gao *et al*. [2002] used a different method for purifying the AAV-2 vector, than all other vectors, indicating the preparations were not comparable. Moreover, the unique expression kinetics of AAV serotypes probably impacted some results. For example, Chao *et al*. [2000] gathered data at a timepoint where expression from AAV-1 had reached a plateau, while that from AAV-2 was still increasing, suggesting that the reported 1000-fold difference between the vectors was an overestimation. Finally, the vector genome itself appears to be a most crucial parameter for gene expression in the muscle, next to the viral capsid, and again this might have affected some of the studies. For example, the AAV-5 particles used by Chao *et al*. [2000] contained a genome carrying ITRs of AAV-5, while the four other vector stocks contained recombinant AAV-2-based DNA. Nevertheless, the majority of studies are consistent in their conclusions, and it will thus be most important and interesting to soon

translate the experiments, under better controlled conditions, into large animal models. Fortunately, such models are available for several muscle-related diseases, such as Duchenne muscular dystrophy, and for other diseases for which muscle might be an interesting target, such as hemophilia B.

… The Lung

Development of tools for efficient gene transfer to the human lung epithelium is a highly desirable goal, and a most intensively studied subject in human gene therapy. The main targeted disease is cystic fibrosis (CF), caused by mutations in a chloride ion channel (CF transmembrane conductance regulator, CFTR) and affecting one in 3000 Caucasian births. The major cause of morbidity is gradual lung destruction due to microbial obstruction, mainly of airway epithelia cells, which are thus the main target for CF gene therapy. The ultimate goal is to deliver a functional CFTR gene to these cells, hoping to achieve long-term correction of the major defect and ameliorate or prevent the pathophysiological events of CF.

From pre-clinical and clinical studies, AAV-2 has emerged as a modestly promising vector candidate for this goal. When engineered to deliver the CFTR gene, AAV-2 vectors were shown to confer long-term expression in mice, rabbits and rhesus macaques, and were well tolerated in a phase I/II clinical trial [Wagner *et al*., 2002]. However, two main issues with the vector indicate that AAV-2 is not an optimal tool for human lung gene transfer, and warrant investigation of alternative AAV serotypes. First, the AAV-2 transduction rate in the lung is low [Allen *et al*., 2000], and although it can be enhanced, e.g. by tissue injury or adenovirus co-infection, such treatment is not reasonable in humans. Second, gene expression in the human lung will likely not be stable, due to the constant turnover rate of the epithelium, indicating a need for re-administration of vector to achieve lifelong therapy. Re-administration is however not feasible with AAV-2, since antibodies generated after an initial AAV-2 vector dose will block repeated transduction. Later, we will describe how AAV serotype vectors might help circumvent this particular problem (chapter 2.2.2.), here we will focus on their general evaluation in the animal lung.

Beck *et al*. [1999] were first to analyse an alternative AAV serotype, AAV-3, in the rabbit lung. They found no difference in efficiency compared to an AAV-2 vector, when both were bronchoscopically delivered in equal doses. However, as will be described later (chapter 2.2.2.), they obtained evidence for the usefulness of AAV-3 in vector re-administration strategies.

In two consecutive reports, Halbert *et al*. [2000,2001] demonstrated that AAV-6 might hold significant advantages over AAV-2 for gene therapy in the lung. Consistent in both studies was the finding that following nasal aspiration, AAV-6 exhibited better transduction of murine epithelial cells in large and small airways, than the AAV-2 vector, with up to 80% of the cells in some airways being infected by AAV-6. Results were less clear for alveolar cells, which in the first study were significantly better transduced by AAV-6, whereas AAV-2 was superior in the follow-up report. Interestingly, while this discrepancy may be related to the use of largely different vector doses in the two studies, the efficiency of the AAV-6 vector in this particular cell type mainly appeared to be influenced by the presence or absence of AAV-6 Rep proteins and ITRs during vector production. Another serotype evaluated, AAV-3, was least efficient in epithelial and alveolar cells. Surprisingly, it gave strongest expression amongst all vectors in airway and vasculature smooth muscle cells, although the potential therapeutic benefit remained unclear.

A series of reports from three independent groups established AAV serotype 5 as another highly promising candidate for lung gene transfer, and provided a molecular model for the vector's high efficiency. Zabner *et al*. [2000] were first to demonstrate up to 50-fold better transduction of cultured human airway epithelia with AAV-5 as compared to AAV-2, as well as of murine lung *in vivo*, where the majority of cells transduced by AAV-5 were alveolar cells. Interestingly, the opposite was found for AAV-2, which in contrast to the reports from Halbert *et al*. [2000,2001] transduced more airway epithelia than alveolar cells.

Higher efficiency from AAV-5 in the murine lung was confirmed by Auricchio *et al*. [2002b], who repeated the initial experiments by Halbert *et al*. [2000,2001] and Zabner *et al*. [2000] with some modifications. Nasal instillation of reporter gene-expressing vectors of serotypes 1, 2, and 5 showed that AAV-5 efficiently transduced alveolar, and to a lesser extent, airway epithelia cells. The same was observed for AAV-1, although at overall reduced efficiency. Surprisingly, transduction by the AAV-2 vector was negligible even when applied in high doses, which conflicts with earlier findings [Halbert *et al*., 2000,2001; Zabner *et al*., 2000], but was perhaps related to the different method used for AAV-2 purification. It moreover remains unclear whether the AAV-1 results are directly comparable to previous findings with AAV-6, as was suggested, since vectors based on AAV-1 and -6 differ significantly *in vitro* and *in vivo* [Grimm *et al*., submitted,in press], despite an only six amino acid divergence in capsid proteins.

Additional experiments reported by Auricchio *et al*. [2002b] were based on the rationale that lung, like muscle, might be useful as a factory for expression of secreted therapeutic proteins. This approach is in fact highly reasonable, since lung can be accessed by non-invasive means, while providing a large, highly vascularized surface area and thus a great capacity for solute exchange. Auricchio *et al*. [2002b] demonstrated proof-of-principle with intranasally administered AAV serotype vectors, using erythropoietin or canine factor IX transgenes. Again AAV-5 was most efficient at expressing these genes, followed by AAV-1 and -2. Importantly, the mice tested were hemophilic, and AAV-5 gave plasma FIX levels high enough to achieve partial correction of the bleeding disorder. The levels were however only slightly higher than the therapeutic threshhold in humans (1% of normal FIX), leaving it unclear whether the approach will be clinically relevant. In particular, it remains to be determined whether similar to muscle, FIX expression from lung induces FIX inhibitor. Thus, liver might still provide the optimal tissue for AAV-mediated expression of FIX, since inhibitor is not observed, and greater than 5% of normal FIX levels (curative threshhold) are readily obtained from low doses of AAV serotypes [Grimm *et al*., submitted].

Molecular level analysis of these results began with a report by Duan *et al*. [1998b], demonstrating that the inability of AAV-2 to efficiently infect polarized human airway cells is related to a lack of AAV-2 receptor, HSPG, on the apical surface of the cells. HSPG is however abundant on the cells' basolateral surface, and indeed, AAV-2 vectors transduced 200-fold more efficiently from this side [Duan *et al*., 1998b]. Zabner *et al.* [2000] confirmed and extended these findings by showing that AAV-5 was more efficient than AAV-2 only when applied apically, while both viruses transduced equally well from the basolateral side of human airway cells. Moreover, better infection with AAV-5 from the apical side correlated with increased cellular binding of the virus. Surprisingly however, AAV-4 also bound more efficiently than AAV-2, but like AAV-2 resulted in poor transgene expression. This puzzle was resolved by three subsequent reports from one group. First, Walters *et al*. [2001] and Kaludov *et al*. [2001] showed in a series of biochemical experiments, that the apical side of human airway epithelial cells contains abundant high affinity receptors for AAV-4 and -5, identified as 2,3-linked sialic acid. However, the two serotypes differ in linkage specificity : AAV-4 binds to O-linked, and AAV-5 to N-linked sialic acid, and this provides the basis for the difference between the two viruses in their efficiency to infect airway epithelia cells. As shown by Walters *et al*. [2002], these cells selectively express and secret mucins, proteins rich in O-linked oligosaccharides, through the apical membrane. These mucins inhibit infection by AAV-4, but have no effect on AAV-5. Interestingly and consistent with the previous reports, mucins do not interfere with AAV-4 binding to the cells, but instead hamper internalization of the virus. Thus, when applied from the mucin-lacking basolateral side, AAV-4 infected airway epithelial cells equally efficiently as AAV-2 and -5.

In the context of this model, it is noteworthy that our recent analyses of AAV-1 to 6 vectors in the murine liver gave evidence for a possible means to circumvent the apical block of AAV-4 infection. Following hepatic or systemic administration of AAV-4 particles, the vast majority of vector genomes accumulated in the lung (amongst a total of six tissues studied), whereas liver and/or spleen were main targets of the five other serotypes [Grimm *et al*., submitted]. This was confirmed in a second preliminary experiment, where tail vein infusion of *lacZ*-expressing AAV-1 to -6 vectors resulted in lung expression of β-Galactosidase only for AAV-4 [Grimm, D., Storm, T., Huang, Z., and Kay, M.A.; unpublished]. The efficiency of gene transfer was poor however, and it remains to be determined which cell types were transduced, and by which mechanism/receptor.

In sum, evaluation of AAV serotypes in the lung thus far yielded results which are as promising as those for muscle, but in some cases also equally difficult to interpret, for the same reasons discussed before. For instance, Halbert *et al*. [2000,2001] used doses for their AAV-2 and -6 vectors that varied over three orders of magnitude in the two studies, without establishing a clear correlation between particle dose and expression levels. This leaves it unclear to which extent their different data are comparable. Nevertheless, the important conclusion from this and the other studies is that three of the known eight AAV isolates, AAV-1, -5 and -6, appear more efficient than AAV-2 at infecting target cells within the murine lung, or cells isolated from the human lung. It is also important to recap that a great amount of current basic knowledge about AAV serotypes was gained in the lung or isolated airway cells, including the discovery of the AAV-4 and -5 receptors, and steps involved in infection with the viruses. Like for the muscle, it will now be crucial to translate the pre-clinical analyses from small into large animals. There it should be interesting to compare AAV serotypes to adenoviral vectors, another promising vector in this tissue. Noteworthy in this respect is that Rooney *et al*. [2002] recently reported that adenoviral gene transfer to isolated airways was significantly impaired by bronchoalveolar lavage liquid (BAL) from healthy subjects. In contrast, no effect was found on an AAV-5 vector, indicating yet another potential benefit from AAV-5 for lung gene transfer in humans.

… The Liver

The final organ to be reviewed is the liver, whose appeal as a target for gene therapy with AAV serotypes is due to the fact that a variety of human disorders originate from genetic defects of hepatocytes. Amongst these, the hemophilias type A and B, resulting from deficiency of or mutations in the genes for blood clotting factor VIII or IX, respectively, are most intensively targeted. As mentioned above, these genes can also be delivered to non-liver tissues such as muscle or lung, since blood clotting factors are secreted from the producing cells into the blood. However, besides the before mentioned problem of FIX inhibitor formation, there is additional concern that posttranslational modifications of clotting factors might differ in tissues other than the liver, which is the physiological site of production. Importantly, although direct liver accessibility is limited without surgical intervention, gene transfer to this organ is achievable by systemic administration of viral vectors. The efficiency of this route is however usually reduced as compared to vector administration into the portal circulation.

In vivo liver gene transfer with AAV-2 vectors was first established by Snyder *et al*. [1997] and subsequently demonstrated in a large number of pre-clinical studies, and is a main matter of interest in our group. Transduction of hepatocytes with AAV-2 is feasible in principle, but our work and studies from others showed that even when administered in high doses, the vectors do not express in more than 5% of all liver cells, although the viral genomes are found in almost all cells [Miao *et al.*, 2000]. This indicates that rather than vector uptake, intracellular steps are limiting AAV-2 vector-mediated transduction of the liver. Expression in only 5% of hepatocytes might still be sufficient to reach therapeutic levels of blood clotting factor. Yet there is hope that the use of alternative serotypes of AAV will increase the efficiency of gene delivery, and eventually allow for the use of lower vector doses to achieve the same goal.

The first report in this direction was by Xiao *et al*. [1999], who injected mice via the portal vein with equal doses of AAV-1 or -2 vectors expressing two secreted reporter proteins. As compared to AAV-1, the AAV-2 vector produced 10- to 50-fold more protein, thus indicating that AAV-1 might not provide beneficial. This result is however difficult to reconcile with three later studies (see below),

which consistently provided evidence that AAV-1 is at least equally efficient at expressing from the liver.

In fact, Rabinowitz *et al*. [2002] reported that following intraportal or intravenous injection of mice with AAV-1 to -5 vectors, also encoding a secreted reporter protein, AAV-1 gave highest levels of expression, followed by AAV types 5, 3, 2 and 4 (note that the hierarchy of the latter three differed in muscle, see previous chapter). However, the exact protein levels were unfortunately not provided, leaving unclear to what extent the serotypes differed from each other. Moreover, it was questionable whether the protein measured actually originated in the livers of the animals, since the CMV promoter driving the transgene is usually rapidly silenced in hepatocytes [e.g., Loser *et al*., 1998].

Better efficiency of liver gene transfer with AAV serotype 5 was confirmed by Mingozzi *et al*. [2002], who injected human *fIX* gene-expressing AAV-2 or -5 vectors into the splenic capsule (for liver-directed gene transfer) or the tail vein of mice. Measurement of plasma hFIX protein showed that the AAV-2 vector reached a plateau of expression by 4 weeks, whereas AAV-5 continued to express up to three-fold higher hFIX levels (1 μg/ml). Consistent with previous data on AAV-2, hepatic administration resulted in up to five-fold higher hFIX levels for both vectors. An important finding was that stronger expression from AAV-5 correlated with an increase in the proportion of hepatocytes transduced, from only 5% for AAV-2 to up to 15% of total liver cells for AAV-5. This indicated that AAV-5 can overcome the intracellular limitations that hamper AAV-2 transduction. This property of AAV-5 might be particularly useful for treating liver diseases such as familial hypercholesterolemia, which require transduction of a maximum proportion of hepatocytes, rather than strong expression per cell. A second interesting observation was that AAV-2 transduction led to clustering of hFIX-expressing hepatocytes near blood vessels, whereas AAV-5-transduced hepatocytes were found spread throughout the liver parenchyma. This was reminiscent of the findings in the CNS, where AAV-5 gene transfer also resulted in a wider spread of transduction as compared to AAV-2 (see above). It is however currently unknown whether these phenomena are related and thus indicate a general quality of AAV-5. In another interesting experiment, it was analysed whether co-administering AAV-2 and -5 vectors has an additive effect in the liver. Co-injection of the two vectors expressing two different transgenes into the splenic capsule, and quantification of cells expressing both transgene products demonstrated that the vectors transduced widely overlapping populations of hepatocytes (greater than 90% of cells expressed both proteins). Although AAV-5 transduced additional cells, their proportion was too small to support the idea of an additive effect. It might however be worth repeating this experiment, since the impact of the different transgenes and promoters used in the two vectors on the result was unclear. Finally, it was demonstrated that intravenous administration of a high dose of FIX-expressing AAV-5 vectors yielded levels of FIX protein in a range of 20% of normal, whereas AAV-2-injected animals expressed 25-fold less. This suggested that the high efficiency of AAV-5 alleviates the need for direct hepatic administration. However, similar to the study by Rabinowitz *et al*. [2002], it was questionable whether the FIX protein originated in the liver, since the promoter (EF1α) used in the vector is ubiquitously active, indicating the *fIX* gene could have been expressed in additional tissues.

A comparison of AAV-7 and -8 to other serotypes in the murine liver was presented by Gao *et al*. [2002]. In this study, AAV-2 vectors, encoding various reporter genes controlled by two different liver-specific promoters, were pseudotyped with capsids of serotypes 1, 2, 5, 7 and 8, and injected into mice via their portal vein. In all cases, the AAV-8 vector gave strongest expression, at levels up to 110-fold higher than those from AAV-2. In contrast, the AAV-1 vector performed almost as poorly as AAV-2, and expression from AAV-5 and -7 was intermediate. Highest protein levels from AAV-8 were corroborated by the findings that this vector also transduced the highest proportion of hepatocytes, and yielded highest amounts of vector DNA in the liver. However, the data were obtained with different vector genomes and are thus not necessarily comparable. Their interpretation is moreover made difficult by the fact that the corresponding data reported for the AAV-2 vector were largely inconsistent with previous publications, as well as with each other. For instance, there was a lack of correlation between vector genome copy numbers per cell and transgene expression levels. It must thus be assumed that the AAV-2 preparation used was impaired in infectivity or functionality, perhaps related to the purification method used (heparin chromatography, all other serotypes were purified by CsCl sedimentation). This suggests that the finding of greater efficiency from AAV-8 as compared to AAV-2 should be independently confirmed.

Finally, a detailed side-by-side evaluation of AAV vectors of types 1 to 6 in the murine liver was recently provided by our group [Grimm *et al*., submitted]. We cross-packaged an AAV-2 vector, containing our most robust human *fIX* gene expression cassette including a strong liver-specific promoter, into the six AAV capsids. The resulting particles were then injected either intraportally or intravenously into immunocompetent mice, in doses ranging over four orders of magnitude. Analyses of plasma hFIX protein levels for up to 12 weeks after injection, and of vector genome tissue distribution showed the following : first, all six AAV vectors were able to direct high levels of hFIX protein from the liver, in a potentially therapeutic range, i.e., greater than 1% of normal hFIX in humans (100% = 5 μg/ml). AAV types 1, 2 and 6 gave strongest expression with peak hFIX levels between 104 and 135 μg/ml, which in hemophilic humans would correspond to 2000% up to 2700% of normal and thus be in great excess of curative levels (5% of normal). The three other vectors expressed lower levels, however still reached a peak of 36% of normal for the least efficient AAV serotype, AAV-4. This hierarchy of AAV types for protein expression was corroborated by analyses of vector genome copy numbers in hepatocytes. This assay also revealed that in most cases, hepatic or systemic vector administration directed the majority of particles to the liver. Interestingly, for AAV-2 and -5, at least equal numbers of vector genomes were present in the spleen, and found in even more tissues for the AAV-3 or -4 vectors. Second, consistent with each other, DNA and hFIX protein analyses showed that direct hepatic particle delivery resulted in up to 84-fold stronger expression of hFIX protein from the liver, as compared to systemic

delivery. Intriguing exceptions were provided by infusion of high particle doses of AAV types 1, 4 and 6, where expression levels from the hepatic and systemic route were comparable. This is interesting from a clinical standpoint, since it indicates that these serotypes allow for highly efficient expression of hFIX protein from the liver following intravenous injection, which is less complex than direct hepatic administration. Finally, liver expression from all vectors was time- and dose-dependent. However, AAV-6 gave fastest and strongest transduction at all particle doses, indicating a most efficient virus uptake or intracellular processing. This is interesting from a virological standpoint, since AAV-6 differs in only six amino acids from AAV-1, which did not show these kinetics. The finding is also clinically relevant, since faster transduction from the vector shortens the lag phase between treatment and expression of therapeutic hFIX levels.

In conclusion, the sum of available results strongly indicate that amongst all potential targets for AAV serotype-mediated gene therapy investigated thus far, the liver might represent the most promising one. This is mainly because it is the only tissue for which highly efficient gene transfer with vectors based on all eight known AAV serotypes was achieved. This trait is crucial for a patient exhibiting a serological profile that precludes transduction by a subset of available AAV serotypes. This is most likely for AAV-2, against which many humans carry neutralizing antibodies, but immunity to other serotypes might also exist and thus hamper gene transfer with selected vectors (see next chapter). Furthermore, data from several studies in mice suggest that in a dose- and time-dependent manner, at least six of the AAV types (1 to 6) can direct supra-physiological hFIX levels in great excess of the therapeutic threshold. This raises reasonable and exciting hopes that this approach can be successfully translated into humans. Prior to this, the immediate step should be to confirm efficient transduction by non-type-2 vectors in larger animal models of the disease, such as the available strain of hemophilic dogs. This should help to delineate the exact parameters, such as vector dose and route of administration, that will result in maximum efficacy in human patients.

2.2. Immunological Aspects of AAV Serotype Vectors

A second hope associated with AAV serotypes, in addition to the feasibility to transduce cells not accessible by AAV-2, is that they are serologically distinct enough to evade host immune responses directed against AAV-2. This would allow the use of AAV serotype vectors in patients carrying neutralizing antibodies to AAV-2, either from a naturally acquired infection with the virus, or from previous treatment with AAV-2 vectors. More generally, AAV serotypes might be distinct enough from each other, to allow repeated patient treatment with two different members of the AAV family.

There is increasing pre-clinical evidence, albeit from a limited number of studies thus far, that AAV serotype vectors might indeed meet these goals. In the following, we will describe serological assays utilized to address this issue and review the data available for both settings, i.e., naturally acquired (2.2.1.) or vector treatment-caused (2.2.2.) immunity to AAV serotypes. We will also address the potential therapeutic strategies emerging from these findings, and eventually summarize the reviewed assays and data in Fig. **4**.

2.2.1. Setting A : Naturally acquired (pre-existing) Immunity to AAV serotypes

Initial data from the early years of AAV-2 research indicated that infection with this serotype (and also AAV-3) is highly common in humans. It usually occurs early in childhood, with the prevalence of antibodies reaching a maximum at 5 to 10 years [Blacklow *et al*., 1968]. Subsequent studies on seroprevalence in adults reported variations between 35 and 80% positive, most likely due to the different serologic assays used, such as complement fixation or Elisa. The variations probably further related to other parameters like the subjects' health status and gender [Erles *et al*., 1999]. The initial data also largely over-estimated the proportion of individuals who are actually resistant to AAV-2 infection, i.e. carry neutralizing antibodies to the virus, since methods used were sensitive, but little specific for a neutralizing immunological response. However, in the context of using AAV-2 and other AAV serotypes as human vectors, identifying the sub-population of individuals who are resistant to these viruses is obviously crucial.

The appropriate serologic *in vitro* assay to detect these subjects, and to measure their titer of neutralizing antibodies (nAb) to AAV serotypes, is explained in Fig. **4A**. Xiao *et al*. [1999] were first to use this assay to screen a total of 77 normal human subjects for naturally prevalent nAb to an AAV serotype other than 2, AAV-1. Interestingly, 71% of the subjects scored negative for AAV-1 and -2, and only 20% had nAb to AAV-1. Similarly, 27% had nAb to AAV-2, which exemplifies that the initially assumed high frequency of seropositivity over-estimated the actual number of immune individuals. Of the positives, 6 had nAb that were mono-specific for AAV-2 (i.e., did not bind AAV-1), while only one had a mild titer of mono-specific anti-AAV-1 nAb. This was counted as proof that AAV-1, like AAV-4, does not infect humans to any appreciable degree, but is endemic in monkeys. The latter was supported by a simultaneous study in 33 rhesus monkeys, 20 of which had nAb to AAV-1, and one-third carried mono-specific nAb to AAV-1. In conclusion, the study showed that AAV-2 infections are present in the human population, and indicated a marginal pre-existing immunity to AAV-1, making the latter serotype particularly attractive as vector for human gene therapy.

Halbert *et al*. [2000] tested seven human volunteers for nAB to AAV-2 and also serotypes 3 and 6. Surprisingly, five subjects had antibodies that inhibited all three viruses, with titers of nAb to AAV-2 being up to 8-fold higher than those of nAb to AAV-3, and 16-fold higher than those to AAV-6. It was unclear why the proportion of AAV-2 positive individuals was higher than reported by Xiao *et al*. [1999], but perhaps the number of seven subjects was too small to be considered significant. Nevertheless, the authors concluded that AAV-6 might be less immunogenic in humans, and/or that the immune response detected resulted from cross-reacting antibodies to the other two AAV types. It was also hypothesized that the finding of 71% subjects mildly positive for AAV-6, as compared to 20% for the closely related AAV-1 [Xiao *et al*., 1999], might be due to the six amino acid difference in the capsid proteins of the two viruses.

However, later studies from Rabinowitz *et al*. [2002] and our group [Grimm *et al*., submitted] rather suggest that AAV-1 and -6 represent the same serotype.

A cohort of 85 human volunteers was screened for nAb to AAV-1, -2 and -5 by Hildinger *et al*. [2001]. Interestingly, despite the presence of nAb to AAV-1 or -2 in 19 or 25% of the subjects, confirming previous findings (see above), not a single individual had nAb to AAV-5. This was in line with an earlier report by Tobiasch *et al*. [1998], who could not detect AAV-5 DNA in 30 different biopsies from human tissue. In contrast, AAV-2 DNA was highly prevalent, which together supports the notion that AAV-5 infections in humans are rare. On the other hand, earlier data indicated seroprevalence for this serotype of up to 60% [Georg-Fries *et al*., 1984], although as mentioned, they might over-estimate the number of immune subjects. However, in a small study, Erles *et al*. [1999] detected nAb to AAV-5 in 50% of human sera tested, and one of the sera was even considered mono-specific for AAV-5, since it did not cross-react with AAV-2 and -3. Last but not least, AAV-5 was detected in a human clinical specimen, showing that infections with the virus occur, although at unknown frequencies [Georg-Fries *et al*., 1984]. Thus, reports on pre-existing immunity to AAV-5 so far are highly controversial, leaving open whether or not administration of AAV-5 vectors in humans will be affected by primary immune status.

Finally, Gao *et al*. [2002] screened sera from 52 human subjects and found low titers of nAb to AAV-7 and -8 in three individuals only, whereas 20% showed strong immune response to AAV-2, which is roughly consistent with previous studies. In contrast, as expected, 30 to 45% individuals in rhesus monkey colonies, from which AAV-7 and -8 were isolated, had high titers of nAb to the two viruses. Thus, similar to AAV-1, the simian AAV serotypes 7 and 8 might hold particular promise for use in human patients, due to the virtual lack of pre-existing immunity. It is tempting to speculate that this is also true for the fourth serotype of non-human origin, AAV-4, for which data on naturally occurring infections in humans are not available yet.

2.2.2. Setting B : Vector treatment-caused Immunity to AAV serotypes

To understand the importance of this setting, it is helpful to recap that there is compelling evidence suggesting that vector genomes delivered by AAV-2 can persist for months to years in the host, as either integrated or extrachromosomal DNAs, and provide long-term expression of encoded proteins [Grimm and Kleinschmidt, 1999]. However, the ultimate goal of life-long therapeutic expression is probably difficult to meet, in particular in one of the most interesting tissues, the lung. This is due to the low but constant turnover rate of the human epithelium, with a cellular life-span of normally 120 days, and even less in CF patients [Beck *et al*., 1999]. Thus, multiple vector administrations are likely going to be required to maintain gene expression in a high enough number of cells to guarantee long-term correction of the electrophysiological defect in CF patients. However, repeated delivery of AAV-2 vectors is hampered by neutralizing antibodies emerging after initial vector administration, raising a need to block or circumvent this immune reponse. A more elegant solution for re-administration of a given AAV vector genome is provided by cross-packaging the transgene into capsids from another serotype, showing no or only mild cross-reactivity with AAV-2, or in general, by using capsids from two immunologically divergent AAV serotypes.

Fig. **4B** shows the two assays which can be used to either predict the success of a re-administration strategy *in vitro*, by evaluating blood from animals that received an initial AAV vector dose and quantifying titers of neutralizing antibodies cross-reacting amongst the AAV serotypes (type I assay). Alternatively, the physiologically more relevant type II assay directly assesses feasibility to re-administer AAV serotypes to previously infected animals *in vivo*. In the following, we will review how these two assays were used, and which results were obtained thus far, in …

… The Eye

An intriguing hypothesis concerning immune reponse to AAV serotypes in the eye was raised by Auricchio *et al*. [2001a]. They found that although subretinal injection of AAV-1, -2 or -5 vectors induced systemic humoral immune response in mice, characterized by developing Th2 isotype antibodies, this response was not neutralizing in nature. It was concluded that the subretinal space can evoke an immune-deviant response, thus representing a unique environment with respect to antigen-specific immunity and being a highly interesting target for vector re-administration strategies. A particularly promising candidate should be AAV-6, which is not only efficient in the eye (see above), but perhaps overall less immunogenic [Halbert *et al*., 2000].

… The CNS

As for the eye, immune response to non-type-2 AAV serotypes in the CNS was addressed in only one study thus far. Mastakov *et al*. [2002b] showed that in the rat striatum, it was feasible to re-administer a recombinant AAV genome through subsequent pseudotyping with capsids from AAV-2 and AAV-5, whereas second administration of AAV-2 particles was blocked. Surprisingly, it was only hampered within the first three months, consistent with low levels of anti-AAV-2 nAb, but feasible later. It moreover became possible when the vector DNA was altered in the promoter sequences. This led to the provocative hypothesis that the vector DNA sequence influences epitopes of the AAV-2 capsid, allowing for two ways to circumvent the modest immune response in the brain after AAV vector delivery : exchanging the viral capsid, or modifying the vector genome. The latter idea however requires further validation, in particular regarding the fact that in this study and in our hands, AAV-2 vector preparations carrying different vector genomes could be purified and titrated equally efficiently by methods which largely rely on the presence of conformational epitopes on the AAV-2 capsid [Grimm *et al*., 1998,1999].

… The Muscle

In contrast to retina and CNS, more data on vector-induced immunity are available for muscle, which was studied in three different reports thus far. First, Xiao *et al*. [1999] investigated the feasibility to re-administer AAV-1 or -2 to mice following initial intramuscular injection of either

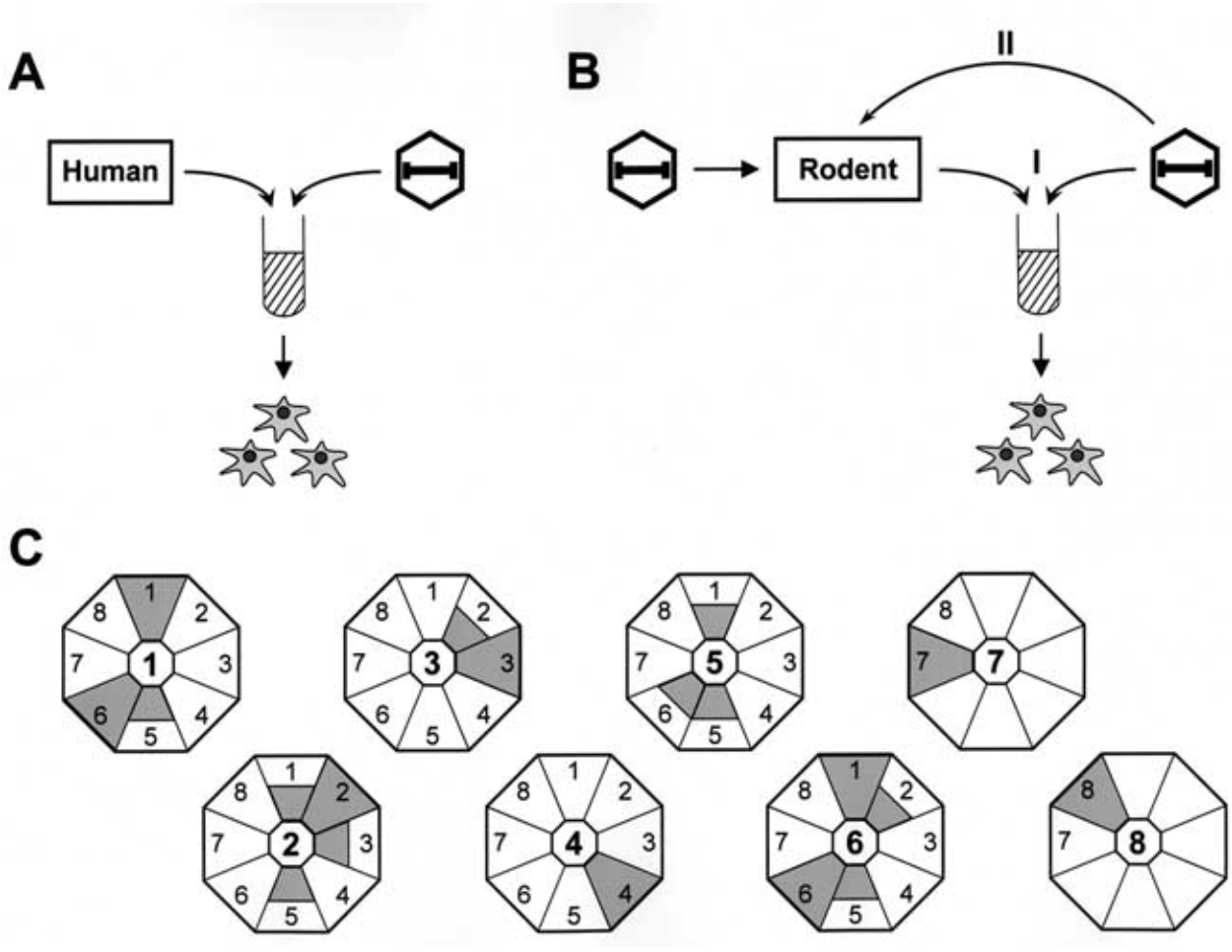

Fig. (4). Qualitative and quantitative evaluation of immune responses to AAV serotypes. (**A**) Analysis of human sera. Sera are assessed for the presence of neutralizing antibodies (nAb) to a particular AAV serotype, by measuring their ability to inhibit transduction of cultured cells with a vector based on the AAV serotype, typically carrying a reporter gene. The vector is pre-incubated with heat-inactivated sera, then added to and left on the cells for a few days, after which the number of reporter gene-expressing cells is determined. The titer of nAb is calculated as the highest sera dilution inhibiting transduction by 50% of that seen in a control reaction (vector plus sera from naive animals). (**B**) Analyses in animals. In a **type I** *in vitro* assay, animals are injected with an AAV serotype vector, and a few weeks later their sera is analysed for nAb to the same or another serotype as outlined in (**A**). In a **type II** *in vivo* assay, the animals are instead re-injected with the same or another serotype, expressing a different transgene than the first vector. After a second incubation, transgene expression in the animals is quantified and compared to control animals (initially injected with saline instead of a vector). (**C**) Cross-reactivity between AAV serotypes. Shown is a summary of data from type I and II assays (for details and references, see text). The numbers in the middle of each figure indicate the vector serotype which the animals were initially challenged with, while surrounding numbers show the serotypes subsequently tested for cross-reactivity, *in vitro* or *in vivo*. A greater degree of grey-shading depicts a larger extent of inhibition of the second vector. Note that the reactivity of AAV type 6 with itself is currently a matter of great controversy (see text).

virus. As expected, re-administration of the same serotype was blocked, while interestingly, an intial AAV-2 dose led to an only modest diminution in second transduction by AAV-1. Prior administration of AAV-1 even resulted in mono-specific nAB that did not interfere with second transduction by AAV-2. This suggested that AAV-1 is a preferred initial vector for muscle-directed gene therapy, since it is more efficient (see above), does not preclude re-treatment with AAV-2, and antibodies to AAV-1 are less prevalent in the human population (see above).

Similar conclusions were reached for vectors based on three other serotypes, AAV-5, -7 and -8. Hildinger *et al*. [2001] showed that intramuscular injection of AAV-2 or -5 vectors dimished the effectiveness of re-administration of the same vector 10- to 20-fold, but did not impact the other vector. This indicated that like AAV-1, AAV-5 vectors not only provide higher efficiency in muscle, but offer potential for repeated treatment. Likewise, Gao *et al*. [2002] found that amongst mice that received intramuscular injection of vectors based on serotypes 1, 2, 7 or 8, only the AAV-8 group became resistant to a second transduction from an

intraportally delivered AAV-8 vector. Moreover, analyses of sera from mice that were intramuscularly injected with vectors based on each of the eight AAV serotypes showed no cross-reactivity of AAV-7 or -8, i.e., the two vectors were only inhibited by samples from mice injected with the same serotype. Together with the low pre-existing immunity in humans (see above), this identified AAV-7 and -8 as two further promising serotypes for repeated use in muscle, in particular AAV-7 which is equally efficient as AAV-1 in this organ.

In conclusion, four of the eight AAV serotypes, 1, 5, 7 and 8, are probably good candidates for use in vector re-administration strategies in muscle. It will now be important to delineate the optimal parameters for this purpose, such as choice of first and second vector, and time frame for injections. This should first be attempted in small animals, but then will be even more interesting in large animal models of muscular diseases, e.g. Duchenne muscular dystrophy, which are clinically more relevant.

… The Lung

Since strategies for vector re-administration are most needed for the lung, it is not surprising that up to now the majority of respective data were obtained in this tissue, although most of the work was done with AAV-2. An initial report on alternative serotypes by Beck *et al*. [1999] was promising, showing that re-administering an AAV-2 vector in the rabbit lung was feasible, and that even a third appliction of a vector based on AAV-2 or -3 was tolerated and led to expression. However, this study was controversial, since it was difficult to explain why a significant rise in neutralizing antibodies to the first AAV-2 vector did not interfere with two subsequent vector deliveries. Moreover, the results were in sharp contrast to earlier reports demonstrating failure to re-transduce rabbit or mouse lung with AAV-2 [Halbert *et al*., 1997,1998].

The latter was further supported by Halbert *et al*. [2000], who presented a series of extensive experiments including type I and II assays (Fig. **4B**). Briefly, it was shown *in vitro* that mice intranasally infused with AAV-2 or -3 elicited a strong immune response against the same serotype, and a weaker response to the other, while there was no reaction with AAV-6. The same was observed vice versa, i.e., sera from AAV-6-infused mice did not cross-react with AAV-2 or -3. Interestingly, they also had only a minor effect on the same serotype, indicating that AAV-6 is immunologically distinct. *In vivo*, AAV-2 infusion did not hamper transduction from a second AAV-6 vector, and the same was true for AAV-2 administration following initial AAV-6 exposure. Moreover, in contrast to AAV-2, the AAV-6 vector-induced neutralizing response was weak, allowing for a second AAV-6 delivery, albeit resulting in reduced transduction. Together with the presumably low pre-existing anti-AAV-6 immunity in the population (see above), this led the authors to hypothesize that AAV-6 might be the most versatile AAV serotype for use in lung re-administration protocols. This idea and the other results however require confirmation, since recent *in vitro* evidence suggests that immunologically, AAV-6 is identical to AAV-1 rather than being unique [Grimm *et al*., submitted; Rabinowitz *et al*., 2002].

Finally, Auricchio *et al*. [2002b] found re-administration of AAV-5 vectors feasible in the murine lung, despite the induction of serum anti-AAV-5 nAb by the first virus dose. This finding was encouraging and reminiscent of the report by Beck *et al*. [1999], but also similarly difficult to explain in view of other reports showing failure to re-administer the same serotype (see above). Perhaps this study profited from the use of AAV serotype 5, which from our recent experience appears to be not very immunogenic [Grimm *et al*., submitted]. This would be consistent with the low anti-AAV-5 nAb titers observed by Gao *et al*. [2002], despite the use of a rather high vector dose ($3x10^{11}$ particles).

In sum, it is unfortunately impossible to conclude on the prospect to re-transduce the lung with different AAV serotypes, since the bulk of data available are too controversial and difficult to explain. At least under some circumstances, re-administration even of the same serotype might be feasible, and AAV-6 might hold particular promise for this purpose, but these are hypotheses requiring further validation.

… The Liver

Although, as mentioned earlier, the liver represents one of the most promising targets for AAV serotype-mediated gene therapy, data on immune responses to the vectors are very limited, and so far only provided by two reports. This is surprising in view of the potential large benefit of vector re-administration in this tissue, since at least with AAV-2, only a small subpopulation of hepatocytes is transducible at a time [Miao *et al*., 2000], requiring either more efficient vectors (see above) and/or options to re-infect the liver.

Xiao *et al*. [1999] first addressed this issue and found that re-administration of AAV-1 or -2 to the murine liver was inefficient when using the same vector for the first and second transduction, but feasible when alternating the serotypes. Interestingly, AAV-2 elicited high levels of nAb suppressing second the gene transfer by AAV-1 up to 20-fold, whereas the effect was much weaker in muscle (see above). This showed that the extent of cross-reactivity depends on the tissue and/or route of particle administration.

This is in fact strongly supported by our results from the analysis of AAV-1 to -6 vectors in the murine liver [Grimm *et al*., submitted]. We could show that intraportal vector administration results in lower nAb titers to AAV serotype capsids, than intravenous injection. Moreover, independent of the route of administration, we found that *in vitro* cross-reactivity with other serotypes was low for AAV-5, and even absent with AAV-4. AAV-1 and -6 were equally efficiently neutralized by nAb to the same or the other virus, supporting the notion that these two AAV types are immunologically identical. Taken together, our results predict that AAV serotypes 4 and 5 might be the most promising candidates for re-administration in the liver, and that generally, direct particle delivery to the hepatic circulation will help to reduce the extent of immune response.

PART III : CONCLUDING REMARKS

Without doubt, some of the most stirring progress currently being made in the area of human gene therapy relates to work on serotypes of adeno-associated virus. The

recent re-discovery of these viruses, and the additional cloning of new members of the virus family, has initiated an entire new era of research in the field of AAV vectorology. As young as this era still is, with the first report of a vector based on an alternative AAV serotype just 6 years ago, the progress already made is remarkable. On one hand, we have begun to learn about the viral biology, to elucidate the structure and function of the different viral genomes, to unravel the molecular interactions taking place in virus binding to targets, and to identify the events occurring after viral crossing of the cellular membrane. On the other hand, efforts are on their way to translate our deeper understanding of virus evolution into a vector revolution. The first steps have already been completed : production methods for vectors based on AAV serotypes have been developed, assays to evaluate the particles *in vitro* and *in vivo* have been established, initial data on their immunological properties have been gathered, and advanced strategies for vector use in various tissues have been worked out.

Importantly, the results from these initial studies thus far are highly encouraging, as they demonstrate that AAV serotype vectors allow for better transduction of old and novel targets, tissues or cell types, which are difficult or impossible to access with conventional AAV-2 vectors. Beyond that, data suggest that particular combinations of AAV serotypes will be useful for the development of innovative gene transfer strategies, involving vector co- and re-administration, which were previously unthinkable with AAV-2 as the only available AAV vector species. Last but not least, findings indicate that the use of AAV serotypes might render a larger proportion of human subjects candidates for AAV-based gene therapy, due to the viruses' potential to evade pre-existing neutralizing immunity against the most prevalent AAV family member, AAV-2.

It must however not been overlooked, that a majority of the initial efforts to establish AAV serotypes as improved vectors in small animal models were not always consistent in their conclusions. The reasons for this are many, and include irregularities in vector production, such as the use of different methods for particle generation and purification between the serotypes. They frequently also relate to discrepancies in the serotype origin of the ITRs in vector genomes compared, large differences in particle numbers used, and the often limited consideration of serotype-specific dose-response and kinetics of transgene expression.

Although the time is right to start analysing the vectors in large animals, it should be obvious to everyone in the field that these inconsistencies must first be reconciled, to eventually justify the step from small to large models of human diseases. Ideal and most important would be to try and find an agreement on unanimous conditions for vector design, production and quality control, but considering the large number of groups involved and approaches described already, this will not be readily achieved. Nevertheless, one should be aware that only when properly developed and characterized, AAV serotype vectors might in the end succeed and revolutionize the areas of AAV research and human gene therapy; the potential is certainly there. In fact, we are convinced that vectors based on AAV serotypes hold many further surprises and benefits awaiting their discovery and exploitation, and thus are confident that this is the dawn of even more exciting times to come.

ACKNOWLEDGEMENTS

The authors wish to thank Theresa A. Storm for critical reading of the manuscript. M.A.K. was supported by grant HL66948 from the National Institutes of Health (NIH).

ABBREVIATIONS

AAV	=	Adeno-associated virus
CNS	=	Central nervous system
CsCl	=	Cesium chloride
fIX/FIX	=	Factor IX gene/protein
HSPG	=	Heparan sulfate proteoglycans
ITR	=	Inverted terminal repeat
nAb	=	Neutralizing antibodies
SA	=	Sialic acid

REFERENCES

Acland, G.M., Aguirre, G.D., Ray, J., Zhang, Q., Aleman, T.S., Cideciyan, A.V., Pearce-Kelling, S.E., Anand, V., Zeng, Y., Maguire, A.M., Jacobson, S.G., Hauswirth, W.W. and Bennett, J. (2001) Gene therapy restores vision in a canine model of childhood blindness. *Nat. Genet*., **28**: 92-95.

Alisky, J.M., Hughes, S.M., Sauter, S.L., Jolly, D., Dubensky, T.W. Jr, Staber, P.D., Chiorini, J.A. and Davidson, B.L. (2000) Transduction of murine cerebellar neurons with recombinant FIV and AAV5 vectors. *Neuroreport*, **11**: 2669-2673.

Allen, J.M., Halbert, C.L. and Miller, A.D. (2000) Improved adeno-associated virus vector production with transfection of a single helper adenovirus gene, E4orf6. *Mol. Ther*., **1**: 88-95.

Auricchio, A., Behling, K.C., Maguire, A.M., O'Connor, E.M., Bennett, J., Wilson, J.M. and Tolentino, M.J. (2002a) Inhibition of retinal neovascularization by intraocular viral-mediated delivery of anti-angiogenic agents. *Mol. Ther*., **6**: 490-494.

Auricchio, A., O'Connor, E., Weiner, D., Gao, G.P., Hildinger, M., Wang, L., Calcedo, R. and Wilson, J.M. (2002b) Noninvasive gene transfer to the lung for systemic delivery of therapeutic proteins. *J. Clin. Invest*., **110**: 499-504.

Auricchio, A., Kobinger, G., Anand, V., Hildinger, M., O'Connor, E., Maguire, A.M., Wilson, J.M. and Bennett J. (2001a) Exchange of surface proteins impacts on viral vector cellular specificity and transduction characteristics: the retina as a model. *Hum. Mol. Genet.*, **10**: 3075-3081.

Auricchio, A., O'Connor, E., Hildinger, M. and Wilson, J.M. (2001b) A single-step affinity column for purification of serotype-5 based adeno-associated viral vectors. *Mol. Ther*., **4**: 372-374.

Bantel-Schaal, U., Hub, B. and Kartenbeck, J. (2002) Endocytosis of adeno-associated virus type 5 leads to accumulation of virus particles in the Golgi compartment. *J. Virol*., **76**: 2340-2349.

Bantel-Schaal, U., Delius, H., Schmidt, R. and zur Hausen, H. (1999) Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent parvoviruses. *J. Virol.*, **73**: 939-947.

Bantel-Schaal, U. and zur Hausen, H. (1984) Characterization of the DNA of a defective human parvovirus isolated from a genital site. *Virology*, **134**: 52-63.

Bartlett, J.S., Wilcher, R. and Samulski, R.J. (2000) Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J. Virol*., **74**: 2777-2785.

Bartlett, J.S., Kleinschmidt, J., Boucher, R.C. and Samulski, R.J. (1999) Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'gamma)2 antibody. *Nat. Biotechnol*., **17**:181-186.

Beck, S.E., Jones, L.A., Chesnut, K., Walsh, S.M., Reynolds, T.C., Carter, B.J., Askin, F.B., Flotte, T.R. and Guggino, W.B. (1999) Repeated delivery of adeno-associated virus vectors to the rabbit airway. *J. Virol*., **73**: 9446-9455.

Berns, K.I. and Linden, R.M. (1995) The cryptic life style of adeno-associated virus. *Bioessays*, **17**: 237-245.

Blacklow, N.R., Hoggan, M.D. and Rowe, W.P. (1968) Serologic evidence for human infection with adenovirus-associated viruses. *J. Natl. Cancer Inst.*, **40**: 319-327.

Brument, N., Morenweiser, R., Blouin, V., Toublanc, E., Raimbaud, I., Cherel, Y., Folliot, S., Gaden, F., Boulanger, P., Kroner-Lux, G., Moullier, P., Rolling, F. and Salvetti, A. (2002) A versatile and scalable two-step ion-exchange chromatography process for the purification of recombinant adeno-associated virus serotypes-2 and -5. *Mol. Ther*., **6**: 678-686.

Chao, H., Monahan, P.E., Liu, Y., Samulski, R.J. and Walsh, C.E. (2001) Sustained and complete phenotype correction of hemophilia B mice following intramuscular injection of AAV1 serotype vectors. *Mol. Ther*., **4**: 217-222.

Chao, H., Liu, Y., Rabinowitz, J., Li, C., Samulski, R.J. and Walsh, C.E. (2000) Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. *Mol. Ther.*, **2**: 619-623.

Chiorini, J.A., Afione, S. and Kotin, R.M. (1999a) Adeno-associated virus (AAV) type 5 Rep protein cleaves a unique terminal resolution site compared with other AAV serotypes. *J. Virol.*, **73**: 4293-4298.

Chiorini, J.A., Kim, F., Yang, L. and Kotin, R.M. (1999b) Cloning and characterization of adeno-associated virus type 5. *J. Virol*., **73**: 1309-1319.

Chiorini, J.A., Yang, L., Liu, Y., Safer, B. and Kotin, R.M. (1997) Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. *J. Virol*., **71**: 6823-6833.

Clark, K.R., Liu, X., McGrath, J.P. and Johnson, P.R. (1999) Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. *Hum. Gene Ther*., **10**: 1031-1039.

Davidson, B.L., Stein, C.S., Heth, J.A., Martins, I., Kotin, R.M., Derksen, T.A., Zabner, J., Ghodsi, A. and Chiorini, J.A. (2000) Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc. Natl. Acad. Sci. USA*, **97**: 3428-3432.

Duan, D., Yan, Z., Yue, Y., Ding, W. and Engelhardt, J.F. (2001) Enhancement of muscle gene delivery with pseudotyped adeno-associated virus type 5 correlates with myoblast differentiation. *J. Virol.*, **75**: 7662-7671.

Duan, D., Yue, Y., Yan, Z., Yang, J. and Engelhardt, J.F. (2000) Endosomal processing limits gene transfer to polarized airway epithelia by adeno-associated virus. *J. Clin. Investig*., **105**: 1573-1587.

Duan, D., Sharma, P., Yang, J., Yue, Y., Dudus, L., Zhang, Y., Fisher, K.J. and Engelhardt, J.F. (1998a) Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J. Virol*., **72**: 8568-8577.

Duan, D., Yue, Y., Yan, Z., McCray, P.B. Jr, and Engelhardt, J.F. (1998b) Polarity influences the efficiency of recombinant adenoassociated virus infection in differentiated airway epithelia. *Hum. Gene Ther*., **10**: 2761-2776.

Duverger, V., Sartorius, U., Klein-Bauernschmitt, P., Krammer, P.H. and Schlehofer, J.R. (2002) Enhancement of cisplatin-induced apoptosis by infection with adeno-associated virus type 2. *Int. J. Cancer*, **97**: 706-712.

Erles, K., Sebokova, P. and Schlehofer, J.R. (1999) Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). *J. Med. Virol.*, **59**: 406-411.

Flotte, T., Agarwal, A., Wang, J., Song, S., Fenjves, E.S., Inverardi, L., Chesnut, K., Afione, S., Loiler, S., Wasserfall, C., Kapturczak, M., Ellis, T., Nick, H. and Atkinson, M. (2001) Efficient ex vivo transduction of pancreatic islet cells with recombinant adeno-associated virus vectors. *Diabetes*, **50**: 515-520.

Fraites, T.J. Jr, Schleissing, M.R., Shanely, R.A., Walter, G.A., Cloutier, D.A., Zolotukhin, I., Pauly, D.F., Raben, N., Plotz, P.H., Powers, S.K., Kessler, P.D. and Byrne, B.J. (2002) Correction of the enzymatic and functional deficits in a model of Pompe disease using adeno-associated virus vectors. *Mol. Ther*., **5**: 571-578.

Gao, G., Alvira, M.R., Wang, L., Calcedo, R., Johnston, J. and Wilson, J.M. (2002) Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl. Acad. Sci. USA*, **99**: 11854-11859.

Georg-Fries, B., Biederlack, S., Wolf, J. and zur Hausen, H. (1984) Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. *Virology*, **134**: 64-71.

Grimm, D., Kay, M.A. and Kleinschmidt, J.A. (2003) Helpervirus-free, optically-controllable and two-plasmid-based (HOT) production of adeno-associated virus (AAV) vectors of serotypes 1 to 6. *Mol. Ther*., in press.

Grimm, D. (2002) Production methods for gene transfer vectors based on adeno-associated virus serotypes. *Methods*, **28**: 146-157.

Grimm, D. (2000) Adeno-associated virus (AAV) serotypes as vectors for human gene therapy. In: Research Advances in Virology 1, Eds. Samal, S.K., Liebler-Tenorio, E., Delsert, C. and Paton, D. Global Research Network, Puthen Chalai, India, pp91-114.

Grimm, D. and Kleinschmidt, J.A. (2000) Strategies for production of adeno-associated virus vectors. In: Viral Vectors: Basic Science & Gene Therapy, Eds. Cid-Arregui, A. and Garcia, A. Eaton Publishing, Natick, MA, USA, pp27-57.

Grimm, D. and Kleinschmidt, J.A. (1999) Progress in adeno-associated virus type 2 vector production: promises and prospects for clinical use. *Hum. Gene Ther.*, **10**: 2445-2450.

Grimm, D., Kern, A., Pawlita, M., Ferrari, F., Samulski, R. and Kleinschmidt, J. (1999) Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. *Gene Ther*., **6**: 1322-1330.

Grimm, D., Kern, A., Rittner, K. and Kleinschmidt, J.A. (1998) Novel tools for production and purification of recombinant adenoassociated virus vectors. *Hum. Gene Ther.*, **9**: 2745-2760.

Halbert, C.L., Allen, J.M. and Miller, A.D. (2001) Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. *J. Virol.*, **75**: 6615-6624.

Halbert, C.L., Rutledge, E.A., Allen, J.M., Russell, D.W. and Miller, A.D. (2000) Repeat transduction in the mouse lung by using

adeno-associated virus vectors with different serotypes. *J. Virol.*, **74**: 1524-1532.

Halbert, C.L., Standaert, T.A., Wilson, C.B. and Miller, A.D. (1998) Successful readministration of adeno-associated virus vectors to the mouse lung requires transient immunosuppression during the initial exposure. *J. Virol*., **72**: 9790-9805.

Halbert, C.L., Standaert, T.A., Aitken, M.L., Alexander, I.E., Russell, D.W. and Miller, A.D. (1997) Transduction by adeno-associated virus vectors in the rabbit airway: efficiency, persistence, and readministration. *J. Virol*., **71**: 5931-5941.

Handa, A., Muramatsu, S., Qiu, J., Mizukami, H. and Brown, K.E. (2000) Adeno-associated virus (AAV)-3-based vectors transduce haematopoietic cells not susceptible to transduction with AAV-2-based vectors. *J. Gen. Virol*., **81**: 2077-2084.

Harris, E.W. (2001) Leber's congenital amaurosis and RPE65. *Int. Ophthalmol. Clin*., **41**: 73-82.

Hildinger, M., Auricchio, A., Gao, G., Wang, L., Chirmule, N. and Wilson, J.M. (2001) Hybrid vectors based on adeno-associated virus serotypes 2 and 5 for muscle-directed gene transfer. *J. Virol.*, **75**: 6199-6203.

Hughes, S.M., Moussavi-Harami, F., Sauter, S.L. and Davidson, B.L. (2002) Viral-mediated gene transfer to mouse primary neural progenitor cells. *Mol. Ther*., **5**: 16-24.

Kaludov, N., Handelman, B. and Chiorini, J.A. (2002) Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography. *Hum. Gene Ther*., **13**: 1235-1243.

Kaludov, N., Brown, K.E., Walters, R.W., Zabner, J. and Chiorini, J.A. (2001) Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *J. Virol*., **75**: 6884-6893.

Kaspar, B.K., Vissel, B., Bengoechea, T., Crone, S., Randolph-Moore, L., Muller, R., Brandon, E.P., Schaffer, D., Verma, I.M., Lee, K.F., Heinemann, S.F. and Gage, F.H. (2002) Adeno-associated virus effectively mediates conditional gene modification in the brain. *Proc. Natl. Acad. Sci. USA*, **99**: 2320-2325.

Kay, M.A., Manno, C.S., Ragni, M.V., Larson, P.J., Couto, L.B., McClelland, A., Glader B., Chew, A.J., Tai, S.J., Herzog, R.W., Arruda, V., Johnson, F., Scallan, C., Skarsgard, E., Flake, A.W. and High, K.A. (2000) Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat. Genet.*, **24**: 257-261.

Kessler, P.D., Podsakoff, G.M., Chen, X., McQuiston, S.A., Colosi, P.C., Matelis, L.A., Kurtzman, G.J. and Byrne, B.J. (1996) Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc. Natl. Acad. Sci. USA*, **26**: 14082-14087.

King, J.A., Dubielzig, R., Grimm, D. and Kleinschmidt, J.A. (2001) DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. *EMBO J.*, **20**: 3282-3291.

Loser, P., Jennings, G.S., Strauss, M. and Sandig, V. (1998) Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: involvement of NFkappaB. *J. Virol*., **72**: 180-190.

Mastakov, M.Y., Baer, K., Kotin, R.M. and During, M.J. (2002a) Recombinant adeno-associated virus serotypes 2- and 5-mediated gene transfer in the mammalian brain: quantitative analysis of heparin co-infusion. *Mol. Ther*., **5**: 380-381.

Mastakov, M.Y., Baer, K., Symes, C.W., Leichtlein, C.B., Kotin, R.M. and During, M.J. (2002b) Immunological aspects of recombinant adeno-associated virus delivery to the mammalian brain. *J. Virol*., **76**: 8446-8454.

Miao, C.H., Nakai, H., Thompson, A.R., Storm, T.A., Chiu, W., Snyder, R.O. and Kay, M.A. (2000) Nonrandom transduction of recombinant adeno-associated virus vectors in mouse hepatocytes *in vivo*: cell cycling does not influence hepatocyte transduction. *J. Virol*., **74**: 3793-3803.

Mingozzi, F., Schuttrumpf, J., Arruda, V.R., Liu, Y., Liu, Y.L., High, K.A., Xiao, W. and Herzog, R.W. (2002) Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. *J. Virol*., **76**: 10497-10502.

Muramatsu, S., Mizukami, H., Young, N.S. and Brown, K.E. (1996) Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. *Virol*., **221**: 208-217.

Muzyczka, N. (1992) Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr. Top. Microbiol. Immunol*., **158**: 97-129.

Prasad, K.M., Yang, Z., Bleich, D. and Nadler, J.L. (2000) Adeno-associated virus vector mediated gene transfer to pancreatic beta cells. *Gene Ther*., **7**: 1553-1561.

Qiu, J., Nayak, R., Tullis, G.E. and Pintel, D.J. (2002) Characterization of the transcription profile of adeno-associated virus type 5 reveals a number of unique features compared to previously characterized adeno-associated viruses. *J. Virol*., **76**: 12435-12447.

Qiu, J., Mizukami, H. and Brown, K.E. (1999) Adeno-associated virus 2 co-receptors? *Nat. Med*., **5**: 467-468.

Rabinowitz, J.E., Rolling, F., Li, C., Conrath, H., Xiao, W., Xiao, X. and Samulski, R.J. (2002) Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J. Virol.*, **76**: 791-801.

Rooney, C.P., Denning, G.M., Davis, B.P., Flaherty, D.M., Chiorini, J.A. and Zabner, J. (2002) Bronchoalveolar fluid is not a major hindrance to virus-mediated gene therapy in cystic fibrosis. *J. Virol*., **76**: 10437-10443.

Rutledge, E.A., Halbert, C.L. and Russell, D.W. (1998) Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. *J. Virol.*, **72**: 309-319.

Sarra, G.M., Stephens, C., Schlichtenbrede, F.C., Bainbridge, J.W., Thrasher, A.J., Luthert, P.J. and Ali, R.R. (2002) Kinetics of transgene expression in mouse retina following sub-retinal injection of recombinant adeno-associated virus. *Vision Res*., **42**: 541-549.

Smith, R.H., Afione, S.A. and Kotin, R.M. (2002) Transposase-mediated construction of an integrated adeno-associated virus type 5 helper plasmid. *Biotechniques*, **33**: 204-211.

Snyder, R.O., Miao, C.H., Patijn, G.A., Spratt, S.K., Danos, O., Nagy, D., Gown, A.M., Winther, B., Meuse, L., Cohen, L.K., Thompson, A.R. and Kay, M.A. (1997) Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat. Genet*., **16**: 270-276.

Summerford, C. and Samulski, R.J. (1998) Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.*, **72**: 1438-1445.

Tobiasch, E., Burguete, T., Klein-Bauernschmitt, P., Heilbronn, R. and Schlehofer, J.R. (1998) Discrimination between different types of human adeno-associated viruses in clinical samples by PCR. *J. Virol. Meth.*, **71**: 17-25.

Wagner, J.A., Nepomuceno, I.B., Messner, A.H., Moran, M.L., Batson, E.P., Dimiceli, S., Brown, B.W., Desch, J.K., Norbash, A.M., Conrad, C.K., Guggino, W.B., Flotte, T.R., Wine, J.J., Carter, B.J., Reynolds, T.C., Moss, R.B. and Gardner, P. (2002) A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. *Hum. Gene Ther*., **13**: 1349-1359.

Walters, R.W., Pilewski, J.M., Chiorini, J.A. and Zabner, J. (2002) Secreted and transmembrane mucins inhibit gene transfer with AAV4 more efficiently than AAV5. *J. Biol. Chem*., **277**: 23709-23713.

Walters, R.W., Yi, S.M., Keshavjee, S., Brown, K.E., Welsh, M.J., Chiorini, J.A. and Zabner, J. (2001) Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. *J. Biol. Chem*., **276**: 20610-20616.

Wustner, J.T., Arnold, S., Lock, M., Richardson, J.C., Himes, V.B., Kurtzman, G. and Peluso, R.W. (2002) Production of recombinant adeno-associated type 5 (rAAV5) vectors using recombinant herpes simplex viruses containing rep and cap. *Mol. Ther*., **6**: 510-518.

Xiao, W., Chirmule, N., Berta, S.C., McCullough, B., Gao, G. and Wilson, J.M. (1999) Gene therapy vectors based on adeno-associated virus type 1. *J. Virol*., **73**: 3994-4003.

Yan, Z., Zak, R., Luxton, G.W., Ritchie, T.C., Bantel-Schaal, U. and Engelhardt, J.F. (2002) Ubiquitination of both adeno-associated virus type 2 and 5 capsid proteins affects the transduction efficiency of recombinant vectors. *J. Virol*., **76**: 2043-2053.

Yang, G.S., Schmidt, M., Yan, Z., Lindbloom, J.D., Harding, T.C., Donahue, B.A., Engelhardt, J.F., Kotin, R. and Davidson, B.L. (2002) Virus-mediated transduction of murine retina with adeno-associated virus: effects of viral capsid and genome size. *J. Virol*., **76**: 7651-7660.

Zabner, J., Seiler, M., Walters, R., Kotin, R.M., Fulgeras, W., Davidson, B.L. and Chiorini, J.A. (2000) Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. *J. Virol*., **74**: 3852-3858.

Zolotukhin, S., Potter, M., Zolotukhin, I., Sakai, Y., Loiler, S., Fraites, T.J. Jr, Chiodo, V.A., Phillipsberg, T., Muzyczka, N., Hauswirth, W.W., Flotte, T.R., Byrne, B.J. and Snyder, R.O. (2002) Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods*, **28**: 158-167.