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## 6 Adeno-Associated Viral Vectors

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### 6.1 INTRODUCTION

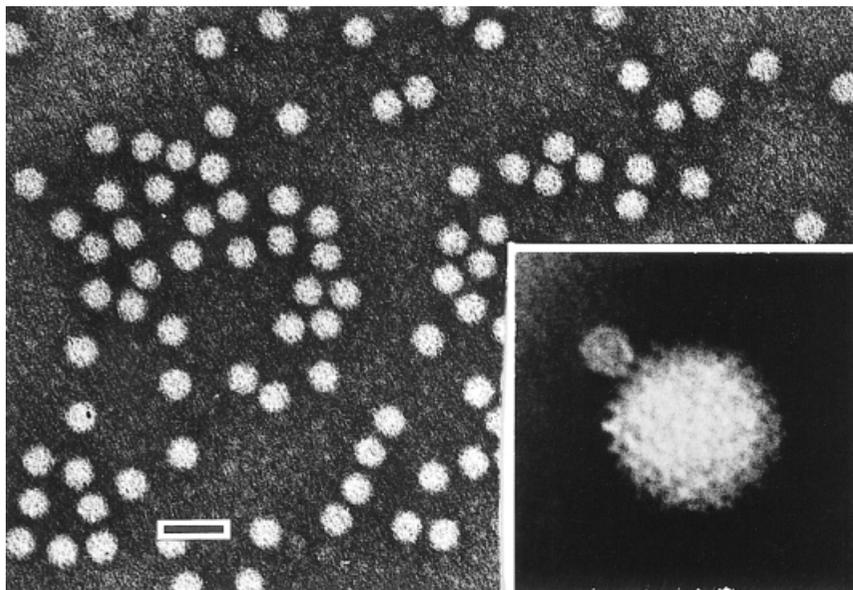
Adeno-associated virus (AAV) is a non-pathogenic human DNA virus with a unique profile of biological properties that have been of interest to molecular virologists for many years (Berns, 1990; Carter, 1990; Carter *et al.*, 1990). Recently, AAV has also attracted interest as a vector for gene transfer (Carter, 1992; Flotte, 1993a; Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1984). In a general sense, AAV is unique among viruses currently being used for gene transfer in that it is a native human virus which is not known to cause disease, and may, in fact, suppress the induction of tumors by other viruses (Cukor *et al.*, 1975; DeLaMaza and Carter, 1981; Hermonat, 1989, 1991; Khelif *et al.*, 1991; Kirschstein *et al.*, 1968; Labow *et al.*, 1987; Mayor *et al.*, 1973; Ostrove *et al.*, 1981). There is a natural enthusiasm to develop therapeutic applications of a virus which is naturally symbiotic with its human host. This must be tempered by careful attention to how the biology of the virus may be altered as its genome is re-engineered to make it into a vector.

### 6.2 BIOLOGY OF AAV

#### 6.2.1 AAV TAXONOMY AND NATURAL HISTORY

AAV was originally identified as a contaminant of adenovirus cultures. Multiple serotypes of AAV have since been identified, including human AAV serotypes 1, 2, 3, and 5, simian AAV serotype 4, as well as bovine, canine and avian AAV (Blacklow, 1988). AAV is a member of the dependovirus genus of the family Parvoviridae. Like other parvoviruses, AAV exists as a non-enveloped icosahedral particle with a diameter of approximately 20 nm (Figure 6.1).

AAV has never been shown to cause any human disease, despite a high seroprevalence rate. AAV serotypes 2 and 3 have been identified in throat

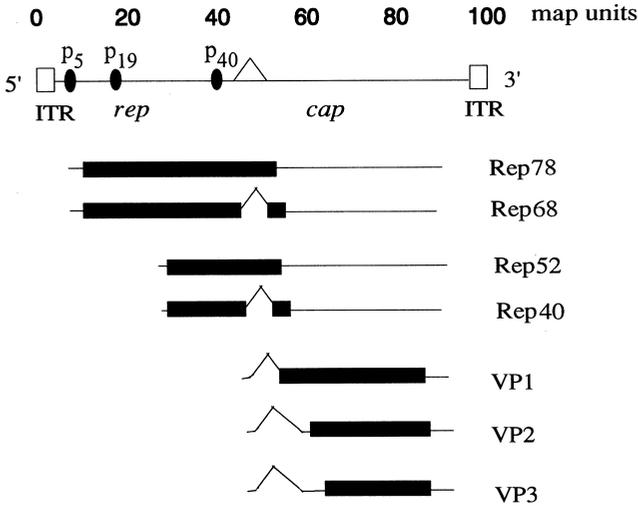


**Figure 6.1.** Electron micrograph of AAV particles. A field of CsCl gradient-purified ( $\rho = 1.41$  g/ml) AAV2 particles is shown, demonstrating the icosahedral shape of the 20 nm virions (magnification = 40 000X). The inset shows a larger adenoviral particle adjacent to an AAV particle from the 1.36 g/ml band (magnification = 120 000X).

and anal swab specimens from otherwise healthy children during a concomitant nursery school outbreak of adenovirus- (Ad-) induced diarrhea (Blacklow *et al.*, 1971). There were no differences observed between the clinical syndromes in individuals infected with AAV and Ad as compared with those infected with Ad alone. Because AAV can also exist in a latent state in human cells, its potential role in neoplastic processes has also been investigated. Surprisingly, AAV seropositivity was inversely correlated with risk for virus-induced cervical carcinoma (Cukor *et al.*, 1975). Studies in animal and tissue culture models of tumorigenesis have confirmed that the AAV-*rep* gene can function as a tumor suppressor (Hermonat, 1991; Kleif *et al.*, 1991; Labow *et al.*, 1987).

## 6.2.2 THE STRUCTURE OF AAV AND ITS GENOME

The AAV2 genome has been cloned (Laughlin *et al.*, 1983; Samulski *et al.*, 1982), sequenced (Srivastava *et al.*, 1983), and characterized in detail (Figure 6.2). The termini consist of the 145-nucleotide inverted repeat sequences (inverted terminal repeats; ITRs). The outer 125 nucleotides of each ITR form a palindrome which can assume a hairpin configuration in the single-stranded state.



**Figure 6.2.** Structure of the AAV genome. The AAV2 genome is represented with a 100 map-unit scale (1 map-unit = 1% of genome size, approximately 47 bp). The open boxes represent the inverted terminal repeats (ITRs). The transcription promoters (p<sub>5</sub>, p<sub>19</sub>, p<sub>40</sub>) are depicted as solid ellipses. The polyadenylation signal is at map position 96. RNA transcripts from AAV promoters are shown below the DNA map with the introns indicated by carets. Protein coding regions are depicted as solid boxes. The three capsid proteins are VP1, VP2, and VP3; the four Rep proteins are Rep78, Rep68, Rep52, and Rep40.

The ITRs contain all *cis*-acting functions required for DNA replication, packaging, integration, and subsequent excision and rescue (Samulski *et al.*, 1989). Also within the ITRs are several transcriptional elements, including Sp1 sites and an initiator (*inr*) site for transcription of RNA (Flotte *et al.*, 1993a). The role of these functions in the natural virus life cycle is unknown. The ITRs also contain binding sites for Rep68 protein (see below), which may be important for the processes of terminal resolution and site-specific integration.

Internal to the ITRs are two viral genes: *rep*, which encodes functions required for replication, and *cap*, which encodes structural proteins of the capsid. The *rep* gene is transcribed from two promoters, the p<sub>5</sub> promoter and the internal p<sub>19</sub> promoter. By utilizing each of these promoters with both spliced and unspliced RNA transcripts, a total of four Rep proteins are produced. These have been designated Rep78, Rep68, Rep52, and Rep40 based on their apparent molecular weights. Rep78 and Rep68 have a number of properties, including: (i) DNA binding to a specific Rep-recognition sequence (*rrs*) within each ITR (McCarty *et al.*, 1994a,b), (ii) DNA helicase activity, (iii) site-specific, strand-specific endonuclease activity for AAV-ITRs during viral DNA replication and rescue, (iv) DNA binding to *rrs* sequences

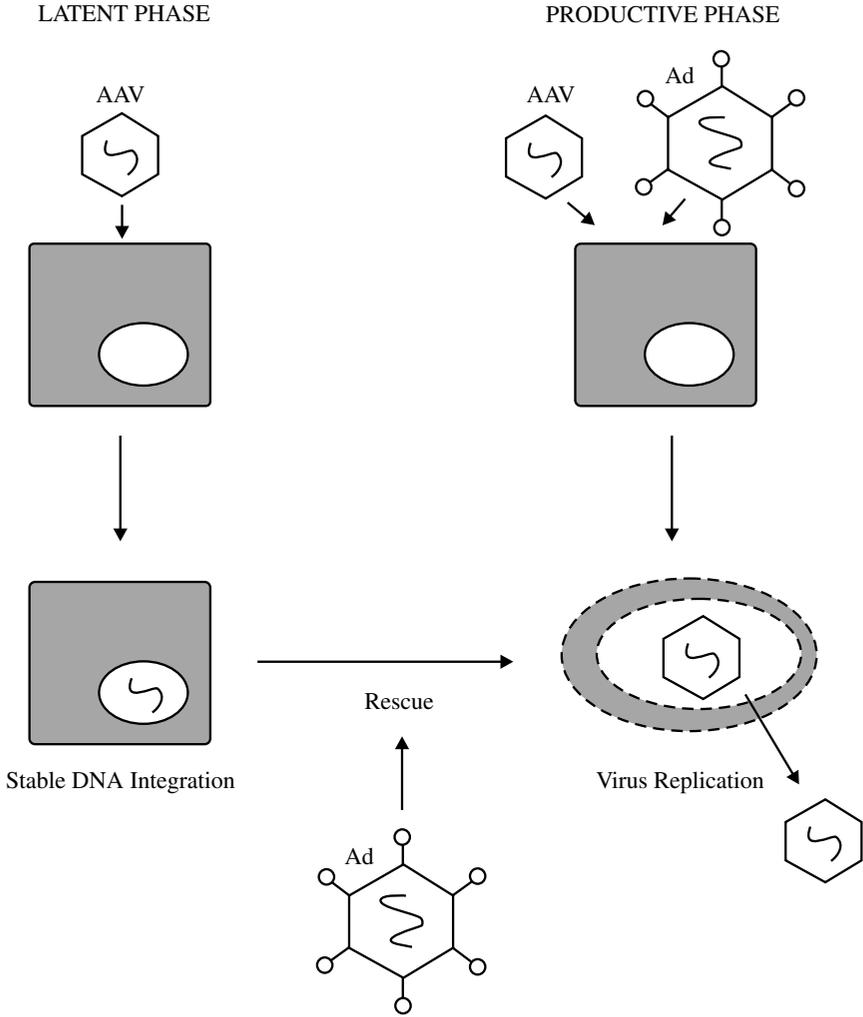
within the preferred chromosome 19 integration sequence (Weitzman *et al.*, 1994), and (v) transcriptional repressor and activator functions (Antoni *et al.*, 1991; Beaton *et al.*, 1989; Kyostio *et al.*, 1994). The first three of these functions appear to be important for normal replication in a productive life cycle. Binding to the rrs on chromosome 19 may be important in the latent phase of the life cycle. The transcription regulation functions are important in suppressing viral gene expression during latency and activating it during the replicative phase. Rep78 and Rep68 also modulate transcription from heterologous promoters of other viruses and from cellular genes. This latter activity may also be responsible for the Rep78/68 effect as a suppressor of tumorigenesis (Hermonat, 1991; Khelif *et al.*, 1991). The biochemical functions of Rep52 and Rep40 are less well defined, although these gene products are required for accumulation of single-stranded DNA copies during a productive infection.

The *cap* gene is transcribed from the p<sub>40</sub> promoter to generate three protein products, VP1, VP2, and VP3, with approximate molecular weights of 85 kDa, 72 kDa, and 61 kDa, respectively. By use of two different splice acceptor sites, two different transcripts are produced: a minor transcript which codes for VP1 and a major transcript which codes for VP2 and VP3 (Trempe and Carter, 1998). These three proteins differ in the length of their amino terminus, but are identical throughout the VP3 coding region. While VP3 accounts for 84% of the capsid protein, all three are required for complete particle assembly.

### 6.2.3 THE AAV LIFE CYCLE

The AAV life cycle consists of two phases, the productive or replication phase and the latent phase (Figure 6.3) (Berns, 1990; Carter, 1990). In the productive life cycle, AAV co-infects the host cell with a helper virus (adenovirus or herpesvirus). As the helper virus replicates, AAV replication also occurs, along with encapsidation of progeny virions. These virions are released if and when the helper virus lyses the cell. The helper virus effects are indirect. In some cells under special conditions cellular factors can support AAV replication without helper virus following treatment with genotoxic agents such as ultraviolet (UV) irradiation, gamma irradiation, or hydroxyureas (Schlehofer *et al.*, 1986).

If cells are infected with AAV in the absence of helper virus, AAV enters the latent phase of its life cycle. This generally involves stable integration of tandem head-to-tail dimers of the AAV genome. However, episomal forms of AAV have also been found to persist in chronically infected cells for at least 100 passages (Cheung *et al.*, 1980; Hoggan *et al.*, 1972). The morphology and growth characteristics of cells are not overtly affected by AAV latency. If latently infected cells are subsequently infected with helper virus, AAV can be rescued, i.e. it can re-enter the productive phase of the life cycle.



**Figure 6.3.** AAV life cycle. The latent and productive phases of the AAV life cycle are depicted. See text for details.

### 6.2.4 SITE-SPECIFICITY OF AAV INTEGRATION

One of the unusual features of AAV latency is the tendency for AAV to integrate within a specific region of human chromosome 19, the AAV-S1 site. In studies of immortalized cell lines infected with AAV, several groups found AAV integrants within the same region of chromosome 19 (q13.3-qter) in approximately 65–70% of cell clones (Kotin *et al.*, 1990, 1991, 1992;

Samulski, 1993; Samulski *et al.*, 1991). This 8.2 kb-AAV-S1 site has been sequenced and found to contain a number of important elements including homologues of the AAV rrs and terminal recognition sequence (trs). Giraud *et al.* (1994), have demonstrated that a 0.5 kb fragment of the S1 site, when incorporated into an episomal Epstein-Barr virus (EBV) plasmid, is sufficient as a target for AAV integration. The mechanism for integration may involve the Rep68 protein, as recent data from Weitzman *et al.* (1994), indicate. In those studies, Rep68 was found to bind to both the AAV-ITR and the AAV-S1 sequence simultaneously, forming a complex which could serve as a pre-integration intermediate.

### 6.3 AAV-DERIVED RECOMBINANT VECTORS

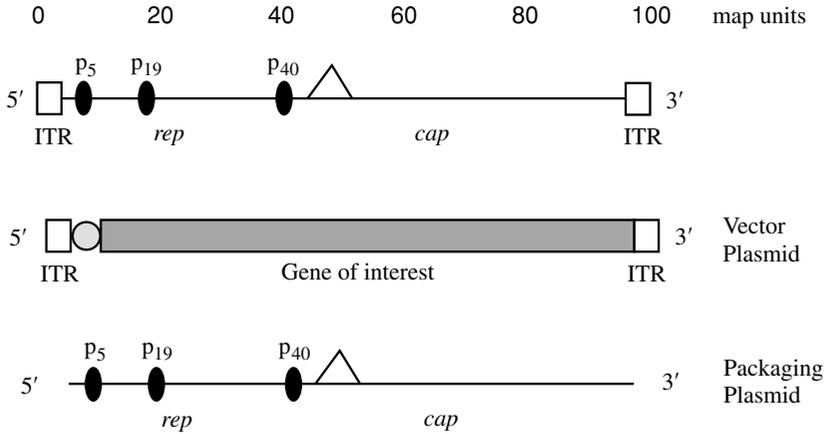
#### 6.3.1 STRUCTURE OF RECOMBINANT AAV VECTORS

In an effort to exploit the unique features of the AAV life cycle in a gene transfer vector, several groups constructed recombinant vectors by deleting internal portions of the AAV genome within plasmids and inserting transgenes of interest (Flotte *et al.*, 1992; Hermonat and Muzyczka, 1984; Samulski *et al.*, 1989; Tratschin *et al.*, 1984). In early experiments, AAV vectors contained substantial portions of the *rep* and/or *cap* genes, and were complemented either with wild-type AAV or with overlapping partially deleted constructs. These vectors demonstrated the feasibility of using AAV as a eukaryotic vector, but were limited by the presence of wild-type virus, which appeared to exert transcriptional suppressor effects mediated by the *rep* gene.

Samulski *et al.* (1989), demonstrated that preparations of AAV vectors substantially free of wild-type AAV could be generated if non-overlapping constructs were used (Figure 6.4). In this packaging procedure, vector constructs contained the gene of interest flanked by AAV-ITRs, while the complementing 'packaging' plasmid expressed the AAV *rep* and *cap* genes from a second plasmid co-transfected into adenovirus-infected cells. Since the ITRs contain the packaging signals, any suitably sized vector construct ( $\leq 5$  kb from ITR to ITR) would be packaged, while the complementing *rep/cap* expression construct would not.

#### 6.3.2 STRATEGIES FOR PACKAGING AAV RECOMBINANT VECTORS

In order to encapsidate recombinant AAV vector DNA into infectious virions, five elements are generally required: (i) cells permissive for AAV replication (e.g. 293 cells), (ii) a helper virus (e.g. adenovirus), (iii) a recom-



**Figure 6.4.** Organization of AAV2-based vectors. The AAV genome with a map-unit scale is depicted above, with the ITRs as open boxes and the transcription promoters ( $p_5$ ,  $p_{19}$ ,  $p_{40}$ ) as dark shaded ellipses. Vector plasmids (middle diagram) are constructed by inserting the foreign gene of interest (lightly-shaded bar) and a promoter (lightly-shaded circle) between the ITRs, which serve as replication origins and packaging signals. Vector DNA is packaged into infectious AAV particles after co-transfection with an ITR-deleted packaging plasmid (bottom diagram) into adenovirus-infected cells.

binant AAV vector of 5 kb or less, including ITRs flanking the transgene and any promoter, enhancer, intron, or polyadenylation elements, (iv) a source of Rep proteins, and (v) a source of capsid proteins. These elements can be supplied by co-transfecting adenovirus-infected cells with two plasmids, one containing the recombinant AAV vector DNA (ITR+, rep-, cap-) and the other containing the complementing AAV genes (ITR-, rep+, cap+). Cells must then be lysed by serial freeze-thaw or other physical methods to release the packaged virions. Packaged AAV vector can then be separated from cellular debris and helper virus by CsCl gradient ultracentrifugation.

There are a number of potential limitations on the efficiency of packaging from a simple two-plasmid co-transfection technique. First, there is potential inefficiency of transfection, which could be multiplied by having to have two plasmids independently enter the packaging cells. Cell lines have been produced which either contain a stable copy of the vector DNA (in rescuable form) (Flotte *et al.*, 1995), and/or the rep/cap expression plasmid (Clark *et al.*, 1995). The level of Rep expression in the target cell is also a potential limiting factor. The fact that Rep68 can downregulate its own expression from the AAV  $p_5$  promoter has led to a strategy in which Rep is expressed from the HIV long terminal repeat (LTR) promoter in 293 cells, where this promoter is constitutively active (Flotte *et al.*, 1995).

### 6.3.3 PERSISTENCE OF VECTOR DNA IN TARGET CELLS

The use of AAV as a transducing vector is based on the assumption that recombinant AAV retains certain characteristics of wild-type AAV in its ability to establish persistence. In fact, a number of studies have indicated that wild-type-free, *rep*-deleted AAV vectors can mediate stable transgene expression *in vitro* and *in vivo*. Recent evidence indicates, however, that the mechanism of persistence of *rep*-deleted AAV vectors may be different from that of wild-type AAV (Afione *et al.*, 1996; Goodman *et al.*, 1994; Kearns *et al.*, 1994). *In vitro* studies indicate that AAV-CFTR (cystic fibrosis transmembrane conductance regulator) vectors integrate into the AAV-S1 locus much less frequently than wild-type AAV (Kearns *et al.*, 1994). The total number of integration events appears to be decreased, and those integrations which do occur do not appear to share the same preference for chromosome 19. In a related *in vivo* study in rhesus macaques, AAV-CFTR vector DNA persistence in bronchial epithelial cells was once again observed for up to 3 months (Afione *et al.*, 1996). In this instance, there was episomal persistence of double-stranded DNA copies of the vector. These observations support the hypothesis that specific interactions between Rep68, the AAV-ITR, and the AAV-S1 sequence may be involved in site-specific integration by wild-type AAV.

### 6.3.4 HOST CELL FACTORS AFFECTING AAV VECTOR TRANSDUCTION

The effects of host cell proliferation and helper virus gene expression on AAV vector transduction have also been examined. With regard to cell division, several studies have indicated that vector DNA entry can occur in quiescent cells (Flotte *et al.*, 1994; Podsakoff *et al.*, 1994; Russell *et al.*, 1994). The effects on transgene expression are variable. One study indicated that when cells are infected with vector while quiescent and then allowed to re-enter the cell cycle to undergo *neo*-selection, there was little change in transduction efficiency as compared with proliferating cells (Podsakoff *et al.*, 1994). In another study, it was found that a much higher multiplicity of infection was required in slowly dividing cells in order to achieve a similar level of transgene expression as compared with rapidly proliferating cells (Flotte *et al.*, 1994). In a third study, there was a marked decrease in transduction efficiency in quiescent cells as compared with proliferating cells, but only one multiplicity of infection was used (Russell *et al.*, 1994). Related studies have indicated differences in transduction efficiency between immortalized and primary cells (Halbert *et al.*, 1995), although several other studies *in vitro* and *in vivo* found no such differences (Kaplitt *et al.*, 1994). Finally,

enhancement of expression by UV irradiation has recently been described (Alexander *et al.*, 1994).

Another recent study has indicated that AAV vector expression can be enhanced by concurrent adenovirus infection or expression of the adenovirus E4-orf6 gene product (Fisher *et al.*, 1996). There is a suggestion that this effect is mediated by enhancement of leading strand synthesis in the conversion of the single-stranded DNA to a double-stranded DNA version of the vector. Unfortunately, this study did not examine the role of multiplicity of infection or the kinetics of leading strand synthesis in the absence of Ad E4-orf6 expression.

### 6.3.5 SUMMARY OF ADVANTAGES AND DISADVANTAGES OF AAV TRANSDUCING VECTORS

The potential advantages and disadvantages of current AAV-based vectors for gene therapy are summarized in Table 6.1. The principal advantage of AAV over other DNA virus vectors, such as adenovirus, is the lack of any viral coding sequence within the vectors, which prevents transduced cells from being recognized and rejected by the immune system. The AAV virion itself also appears to be less pro-inflammatory on initial exposure than the adenovirus virion. These two factors probably account for the very favorable safety profile of AAV vectors in animals (Conrad *et al.*, 1994; Flotte *et al.*, 1993b). Recombinant AAV is also efficient at cell entry, and tends to persist in cells over long periods of time. The principal disadvantages of AAV relate to the fact that it enters the cell as single-stranded DNA and must be converted to double-stranded DNA prior to expression of the transgene. This may limit the level of expression in some cells. Also, as mentioned above, AAV vectors which lack the *rep* gene do not appear to integrate at as high a frequency as wild-type AAV. This could ultimately limit the duration of expression. The packaging limit of AAV is relatively small, at 5 kb. Since vectors must contain the ITRs (0.3 kb total), this leaves only 4.7 kb for the entire insert, including the transgene coding region, the promoter, the polyadenylation signal, and any other regulatory elements. Although AAV vector production is still a relatively inefficient process, this will benefit from further refinements to allow potential widespread clinical use of these agents.

## 6.4 APPLICATIONS OF AAV VECTORS

Table 6.2 summarizes some of the published applications of AAV gene transfer vectors to specific cell targets or disease models. These include both *in vitro* and *in vivo* experiments.

**Table 6.1** Potential advantages and disadvantages of AAV vectors for gene therapy

Advantages	Disadvantages
1. Non-immunogenic (no viral coding sequences)	1. Requires conversion to double-stranded DNA (may delay expression)
2. No host inflammatory reaction to capsid components	2. Decreased integration frequency in absence of Rep proteins
3. Efficient entry of DNA into target cell	3. Small packaging limit (4.7 kb insert)
4. Long-term DNA persistence in target cell	

**Table 6.2.** Applications of AAV vectors

<i>In vitro</i>		<i>In vivo</i>	
Cell targets	Disease models	Cell targets	Disease models
K562 (erythroid) CD34+ PBLs	Thalassemias Sickle cell disease	Rabbit bronchus Rhesus bronchus	Cystic fibrosis Cystic fibrosis
Murine bone marrow cells IB3-1 (bronchial) Human fibroblasts	Hemoglobinopathies Cystic fibrosis Gaucher's disease and metachromatic leukodystrophy AIDS	Rat brain	Parkinson's disease
CD4+ T lymphocyte lines Immortalized B lymphocytes	Chronic granulomatous disease		

#### 6.4.1 *IN VITRO* APPLICATIONS

AAV transducing vectors were initially studied in immortalized cell lines, such as HeLa, 293, and KB, using reporter genes, such as neomycin phosphotransferase (*neo*) and chloramphenicol acetyltransferase (CAT) (Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1984, 1985). The utility of these vectors has been confirmed in more differentiated cell lines, including the K562 erythroleukemia cell line (Walsh *et al.*, 1992), the IB3-1 cystic fibrosis (CF) bronchial epithelial cell line (Flotte *et al.*, 1992), and several CD4+ lymphoid cell lines (Chatterjee *et al.*, 1992). AAV vectors have also been used in primary cell types and in *in vitro* models of specific disease applications.

A number of studies have focused on the use of AAV vectors in bone marrow-derived cells as a potential treatment for hemoglobinopathies. Walsh *et al.* (1992), initially demonstrated regulated high-level expression of a human  $\gamma$  globin gene in the K562 erythroleukemia cell line. Subsequently, the same group demonstrated the feasibility of using their AAV-globin vector to transduce CD34+ progenitor cells derived from the peripheral blood of a patient with sickle cell anemia (Miller *et al.*, 1994). Similar results were obtained with CD34+ cells from rhesus monkeys and humans infected with AAV- $\beta$ -galactosidase vectors or wild-type AAV (Goodman *et al.*, 1994). AAV vectors have also been used to express antisense to the  $\alpha$  globin gene, which could have therapeutic effects in  $\beta$  thalassemia, where an imbalance of  $\alpha$  and  $\beta$  globin contributes to abnormal erythroid maturation (Ponnazhagan *et al.*, 1994).

AAV vectors have also been used in other models of primary hematopoietic disease. AAV reporter vectors have been used in murine hematopoietic precursors (Zhou *et al.*, 1993). Another important study utilized cells from a patient with Fanconi's anemia, complementation group C (FACC) (Walsh *et al.*, 1994). Transduction of progenitor cells with an AAV-FACC vector resulted in phenotypic correction of the basic defect in DNA repair and restoration of the colony forming ability which is deficient in this disease. AAV vectors have also been used to transfer the NADPH-oxidase gene, which is deficient in chronic granulomatous disease (Thrasher *et al.*, 1995), the glucocerebrosidase gene, which is deficient in Gaucher's disease, and the arylsulfatase A gene, which is deficient in metachromatic leukodystrophy (Wei *et al.*, 1994). Another report described the use of an AAV antisense vector for inhibition of HIV-1 replication in lymphoid cell lines as a potential treatment for the acquired immunodeficiency syndrome (AIDS) (Chatterjee *et al.*, 1992).

Several studies have examined the potential utility of AAV as a gene transfer vector for CF. AAV vectors for expressing the CFTR were constructed using small endogenous AAV promoter elements in order to facilitate packaging of the 4.5 kb CFTR coding region, which along with the mandatory 0.3 kb for the ITRs produces a vector size near the packaging limit of AAV (Flotte *et al.*, 1993a). The AAV-CFTR vector constructs produced were able to be packaged and were used to transduce the CF-defective IB3-1 cell line, resulting in phenotypic correction of the chloride transport defect. Interestingly, CFTR expression resulted in both the appearance of a small linear chloride conductance associated with recombinant CFTR expression and the restoration of cAMP-responsiveness of the outwardly-rectifying chloride channel (Egan *et al.*, 1992; Schwiebert *et al.*, 1994). The demonstration of phenotypic correction of a bulk culture of cells without selection encouraged pursuit of further *in vivo* studies of AAV-CFTR gene transfer.

#### 6.4.2 *IN VIVO* APPLICATIONS

Published reports of *in vivo* gene transfer with AAV vectors have focused on the brain and the lung as potential target organs. Kaplitt *et al.* (1994) demonstrated that AAV vectors expressed the *lacZ* gene for over 3 months after direct injection into the rat brain. They also showed that an AAV vector expressing the tyrosine hydroxylase (TH) gene effected a partial phenotypic correction in a rat model of Parkinson's disease. AAV-CFTR vectors have been studied in the lungs of rabbits (Flotte *et al.*, 1993a) and rhesus monkeys (Afione *et al.*, 1996) after delivery via fiberoptic bronchoscopy. In each case, long-term vector DNA persistence and RNA expression were observed without overt toxicity. These studies have served as a basis for a phase I clinical trial of AAV-CFTR administration to the nose and lung of adult CF patients with mild lung disease which has recently been initiated (Flotte *et al.*, 1996).

### 6.5 SAFETY ISSUES

AAV is based on a virus which commonly infects humans without causing disease, and AAV vectors have had a remarkably favorable safety profile in *in vivo* tests. Nevertheless, it is important to consider potential safety issues to be assessed in future preclinical and clinical trials. Safety issues with AAV vectors may be considered in terms of (i) potential risks to the intended recipient of the gene therapy vector, i.e. the subject, and (ii) potential risks associated with spread of the recombinant virus to other individuals, i.e. environmental contacts.

#### 6.5.1 SUBJECT SAFETY

Recent *in vivo* data suggests that there is no vector-related toxicity associated with AAV vector administration to the lungs of rabbits and rhesus monkeys or to the brains of rats. Therefore, safety concerns with AAV vectors remain largely theoretical, and are based on the experience with other viral vectors whose biological characteristics differ substantially from those of AAV.

The possibility of insertional mutagenesis and subsequent tumorigenesis was considered because *rep*-deleted AAV vectors have been found to integrate non-specifically into some cells within the target population. However, DeLaMaza and Carter (1981) examined the tumorigenic potential of both *rep*<sup>+</sup> and *rep*<sup>-</sup> AAV in a newborn hamster model of tumorigenesis. There was no evidence of enhanced tumorigenesis with either virus. In fact, both *rep*<sup>+</sup> and *rep*<sup>-</sup> AAV were found to suppress the tumorigenic potency of adenovirus type 12. Furthermore, there has been no evidence of neoplastic changes on long-term follow-up of the animals involved in the *in vivo* gene

transfer studies mentioned above (Flotte *et al.*, 1993a; Kaplitt *et al.*, 1994). Based on this experience, the mutagenesis risk from these vectors appears to be low.

The possibility of vector-induced inflammation and cell-mediated immune responses was raised because adenovirus vector administration to the lung is associated with both a dose-related inflammatory response (Simon *et al.*, 1993) and subsequent immune-mediated elimination of transduced cells (Yang *et al.*, 1994). These issues have been directly studied with AAV vectors in the rhesus macaque (Conrad *et al.*, 1994). Bronchoscopic delivery of AAV-CFTR to the bronchial epithelium was not associated with any detectable inflammation as judged by bronchoalveolar lavage fluid analysis (including cell counts, and interleukin-6 (IL-6) and interleukin-8 (IL-8) levels), radiographic studies, pulmonary function studies, and histopathological examination. These studies included doses of vector as high as  $1 \times 10^{11}$  total particles and time points ranging from 10 to 180 days. The two key differences between AAV and Ad in this regard would seem to be: (i) that AAV capsid proteins have less cytotoxicity and pro-inflammatory effects and (ii) that the absence of any viral coding sequences within AAV vectors prevents transduced cells from becoming targets for cellular immune surveillance.

### 6.5.2 ENVIRONMENTAL SAFETY

Until it is established in clinical trials that AAV vectors are safe in humans, it is important to prevent the exposure of individuals other than the subjects themselves. For *ex vivo* manipulations, standard biosafety level 1 precautions have been sufficient for this agent. There are additional issues related to *in vivo* gene transfer. These include: (i) shedding of recombinant AAV from individuals immediately after vector administration, and (ii) rescue or subsequent shedding from vector-treated individuals who may be later infected with wild-type AAV and adenovirus. Each of these issues was studied in the rhesus macaque model (Afione *et al.*, 1996). Shedding after initial exposure was found to be undetectable by 3 days in the nasal fluid, bronchial lavage, urine, and stool. Rescue was studied in several different ways, varying the site and sequence of administration of Ad, wt-AAV, and AAV-CFTR vector. In most instances, no subsequent vector shedding was observed. When a large inoculum ( $10^{10}$  total particles) of wild-type AAV and then AAV-CFTR were administered to the same site in the lower respiratory tract, followed by Ad infection, a very low level of AAV-CFTR shedding was detectable in the lung, which lasted for 6 days. These findings indicate that the risk of environmental exposure will be low. Shedding studies are currently under way as part of phase I trials of AAV-CFTR administration.

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