Current Gene Therapy, 2001, *1*, 137-147

Rate Limiting Steps of AAV Transduction and Implications for Human Gene Therapy

S. Sanlioglu[1,2,4]*, M. M. Monick[1], G. Luleci[4], G. W. Hunninghake[1,2] and J. F. Engelhardt[1,2,3]

[1]*Department of Internal Medicine, Division of Pulmonary and Critical Care,* [2]*the Center for Gene Therapy,* [3]*Department of Anatomy and Cell Biology, University of Iowa, College of Medicine, Iowa City, IA 52242, USA,* [4]*Department of Medical Biology and Genetics, Akdeniz University, College of Medicine, Antalya, 07070, Turkey*

Abstract: Despite the fact that adeno-associated virus type 2 (AAV2) is an extremely attractive gene therapy vector, its application has been limited to certain tissues such as muscle and the brain. In an attempt to broaden the array of target organs for this vector, molecular studies on the mechanism(s) of AAV transduction have expanded over the past several years. These studies have led to the development of innovative strategies capable of overcoming intracellular barriers to AAV2 transduction. The basis of these technologic breakthroughs has stemmed from a better understanding of the molecular processes that control AAV entry and intracellular trafficking to the nucleus. This review will focus on the identification of molecular components important for recombinant AAV (rAAV) transduction while highlighting the techniques used to discover them and potential clinical application of research findings.

INTRODUCTION

Adeno-associated virus (AAV) is a helper-dependent parvovirus with a single-stranded DNA genome of 4680 nucleotides (Samulski *et al*., 1983; Srivastava, 1994). Its diverse organ tropism, capacity for establishing latency by integration into the host chromosome, and nonpathogenic characteristics support the great potential for its use as a vector for gene therapy (Carter *et al*., 1992; During, 1997; Flotte and Carter, 1995; Grant *et al*., 1997; Kotin, 1994; Muzyczka, 1992). Moreover, the unique capability of AAV to infect quiescent cells, in contrast to retroviruses that require cell division for efficient infection, (Lewis and Emerman, 1994; Miller *et al*., 1990) has made AAV more versatile for gene therapy applications (Alexander *et al*., 1994; Kaplitt *et al*., 1994; Xiao *et al*., 1996). Despite its many attractive features, further optimization of AAV-mediated gene delivery (ie. transduction) is needed before AAV-mediated gene transfer becomes therapeutically useful. Although, AAV transduction in organs such as muscle (Duan *et al*., 1998a; Fisher *et al*., 1997; Kessler *et al*., 1996; Xiao *et al*., 1996; Yang *et al*., 1999) is highly efficient and stable, transduction of tissues such as lung by AAV2 remains very inefficient (Duan *et al*., 1998b; Hillgenberg *et al*., 1999). Early studies have attributed inefficient transduction to the inability of the cells to convert the single-stranded AAV viral DNA to the transcriptionally active double-stranded form typically associated with the replication phase of wild type AAV (Ferrari *et al*., 1996; Fisher *et al*., 1996a). However, more recent studies have demonstrated that conversion of single-stranded AAV genomes to circular inter-mediates is the critical step in gene conversion during latent phase infection with recombinant AAV (Duan *et al*., 1998a; Sanlioglu *et al*., 1999).

Following infection of a cell, wild type AAV undergoes either a latent phase (dormant infection) or a lytic phase (active replication). Productive active replication of AAV requires helper functions supplied by a second co-infecting virus, such as adenovirus or herpes virus [Berns, 1990). Once co-infected, AAV replicates and then can be rescued from cells upon the completion of the lytic phase (Berns and Linden, 1995). This aspect of the AAV life cycle is the foundation of approaches developed by Samulski and colleagues (Samulski *et al*., 1987; Samulski *et al*., 1989).

Recombinant AAV (rAAV) is produced by the expression of viral Rep and Cap genes, which are provided in trans. Rep is required for AAV replication and Cap is for capsid production. Two 145-base pair inverted terminal repeats (ITRs) are the only necessary viral sequences for packaging and integration of rAAV (Kearns *et al*., 1996; Kotin *et al*., 1990; Ponnazhagan *et al*., 1997a; Weitzman *et al*., 1994; Yang *et al*., 1997). Therapeutic cDNA sequences are substituted for all viral open reading frames, generating rAAV vectors.

Wild type AAV specifically integrates at an AAVS1 loci on chromosome 19 (Kotin *et al*., 1992; Kotin *et al*., 1991; Samulski *et al*., 1991). Site-specific integration into the host chromosome is desirable in the design of viral vectors. This minimizes the risk of insertional-mutagenesis while the integration capabilities of the vector remain intact. However, since Rep is required for the site-specific integration of AAV, recombinant vectors have lost their ability to integrate site specifically into the host chromosome (Ponnaz-hagan *et al*., 1997a). Nonetheless, with the knowledge that AAV specifically integrates at AAVS1 loci on chromosome 19, (Kotin *et al*., 1992; Kotin *et al*., 1991; Samulski *et al*., 1991)

*Address correspondence to this author at the Department of Internal Medicine, Division of Pulmonary and Critical Care, 100 EMRB, University of Iowa, College of Medicine, Iowa City, IA, 52242, USA; Tel: (319) 335-7590; E-mail: sanlioglus@mail.medicine.uiowa.edu

1566-5232/00 $20.00+.00 © 2001 Bentham Science Publishers Ltd.

several studies have been initiated to design rAAV vectors for targeted site-specific integration and/or gene correction (Russell and Hirata, 1998).

Although rAAV2 has been a promising gene therapy vector, its potential for therapeutic use has been limited due to its inefficient transduction in certain tissue types, such as the liver or the lung (Duan *et al*., 1998b; Xiao *et al*., 1998). To further increase the utility of rAAV2 for gene therapy, studies were conducted to elucidate the underlying mechanism(s) and barriers to rAAV transduction. It is the aim of this review to discuss current developments in the area of transduction biology of rAAV2 vectors.

STAGES OF AAV TRANSDUCTION

Based on our current knowledge, AAV transduction can be categorized into five discrete stages including: 1) Binding, 2) Endocytosis, 3) Trafficking to the nucleus, 4) Nuclear entry and uncoating of the virus, and 5) Nuclear gene conversion events. Our current understanding of each of these stages will be highlighted in the below sections.

Viral Binding

Heparan sulfate proteoglycan (HSPG), the first identified receptor for AAV-2 (Summerford and Samulski, 1998), serves primarily as an anchor for viral attachment to the cell surface. The abundance of the membrane associated heparin sulfate proteoglycan was recently identified as one of the critical aspects, which determines the efficiency of rAAV infection in various cell types (Summerford and Samulski, 1998). For instance, the low abundance of HSPG on the apical surface of airway epithelial cells has been implicated for the inefficient AAV transduction in the lung (Duan *et al*., 1998b). However, from these studies it is also clear that other low affinity receptors must also exist at the apical surface of airway cells. In a recent study, fluorescent-labeled rAAV (Cy3AAV) was used in order to characterize the binding properties of AAV-2 (Sanlioglu *et al*., 2000b). In this study, heparin was used as a specific competitor of AAV-2 to block the viral particles from binding to the HSPG receptor. The result suggested that increasing concentrations of heparin competed with Cy3AAV for binding to Hela cells. Although the majority of AAV-2 binding was competitively inhibited with 40μM heparin, some residual binding of rAAV was observed even with higher concentrations of heparin (1 mM). Since other types of rAAV co-receptors have been reported, (Qing *et al*., 1999; Summerford *et al*., 1999) this residual binding was attributed to the presence of other rAAV2 receptors on the cell surface. Consequently, further investigation showed that although HSPG is the main receptor necessary for AAV attachment, the expression of HSPG alone was insufficient for the optimum AAV transduction. Successful viral internalization was only achieved when additional co-receptors, such as fibroblast growth factor 1 (FGFR-1) (Qing *et al*., 1999) and V 5 integrin (Summerford *et al*., 1999) were also present on the cell surface.

Viral Internalization and Endocytosis

The mechanism of AAV endocytosis has recently been investigated using fluorescent-tagged AAV virions (Bartlett and Samulski, 1998; Bartlett *et al*., 1998; Bartlett *et al*., 2000; Hansen *et al*., 2000; Sanlioglu *et al*., 2000b). A co-localization study using Cy3AAV with a fluid phase marker (FITC-labeled Dextran) (Shurety *et al*., 1998) and a clathrin-dependent endocytic marker (FITC-labeled transferrin) (Daro *et al*., 1996; Duan *et al*., 1999) was conducted to examine whether AAV endocytosis occurred through clathrin-mediated mechanisms. These experiments demonstrated that the majority of Cy3AAV particles were co-endocytosed with FITC-labeled transferrin (Fig. **1**, Panel B). Little overlap was seen between Cy3AAV and the fluid phase marker FITC-labeled Dextran (Fig **1**, Panel A). These results suggest that similar to the adenovirus, AAV is predomin-antly endocytosed through clathrin-coated pits. To further demonstrate conclusively that AAV endocytosis occurs through clathrin-mediated mechanisms, a dominant-negative mutant form of dynamin encoded in a recombinant adenovirus (Ad.K44Adynamin) (Ceresa *et al*., 1998; Kao *et al*., 1998) was used to modulate rAAV endocytosis. Hela cells were infected at an MOI of 1000 DNA particles/cell with Ad.K44Adynamin or Ad.CMVLacZ, which was used as a negative control. Following infection with the adenovirus for 48hours, Cy3AAV trafficking was assessed. As seen in (Fig. **1**), only Ad.K44Adynamin was capable of inhibiting endocytosis of Cy3AAV (Fig. **1**, Panels I and J). In contrast, no effect on endocytosis of AAV was seen in cells infected with Ad.CMVLacZ (Fig. **1**, Panels G and H). However, expression of neither K44Adymamin (Panels E and F) nor LacZ (Panels C and D) affected the binding of Cy3AAV to cells. These morphologic results were consistent with clathrin-mediated endocytosis of AAV. To assess whether membrane-associated Cy3AAV in K44Adynamin expressing cells remains externally on the cell surface or resides in a sub-membrane intracellular compartment, a selective trypsinization assay followed by Hirt Southern blot analysis of viral DNA was performed. As shown in (Fig. **1**), Panel K, only Ad.K44Adynamin infected cells demonstrated a reduction in trypsin resistant, internalized viral genomes after incubation for 2 hrs at 37°C. These studies also demonstrated that viral binding at 4°C remained unaffected by expression of either dynamin or LacZ transgenes. In summary, using both morphologic and molecular criteria, these observations support the notion that AAV endocytosis is mediated through clathrin-dependent mechanisms. Other studies have also shown that overexpression of the dynamin mutant (Ad.K44Adynamin) significantly reduced the endocytosis of rAAV virus (Bartlett *et al*., 2000; Duan *et al*., 1999).

Recent reports have implicated other receptors, such as Human Fibroblast Growth Factor Receptor-1 (hFGF-1) (Qing *et al*., 1999) or V 5 integrin (Summerford *et al*., 1999) for efficient AAV-2 infection. Integrins are molecules involved in cell adhesion and motility (Brooks *et al*., 1997; Klemke *et al*., 1997). Cy3AAV studies using blocking monoclonal antibodies developed against the human V 5 integrin suggested that although V 5 integrin is required for the internalization of AAV-2, it is not solely necessary for the binding of AAV virus to Hela cells (Sanlioglu *et al*.,

2000b). In addition, it is well known that integrins are associated with multiple small intracellular signaling molecules, including Rho, Rac and Cdc42, GTPases through which actin fibers facilitate motility and the endocytic pathways (Nobes and Hall, 1995; Parsons, 1996). Dominant negative mutants of these proteins were tested in order to examine the potential role of these small GTP binding proteins in AAV endocytosis. In this context, inactivation of Rac1 by expression of a dominant negative form of Rac1, Ad.N17Rac1 (Kim *et al*., 1998), led to the inhibition of AAV endocytosis (Sanlioglu *et al*., 2000b). These studies suggested that the endocytosis of rAAV is dependent on the Rac1.

Intracellular Trafficking to the Nucleus

A pioneering study describing impaired intracellular trafficking of AAV in NIH 3T3 cells was first reported using Cy3 labeled AAV virions (Hansen *et al*., 2000). In this study, despite the efficient binding and endocytosis of AAV in NIH 3T3 cells, the trafficking of rAAV to the nucleus appeared to be significantly impaired, representing a barrier to rAAV transduction. In contrast, infection of cells such as 293 and Hela cells, which are readily transducible by AAV vectors, demonstrated rapid and efficient viral trafficking to nucleus (Bartlett *et al*., 2000; Sanlioglu *et al*., 2000b). Cellular signaling pathways involved in the intracellular trafficking of rAAV virus have not yet been fully elucidated. Nonetheless, the knowledge of how AAV2 co-receptors such as integrins act in other systems, leads to several hypotheses as to how AAV2 traffics to the nucleus. Integrins activate focal adhesion kinases (FAK), also known as $pp125^{FAK}$, through tyrosine phosphorylation (Miyamoto *et al*., 1995). Subsequently, tyrosine phosphorylated FAK recruits Phoshatidylinositol 3-Kinase (PI3K) for activation (Li *et al*., 1998b). Activation of this PI3K pathway leads to the generation of phosphoinositol–3,4-biphosphate (PIP_2) and phosphatidylinositol-3,4,5-triphosphate (PIP_3). These messengers are known to regulate vesicular trafficking (Odorizzi *et al*., 1998) and the rearrangement of cytoskeletal proteins such as actin (Kapeller and Cantley, 1994). It has been reported that PI3-kinase activity is required for the endocytosis and sorting of integrin-linked receptors (Ng *et al*., 1999). Despite the fact that active Rac1 and PI3-Kinase pathways are required for the internalization of adenovirus

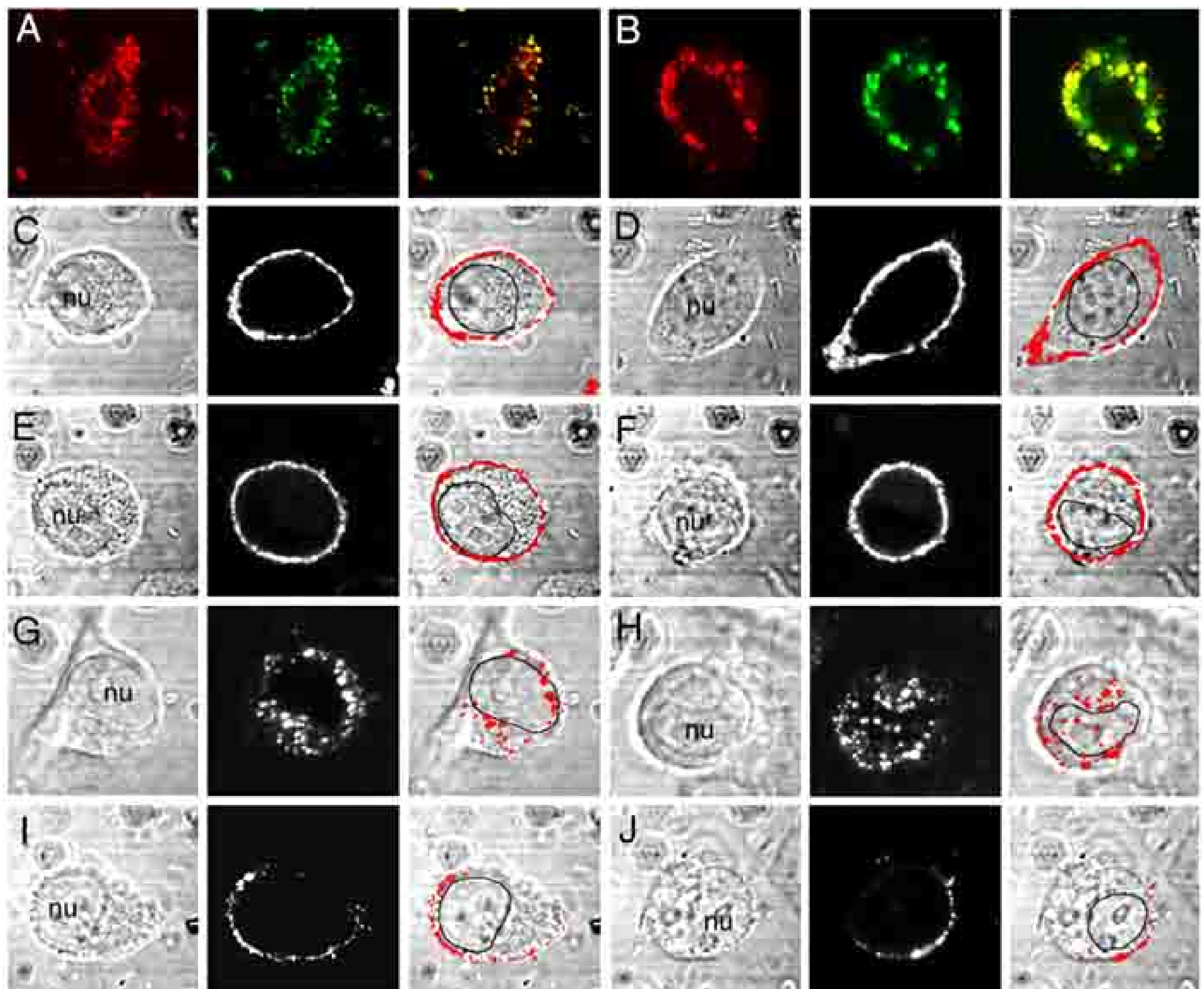

Fig. (1). contd….

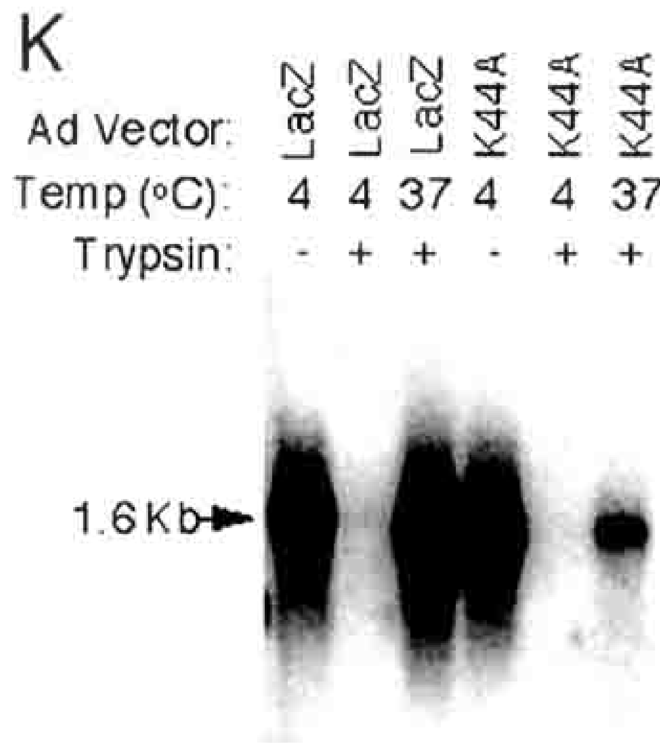

Fig. (1). Endocytosis of AAV occurs through clathrin-coated vesicles. Hela cells were infected with Cy3AAV for 30 minutes at 37^{0} C in the presence of either FITC-dextran (Panel A) or FITC-transferrin (Panel B) . Cells were then fixed and evaluated by confocal fluorescent microscopy. Images in each panel represent Cy3AAV (left in red), FITC (middle in green), and superimposed red and green channels (right). These photomicrographs represent a single 0.5 uM layer. Only one representative example is given from the twenty cells analyzed for each condition. Hela cells were infected with either Ad.K44Adynamin (Panels E, F, I, and J) or Ad.CMVLacZ virus (Panels C, D, G and H) at an MOI of 1000 DNA particles/cell 48 hours prior to infection with Cy3AAV. The cells were incubated with Cy3AAV at 4^{0}C for 1 hour to examine viral binding and were either fixed immediately for analysis (Panels C-F) or were shifted to 37 ^{0}C for 45 minutes to examine effects on endocytosis (Panels G-J). Representative confocal photomicrographs from two cells are shown for each condition. Each of the panels shows the confocal phase contrast image (left), gray scale stack of Cy3AAV images (middle) and Cy3-labeled AAV particles superimposed on the phase contrast image (right). Each confocal image is a stack of six consecutive 0.5 um layers that intersects the nucleus. The nucleus (nu) of each cell is marked and circled for clarity. Southern blotting of Hirt DNA isolated from rAAV infected cells was performed as an alternative approach for evaluating rAAV endocytosis in Ad.K44Adynamin and Ad.CMVLacZ infected (1000 DNA particles/cell) Hela cells (Panel K). 48 hrs following adenoviral infection, Hela cells were infected with unlabeled AV.GFP3ori virus (MOI of 1000 DNA particles/cell) for 1 hour at 4^{0}C, washed, and shifted to 37°C for 2 hrs. Cells were harvested by either direct scraping to determine viral binding (- trypsin) or by trypsinization to remove extracellular virus (+ trypsin). Hirt DNA was prepared from cells subjected to the various conditions and Southern blots were hybridized with P^{32}-labeled EGFP cDNA probe. Single stranded rAAV genomes are marked by a 1.6 Kb-hybridizing band.

(Li *et al*., 1998a; Li *et al*., 1998b), the inhibition of PI3-Kinase pathways in Hela cells did not appear to influence the endocytosis of AAV virus. However, the movement of AAV from the cytosol to the nucleus was significantly impaired when cells were treated with PI3K inhibitors (Sanlioglu *et al*., 2000b). Furthermore, the expression of a dominant negative mutant of Rac1 (N17Rac1) blocked rAAV-mediated induction of PI3K activity. Therefore, Rac1 appears to be localized upstream to PI3K in the AAV2 infection scheme. These studies suggest that Rac1 and PI3K pathways are linked and both appear to affect distinct stages of rAAV transduction (Sanlioglu *et al*., 2000b). The lack of efficient endocytosis and intracellular trafficking of rAAV2 could be potential barriers to transduction in certain cell types.

Modification of AAV capsids via ubiqui-tination during the endosomal processing of AAV has also been suggested to pose yet another barrier for gene therapy in the liver and lung (Duan *et al*., 2000b). In this study, it was suggested that despite the absence of HSPG and other co-receptor(s) for AAV2, the inefficient transduction from the apical surface of airway cells was not due to the viral uptake. Instead, reduced gene expression from recombinant AAV2 vectors was attributed to the ubiquitination of AAV capsids following endocytosis, which reduced nuclear trafficking of AAV2 virus. Interestingly, this barrier appeared to be a concern only when virus entered polarized epithelia from the apical membrane. In contrast, infection, nuclear trafficking, and gene expression from the basolateral membrane with AAV2 was efficient. Such findings suggest that polarity significantly affects the cellular processes of AAV infection. Interestingly, proteosome (Calpain inhibitor I and MG132) and ubiquitin ligase (E3) inhibitors significantly increased the efficiency of AAV gene delivery from the apical surface of polarized airway epithelia. These studies suggested new innovative strategies to escape intracellular processing barriers and increase the efficacy of rAAV mediated gene

delivery in the airway. Interestingly, these proteasome barriers are not present in tissues such as cardiac and skeletal muscle for which rAAV transduction proceeds at high efficiency. These inhibitors however are proven effective in increasing rAAV transduction in liver (Duan *et al*., 2000b).

The timing of AAV escape from the endosome is another unresolved issue. The question remains as to whether AAV breaks free of the endosomes immediately after it enters into the cytoplasm (as is known for adenovirus), or if AAV is transported to the nucleus prior to being liberated from the endosomal compartment. Co-localization of Cy3-labeled AAV with FITC-transferrin containing endosomes near the nucleus suggests that unlike adenovirus, AAV may travel to the nucleus in endosomes (Fig. **1**, Panels A and B). The concen-trated localization of AAV to microtubule organizing centers near the nucleus also support the notion that this region may be important in sorting AAV loaded endosomes prior to nuclear transport.

Most mammalian viruses must reach the nucleus for productive transduction. Cytoskeletal proteins such as actin and tubulin prevent the free diffusion of large particles in the cytoplasm (Seksek *et al*., 1997). Interestingly, microtubules and microfilaments also facilitate the trafficking of viral particles to specific destinations, such as the nucleus or lysosomes (Cudmore *et al*., 1997; Dramsi and Cossart, 1998; Luftig and Lupo, 1994). For instance, nuclear targeting of adeno-virus requires functional microtubules and microfilaments (Fasbender *et al*., 1998; Leopold *et al*., 1998; Li *et al*., 1998a; Patterson and Russell, 1983; Qiu *et al*., 1998; Suomalainen *et al*., 1999). Studies with Cy3AAV indicated that AAV movement to the nucleus is dependent on intact cytoarchitecture of microtubules and microfila-ments. In contrast, the disruption of microtubules and microfilaments did not appear to significantly affect the endocytosis of AAV (Sanlioglu *et al*., 2000b). Further studies are needed to better understand how specific receptor entry pathways are linked to barriers such as ubiquitination, which control intracellular trafficking of AAV2.

Nuclear Transport and Viral Uncoating

It is well known that adenovirus uncoats in the nuclear pores before its genome enters the nucleus. Obviously, this represents another barrier for adenovirus transduction. In case of AAV, however, it is unclear whether it uncoats in the nuclear pores or enters the nucleus uncoated. In fact, 3D recons-truction of Cy3AAV confocal images suggests that AAV can move through the nuclear pores prior to uncoating (Sanlioglu *et al*., 2000b). A similar study conducted by Bartlett et al., (Bartlett *et al*., 2000) suggested that the majority of the Cy3AAV virions are retained in the perinuclear compartment at the time of transgene expression. These observations suggest that the nuclear entry of rAAV might represent another rate-limiting step in rAAV transduction.

Nuclear Gene Conversion

After AAV uncoats in the nucleus, its DNA is converted to a double stranded DNA form. Conversion from the single stranded DNA (ssDNA) to duplex form has been introduced as one of the most important rate limiting steps in AAV transduction (Ferrari *et al*., 1996; Fisher *et al*., 1996a). An alteration in the phosphorylation status of a single-stranded D sequence-binding protein (ssD-BP) has been implicated in this conversion process (Qing *et al*., 1998; Qing *et al*., 1997). Even though the mechanism of this conversion process is not yet clear, both protein tyrosine kinase pathways (Qing *et al*., 1997) and protein tyrosine phosphatase pathways (Sanlioglu and Engelhardt, 1999) are involved in the regulation of this conversion process. Several types of environmental damage, such as UV, hydroxyurea (HU) and ionizing irradiation, have been shown to augment rAAV transduction in cell types exhibiting inefficient transduction (Alexander *et al*., 1994; Hillgenberg *et al*., 1999; Sanlioglu *et al*., 1999; Yakinoglu *et al*., 1988). It is believed that in addition to DNA damage, these environmental stimuli generate cellular factors important in the conversion of single stranded DNA genomes to the expressible duplex forms. Recently, it was suggested that these genotoxic agents augment rAAV transduction by a common mechanism involving the generation of reactive oxygen intermediates (ROIs) (Sanlioglu and Engelhardt, 1999). Furthermore, the production of ROI was also correlated with an alteration in the phosphorylation status of ssD-BP. These results suggest that the cellular redox state plays a crucial role in the regulation of rAAV transduction.

In addition to genotoxic agents, the adenoviral gene product E4orf6 has been implicated in the modulation of rAAV transduction via enhancement of double-stranded DNA synthesis (Ferrari *et al*., 1996; Fisher *et al*., 1996a). Consequently, AAV replication form (Rf) intermediates are generated as byproducts of the AAV lytic phase life cycle. This is expected given that adenovirus is a helper virus, which is required for wt AAV replication (Berns, 1990; Richardson and Westphal, 1981; Straus *et al*., 1976; Ward *et al*., 1998). Lytic phase replication of wt AAV occurs through head-to-head and tail-to-tail replication form double stranded DNA intermediates (Berns, 1990; Richardson and Westphal, 1981; Ward *et al*., 1998). However, genotoxic agents such as hydroxyurea (Yakobson *et al*., 1987) and heat shock treatment (Yakinoglu *et al*., 1988) have also induced helper free wild type AAV DNA synthesis by an unknown mechanism. In contrast to the lytic phase replication of AAV, latent phase persistence of AAV has been predominantly associated with either episomal or integrated rAAV head-to-tail genomes (Duan *et al*., 1998a; Sanlioglu *et al*., 1999; Wu *et al*., 1998; Yang *et al*., 1997). Interestingly, differences exist in the mechanisms by which genotoxic agents and viral genes influence this conversion process. For instance, UV irradiation has been demonstrated to augment the generation of rAAV circular intermediates (Sanlioglu *et al*., 1999). In contrast, adenoviral E4orf6 gene expression has led to augmentation of Rf intermediates. These results suggest that these two pathways may compete for the conversion of the single stranded AAV genomes to expressible forms. Nonetheless, circularized rAAV genomes are the major detectable molecular conversion products seen in the absence of E4orf6 expression in Hela cells. Therefore, cellular genes involved in the formation of rAAV circular forms might be important tools for modulating latent rAAV gene conversion in cells with poor rAAV transduction.

The integration of the viral genomes is essential for prolonged transgene expression. Despite the fact that wild type virus (AAV) integrates at the AAVS1 loci on chromosome 19 (Kotin *et al*., 1992; Kotin *et al*., 1991; Samulski *et al*., 1991), little is known about the mechanism of rAAV integration in the cell. In fact, strong evidence exists that rAAV is mostly retained as large episomal circular concatamers in muscle (Duan *et al*., 1998a; Yang *et al*., 1999), while studies in liver suggest that these large circular concatamers may integrate into host chromosomes at a higher efficiency (Ponnazhagan *et al*., 1997b; Xiao *et al*., 1998). Cellular genes involved in rAAV integration have not yet been identified. However a recent study has suggested that Ataxia-Telangiectasia defective (ATM) cell lines possess enhanced capacity for rAAV2 transduction and stable integration (Sanlioglu *et al*., 2000a). It is presently unclear whether this enhanced capacity for integration in AT cell lines is due to the higher abundance of viral circular form genomes, which may be the direct precursors to integration or whether the enhanced integration is directly linked to the genetic defect. Interestingly, UV irradiation also increases the abundance of rAAV circular form genomes and the extent of rAAV integration in wild type but not in AT cell lines (Sanlioglu *et al*., 1999). This finding suggests that the mechanism of UV induced rAAV transduction might already be maximally elevated in AT cells. Since both ATM defects and UV irradiation enhance the abundance of rAAV circular forms and the integration, it is thought that the rAAV circular forms may represent a pre-integration complex for rAAV.

In addition to DNA synthesis and topoiso-merase inhibitors, several genotoxic agents, such as UV and -irradiation, have been demonstrated to augment rAAV transduction (Alexander *et al*., 1994; Alexander *et al*., 1996; Russell *et al*., 1995; Sanlioglu and Engelhardt, 1999). The mechanism of action for these agents is not clearly understood but it is thought to involve the DNA repair machinery, which is activated by DNA damage. In order to identify cellular genes that are involved in rAAV transduction, a number of cell lines defective in DNA repair were screened. Among them, only ATM exhibited an enhancement in rAAV transduction (Sanlioglu *et al*., 2000a). The ATM gene has been suggested to be an oxidative stress sensor and the absence of ATM function leads to chronic oxidative stress (Rotman and Shiloh, 1997). Since ROIs have been shown to be important for rAAV transduction, the enhanced rAAV transduction present in AT cells appears to substantiate the importance of ROIs for rAAV transduction.

OVERCOMING BARRIERS TO AAV TRANSDUCTION

Based on the above discussions, a model summarizing the rate limiting steps involved in rAAV2 transduction is proposed in (Fig. **2**). These include the binding, internalization, intracellular trafficking (endosomal processing), nuclear targeting, nuclear import, conversion from single stranded DNA to the duplex form (circular or linear) and integration (site specific or random). Depending on the cell type, one or more of these steps might pose a barrier to rAAV transduction. Based on the field's increased understanding of barriers to AAV2 transduction, numerous advances in rAAV technology have been accomplished which have renewed enthusiasm for this vector as a gene therapy tool. Several of these advances are further highlighted below.

In an effort to overcome inefficient transduction associated with the lack of AAV2 receptors in certain target organs, AAV2 capsid was modified by insertion of a 14-amino-acid targeting peptide (L14) into six different putative loops of the AAV2 capsid protein (Girod *et al*., 1999). Certain modifications were capable of redirecting the binding of the virus to an integrin receptor and increased AAV transduction in rAAV resistant cell lines. In addition, a targeted delivery system for AAV2 vector has been developed using a bispecific F(ab')2 antibody (Bartlett *et al*., 1999). In this system, bispecific F(ab')2 antibody mediates a novel interaction between the AAV vector and a specific cell-surface receptor expressed on human megakaryocytes. A 70-fold increase in rAAV2 transduction was observed by this approach. Therefore, in addition to targeting rAAV2 to specific cell types, these studies might be useful to increase rAAV2 transduction in non-permissive cells via redirecting

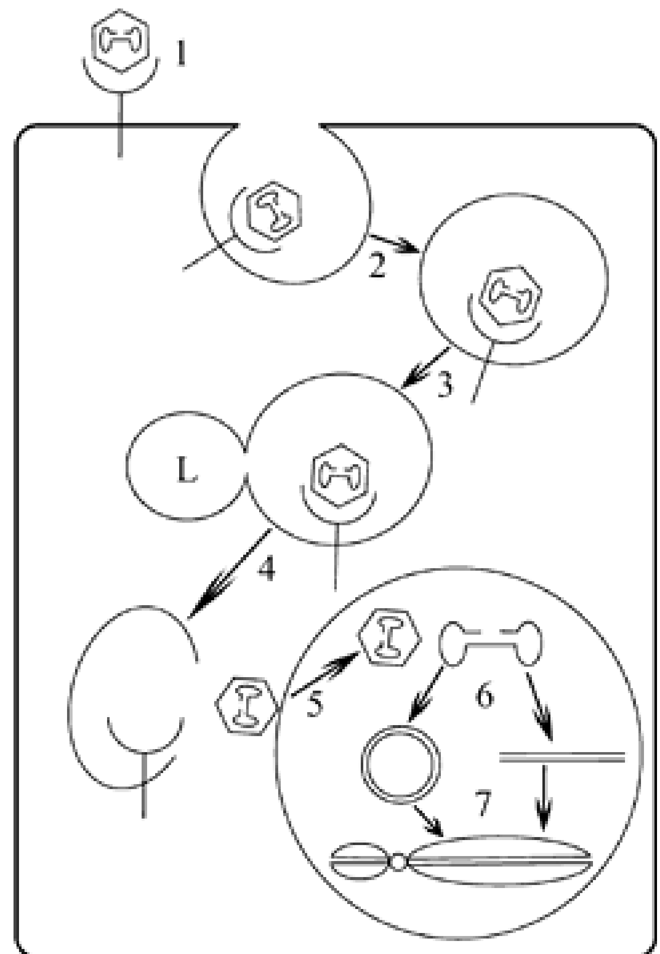

Fig. (2). A schematic model of the rate limiting steps in rAAV transduction. Potential rate limiting steps in rAAV transduction include: 1. Binding, 2. Internalization, 3. Intracellular trafficking, 4. Nuclear targeting, 5. Nuclear import, 6. Conversion from single stranded DNA forms to duplex forms 7. Integration.

the virus to other receptors present on the cell surface. In addition to rAAV2, several other rAAV serotypes (rAAV4, rAAV5) (Chiorini *et al*., 1999; Chiorini *et al*., 1997) are currently being tested for their tropisms and transduction efficiencies in the central nervous system (CNS) (Alisky *et al*., 2000; Davidson *et al*., 2000). Approximately a 10-fold higher trans-duction efficiency was achieved with rAAV4 and/or rAAV5 vectors in the ependymal cells of the lateral ventricle compared to rAAV2 vectors. In addition, AAV5 was also able to transduce not only neuronal cells but also astrocytes. Furthermore, in a different study, it was demonstrated that recombinant AAV5 bound to airway epithelia and mediated gene transfer 50-fold more efficiently than AAV2 (Zabner *et al*., 2000). Therefore, testing different serotypes of rAAV might be useful to improve gene therapy for CNS and lung disorders.

Understanding the molecular mechanism of rAAV transduction has provided opportunities to artificially manipulate the cellular milieu, which controls the efficiency of viral transduction. Since the activation of the Rac1 pathway was necessary for rAAV endocytosis, (Sanlioglu *et al*., 2000b) it was feasible to test whether the expression of a dominant active form of Rac1 (V12Rac1) might be an avenue by which to enhance rAAV transduction. As expected, the expression of V12Rac1 using a recombinant adenovirus (Ad.V12Rac1) in Hela cells augmented rAAV transduction 10-fold as compared to LacZ (Ad.CMVLacZ) expressing cells (data not shown). In the future, this finding might prove useful to pharmacologically overcome problems associated with low transduction due to poor endocytosis in certain cell types. By the same token, since the PI3K pathway is also important for the nuclear targeting of rAAV, dominant active forms of PI3K (or chemical agonists) might enhance transduction in cell types for which impaired intracellular trafficking is a major limiting barrier.

In order to increase the infectious propensity of rAAV, hybrid rAAV vectors such as AAV-B19 (Srivastava *et al*., 1989), HSV-AAV (Fraefel *et al*., 1997; Johnston *et al*., 1997) and adenovirus-AAV (Fisher *et al*., 1996b) have also been generated. These hybrid vectors exhibited a higher level of transduction and larger transgene capacity, while maintaining long-term transgene expression provi-ded by the AAV vector backbone. Considering the fact that HSV (Brehm *et al*., 1999) and adeno-viruses (Chirmule *et al*., 1999) generate an immune response in vivo, administration of such hybrid viruses for gene therapy might be most advantageous in diseases such as cancer (Dachs *et al*., 1997).

The inability to package transgenes larger than 5 Kb into AAV has been one of the biggest drawbacks in AAV mediated gene therapy applica-tions. This size restriction of current rAAV vectors has prevented investigators from using AAV for the treatment of diseases with large therapeutic genes such as Duchenne muscular dystrophy. Key findings which have led to the development of new AAV vectoring technologies capable of increasing the capacity of AAV packaging, include the demonstration that circular concatamerization of AAV genome in muscle occurs through a mechan-isms of intermolecular concatamerization (Yang *et al*., 1999). These studies have lead to the develop-ment of dual vector technologies, which allow for the expression of trans-spliced cDNAs up to 9.4kb. In these studies, heterodimerization of two inde-pendent viral genomes was the key to expressing large intact genomic loci such as the erythopoietin gene (Yan *et al*., 2000) or a split cDNA LacZ reporter gene (Sun *et al*., 2000). Using this same strategy, a dual vector approach was also exploited to augment rAAV mediated gene expression by co-infection with an enhancer carrying vector and promoters or minimal promoter transgene expre-ssing vector (Duan *et al*., 2000a). This particular strategy may be particularly useful for enhancing expression from cDNAs, which just fit into rAAV vectors leaving no room for promoter/enhancer sequences. Consequently, both of these strategies have opened up new avenues to increase the utility of rAAV vectors for gene therapy.

AAV vectors are being tested for their potential to correct genetic defects through mismatch recognition and repair (Inoue *et al*., 1999). In fact, AAV with the titer of 10^4 particles/cell was sufficient to correct all the retrovirally induced mutations in a *neo* gene. This strategy widens the utilization of AAV vectors for gene targeting purposes. The ability to regulate the rate of rAAV integration and targeted integration is essential to generate safer AAV vectors for human gene therapy. Since the mechanism of rAAV integration is not understood, the manipulation of rAAV integration poses a challenge in AAV mediated gene therapy. Some studies to resolve this issue are already underway. For example, transposable elements were tested for their potential to augment rAAV integration (Carter and Samulski, 2000). In this study, Sleeping Beauty transposase clearly enhanced the integration of AAV vector carrying puromycin^r gene in Hela cells. Despite the enhanced rAAV integration, site directed insertion was not achieved due to the nature of the transposase used. However, recent work using a hybrid system could hold the key to achieve this goal. Infection of Hepatoma cells with two helper-dependent adenoviral vectors, one carrying the Rep 78 (Chiorini *et al*., 1996) and the other a transgene flanked by AAV ITRs, resulted in a site specific integration of the transgene at the AAVS1 loci (Recchia *et al*., 1999).

In the absence of substantial integration into stem cells, readministration of viral vectors may be necessary to achieve sustained therapeutic correc-tion. Repeated administration of rAAV vectors has traditionally failed due to the appearance of neutralizing antibodies developed against AAV capsid proteins (Chao *et al*., 1999; Halbert *et al*., 1997; Hernandez *et al*., 1999; Moskalenko *et al*., 2000; Xiao *et al*., 1996). Therefore, the existence of humoral immunity against AAV represents another barrier for AAV mediated gene therapy applications. Transient immunosuppression using anti-CD40 ligand antibody (MR1) and/or CTLA4 immunoglobulin fusion protein (CTLA4Ig) has allowed readministration of rAAV vectors in the lung by suppressing the production of neutralizing antibodies (Halbert *et al*., 1998; Manning *et al*., 1998). By the same token, transient inhibition of CD4+ T-cell function using CD4 antibody prevented activation of memory B cells, thereby allowing productive transduction following repea-ted administration of virus (Chirmule *et al*., 2000). These results suggest that immuno-modulation prior to AAV administration might be necessary to avoid humoral immunity against AAV. In addition, existence of at least six

different AAV serotypes (AAV1 to 6) has been reported (Halbert *et al*., 2000; Rutledge *et al*., 1998; Xiao *et al*., 1999). Considering the fact that each AAV serotype elicits a distinct immune response, combinatorial use of different AAV serotypes was suggested in order to prevent the development of a humoral immune response against AAV (Rutledge *et al*., 1998). Successful repeated transduction of mouse lung using different AAV serotypes has been achieved without the production of cross-reactive neutralizing antibodies (Halbert *et al*., 2000; Rutledge *et al*., 1998). In summary, immuno-modulation and the use of different AAV serotypes are two ways of circumventing the pro-duction of neutralizing antibodies against AAV.

Although the field has made significant progress in understanding how signaling processes influence AAV transduction, further studies are needed to fully understand these molecular path-ways. Nonetheless, these results are encouraging and may provide the means for enhancing AAV transduction in tissues, which are not susceptible to AAV infection. Consequently, the modulation of these molecular pathways and/or the virus itself is essential for increasing the effectiveness of AAV mediated gene transfer for the human gene therapy.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NHLBI) grant HL58340 (J.F.E), the Center for Gene Therapy of Cystic Fibrosis and other Genetic Diseases (J.F.E) co-funded by the National Institute of Health (P30 DK54759) and the Cystic Fibrosis Foundation, and VA Merit Review grant; NIH grant #ES-09607, NIH grant #HL-60316 to G. W. Hunninghake. We also gratefully acknowledge the University of Iowa DERC (NIDDK) for tissue culture media supplies.

REFERENCES

Alexander, I.E., Russell, D.W. and Miller, A.D. (1994) DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors, *J. Virol.,* **68**: 8282-8287.

Alexander, I.E., Russell, D.W., Spence, A.M. and Miller, A.D. (1996) Effects of gamma irradiation on the transduction of dividing and nondividing cells in brain and muscle of rats by adeno-associated virus vectors, *Hum. Gene Ther.,* **7**: 841-850.

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 [In Process Citation], *Neuroreport*, **11**: 2669-2673.

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 [published erratum appears in Nat Biotechnol 1999 Apr;17(4):393], *Nat. Biotechnol.,* **17**: 181-186.

Bartlett, J.S. and Samulski, R.J. (1998) Fluorescent viral vectors: a new technique for the pharmacological analysis of gene therapy, *Nat. Med.*, **4**: 635-637.

Bartlett, J.S., Samulski, R.J. and McCown, T.J. (1998) Selective and rapid uptake of adeno-associated virus type 2 in brain, *Hum. Gene Ther.,* **9**: 1181-1186.

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.

Berns, K.I. (1990) Parvovirus replication, *Microbiol. Rev.,* **54**: 316-329.

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

Brehm, M., Samaniego, L.A., Bonneau, R.H., DeLuca, N.A. and Tevethia, S.S. (1999) Immunogenicity of herpes simplex virus type 1 mutants containing deletions in one or more alpha-genes: ICP4, ICP27, ICP22, and ICP0, *Virology*, **256**: 258-269.

Brooks, P.C., Klemke, R.L., Schon, S., Lewis, J.M., Schwartz, M.A. and Cheresh, D.A. (1997) Insulin-like growth factor receptor cooperates with integrin alpha v beta 5 to promote tumor cell dissemination in vivo, *J. Clin. Invest.*, **99**: 1390-1398.

Carter, B.J., Antoni, B.A. and Klessig, D.F. (1992) Adenovirus containing a deletion of the early region 2A gene allows growth of adeno-associated virus with decreased efficiency, *Virology*, **191**: 473-476.

Carter, P.J. and Samulski, R.J. (2000) Adeno-associated viral vectors as gene delivery vehicles, *Int. J. Mol. Med.*, **6**: 17-27.

Ceresa, B.P., Kao, A.W., Santeler, S.R. and Pessin, J.E. (1998) Inhibition of clathrin-mediated endocytosis selectively attenuates specific insulin receptor signal transduction pathways, *Mol Cell Biol*, **18**: 3862-3870.

Chao, H., Samulski, R., Bellinger, D., Monahan, P., Nichols, T. and Walsh, C. (1999) Persistent expression of canine factor IX in hemophilia B canines, *Gene Ther.,* **6**: 1695-1704.

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

Chiorini, J.A., Wiener, S.M., Yang, L., Smith, R.H., Safer, B., Kilcoin, N.P., Liu, Y., Urcelay, E. and Kotin, R.M. (1996) The roles of AAV Rep proteins in gene expression and targeted integration, *Curr. Top. Microbiol. Immunol.,* **218**: 25-33.

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.

Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R. and Wilson, J. (1999) Immune responses to adenovirus and adeno-associated virus in humans, *Gene Ther.,* **6**: 1574-1583.

Chirmule, N., Xiao, W., Truneh, A., Schnell, M.A., Hughes, J.V., Zoltick, P. and Wilson, J.M. (2000) Humoral immunity to adeno-associated virus type 2 vectors following administration to murine and nonhuman primate muscle, *J. Virol.,* **74**: 2420-2425.

Cudmore, S., Reckmann, I. and Way, M. (1997) Viral manipulations of the actin cytoskeleton [see comments], *Trends Microbiol.*, **5**: 142-148.

Dachs, G.U., Dougherty, G.J., Stratford, I.J. and Chaplin, D.J. (1997) Targeting gene therapy to cancer: a review, *Oncol. Res.*, **9**: 313-325.

Daro, E., van der Sluijs, P., Galli, T. and Mellman, I. (1996) Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling, *Proc. Natl. Acad. Sci. U S A,* **93**: 9559-9564.

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. U S A,* **97**: 3428-3432.

Dramsi, S. and Cossart, P. (1998) Intracellular pathogens and the actin cytoskeleton, *Annu. Rev. Cell. Dev. Biol.*, **14**: 137-166.

Duan, D., Li, Q., Kao, A.W., Yue, Y., Pessin, J.E. and Engelhardt, J.F. (1999) Dynamin is required for recombinant adeno-associated virus type 2 infection, *J. Virol.,* **73**: 10371-10376.

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 [published erratum appears in J Virol 1999 Jan;73(1):861], *J. Virol.,* **72**: 8568-8577.

Duan, D., Yue, Y., Yan, Z. and Engelhardt, J.F. (2000a) A new dual-vector approach to enhance recombinant adeno-associated virus-mediated gene expression through intermolecular cis activation, *Nat. Med.*, **6**: 595-598.

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.,* **9**: 2761-2776.

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

During, M.J. (1997) Adeno-associated virus as a gene delivery system, *Adv. Drug Deliv. Rev.,* **27**: 83-94.

Fasbender, A., Lee, J.H., Walters, R.W., Moninger, T.O., Zabner, J. and Welsh, M.J. (1998) Incorporation of adenovirus in calcium phosphate precipitates enhances gene transfer to airway epithelia in vitro and in vivo, *J. Clin. Invest.*, **102**: 184-193.

Ferrari, F.K., Samulski, T., Shenk, T. and Samulski, R.J. (1996) Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors, *J. Virol.,* **70**: 3227-3234.

Fisher, K.J., Gao, G.P., Weitzman, M.D., DeMatteo, R., Burda, J.F. and Wilson, J.M. (1996a) Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis, *J. Virol.,* **70**: 520-532.

Fisher, K.J., Jooss, K., Alston, J., Yang, Y., Haecker, S.E., High, K., Pathak, R., Raper, S.E. and Wilson, J.M. (1997) Recombinant adeno-associated virus for muscle directed gene therapy, *Nat. Med.*, **3**: 306-312.

Fisher, K.J., Kelley, W.M., Burda, J.F. and Wilson, J.M. (1996b) A novel adenovirus-adeno-associated virus hybrid vector that displays efficient rescue and delivery of the AAV genome, *Hum. Gene Ther.,* **7**: 2079-2087.

Flotte, T.R. and Carter, B.J. (1995) Adeno-associated virus vectors for gene therapy, *Gene Ther.,* **2**: 357-362.

Fraefel, C., Jacoby, D.R., Lage, C., Hilderbrand, H., Chou, J.Y., Alt, F.W., Breakefield, X.O. and Majzoub, J.A. (1997) Gene transfer into hepatocytes mediated by helper virus-free HSV/AAV hybrid vectors, *Mol. Med.*, **3**: 813-825.

Girod, A., Ried, M., Wobus, C., Lahm, H., Leike, K., Kleinschmidt, J., Deleage, G. and Hallek, M. (1999) Genetic capsid modifications allow efficient re-targeting of adeno- associated virus type 2 [published erratum appears in Nat. Med. 1999 Dec;5(12):1438], *Nat. Med.*, **5**: 1052-1056.

Grant, C.A., Ponnazhagan, S., Wang, X.S., Srivastava, A. and Li, T. (1997) Evaluation of recombinant adeno-associated virus as a gene transfer vector for the retina, *Curr. Eye Res.*, **16**: 949-956.

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., 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**: 5932-5941.

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**: 9795-9805.

Hansen, J., Qing, K., Kwon, H.J., Mah, C. and Srivastava, A. (2000) Impaired intracellular trafficking of adeno-associated virus type 2 vectors limits efficient transduction of murine fibroblasts, *J. Virol.,* **74**: 992-996.

Hernandez, Y.J., Wang, J., Kearns, W.G., Loiler, S., Poirier, A. and Flotte, T.R. (1999) Latent adeno-associated virus infection elicits humoral but not cell- mediated immune responses in a nonhuman primate model, *J. Virol.,* **73**: 8549-8558.

Hillgenberg, M., Schlehofer, J.R., von Knebel Doeberitz, M. and Klein-Bauernschmitt, P. (1999) Enhanced sensitivity of small cell lung cancer cell lines to cisplatin and etoposide after infection with adeno-associated virus type 2, *Eur J Cancer*, **35**: 106-110.

Inoue, N., Hirata, R.K. and Russell, D.W. (1999) High-fidelity correction of mutations at multiple chromosomal positions by adeno-associated virus vectors, *J. Virol.,* **73**: 7376-7380.

Johnston, K.M., Jacoby, D., Pechan, P.A., Fraefel, C., Borghesani, P., Schuback, D., Dunn, R.J., Smith, F.I. and Breakefield, X.O. (1997) HSV/AAV hybrid amplicon vectors extend transgene expression in human glioma cells, *Hum. Gene Ther.,* **8**: 359-370.

Kao, A.W., Ceresa, B.P., Santeler, S.R. and Pessin, J.E. (1998) Expression of a dominant interfering dynamin mutant in 3T3L1 adipocytes inhibits GLUT4 endocytosis without affecting insulin signaling, *J. Biol. Chem.*, **273**: 25450-25457.

Kapeller, R. and Cantley, L.C. (1994) Phosphatidylinositol 3-kinase, *Bioessays*, **16**: 565-576.

Kaplitt, M.G., Leone, P., Samulski, R.J., Xiao, X., Pfaff, D.W., O'Malley, K.L. and During, M.J. (1994) Long-term gene expression and phenotypic correction using adeno- associated virus vectors in the mammalian brain, *Nat. Genet.*, **8**: 148-154.

Kearns, W.G., Afione, S.A., Fulmer, S.B., Pang, M.C., Erikson, D., Egan, M., Landrum, M.J., Flotte, T.R. and Cutting, G.R. (1996) Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line, *Gene Ther.,* **3**: 748-755.

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. U S A,* **93**: 14082-14087.

Kim, K.S., Takeda, K., Sethi, R., Pracyk, J.B., Tanaka, K., Zhou, Y.F., Yu, Z.X., Ferrans, V.J., Bruder, J.T., Kovesdi, I., Irani, K., Goldschmidt-Clermont, P. and Finkel, T. (1998) Protection from reoxygenation injury by inhibition of rac1, *J. Clin. Invest.*, **101**: 1821-1826.

Klemke, R.L., Cai, S., Giannini, A.L., Gallagher, P.J., de Lanerolle, P. and Cheresh, D.A. (1997) Regulation of cell motility by mitogen-activated protein kinase, *J. Cell Biol.*, **137**: 481-492.

Kotin, R.M. (1994) Prospects for the use of adeno-associated virus as a vector for human gene therapy, *Hum. Gene Ther.,* **5**: 793-801.

Kotin, R.M., Linden, R.M. and Berns, K.I. (1992) Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination, *Embo. J.*, **11**: 5071-5078.

Kotin, R.M., Menninger, J.C., Ward, D.C. and Berns, K.I. (1991) Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter, *Genomics*, **10**: 831-834.

Kotin, R.M., Siniscalco, M., Samulski, R.J., Zhu, X.D., Hunter, L., Laughlin, C.A., McLaughlin, S., Muzyczka, N., Rocchi, M. and Berns, K.I. (1990) Site-specific integration by adeno-associated virus, *Proc. Natl. Acad. Sci. U S A,* **87**: 2211-2215.

Leopold, P.L., Ferris, B., Grinberg, I., Worgall, S., Hackett, N.R. and Crystal, R.G. (1998) Fluorescent virions: dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells, *Hum. Gene Ther.,* **9**: 367-378.

Lewis, P.F. and Emerman, M. (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus, *J. Virol.,* **68**: 510-516.

Li, E., Stupack, D., Bokoch, G.M. and Nemerow, G.R. (1998a) Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases, *J. Virol.,* **72**: 8806-8812.

Li, E., Stupack, D., Klemke, R., Cheresh, D.A. and Nemerow, G.R. (1998b) Adenovirus endocytosis via alpha(v) integrins requires phosphoinositide- 3-OH kinase, *J. Virol.,* **72**: 2055-2061.

Luftig, R.B. and Lupo, L.D. (1994) Viral interactions with the host-cell cytoskeleton: the role of retroviral proteases, *Trends Microbiol.*, **2**: 178-182.

Manning, W.C., Zhou, S., Bland, M.P., Escobedo, J.A. and Dwarki, V. (1998) Transient immunosuppression allows transgene expression following readministration of adeno-associated viral vectors, *Hum. Gene Ther.,* **9**: 477-485.

Miller, D.G., Adam, M.A. and Miller, A.D. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection [published erratum appears in Mol Cell Biol 1992 Jan;12(1):433], *Mol. Cell Biol.*, **10**: 4239-4242.

Miyamoto, S., Teramoto, H., Coso, O.A., Gutkind, J.S., Burbelo, P.D., Akiyama, S.K. and Yamada, K.M. (1995) Integrin function: molecular hierarchies of cytoskeletal and signaling molecules, *J. Cell Biol.*, **131**: 791-805.

Moskalenko, M., Chen, L., van Roey, M., Donahue, B.A., Snyder, R.O., McArthur, J.G. and Patel, S.D. (2000) Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure, *J. Virol.,* **74**: 1761-1766.

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

Ng, T., Shima, D., Squire, A., Bastiaens, P.I., Gschmeissner, S., Humphries, M.J. and Parker, P.J. (1999) PKCalpha regulates beta1 integrin-dependent cell motility through association and control of integrin traffic, *Embo. J.*, **18**: 3909-3923.

Nobes, C.D. and Hall, A. (1995) Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility, *Biochem. Soc. Trans.*, **23**: 456-459.

Odorizzi, G., Babst, M. and Emr, S.D. (1998) Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body, *Cell*, **95**: 847-858.

Parsons, J.T. (1996) Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins, *Curr. Opin. Cell Biol.*, **8**: 146-152.

Patterson, S. and Russell, W.C. (1983) Ultrastructural and immunofluorescence studies of early events in adenovirus-HeLa cell interactions, *J. Gen. Virol.*, **64**: 1091-1099.

Ponnazhagan, S., Erikson, D., Kearns, W.G., Zhou, S.Z., Nahreini, P., Wang, X.S. and Srivastava, A. (1997a) Lack of site-specific integration of the recombinant adeno-associated virus 2 genomes in human cells, *Hum. Gene Ther.,* **8**: 275-284.

Ponnazhagan, S., Mukherjee, P., Yoder, M.C., Wang, X.S., Zhou, S.Z., Kaplan, J., Wadsworth, S. and Srivastava, A. (1997b) Adeno-associated virus 2-mediated gene transfer in vivo: organ-tropism and expression of transduced sequences in mice, *Gene*, **190**: 203-210.

Qing, K., Khuntirat, B., Mah, C., Kube, D.M., Wang, X.S., Ponnazhagan, S., Zhou, S., Dwarki, V.J., Yoder, M.C. and Srivastava, A. (1998) Adeno-associated virus type 2-mediated gene transfer: correlation of tyrosine phosphorylation of the cellular single-stranded D sequence- binding protein with transgene expression in human cells in vitro and murine tissues in vivo, *J. Virol.,* **72**: 1593-1599.

Qing, K., Mah, C., Hansen, J., Zhou, S., Dwarki, V. and Srivastava, A. (1999) Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2 [see comments], *Nat. Med.*, **5**: 71-77.

Qing, K., Wang, X.S., Kube, D.M., Ponnazhagan, S., Bajpai, A. and Srivastava, A. (1997) Role of tyrosine phosphorylation of a cellular protein in adeno- associated virus 2-mediated transgene expression, *Proc. Natl. Acad. Sci. U S A,* **94**: 10879-10884.

Qiu, C., De Young, M.B., Finn, A. and Dichek, D.A. (1998) Cationic liposomes enhance adenovirus entry via a pathway independent of the fiber receptor and alpha(v)-integrins, *Hum. Gene Ther.,* **9**: 507-520.

Recchia, A., Parks, R.J., Lamartina, S., Toniatti, C., Pieroni, L., Palombo, F., Ciliberto, G., Graham, F.L., Cortese, R., La Monica, N. and Colloca, S. (1999) Site-specific integration mediated by a hybrid adenovirus/adeno- associated virus vector, *Proc. Natl. Acad. Sci. U S A,* **96**: 2615-2620.

Richardson, W.D. and Westphal, H. (1981) A cascade of adenovirus early functions is required for expression of adeno-associated virus, *Cell*, **27**: 133-141.

Rotman, G. and Shiloh, Y. (1997) Ataxia-telangiectasia: is ATM a sensor of oxidative damage and stress?, *Bioessays*, **19**: 911-917.

Russell, D.W., Alexander, I.E. and Miller, A.D. (1995) DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors, *Proc. Natl. Acad. Sci. USA,* **92**: 5719-5723.

Russell, D.W. and Hirata, R.K. (1998) Human gene targeting by viral vectors [see comments], *Nat. Genet.*, **18**: 325-330.

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.

Samulski, R.J., Chang, L.S. and Shenk, T. (1987) A recombinant plasmid from which an infectious adeno-associated virus genome can be excised in vitro and its use to study viral replication, *J. Virol.,* **61**: 3096-3101.

Samulski, R.J., Chang, L.S. and Shenk, T. (1989) Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression, *J. Virol.,* **63**: 3822-3828.

Samulski, R.J., Srivastava, A., Berns, K.I. and Muzyczka, N. (1983) Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV, *Cell*, **33**: 135-143.

Samulski, R.J., Zhu, X., Xiao, X., Brook, J.D., Housman, D.E., Epstein, N. and Hunter, L.A. (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19 [published erratum appears in EMBO J 1992 Mar;11(3):1228], *Embo. J.*, **10**: 3941-3950.

Sanlioglu, S., Benson, P. and Engelhardt, J.F. (2000a) Loss of ATM function enhances recombinant adeno-associated virus transduction and integration through pathways similar to UV irradiation, *Virology*, **268**: 68-78.

Sanlioglu, S., Benson, P.K., Yang, J., Atkinson, E.M., Reynolds, T. and Engelhardt, J.F. (2000b) Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation, *J. Virol.,* **74**: 9184-9196.

Sanlioglu, S., Duan, D. and Engelhardt, J.F. (1999) Two independent molecular pathways for recombinant adeno-associated virus genome conversion occur after UV-C and E4orf6 augmentation of transduction, *Hum. Gene Ther.,* **10**: 591-602.

Sanlioglu, S. and Engelhardt, J.F. (1999) Cellular redox state alters recombinant adeno-associated virus transduction through tyrosine phosphatase pathways, *Gene Ther.,* **6**: 1427-1437.

Seksek, O., Biwersi, J. and Verkman, A.S. (1997) Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus, *J. Cell Biol.*, **138**: 131-142.

Shurety, W., Stewart, N.L. and Stow, J.L. (1998) Fluid-phase markers in the basolateral endocytic pathway accumulate in response to the actin assembly-promoting drug Jasplakinolide, *Mol. Biol. Cell*, **9**: 957-975.

Srivastava, A. (1994) Parvovirus-based vectors for human gene therapy, *Blood Cells*, **20**: 531-536.

Srivastava, C.H., Samulski, R.J., Lu, L., Larsen, S.H. and Srivastava, A. (1989) Construction of a recombinant human parvovirus B19: adeno-associated virus 2 (AAV) DNA inverted terminal repeats are functional in an AAV- B19 hybrid virus, *Proc. Natl. Acad. Sci. U S A,* **86**: 8078-8082.

Straus, S.E., Sebring, E.D. and Rose, J.A. (1976) Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis, *Proc. Natl. Acad. Sci. U S A,* **73**: 742-746.

Summerford, C., Bartlett, J.S. and Samulski, R.J. (1999) AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection [see comments], *Nat. Med.*, **5**: 78-82.

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.

Sun, L., Li, J. and Xiao, X. (2000) Overcoming adeno-associated virus vector size limitation through viral DNA heterodimerization, *Nat. Med.*, **6**: 599-602.

Suomalainen, M., Nakano, M.Y., Keller, S., Boucke, K., Stidwill, R.P. and Greber, U.F. (1999) Microtubule-dependent plus- and minus end-directed motilities are competing processes for nuclear targeting of adenovirus, *J. Cell Biol.*, **144**: 657-672.

Ward, P., Dean, F.B., O'Donnell, M.E. and Berns, K.I. (1998) Role of the adenovirus DNA-binding protein in in vitro adeno-associated virus DNA replication, *J. Virol.,* **72**: 420-427.

Weitzman, M.D., Kyostio, S.R., Kotin, R.M. and Owens, R.A. (1994) Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA, *Proc. Natl. Acad. Sci. U S A,* **91**: 5808-5812.

Wu, P., Phillips, M.I., Bui, J. and Terwilliger, E.F. (1998) Adeno-associated virus vector-mediated transgene integration into neurons and other nondividing cell targets, *J. Virol.,* **72**: 5919-5926.

Xiao, W., Berta, S.C., Lu, M.M., Moscioni, A.D., Tazelaar, J. and Wilson, J.M. (1998) Adeno-associated virus as a vector for liver-directed gene therapy, *J. Virol.,* **72**: 10222-10226.

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.

Xiao, X., Li, J. and Samulski, R.J. (1996) Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector, *J. Virol.,* **70**: 8098-8108.

Yakinoglu, A.O., Heilbronn, R., Burkle, A., Schlehofer, J.R. and zur Hausen, H. (1988) DNA amplification of adeno-associated virus as a response to cellular genotoxic stress, *Cancer Res.*, **48**: 3123-3129.

Yakobson, B., Koch, T. and Winocour, E. (1987) Replication of adeno-associated virus in synchronized cells without the addition of a helper virus, *J. Virol.,* **61**: 972-981.

Yan, Z., Zhang, Y., Duan, D. and Engelhardt, J.F. (2000) From the cover: trans-splicing vectors expand the utility of adeno- associated virus for gene therapy [see comments], *Proc. Natl. Acad. Sci. U S A,* **97**: 6716-6721.

Yang, C.C., Xiao, X., Zhu, X., Ansardi, D.C., Epstein, N.D., Frey, M.R., Matera, A.G. and Samulski, R.J. (1997) Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration in vivo and in vitro, *J. Virol.,* **71**: 9231-9247.

Yang, J., Zhou, W., Zhang, Y., Zidon, T., Ritchie, T. and Engelhardt, J.F. (1999) Concatamerization of adeno-associated virus circular genomes occurs through intermolecular recombination, *J. Virol.,* **73**: 9468-9477.

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.