

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

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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 intermediates 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 (Ponnazhagan *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)

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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 40 μ M 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 colocalization 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 predominantly 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.CMV Δ LacZ, which was used as a negative control. Following infection with the adenovirus for 48 hours, 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.CMV Δ LacZ (Fig. 1, Panels G and H). However, expression of neither K44Adynamin (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 Phosphatidylinositol 3-Kinase (PI3K) for activation (Li *et al.*, 1998b). Activation of this PI3K pathway leads to the generation of phosphoinositol-3,4-bisphosphate (PIP₂) and phosphatidylinositol-3,4,5-triphosphate (PIP₃). 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

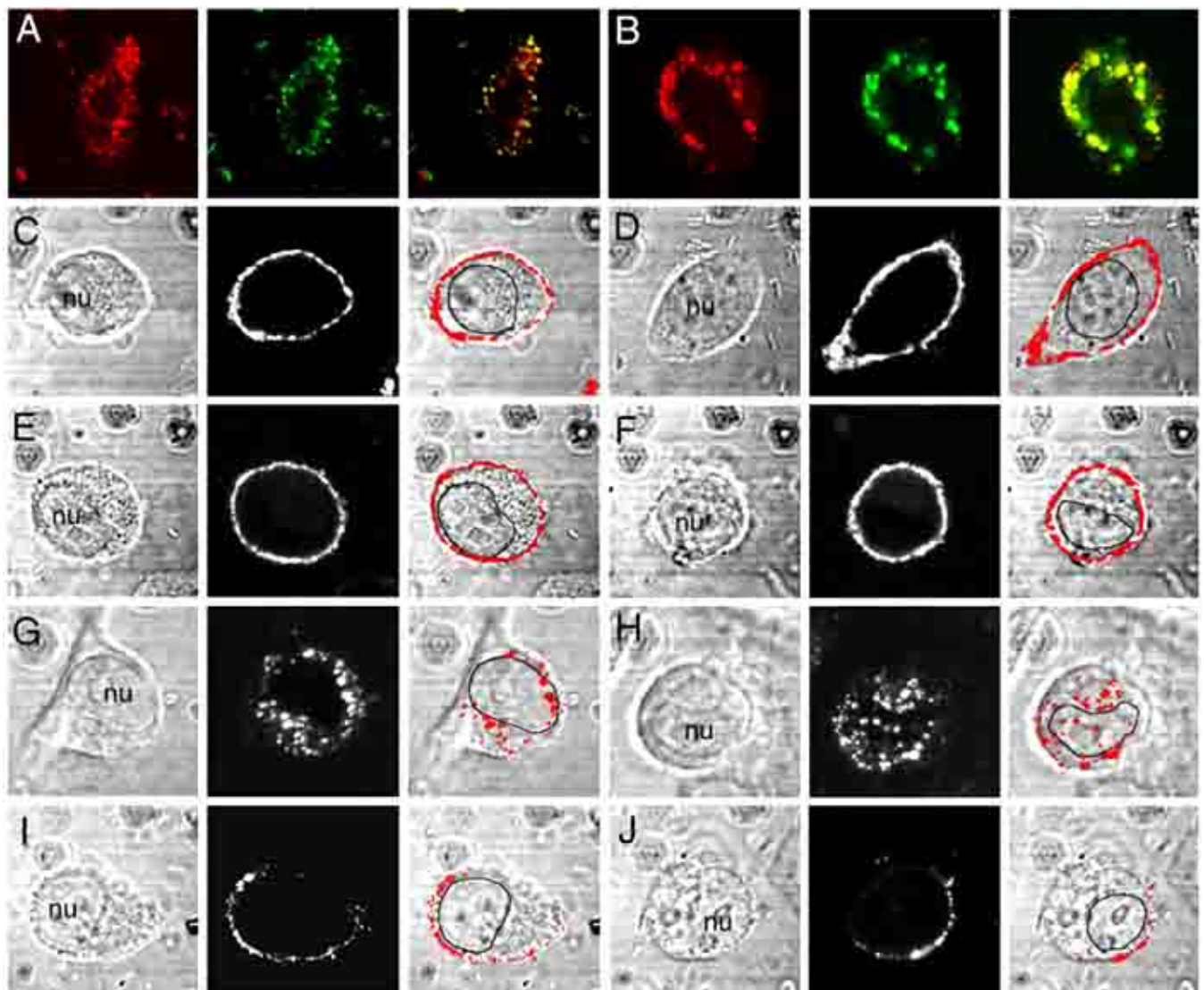


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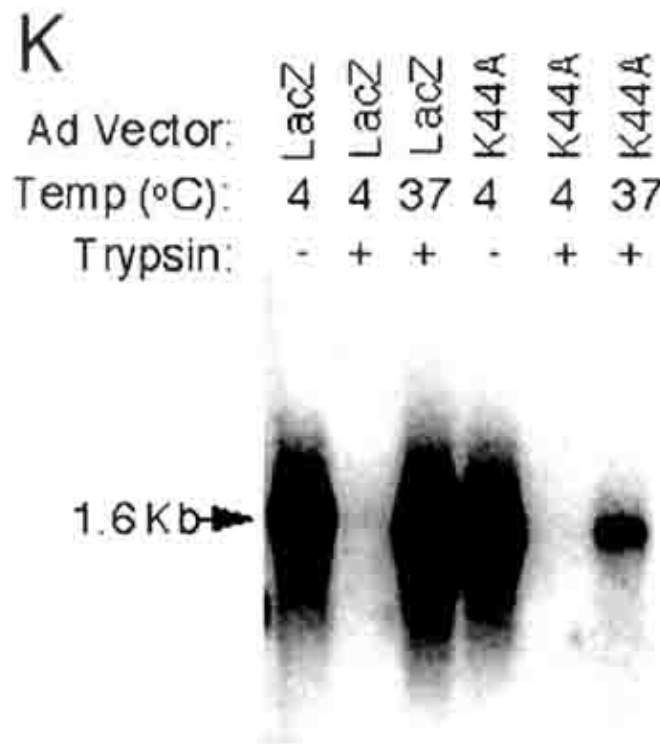


Fig. (1). Endocytosis of AAV occurs through clathrin-coated vesicles. HeLa cells were infected with Cy3AAV for 30 minutes at 37^o C in the presence of either FITC-dextran (Panel A) or FITC-transferrin (Panel B) . Cells were then fixed and evaluated by confocal fluorescent microscopy. Images in each panel represent Cy3AAV (left in red), FITC (middle in green), and superimposed red and green channels (right). These photomicrographs represent a single 0.5 μ M 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^oC for 1 hour to examine viral binding and were either fixed immediately for analysis (Panels C-F) or were shifted to 37^oC 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 μ m 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^oC, washed, and shifted to 37^oC 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 P³²-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 ubiquitination 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, proteasome (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 concentrated 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 microfilaments. 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 reconstruction 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 topoisomerase 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

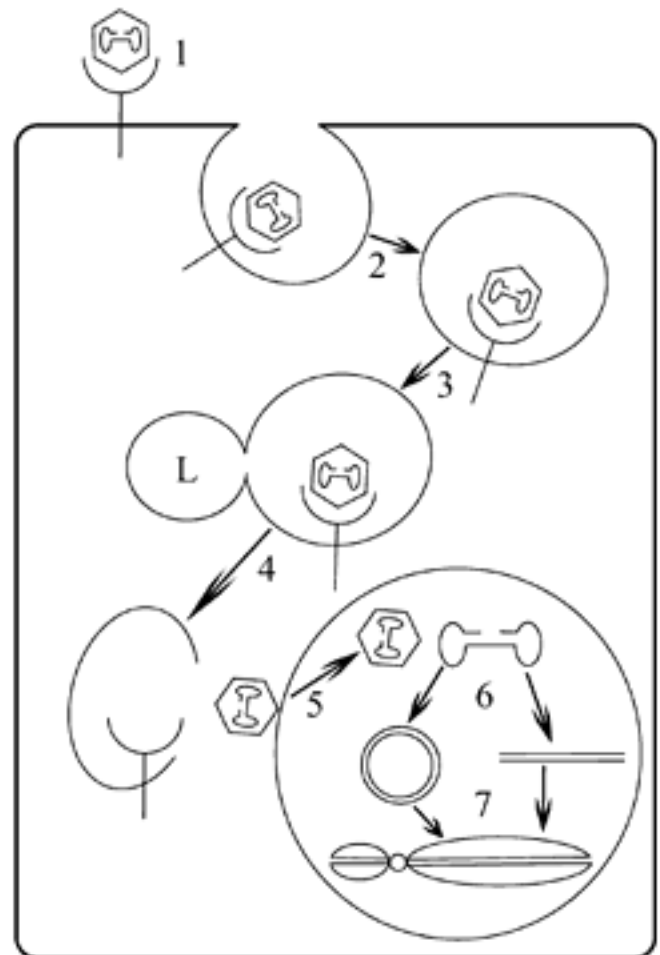


Fig. (2). A schematic model of the rate limiting steps in rAAV transduction. Potential rate limiting steps in rAAV transduction include: 1. Binding, 2. Internalization, 3. Intracellular trafficking, 4. Nuclear targeting, 5. Nuclear import, 6. Conversion from single stranded DNA forms to duplex forms 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 transduction 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 provided by the AAV vector backbone. Considering the fact that HSV (Brehm *et al.*, 1999) and adenoviruses (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 applications. 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 mechanism of intermolecular concatamerization (Yang *et al.*, 1999). These studies have led to the development of dual vector technologies, which allow for the expression of

trans-spliced cDNAs up to 9.4kb. In these studies, heterodimerization of two independent viral genomes was the key to expressing large intact genomic loci such as the erythropoietin 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 expressing 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 correction. 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 repeated administration of virus (Chirmule *et al.*, 2000). These results suggest that immunomodulation 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 production 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 pathways. 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.

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