Nuclear targeting by adeno-associated virus capsid proteins and virions

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ABSTRACT

STEPHEN SNOWDY: Nuclear Targeting by adeno-associated virus capsid proteins and virions
(Under the direction of Richard Jude Samulski)

Adeno-associated virus (AAV) is gaining momentum as a vehicle for delivering genes and DNA material to neuronal tissues. However, there remain impediments to the development of the virus as research and gene therapy tools. One impediment is the difficulty of producing the virus in large amounts and high purity. We developed a protocol for isolating AAV from producer cells that takes advantage of the virus' nuclear assembly. The protocol resulted in recovery of 7-fold more recombinant AAV from producer cells than if the virus was recovered through detergent lysis of producer cells and resulted in AAV that contained far fewer contaminants than was obtainable with other methods. Another impediment in the development of AAV technology is the incomplete understanding of the biology of the virus, including its nuclear targeting by the capsid proteins during initial infection and during progeny viral assembly. We demonstrate that the AAV putative nuclear localization signal, ^{166}PARKRLN^{172}, is not necessary for trafficking the capsid proteins into the nucleus during viral assembly, but the region is necessary for the initial infection by AAV virions. We show that mutation of the ^{166}PARKRLN^{172} region does not interfere with trafficking of AAV capsids or genomes to the nuclear periphery, but virions containing the mutation are unable to facilitate transfer of their encapsulated

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DNA into the nucleoplasm. Virions missing VP2 were fully infectious, demonstrating that the ^{166}PARKRLN^{172} region from VP2 is not required for infectivity. We show that another basic region in the AAV capsid, ^{307}RPKRLN^{311}, plays a role in concentrating AAV capsid proteins in the nucleus during assembly of progeny virions. We mutated several kinase consensus sequences near the AAV ^{166}PARKRLN^{172} region to determine whether the possible phosphorylation sites are important in the infectivity of the virus. We mutated each of three possible kinase phosphorylation targets to either alanine or aspartic acid. We found one site, serine 181, for which transduction efficiency was enhanced by the S181A substitution and for which transduction efficiency was abrogated by the converse S181D substitution. Taken together, the data presented here make strides in AAV production technology and in understanding the molecular biology of the virus.

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
AAVS1	AAV integration site
Ad	Adenovirus
AdDBP	Ad DNA binding protein
ATP	adenosine triphosphate
BR	basic region
BSA	bovine serum albumin
CKII	casein kinase II
CMV	cytomegalovirus
CPV	canine parvovirus
DL	detergent lysis
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
eGFP	enhanced green fluorescent protein
EM	electron micrograph
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HAP	heparin affinity purification

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HSPG	heparin sulfate proteoglycan
HSV	herpes simplex virus
Imp	importin
INM	inner nuclear membrane
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
MVM	minute virus of mice
NE	nuclear envelope
NLS	Nuclear localization signal
nm	nanometers
NPC	nuclear pore complex
NTF	nuclear transport factor
ONM	outer nuclear membrane
ORF	open reading frame
rAAV	recombinant adeno-associated virus
RBE	rep binding protein
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl phosphate polyacrylamide electrophoresis
ssD-BP	single stranded D-sequence binding protein

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ssDNA	single-stranded deoxyribonucleic acid
TR	terminal repeat
wt	wild-type

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I. INTRODUCTION AND BACKGROUND

I.A. Introduction to this thesis

Scientists and physicians have spoken for years of the possibility of replacing faulty or missing genes as a modality for treating genetic diseases of the brain. Through a great deal of effort by researchers, progress towards gene therapy success in the context of several diseases has emerged and will likely reshape medicine of the 21st century. A review of the literature reveals hundreds of citations in which investigators report progress towards successful gene therapy, including cancer, hemophilia, muscular dystrophy, and others. Particularly encouraging progress is being reported in research aimed at employing gene therapy in treatment of neurological diseases such as cancer, Parkinson's Disease, epilepsy, ischemic injury, and liposomal storage disorders.

A progress-limiting aspect of gene therapy in the brain is the lack of methods providing for efficient delivery of genes to the nervous system. Adeno-associated virus (AAV) is emerging as a lead candidate for delivering genes in the treatment of genetic diseases that affect the nervous system. As a gene delivery reagent, AAV offers several advantages over other technologies available for the delivery of genes, which include both virus-based and non virus-based systems for gene delivery. Of all the viruses capable of efficiently delivering genetic material in a gene therapy setting, AAV is the only virus that has never been associated with a human disease. In addition, AAV is one of

only a handful of viruses capable of permanent placement of its recombinant DNA cargo (the others belonging to the retrovirus family) and is capable of delivering and expressing genes in non-dividing cells. AAV's safety, persistent gene expression, and its ease of genetic manipulation make the virus an ideal candidate for gene therapy applications in the brain.

Though there are several non-viral methods available for delivering genes to animal tissues, including naked DNA, lipofection reagents, synthetic nucleophilic protein carriers, and others, these methods are limited in their utility in gene therapy. These limitations include inefficiency of gene transfer, only temporary gene placement, and unknown toxicity and immunogenicity.

The advantages of AAV have precipitated a great body of work demonstrating the virus' utility in transferring genes to several areas of the brain, including neurons of the hypothalamus, inferior colliculus, hippocampus, optic nerve, and the striatum. Some of the diseases being pursued as targets of AAV-mediated gene therapy include epilepsy, Parkinson's Disease, obesity related to leptin deficiency, degenerative retinopathy, Alzheimer's disease, and cancer, though this list is not exhaustive.

Despite its advantages as a gene therapy and gene delivery reagent, AAV has some limitations. For one, the virus is difficult to manufacture in high titer and high purity. Also, the virus' molecular biology is not well understood, which results in difficulties during the regulatory approval process of the virus for clinical trials.

This body of work contributes to removing impediments to AAV's progress as a gene therapy vector and gene delivery reagent. First, it will introduce the reader to the nuclear structure and cytonuclear transport of proteins, as AAV proteins must target the nuclei of its host cells in order to replicate, and the assembly of progeny virions takes place in the nuclei of host cells. The introduction to nuclear structure and cytonuclear protein transport will be followed by an introduction to adeno-associated virus. Data will then be presented to demonstrate an improved method of AAV production that results in virus of titer and purity that represents a great step forward in AAV research, an improvement that was necessary to carry out some of the experiments in this project. Lastly, this work will present data that takes steps towards understanding the trafficking mechanisms through which adeno-associated virus infects its target cells, in particular, how the virus targets the nuclei of its target cells during its infection cycle.

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I.B. Introduction to cytonuclear transport of proteins

The nucleus was identified in the 17th century by Leeuwenhoek and has since been the subject of exhaustive research looking at all levels of nuclear structure function. The possession of a nucleus by eukaryotic cells has far reaching implications. Since the production of RNA messages occurs in the nucleus (requiring nuclear proteins), and the reading and translation of those messages into proteins occurs in the cytoplasm, processes must exist for the transport of proteins from the cytoplasm, across the nuclear envelope, and into the nucleus. Cytonuclear transport is also required for the regulation of transcription by hormones and other regulatory protein factors. The major nuclear structures that are important to the transport of proteins into and out of the nucleus are the nuclear envelope, together with its lamina and matrix, the nuclear pore complex, and several cytoplasmic and nuclear factors that facilitate the bidirectional movement of proteins across the nuclear envelope. Each of these elements will be discussed in turn.

B.1. The nuclear envelope

The nuclear envelope (NE) surrounds and defines the nucleus and is composed of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM). Both the inner and outer membranes are lipid bilayers, though the proteins contained in the layers differ substantially (Figure 1). The outer membrane is continuous with and resembles the rough endoplasmic reticulum and has functional ribosomes attached. The INM is connected to the cytoplasmic

membrane and does not share the characteristics of the endoplasmic reticulum (reviewed by (Forbes and Johnson 1995).

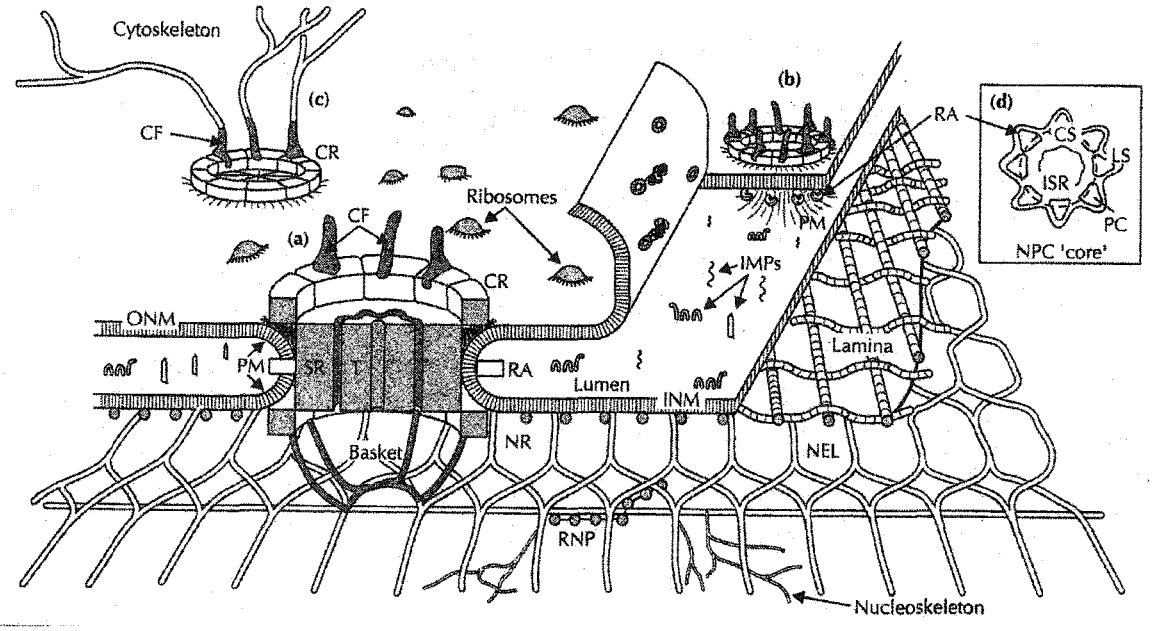

Figure 1. Cutaway diagram of the nuclear envelope. (a), A nuclear pore complex (NPC) cut in half axially. (b) Another nuclear pore complex as viewed from the cytoplasm. (c) A nuclear pore complex emphasizing the cytoplasmic filaments. (d) A cross section through an NPC. Abbreviations: CF, cytoplasmic filaments; CR, ctyplasmic ring; IMPs, integral membrane proteins; INM, inner nuclear membrane; NEL, nuclear envelope lattice; SR, spoke ring; T, transporter; CS, central spoke domain; ISR, inner spoke ring; LS, luminal spoke domain; PC peripheral channel. From Current Opinion in Cell Biology (1995), 7:302

Between the INM and the ONM is the nuclear envelope lumen. The lumen is an aqueous compartment that baths the luminal side of nuclear pore complexes and may communicate with the nucleus, the cytoplasm, and the nuclear pore complex. During cell division, the NE dissociates into NE vesicles, which later reassemble via a calcium signaling mechanism into the intact nuclear membrane.

B.2. The nuclear pore complex

As far as we know, all movement, both passive and active, of molecules into and out of the nucleus occurs through the nuclear pore complex (NPC) (Hanover 1992; Stochaj and Silver 1992; Agutter and Prochnow 1994; Jans and Hubner 1996). The NPC is an organelle-sized structure composed of up to 1000 protein components and has a mass of approximately 10^5 kDa. The NPC is composed of several macromolecular components, including the core, the peripheral channels, cytoplasmic filaments, the cytoplasmic ring, the nucleoplasmic ring, and the basket (Figure 1).

The cytoplasmic filaments are the outermost element of the nuclear pore complex. They extend outward from the nuclear pore complex and attach to the cytoskeleton (Goldberg and Allen 1993). Some evidence suggests that the cytoplasmic filaments may be the initial binding site of proteins that are to be imported through the nuclear pore complex (Richardson, Mills *et al.* 1988). Two proteins have been found to localize on the cytoplasmic filaments, Nup180 and Nup214 (Byrd, Sweet *et al.* 1994; Kraemer, Wozniak *et al.* 1994; Pante, Bastos *et al.* 1994). Evidence for the docking of viral capsid protein to Nup 214 at the

cytoplasmic filament was recently provided for adenovirus (Kraemer, Wozniak *et al.* 1994). Adenovirus was shown to bind Nup214 independent of cytosolic factors immediately before transfer of the viral genome through the NPC. The cytoplasmic filaments are attached at their NPC ends to the cytoplasmic ring of the NPC.

The cytoplasmic ring is held in place by vertical supports that attach to the spoke assembly. It is composed of 8 subunits, has a diameter of 120 nm, a height of 15 nm, and rests atop the nuclear membrane (Hinshaw, Carragher *et al.* 1992; Akey and Radermacher 1993). The protein composition and function of the cytoplasmic ring are unknown, though two candidates are the proteins p62 and Nup180 (Wilken, Kossner *et al.* 1993; Buss, Kent *et al.* 1994). Epitopes of both of these proteins have been localized to the periphery of the NPC, though as mentioned above, it is also possible that Nup180 is a component of the cytoplasmic filaments. p62 appears to be essential for the cytonuclear transport of at least some proteins. Nup180, on the other hand, is not necessary for nuclear transport but may be important in the formation of the NPC.

Working inward from the cytoplasmic ring, the next component of the NPC is the core of the NPC. The major structure of the NPC core is a ring structure within the lumen of the pore. The lumen ring structure consists of eight loops, called radial arms, joined together by eight spokes that traverse the pore membrane (Figure 1). Each spoke has 3 domains, termed the luminal spoke, which is found in the lumen of the NPC and joins the radial arms, the central spoke domain, which crosses the pore membrane, and the spoke ring, which

joins the spokes at the inner spoke domain (see figure, for review see (Goldberg and Allen 1995). Two proteins that are known to localize to the NPC core are gp210 and Pom152. gp210 resembles in structure extracellular transmembrane proteins and has been localized to the lumen of the nuclear envelope (i.e. anchored onto the NPC, but facing outward into the NE lumen versus facing inward to the NPC lumen). Interstingly, antibodies introduced into the NE lumen against gp210 blocked active transport through the nuclear pore complex, suggesting that NPC transport activities may be regulated by events in the NE lumen (Greber and Gerace 1992).

Between each radial arm and the inner spoke ring there are gaps that have been referred to as peripheral channels (Hinshaw, Carragher *et al.* 1992). It has been suggested that these channels are the path through which passive diffusion takes place through the NPC, as the dimensions of the peripheral channels are of similar size to the maximum size of particles that are capable of freely diffusing through the NPC. However, other research suggests that the peripheral channels only exist if the NE is removed from the nuclear pore by detergent.

The nucleoplasmic ring and its associated basket introduces the pore complex to the nucleoplasm. It is of similar structure to the cytoplasmic ring, though its composition and function are unknown (Goldberg and Allen 1995). Between each subunit of the nucleoplasmic ring are filaments (8 filaments per ring) that extend 30-70 nm into the nucleus. These filaments are joined together at their nuclear ends by a ring. The nuclear filaments with the ring attached at

their ends forms a basket structure for which the function is unknown (Goldberg and Allen 1993). It has been suggested that the basket acts as a sieve, preventing the NPC from being clogged by nuclear contents, or that the basket has a role in the regulation of some genes.

The INM is separated from the nuclear contents by the presence of a nuclear envelope lattice and the nuclear lamina. These are both lattice structures that help maintain the morphology and the nucleus, though their other functions remain a mystery.

B.3. Nuclear transport factors

Small molecules are capable of diffusing passively through the NPC. Molecules larger than 25-30 nm, or approximately 20-40 kDa, must be actively and selectively transported across the nuclear membrane (Hall, Hereford *et al.* 1984; Kalderon, Richardson *et al.* 1984; Kalderon, Roberts *et al.* 1984; Lanford and Butel 1984). The selectivity of the NPC transport process requires cellular receptors to identify proteins that are to be transported into the nucleus. These cellular receptors have been termed karyopherins, or importins, and are the first step in identifying and mediating the movement of large proteins and macromolecules into the nucleus. Following identification by importins, the importins then escort the protein through the nuclear pore complex in a process that will be described below.

Proteins that are to be imported into the nucleus are identified to the cell by nuclear localization signals (NLSs), which are short peptide sequences that interact with cytoplasmic receptors (Hall, Hereford et al. 1984; Kalderon,

Richardson et al. 1984; Kambach and Mattaj 1994). There is no clear consensus sequence for NLSs, but there are some trends that help us identify NLSs in the primary and secondary structure of proteins. NLSs tend to be highly concentrated in basic residues (R, K, and L), tend to be very hydrophilic, and tend to be preceded by β-turn/random coil regions (Hall, Hereford *et al.* 1984; Kalderon, Richardson *et al.* 1984; Kambach and Mattaj 1994). NLS may be continuous, or they may be broken into two different regions of a protein, but join together in the folded protein (bipartite NLSs). The NLSs often begin with a proline residue that seems to be a necessary component in NLSs that begin with proline residues. The table below shows a list of NLSs and demonstrates the diversity of NLSs that have been identified to date.

NLS-bearing protein	Putative NLS
SV40 large tumor antigen	PKKKRKV
Polyoma T	VSRKRPRP
	PPKKARED
Lamin L1	VRTTKGKRKRIDV
Lamin A/C	SVTKKRKLE
Cofilin	PEEVKKRKKAV
Human c-myc	PAAKRVKLD
	RQRRNELKRSF
Ad7 E1a	KRPRP
SV40 VP1	APTKRKGS
SV40 VP2/3	PNKKKRK
Human p53	PQPKKKPL
NFkB p50	QRKRQK
NFkB p65	EEKRKR
Chicken v-rel	KSKKQK
Mouse c-abl IV	SALIKKKKKMAP
Influenza virus NS1	DRLRR
	PKQKKRK
Hepatitiis virus delta antigen	RKLKKKIKKL
	PRKRP
Chicken c-ets-1	GKRKNKPK
v-jun	KSRKRKL
Ribosomal protein L29	KTRKHRG
	KHRKHPG
Human DNA ligase I	PKRRTARKQLPKRT
Human hnRNP B1	KTLETVPLERKKREK
Human hnRNP A1	NDFGNYNNQSSNFGPMKGGNFGGRSSGPY
Yeast Histone 2B	GKKRSKAK
Monkey v-sis	RVTIRTVRVRRPPKGKHRK
Human PDGF-A	RESGKKRKRKRLKPT
Mouse Mx1	REKKKFLKRR
Prothymosin a	TKKQKT
VirD2 protein	EYLSRKGKLEL
Maize R protein	GDRRAAPARP
MyoD	CKRKTTNADRRK
	CVNEAFETLKRC
Serum response factor	RRGLKR
CaM d B1	AKKPDGVKKRKS
Yeast Mata2	NKIPIKDLLNPQ
Influenze virus nucleoprotein	AAFEDLRVLS
Hepatitis B virus core protein	SKCLGWLWG

The first step in the nuclear transport of a protein is the identification of the NLS by the cellular importins (Figure 2, step 1). There are two major classes of importins, the α and the β classes, with over a dozen importin alphas and 20 importin betas having been identified in mammalian cells, and several others in yeast (Chook and Blobel 2001). The "classical" nuclear import pathway, which does not include all, or even a majority of nuclear import events, begins with the binding of a preformed importin α_2/importin β_1 heterodimer to the NLS-bearing protein. This heterodimer-mediated binding mechanism is probably due to a unique ability of importin β_1 to lift an autoinhibition from importin α_2 that prevents importin α_2 from binding directly to NLS substrate (Moroianu, Blobel *et al.* 1996). The binding of NLS ligand to heterodimerized importin α and importin β, however, may be the exception, as evidence is starting to accumulate that

suggests that NLS ligands are more likely to bind directly to a member of the importin β group (e.g. importin β2 versus binding an adaptor molecule such as importin α (Chook and Blobel 2001). The heterotrimer then binds to the NPC (Figure 2, steps 2 and 3), possibly at the filament of the cytoplasmic ring via an adaptor called p97 (Adam and Adam 1994), and moves across the NPC via an unknown mechanism (step 4).

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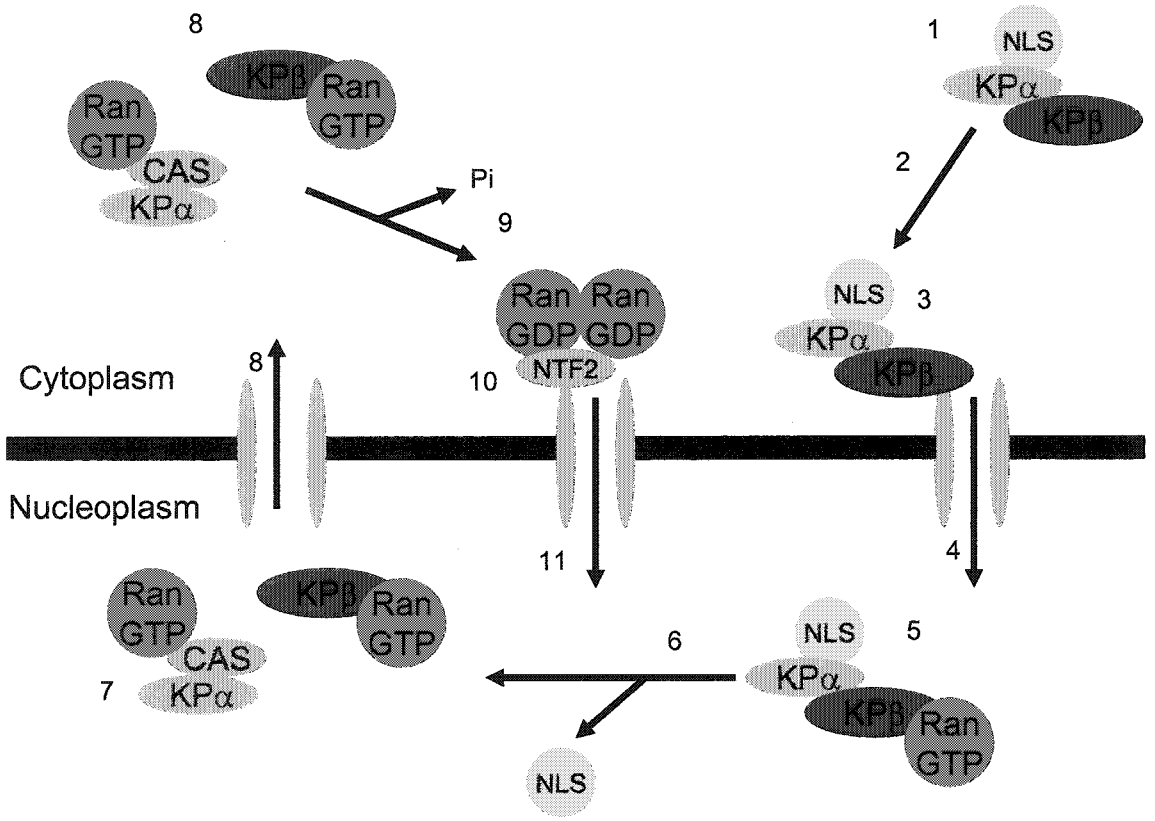

Figure 12. Schematic representation of the nuclear import process. The steps of nuclear import are described in the text.

On arrival in the nucleoplasm, the heterotrimer (or the heterodimer of ligand and importin β if importin α is not involved in the nuclear import) binds to a GTP binding protein called Ran (step 5). RanGTP binds to the incoming complex at importin β and the binding of the complex to RanGTP triggers the dissociation of the NLS-bearing ligand from the rest of the complex, shown as step 6 of figure 2 (Vetter, Arndt *et al.* 1999). RanGTP then escorts the importins

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back into the cytoplasm through the NPC (Schwoebel, Talcott et al. 1998). Once in the cytoplasm, the RanGTP is hydrolyzed to RanGDP (step 9), which signals the readiness of Ran to recycle to the interior of the nucleus. To this end, RanGDP binds to the NPC via nuclear transport factor 2 (NTF2), and is actively transported back into the nucleus (steps 10 and 11), where it is converted to RanGTP in order to prepare it for the next incoming ligand/importin complex (Clarkson, Kent *et al.* 1996; Ribbeck, Lipowsky *et al.* 1998; Smith, Brownawell *et al.* 1998).

B.4. Regulation of nuclear accumulation by phosphorylation status

It is very common for the cytonuclear transport of proteins to be regulated by the phosphorylation status of sites nearby the NLS (Jans and Hubner 1996). Phosphorylation status can affect nuclear transport by masking an NLS, by regulating the affinity of an NLS for nuclear transport factors, or by enhancing cytoplasmic retention of the NLS substrate. The best described NLS, that of the T-antigen of SV40, has been demonstrated to depend on at least two nearby phosphorylation sites for its efficient transport into the nucleus, one for casein kinase II (CKII), and one for *cdc*2 (Jans and Jans 1994; Xiao, Jans *et al.* 1998). In addition, several transcription factors and viral components rely upon phosphorylation status to control cytonuclear transport, such as c-myc, NFkB, c-rel, dorsal, and SW15 from yeast (for review, see (Jans and Hubner 1996).

Studies establishing the importance of phosphorylation sites in regulating nearby NLSs depend on the finding that, at least in the context of regulating the efficiency of NLSs, a phosphorylated serine or threonine can be mimicked by

substitution of the residue with asparagine, resulting in a constitutively phosphorylated residue, and a constitutively dephosphorylated residue can be mimicked by alanine (Jans and Jans 1994).

I.C. Adeno-associated virus

AAV is a single stranded DNA (ssDNA) virus belonging to the subfamily *Parvovirinae* (Berns 1996), a subfamily belonging to the *Parvoviridae* family. The parvoviruses are characterized by the organization of their single-stranded 5 kb DNA genome, which is protected by a non-enveloped icosohedral capsid measuring 25-30 nm in diameter. AAV was first identified in the 1960's as a contaminant of preparations of simian adenovirus, a double stranded DNA virus (hence the name adeno-associated virus), and by 1965, AAV was identified as a parvovirus (Atchison 1965; Berns and Bohenzky 1987). Atchison identified AAV as a defective virus, and soon thereafter, it was determined more specifically that, unlike all parvoviruses identified to date, AAV was incapable of replication in the absence of a helper virus, such as adenovirus (Casto, Armstrong *et al.* 1967; Casto, Atchison *et al.* 1967). This unique quality of AAV required that the virus be placed into a new genus of the *Parvoviridae* family, the genus *Dependovirus*.

To date, 7 distinct AAV serotypes have been described, numbered AAV1-AAV5, AAV7, and AAV8. All of the AAV serotypes were cloned from primates, but only serotypes 2, 3, and 5 have been found in humans (Hoggan, Blacklow *et al.* 1966; Parks, Melnick *et al.* 1967; Bantel-Schaal and zur Hausen 1984; Gao, Alvira *et al.* 2002). Of the 7 known AAV serotypes, AAV2 has been most thoroughly described in the literature.

C.1. The AAV life cycle

The AAV life cycle occurs in two distinct stages, the latent phase and the lytic phase. Upon initial infection, the viral genome site-specifically integrates into the host genome, where it remains latent until rescued by one of several helper viruses (Hoggan 1972; Handa, Shiroki *et al.* 1977; Cheung, Hoggan *et al.* 1980; Berns 1996). In human cells, the integration of the viral genome occurs in the q arm of chromosome 19 at a site that has been termed AAVS1 (Kotin, Siniscalco *et al.* 1990; Kotin, Menninger *et al.* 1991; Samulski, Zhu *et al.* 1991). This ability to integrate site specifically is unique among other latent eukaryotic viruses, such as retroviruses, which integrate randomly into the host genome.

The mechanism of AAV integration into the host genome is not completely understood, though it appears that the integration of AAV into AAVS1 is dependent upon Rep 68 and/or Rep 78. There are Rep binding elements (RBE) in both the AAV genome and in AAVS1. The Rep protein functions by binding to the RBEs situated in both AAV ITRs and at the AAVS1 site (Giraud, Winocour et al. 1994; Weitzman, Kyostio et al. 1994; Chiorini, Wendtner et al. 1995; Giraud, Winocour et al. 1995; Linden, Ward et al. 1996; Weitzman, Young et al. 2003). The endonuclease activity of the two larger Rep proteins allows them to nick at the terminal resolution site, which is positioned 8 or 11 bp away from the RBEs in AAV and AAVS1, respectively. There then follows an interaction between Rep molecules that are bound to the AAV genome and those that are bound to the AAVS1 site, and a nonhomologous recombination event occurs, resulting in integration of the AAV genome (Linden, Ward et al. 1996; Weitzman, Young et

al. 2003). It has been shown previously that head-to-tail concatemers of the wild-type AAV (wtAAV) genome are able to site specifically integrate in this manner. Preferred AAV recombination junction sites have been localized to both left and right AAV ITR elements as well as the p5-*rep* promoter region of the AAV genome.

AAV's lytic cycle begins with superinfection of an AAV-infected cell by a helper virus, such as adenovirus (Atchison 1965; Melnick 1965; Hoggan, Blacklow *et al.* 1966; Parks 1970; Hoggan 1972). The AAV genes become active, the viral genome is excised from the host genome and replicated, and progeny virions are produced. The AAV lytic phase ends with the death of the host cell and subsequent release of progeny virions.

C.2. AAV helper viruses

Several viruses have been identified as AAV helper viruses, including adenovirus (Ad), herpes simplex virus (HSV), cytomegalovirus (CMV), human papillomavirus (HPV), and vaccina virus (Atchison 1965; Melnick 1965; Hoggan, Blacklow *et al.* 1966; Buller, Janik *et al.* 1981; McPherson, Rosenthal *et al.* 1985; Schlehofer, Ehrbar *et al.* 1986; Walz, Deprez *et al.* 1997). Of the known AAV helper viruses, Ad is the best characterized in its ability to facilitate AAV rescue and replication functions.

Ad early gene products are known to aid in AAV mRNA transcription, transport and translation, and Ad DNA binding protein (AdDBP) has been shown to promote AAV replication (Ward, Dean *et al.* 1998)(reviewed by Berns, 1996). There is also evidence that the gene product of Ad E4 ORF 6 enhances second

strand synthesis of the AAV genome (Ferrari, Samulski *et al.* 1996; Fisher, Gao *et al.* 1996). Like Ad E4 ORF 6, several other treatments have been shown to enhance AAV replication. These treatments include hydroxyurea, UV and X-ray irradiation, heat shock, and topoisomerase inhibitors (Alexander, Russell *et al.* 1994; Russell, Alexander *et al.* 1995; Ferrari, Samulski *et al.* 1996; Fisher, Gao *et al.* 1996). Since the commonality between all of these treatments is their upregulation of DNA repair mechanisms, it is assumed that Ad E4 ORF 6 and these genotoxic treatments increase AAV transduction by activating DNA repair mechanisms, hence enhancing second-strand synthesis of the AAV genome.

An additional mechanism of regulating AAV second strand synthesis has been recently described (Qing, Wang *et al.* 1997; Qing, Khuntirat *et al.* 1998). In this mechanism, the single stranded AAV genome is bound by a cellular factor that was originally termed single strand D-sequence binding protein (ssD-BP), as it specifically binds the D region of the AAV genome. When phosphorylated, ssD-BP prevents second strand synthesis of the AAV genome. In the presence of Ad E4 ORF 6, or any of the genotoxic agents shown to enhance AAV transduction, ssD-BP is dephosphorylated and this dephosphorylation is correlated with rapid second strand synthesis of the AAV genome. It was later established that the ssD-BP was a cellular tyrosine phosphoprotein.

It is not completely understood how adenovirus rescues AAV from latently infected cells. The prevailing theory is that Ad infection results in initiation of synthesis of AAV transcripts necessary for rescue of the AAV genome, namely the Rep transcripts (described below).

C.3. The AAV genome and virion

The genome of AAV type 2 has been sequenced and is 4,680 bases in length (Figure 3). The genome has only two open reading frames, termed the

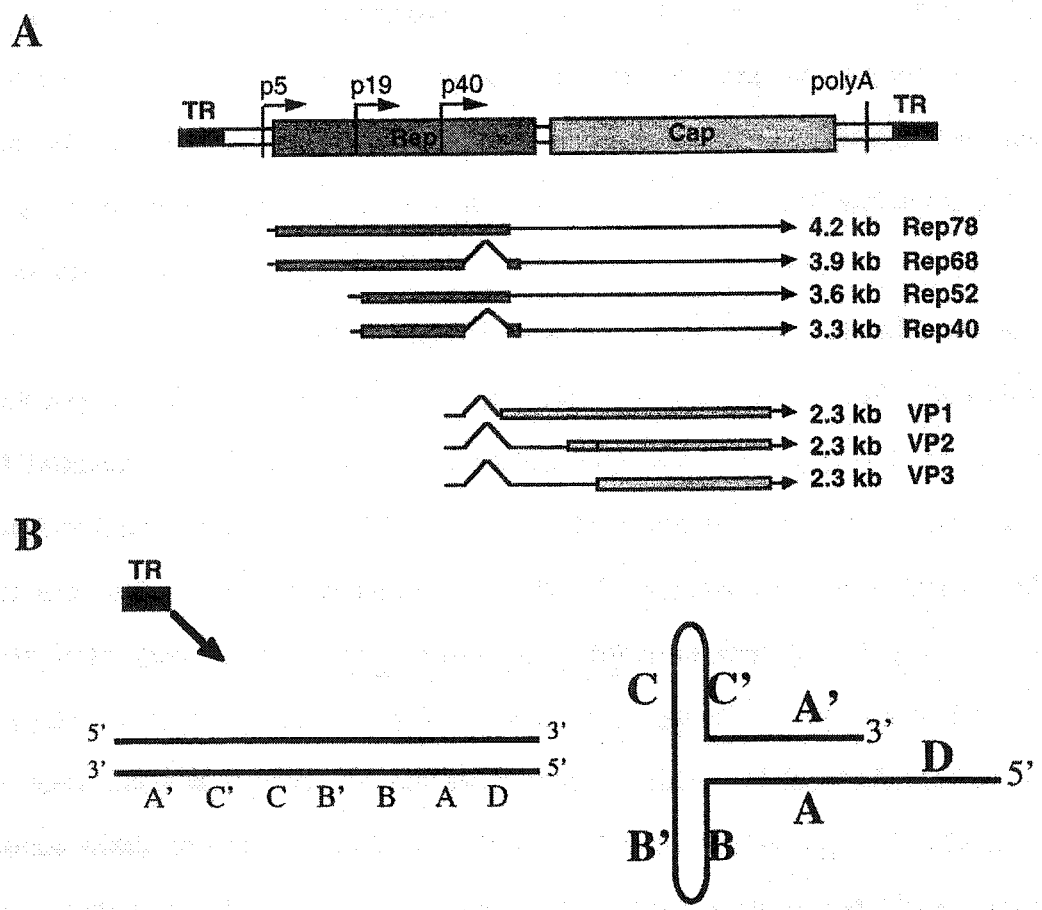

Figure 3. Structure of the AAV genome. A) The genome consists of two large open reading frames (ORF). The non-structural Rep proteins are transcribed from the left hand ORF and the structural Cap proteins from the right hand ORF. The viral mRNA transcripts shown are derived from threee promoters and share a common polyadenylation site. B) A diagrammatic representation of the AAV terminal repeat. In the single-stranded AAV genome, the palindromic sequences AA', BB', and CC', base pair to form a T-shaped hairpin structure. The non-palindromic D sequence lies outside the putative T-shaped structure.

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left open reading frame and the right open reading frame. The left open reading frame contains the Rep gene, while the right open reading frame contains the Cap gene (Srivastava, Lusby *et al.* 1983; Cassinotti, Weitz *et al.* 1988; Berns 1996).

The Rep gene gives rise to four non-structural replication proteins called Rep78, Rep68, Rep52, and Rep42, which are essential for the virus' integration into the host genome and for packaging of progeny viral genomes into their viral capsids (the proteins are named for their molecular weights). The 4 Rep proteins result from alternative start and splice sites within the Rep gene, with Rep 78 and 68 being translated from the p5 promoter and Rep 52 and 40 from the p19 promoter. Rep 78 and 68 share a start site, but are spliced differentially downstream, as is the case for Rep 52 and 40. This results in Rep 78 and 52 being identical in all but their N-termini (Srivastava, Lusby *et al.* 1983; Cassinotti, Weitz *et al.* 1988; Berns 1996).

The cloning and purification of the AAV Rep proteins have allowed for better characterization of these proteins. Some of the functional properties that have been found in the Rep proteins include binding affinity for specific DNA sequences, ATP-dependent endonuclease activity, which the virus uses to nick the AAV genome during replication, and DNA helicase activity. Rep binding elements (RBEs) play a critical role in the life cycle of AAV. Rep has been shown to bind the AAV terminal repeats, the AAV p5 and p19 promoters, chromosome 19 of the primate genome (this is the site of latent AAV integration), and several other human chromosomal sequences (Berns 1996).

Rep 78 and 68 play important roles as regulators of AAV gene expression. During latency, the two proteins act as repressors of AAV gene expression from the p5 and p19 promoters, i.e. they are self-repressing. Additionally, the proteins play an important role in mediating the chromosome 19-specific targeting of genome integration. Rep 78 and 68 have also been implicated in repressing heterologous viral promoters and are responsible for AAV's anit-oncogenic effects (Berns 1996).

The properties of Rep 52 and 40 are less characterized than those of Rep 78 and 68. Rep 52 and 40 have been shown to be unnecessary for AAV genome replication, though Rep 52 is necessary for accumulation of AAV ssDNA, and ssDNA is necessary for the production of progeny virions. Since Rep 52 is not necessary for viral genome replication, but is necessary for the production of ssDNA, it is possible that Rep 52 is responsible for the separation of dsDNA into ssDNA that will be packaged into virions. In fact, Rep 52 has been shown to have helicase activity, though it is unknown whether this helicase activity is necessary for the viral life cycle.

The Cap gene is driven from the p40 promoter of the AAV genome. This gene gives rise to three proteins termed VP1, VP2, and VP3, which are all in the same reading frame, but use different start codons, thus are identical in their C-termini (Figure 3). Together these proteins form the viral capsid shell in a stoichiometric ratio of 1:1:10 and have molecular weights of 87, 73, and 62 kDa for VP1, VP2, and VP3, respectively (Johnson, Ozer *et al.* 1971; Salo and Mayor 1977). The viral capsid is non-enveloped and measures approximately 25 nm in

diameter. It contains within it all of the functions necessary to deliver the AAV genome to the nucleus for replication (Kronenberg, Kleinschmidt *et al.* 2001; Xie, Bu *et al.* 2002).

During the course of an AAV infection, the capsid proteins must twice target the nucleus. The first targeting event is during the initial infection when the virus must deliver the viral genome to the nucleus for replication. Since AAV replicates in the nucleus of its host cell, (Wistuba, Kern *et al.* 1997)the second targeting event occurs when progeny viral capsid proteins enter the nucleus for assembly of progeny virions. It remains unknown how the AAV capsid mediates delivery of the AAV genome to the nucleus, or how the individual capsid proteins enter the nucleus for assembly into progeny virions.

A single nuclear localization has been proposed as a mediator for targeting the AAV virion to the nucleus during initial infection and for targeting individual capsid proteins to the nucleus during progeny virus assembly (Hoque, Ishizu *et al.* 1999). In these experiments, the authors constructed a fusion protein of AAV VP2 and green fluorescent protein (GFP). The fusion protein localized primarily to the nucleus, but when N-terminus deletions were made to VP2, a basic region was found to be necessary for the localization of the fusion protein to the nucleus. The authors concluded that this basic region must be responsible for the AAV's ability to deliver its genome to the nucleus and for the ability of the viral capsid proteins to accumulate in the nucleus for progeny virus assembly.

The Rep and Cap genes of AAV are flanked by terminal repeats (TRs) of 145 nucleotides (Srivastava, Lusby *et al.* 1983). The TRs are rich in GC content and contain a 125 nucleotide palindromic sequence that consists of two smaller palindromic internal palindromes (BB' and CC') flanked by a larger palindromic sequence (AA'). The palindromes are believed to base pair and form stable secondary T-shaped hairpin structures at each end of the genome (Figure 3). This unique structure allows for the terminal 3'-OH to provide an origin of self-primed DNA replication. A non-palindromic sequence, the D sequence, lies outside the putative T-shape structure. This sequence is an important part of the terminal repeat and is required for replication of the AAV genome. The terminal repeats have been shown to be the sole cis-acting sequences that are necessary for AAV integration, rescue of latent provirus, AAV DNA replication, and DNA encapsidation.

The TRs have been suggested to contain promoter activity capable of regulating gene expression inside and outside of the context of AAV. Haberman *et al* demonstrated gene expression from a TR-green fluorescent protein construct without any promoter (other than the upstream AAV terminal repeat), demonstrating that the AAV terminal repeat contains an intrinsic ability to promote gene expression (Haberman, McCown *et al.* 2000).

C.4. Cellular Trafficking of adeno-associated virus-From the cellular membrane to nuclear periphery

As intracellular parasites, viruses must enter target cells to initiate replication and infection, and for the preponderance of DNA viruses, must traffic

across the cytoplasm to facilitate the delivery of their genomes to the nucleus. In general, viruses can enter target cells by either directly crossing the cell membrane, or by internalizing into cellular compartments such as endosomes. The latter pathway, as is thought to be the case for AAV, often begins with the virion making contact with a cell surface receptor, which then triggers internalization of the virus. The virus then enters the cell via one of several possible internalization mechanisms, which include macropinocytosis, clathrin-mediated endocytosis, caveolae, and non-clathrin/non-caveolae mediated internalization.

Adeno-associated virus begins infection of its target cell by binding to a cell surface receptor. For the prototypical AAV serotype 2, this receptor is heparin proteoglycan, while for AAV serotypes 4 and 5, sialic acid is a necessary receptor component (Summerford and Samulski 1998; Kaludov, Brown *et al.* 2001; Walters, Yi *et al.* 2001). Internalization of AAV serotype 2 is enhanced through binding to the co-receptor $\alpha_V\beta_5$ (Summerford, Bartlett *et al.* 1999). AAV2 is internalized via clathrin-coated pits via a dynamin-dependent mechanism and trafficked to early endosomes (Bartlett, Wilcher *et al.* 2000). At this point in the infection, evidence in the literature diverges. It was shown by Bartlett that AAV infection is dependent upon endosomal acidification by HTPase. Bartlett showed that the HTPase inhibitor bafilomycin A blocked AAV infection, and proposed that the virus escapes from early endosomes (Bartlett, Wilcher *et al.* 2000). More recently, though, other groups have performed more thorough examinations of mechanisms of endosomal escape of AAV and have shown that the virus more

likely escapes from late endosomes or proteosomes (Hansen, Qing *et al.* 2000; Douar, Poulard *et al.* 2001). In addition, it was recently demonstrated that the parvovirus minute virus of mice (MVM) traffics to at least the late endosome before gaining access to the cytosol, and may actually depend on the proteosome for further processing (Ros, Burckhardt *et al.* 2002). Regardless of whether the virus escapes from early or late endosomes, its trafficking through the cytoplasm is an energy dependent process that requires microfilaments and microtubulin and is dependent upon the small GTP binding protein Rac1 (Sanlioglu, Benson *et al.* 2000).

At some point during viral trafficking, the AAV virion must escape from the endosome. Thus far, there is no evidence as to how the virus escapes from the endosome. It is possible that the lipase activity identified in VP2 of the virus (Girod, Wobus *et al.* 2002) somehow mediates the virus' escape from the endosome by digesting a hole in the membrane of the endosome, though such a mechanism of endosomal escape has not yet been identified for any virus. Another possible route of escape is a receptor-mediated mechanism. In this process, the virus would bind to a receptor on the inner membrane of the endosome and escape via macropinocytosis or some other membrane budding process.

While it has been accepted that AAV capsid proteins target the nucleus, the mechanism through which AAV targets the nucleus has received very little attention. Some have proposed that AAV enters the nucleus as an intact virion, and uncoats in the nucleus (Bartlett, Wilcher *et al.* 2000; Hansen, Qing *et al.*

2001), though much more evidence will be needed to firmly establish that this is the case . A sequence residing at the N-terminus of AAV2 VP2 has been identified that when attached to the N-terminus of GFP, is capable of driving the GFP into the nucleus of transfected mammalian cells (Hoque, Ishizu *et al.* 1999). The residues found to be important for nuclear localization of VP2-GFP were amino acids 29-34 of VP2, PARKRL (residues 166-171 of VP1). This study, while showing that residues 29-34 of VP2 are capable of localizing a fusion protein to the nucleus, left open the question of what function, if any, the region serves in the context of a wild-type virion and in a natural infection.

Additional regulation of AAV infection may be conferred by importin isoform expression that is dependent upon the cell type and cell cycle. For example, it has been shown that in skeletal muscle, Impα4 is the dominant isoform of Impα. The same study also showed that Impα4 represents 1% of the total protein in skeletal muscle cells (Nachury, Ryder *et al.* 1998). Data from our laboratory, and data emerging in the literature, suggests that AAV serotypes have transduction efficiencies that are dependent on the tissue type. At least part of the tissue dependence may be due to differential abilities of the tissues to support nuclear transport of the virus.

C.5. Production of recombinant AAV

A major obstacle in the progress of AAV technology in both research and clinical contexts is the production of rAAV that is of high-titer and of high purity. The first methods of generating recombinant AAV (rAAV) involved the transfection of E1A-transformed human embryonic kidney cells (293 cells) with a

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plasmid containing the transgene to be packaged, and a plasmid carrying the Rep and Cap genes of AAV (Samulski, Chang *et al.* 1989). The transfected cells were then infected with wild-type Adenovirus (wtAd), which provided additional factors needed for the replication of the Rep and Cap genes and for production of AAV particles. The rAAV generated by this technique were extremely valuable as research reagents, but would not suffice for gene therapy applications, as the contaminating wtAd and contaminating replication-competent AAV rendered the preparations unusable for gene therapy applications. The need for wtAd in the two-plasmid production system was circumvented by Xiao *et al*, who developed an Ad-free production system that relied on a third plasmid to carry the only the Ad genes essential to AAV replication and rAAV generation (Grimm, Kern *et al.* 1998; Matsushita, Elliger *et al.* 1998; Xiao, Li *et al.* 1998).

Since the invention of the Ad-free production system, several improvements have been made towards the goal of improving the efficiency of rAAV production by producer cells. Nearly all improvements are aimed at abrogating the need for transfection of the producer cell lines, as the transfection of producer cells is seen as too inefficient and cumbersome to be used to generate clinical quantities of rAAV. One approach being aggressively pursued is the creation of cell lines that stably express the Rep and Cap genes under inducible promoters, while other systems use viruses to deliver to the producer cells all of the DNA constructs necessary for the production of the rAAV (Clark, Voulgaropoulou *et al.* 1995; Tamayose, Hirai *et al.* 1996; Gao, Qu *et al.* 1998; Inoue and Russell 1998; Conway, Rhys *et al.* 1999; Liu, Clark *et al.* 1999). The

major draw back of cell line-based and viral based production systems is the requirement for wtAd infection of the producer cells, raising the risk of contamination of rAAV preparations with wtAd, though progress has been made towards designing purification schemes that effectively remove the Ad from the rAAV preparation (Gao, Qu *et al.* 2000; O'Riordan, Lachapelle *et al.* 2000). Because of the significant resources that must be employed in establishing an AAV producer cell line and because the research into cell line-based and viral-based rAAV production systems is still maturing, 3- plasmid transfection of producer cells remains the most widely used method of rAAV production.

While improvement in rAAV production technology is an extremely important factor in fully realizing the utility of rAAV, equally important is concomitant improvement in the methods used to recover and purify rAAV vectors. Simplified and accessible methods to obtain rAAV of high titer and purity are critical elements not only in clinical applications, but also in expanding the usefulness of rAAV technology in growing new fields such as functional proteomics and genomics, where methods are needed to facilitate rapid screening of the functions of genes and proteins.

Typically, one begins purification of rAAV by lysing the producer cell line through treatment with detergent, or by several cycles of rapid freezing and thawing between a dry ice/ethanol bath and a warm-water bath. The lysate is then cleared by centrifugation and the virus further purified through one of several methods.

Most of the methods proposed for the purification of the virus away from producer cell lysate contaminants include either centrifugation of the lysate through an iodixonol step gradient followed by either affinity purification over a heparin sepharose affinity column (Hermens, ter Brake *et al.* 1999; Zolotukhin, Byrne *et al.* 1999), or by two 48-hour cycles of centrifugation in a cesium chloride density gradient (Toolan 1968). Unfortunately, centrifugation through a cesium chloride gradient typically yields preparations with considerable amounts of contaminating nonviral proteins, including protein encoded by the transgene (Malik, Monahan *et al.* 2000). It has also been shown that centrifugation of AAV in cesium chloride results in large losses of infectivity over the course of the centrifugation, with less than 15 percent of the infectivity remaining after 72 hours in cesium chloride (Auricchio, Hildinger *et al.* 2001). The purification of rAAV through an iodixonol gradient and a heparin sepharose affinity column results in far cleaner preparations with less loss of infectivity (Zolotukhin, Byrne *et al.* 1999). Unfortunately, though, purification of rAAV by heparin affinity chromatography only works for AAV serotypes 2 and 3, as none of the other serotypes bind to heparin.

Several authors have presented evidence showing that adeno-associated virus begins capsid protein assembly in the cytoplasm, but completes capsid assembly and packaging of viral DNA in the nucleus (McPherson, Ginsberg *et al.* 1982; Trempe, Mendelson *et al.* 1987; Hunter and Samulski 1992; Wistuba, Kern *et al.* 1997). These findings left open the possibility of isolating rAAV from the purified nuclei of producer cells. However, because Adenovirus coinfection with

AAV leaves the infected cells and their nuclei too fragile to routinely isolate the virus-containing nuclei away from cytoplasmic components (unpublished observation), we have been unable to take advantage of this aspect of AAV biology in the isolation and purification of rAAV.

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II. EXTRACTION OF AAV FROM PURIFIED NUCLEI RESULTS IN GREATLY IMPROVED VIRAL VECTOR

II.A. Introduction

Adeno-associated virus (AAV) is an extremely useful gene delivery tool. Because of the virus' lack of pathogenicity, ease of genetic manipulation, and its ability to provide for permanent gene expression, the virus is undergoing a great deal of research as a gene therapy vector and as a molecular biology tool.

Production of large amounts of high purity, high titer recombinant AAV is a critical aspect of AAV research that has limited progress of the field in developing gene delivery vectors and in elucidating the virus' molecular biology. However, current methods of producing and purifying AAV leave the virus contaminated with cellular proteins (discussed above). Studies of the molecular biology of AAV in which the virus is labeled with fluorescent die, or studies in which the virus is bound to cellular proteins in vitro to study protein interactions, have been hampered by the absence of efficient methods for the purification of recombinant AAV.

It has long been known that AAV replicates in the nucleus of its host cells (McPherson, Ginsberg *et al.* 1982; Trempe, Mendelson *et al.* 1987; Hunter and Samulski 1992; Wistuba, Kern *et al.* 1997), making possible the isolation of the virus from purified nuclei of producer cells, which would in theory, result in virus less contaminated by cytoplasmic proteins and membrane proteins from the

producer cells than methods that involve whole cell lysis. Unfortunately, though, Ad coinfection with AAV leaves the nuclei of the producer cells too fragile for routine separation from the rest of the cell (unpublished observation from our laboratory). In our laboratory, we found that the development of Ad-free AAV production systems, in which the AAV producer cells are transfected only with the Ad genes that are essential for AAV replication, has resulted in producer cell nuclei that are of normal durability. This finding led us to revisit the possibility of isolating AAV from purified nuclei as a way to obtain AAV of higher purity and titer than that obtained through whole cell lysis methods.

II.B. Results

B.1. rAAV particles concentrate in the nuclei of producer cells after triple transfection protocol

Since AAV assembles in large arrays in the nucleus (McPherson, Ginsberg *et al.* 1982; Trempe, Mendelson *et al.* 1987; Hunter and Samulski 1992; Wistuba, Kern *et al.* 1997), and Adenovirus-free (Ad-free) production methods leave the nuclear envelope intact (unpublished observation), we reasoned that one might obtain higher titer and higher purity rAAV by isolating the nuclei of rAAV-producing cells before releasing the rAAV into the supernatant. The first step in evaluating this hypothesis was to confirm that when using a triple-transfection protocol, AAV concentrates in the nucleus in a way that could be exploited in rAAV production and purification. By electron microscopy, we found the nuclei of triple-transfected producer cells pervaded by large arrays of virions, identical to those seen previously in Ad/AAV coinfected cells, and by Western blot, found the preponderance of AAV capsid proteins in the nucleus of fractionated producer cells (Figure 4).

B.2. Extraction of rAAV from purified nuclei results in higher titer virus than if isolated from detergent lysis of whole cells

We sought to determine whether rAAV could be recovered in higher titer and purity from purified nuclei than from traditional methods employing whole cell lysis. Nuclei were isolated from producer cells by hypotonic swelling, dounce homogenization, and centrifugation. The virus-containing nuclei were then lysed

by osmotic shock through treatment with high salt. On average (n=6), 7-fold more virus and 25% less total protein were extracted from purified nuclei than from detergent-lysed cells (Figure 5, upper panel). The nuclear extraction (NE)

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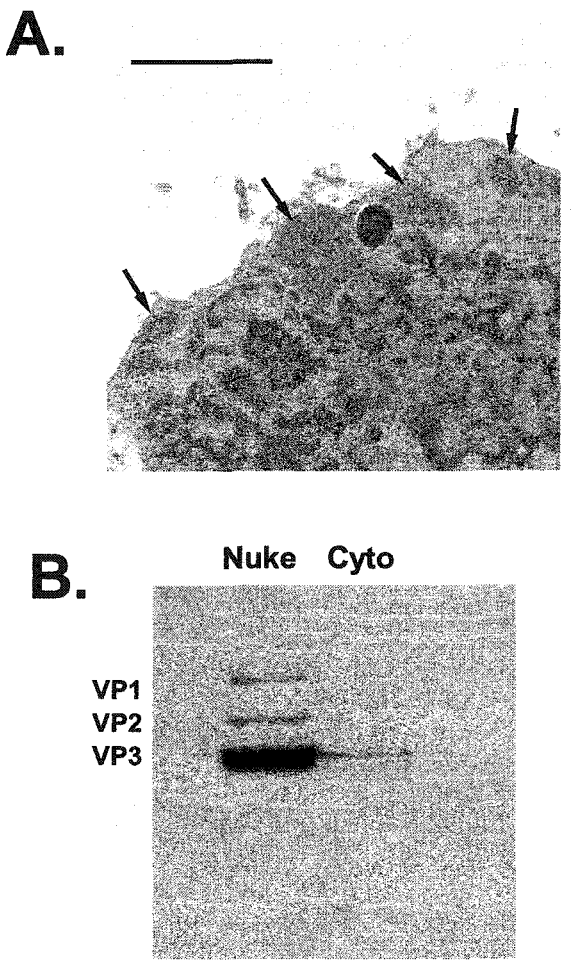

Figure 4. Adenovirus-free assembly of AAV in the nuclei of producing cells. Cells were transfected with AAV helper, Ad helper, and transgene-containing plasmids. 48 hours later, nuclei were either viewed by electron microscopy (panel A), or lysed and assayed by Western blot, along with the cytoplasmic fraction, for the presence of AAV capsid proteins (panel B). In the EM image, the arrows point at arrays of AAV virions. In the Western blot, "Nuke" marks the lane in which nuclear lysate was loaded and "Cyto" marks the lane in which cytoplasmic lysate was loaded.

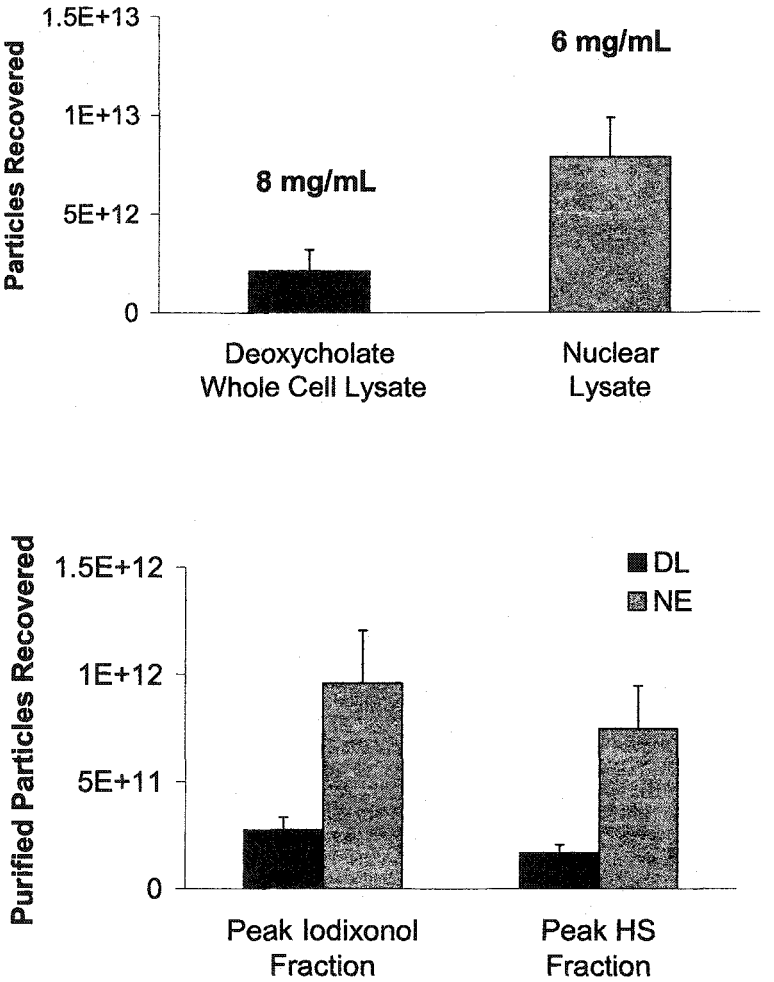

Figure 5. Upper panel, recovery of rAAV from producer cell lysate. Each lysate was analyzed by Southern-blot hybridization to determine the number of genome copies recovered. Bars represent the mean±SEM of 6 virus preparations. The mean fold difference between the two methods is significantly different from 1 ($p<0.05$). The numbers above the bars represent the average total protein concentration of the lysate.
Lower panel, recovery of rAAV from peak iodixonol and heparin affinity purification fractions. Each sample was analyzed by Southern-blot hybridization as described in the methods section. Bars represent the mean±SEM of 6 virus preparations. The mean fold difference between the two methods is significantly different from 1 for both the iodixonol peak fractions and the HAP peak fraction ($p<0.05$)..

method resulted in the peak iodixonol and heparin affinity purified (HAP) fractions containing an average of 6- and 8-fold more virus, respectively, than iodixonol and HAP fractions from the detergent lysis (DL) method (Figure 5, lower panel). The total particles recovered represent only the particles that were in the peak fractions of the iodixonol gradients and the HAP eluate (i.e. side fractions were not included).

The difference in viral titer in the HAP fractions was immediately apparent on elution from the HAP column. When eluding from the HAP column, the virus prepared by NE resulted in several-fold higher absorption readings than virus prepared in parallel by the DL method, and peak height roughly correlated with virus yield (Figure 6). The titers of the NE and DL virus preparations were also verified by infection of HeLa cells. The two methods did not yield virions with significantly different physical particle to infectious unit ratios (not shown).

B.3. Extraction of rAAV from purified nuclei results in higher purity virus than if isolated from detergent lysis of whole cells

Not only did the nuclear extraction method result in higher titer virus, but the virus recovered through nuclear extraction contained far fewer contaminants and particulate than the virus recovered through detergent lysis. Before and after centrifugation, the iodixonol gradient from the NE virus had no visual evidence of particulate or contaminating green fluorescent protein (GFP, the transgene packaged into the rAAV for these experiments). In contrast, particulate and substantial amounts of GFP are visually evident in the iodixonol gradients of the DL virus both before and after centrifugation (Figure 7).

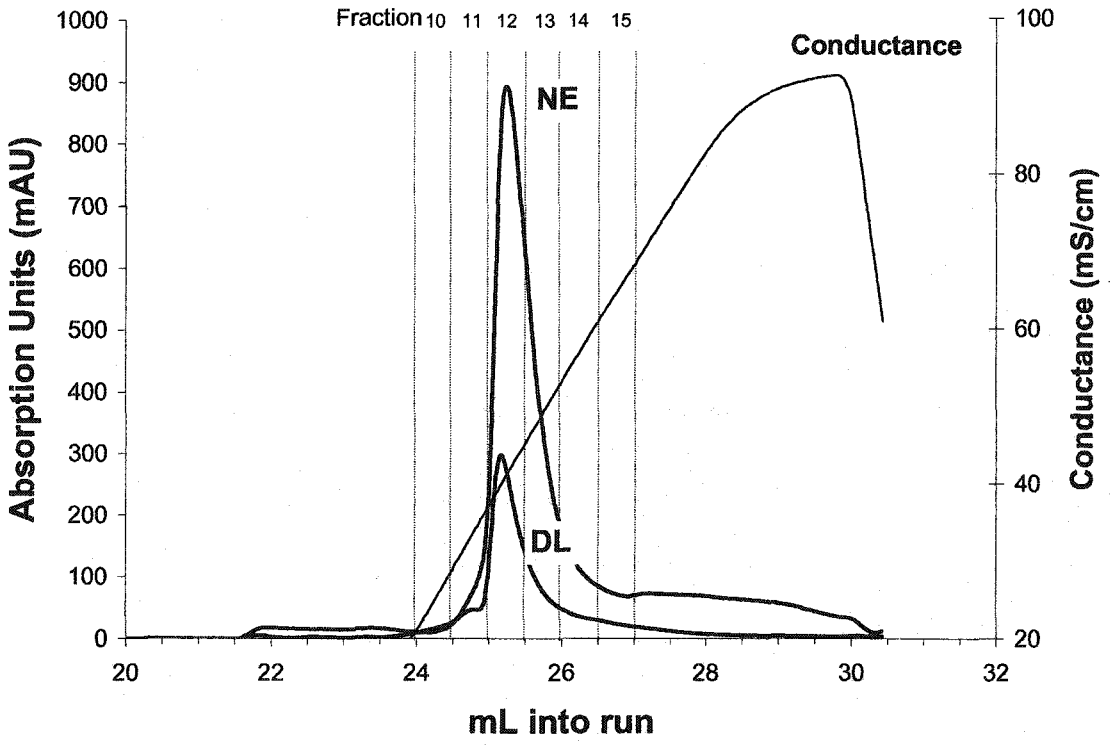

Figure 6. Representative heparin affinity purification of peak iodixonol fractions. Fraction 3 of the iodixonol step gradients of a nuclear extraction preparation (NE) and of a detergent lysis preparation (DL) was bound by FPLC to a heparin sepharose affinity column in separate runs. Virus was eluted by a gradient of high salt (indicated by electrical conductance of elute)

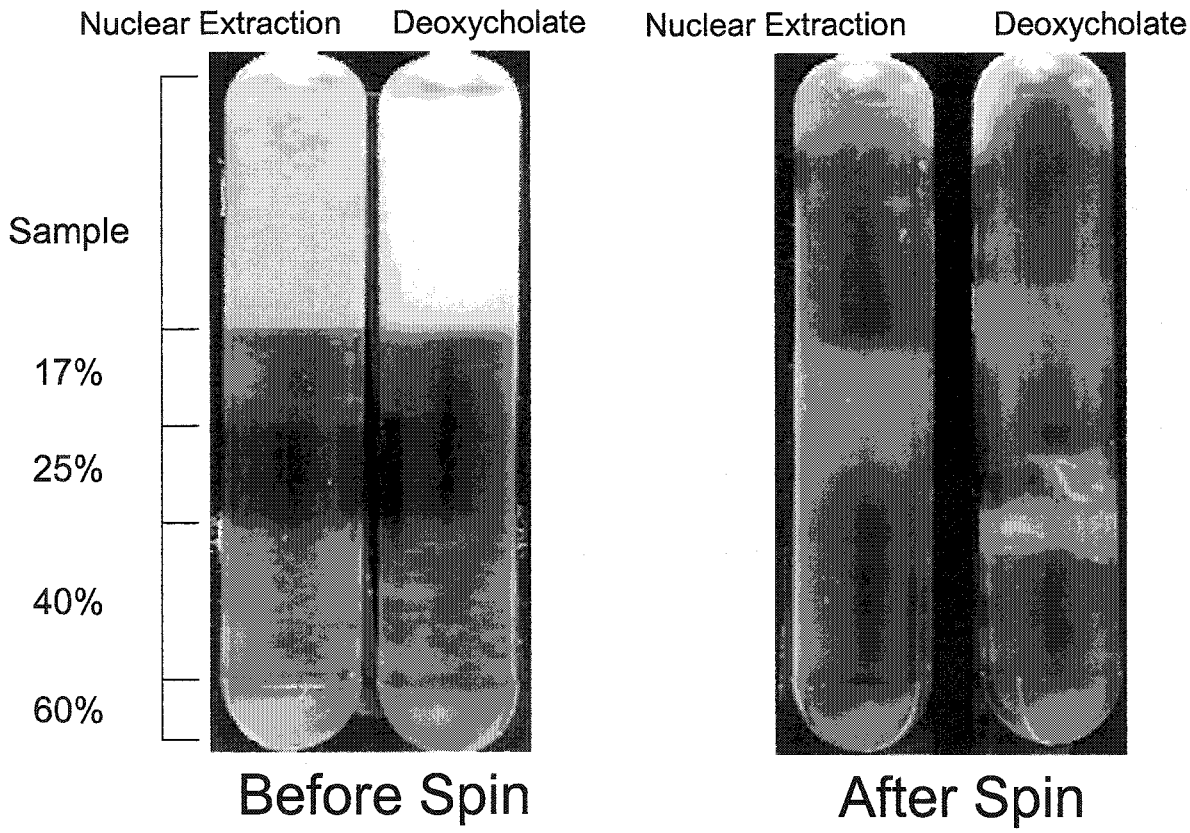

Figure 7. Iodixonol step gradient centrifugation of either whole cell lysate (Deoxycholate) or nuclear lysate (Nuclear extraction) from AAV producer cells. Percentages on left side represent the concentration of iodixonol in the layer indicated. Note the green fluorescent protein visually apparent both before and after centrifugation in the deoxycholate lysis sample, and the debris layer in the deoxycholate sample after centrifugation. The gene for green fluorescent protein (GFP) was transfected into the cells as the transgene, so GFP protein was expressed during the viral production process.

Fractions from the NE virus iodixonol gradients were far more pure than the fractions from the DL virus when analyzed by silver stain, with fraction 3 of the NE iodixonol gradient having no observable contaminants (Figure 8, panel A, right). In the silver stains of iodixonol fractions 4 and 5 of the NE virus, 3 of the 4 contaminating bands are reactive to a monoclonal antibody against VP3 (bands marked by asterisks in silver stain), so these proteins may represent capsid protein breakdown products, or nuclear proteins that cross react with the antibody. In contrast, iodixonol fractions from the DL virus preparation all contain substantial amounts of contaminating protein of all molecular weights (Figure 8, panel A, left).

Not only did the iodixonol fractions from the NE virus contain fewer contaminants than those of the DL virus, but after HAP, the NE virus contained no detectable protein contaminants (Figure 8, panel B). This finding is in contrast to that of the DL virus, which still contained several contaminating proteins following HAP (Figure 8, panel B).

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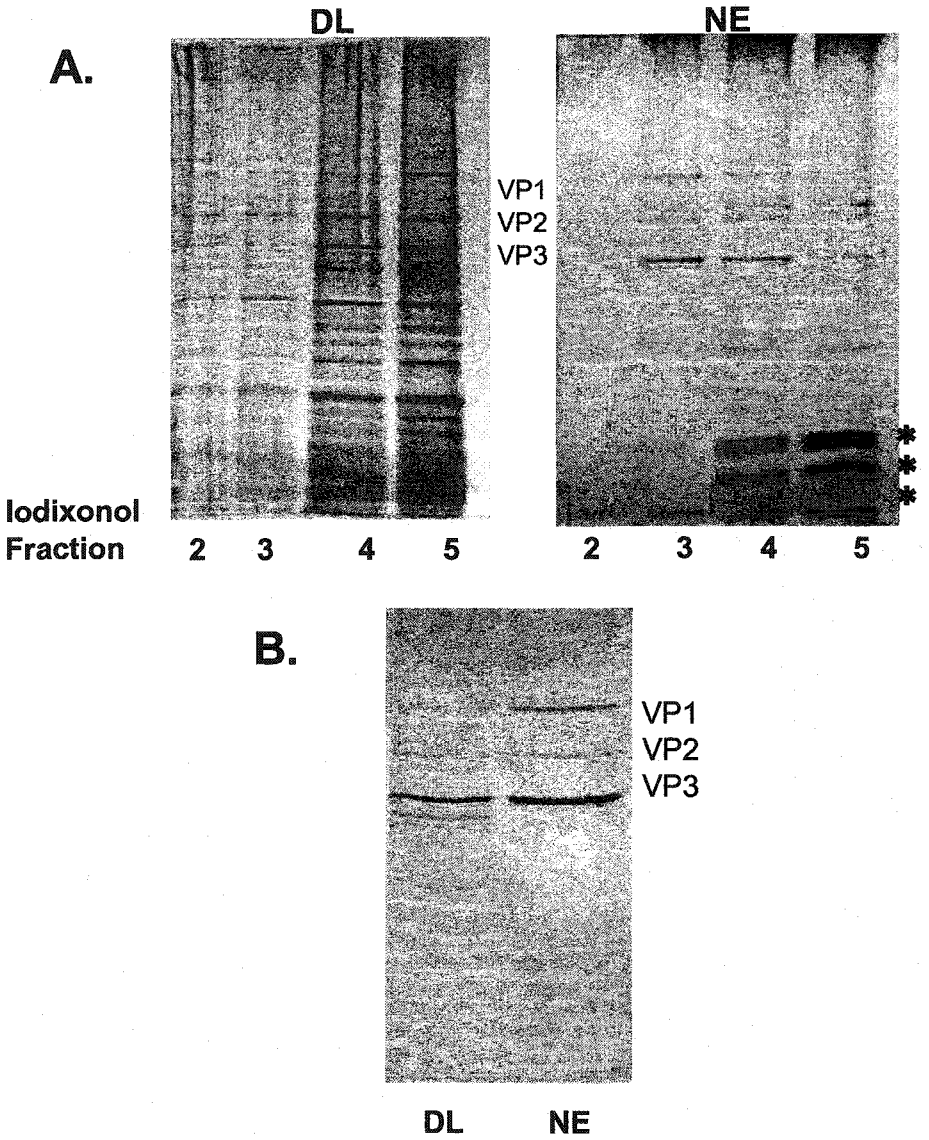

Figure 8. Silver stain analysis of the iodixonol step gradients (panels A and B) and heparin affinity purified virus (panel C). For iodixonol fractions, numbers on bottom represent fraction number. 10 uL of each iodixonol fraction from both the nuclear extraction method (NE, panel A) and detergent lysis method (DL, panel B) were loaded onto a 9% SDS-polyacrylamide gel and resolved by electrophoresis. For heparin-purified virus (panel C), an equal number of particles was loaded in each lane (~10^{10} particles).

II.C. Discussion

Recombinant adeno-associated virus is proving to be a valuable gene transfer tool in gene therapy and in molecular biology. Several obstacles limit the use of rAAV as a gene transfer tool (Monahan and Samulski 2000). One of the barriers is a simple protocol for the consistent production of high-titer, high-purity recombinant virus vector. High production efficiency of rAAV is critical for generating enough reagent for laboratory and human applications and for commercializing rAAV as a gene therapy vector. The purity of the rAAV vector is essential in minimizing immune response and in simplifying interpretation of results obtained in animal studies.

In early AAV research, AAV virions were released from producer cells by detergent lysis. Gradually, the field moved towards the use of freeze/thaw lysis of whole cells because freeze/thaw lysis resulted in virus with fewer contaminants. In our laboratory, side by side comparisons of detergent lysis versus freeze/thaw lysis showed that the detergent lysis method resulted in several-fold more virus recovered from the producer cells. Unfortunately, the detergent lysis method also resulted in far more contaminants in the prepared virus than the freeze/thaw method, and resulted in inconsistent viral titers.

Over the past several years, the titer and purity of rAAV preparations has improved incrementally, with one of the greatest improvements resulting from the discovery that AAV binds to heparin sulfate proteoglycan (HSPG) with high affinity (Summerford and Samulski 1998). Taking advantage of this finding, an affinity purification scheme was developed that used an iodixonol step gradient to

initially purify the rAAV away from the cell lysate and then further purified the virus over a heparin sepharose affinity column (Zolotukhin, Byrne *et al.* 1999).

We report here a simple, modified method of rAAV preparation that exploits the assembly of AAV particles in the nuclei of producer cells during Ad-free AAV production. There have been several methods of rAAV purification proposed over the past several years, all of which included a whole-cell lysis step of either rapid freeze/thaw cycles, or detergent lysis (Xiao, Li *et al.* 1998; Cao, Liu *et al.* 2000; Gao, Qu *et al.* 2000; O'Riordan, Lachapelle *et al.* 2000; Auricchio, Hildinger *et al.* 2001; Drittanti, Jenny *et al.* 2001). In addition, many of the methods rely on concentrator columns to improve purity of the virus and increase its concentration, which can be time-consuming and further decrease the number of viable particles recovered. In this thesis, we present evidence that isolation of the nuclei of the producing cells, followed by high-salt extraction of the rAAV from the isolated nuclei, results in 7-fold better recovery of virus over a method employing detergent lysis. We also show that the virus obtained through nuclear extraction of purified nuclei is nearly free of protein contaminants after iodixonol purification and free of detectable contaminants after heparin-sepharose affinity purification.

Though evidence is not presented in this thesis, we have also found that nuclear extraction of rAAV greatly improves the results obtained when one purifies the virus by cesium chloride density gradients in that one obtains higher titer virus that contains fewer protein contaminants. One simply resuspends the rAAV-containing nuclei into the cesium chloride solution. The osmotic pressure

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resulting from the high concentration of cesium chloride causes bursting of the nuclei, and the rAAV is released directly into the cesium gradient solution. In addition to AAV serotype 2, we were also able to purify AAV serotypes 1, 3, 4, and 5 by extraction from purified nuclei (not shown). When purification of the rAAV required centrifugation in cesium chloride, such as for serotypes that do not bind to heparin, we modified slightly the nuclear extraction protocol. If one is purifying the rAAV by cesium chloride, instead of lysing the nuclei in high salt buffer, one can simply lyse the nuclei by resuspending the nuclear pellet in the cesium chloride solution that will be used for the gradient. However, the solution should still be briefly sonicated before centrifugation.

There are several reasons why isolating the nuclei away from cytoplasmic and membranous components prior to high-salt lysis may increase virus yield. One possible explanation is that one avoids harsh conditions such as rapid freezing and thawing or treatment with detergents. Another possible reason is that there is far less particulate in which the virus may become entangled during clearing of the lysate by centrifugation. A third explanation is that during whole-cell lysis, the virus may bind to heparin proteoglycans, or other proteins present on the cell membrane and in the cytoplasm, and sediment with those components during centrifugation.

The method of rAAV recovery that we present here represents a substantial improvement to current methods, though it requires no equipment other than equipment required for current techniques. Extraction of rAAV from purified nuclei is faster than recovering virus using available methods, easy to

learn, scaleable (we have used the technique successfully for 60-plate preparations) and easily adapted to evolving purification schemes. Importantly, nuclear extracted rAAV is of sufficient purity following iodixanol purification to be used for in vitro studies, without the need for further purification of the virus, especially if the transgene product is not localized to the nucleus (contamination of the rAAV with transgene product generated during virus production has been shown to complicate interpretation of data, (Malik, Monahan *et al.* 2000).

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III. ADENO-ASSOCIATED VIRUS EMPLOYS AT LEAST TWO DOMAINS IN ITS CAPSID PROTEINS TO TARGET THE NUCLEUS OF ITS HOST CELL AT DIFFERENT TIMES DURING THE INFECTIOUS CYCLE

III.A. Introduction

Most DNA viruses use the nuclei of their host cells as a site for replication. The nucleus offers several benefits as a site of replication, including access to cellular machinery for DNA replication, transcription, and RNA processing. An additional benefit gained by nuclear replication is the access to mechanisms for latent integration into the host genome and access to factors for regulating gene expression.

Reliance on the host nucleus for latent infection, replication of the viral genome, transcription, and RNA processing does not come without a great deal of challenge for the virus. The nuclei of animal cells are very specific about which molecules are permitted cytonuclear transport and present a formidable barrier to transfer of the viral genome and viral components. If the virus uses the nucleus as a site of progeny virion assembly, the progeny viral components must find entry into the nucleus. To navigate these challenges, DNA viruses must make use of a very complex repertoire of cellular mechanisms that the host cell uses to transport cellular molecules into the nucleus.

There are several trafficking events that must be successful for a viral infection itself to be successful. First, the virus must enter the cell. For non-enveloped viruses, this involves the binding of a cell surface receptor, followed

by endosomal internalization. After trafficking in the endosome, the virus must somehow escape the endosome, though little is known about how endosomal escape may occur. After endosomal escape, the virus must target the nucleus and deliver to the interior of the nucleus the viral genome.

The mechanisms by which DNA viruses transfer their genomes to the nucleus can follow one of three generalized pathways. One possibility is that the DNA is shuttled into the nucleus by the entire virion, followed by intranuclear uncoating of the virus and release of the viral DNA. Another possibility is that the virion is denatured before reaching the nucleus, and one of the capsid proteins, or some other viral protein, then carries the DNA to the nucleus. Under this scenario, a capsid component would either shuttle the DNA into the nucleus, or release the DNA at the nuclear pore, allowing the DNA to diffuse across the membrane. Alternatively, the genome could piggyback on a cellular factor making its way to the nucleus. A third nuclear entry pathway, which will not be discussed here, as it is used only by some retroviruses, is entering the nucleus during mitosis, when the nuclear membrane becomes fragmented. All three of these possible pathways are represented in the repertoire of known viral genome nuclear import pathways. Examples of each pathway are given below.

A.1. Viruses that enter the nucleus intact

The NPC is believed to have a lumen capable of accommodating particles up to 28 nm in diameter (Feldherr and Akin 1990). With this being the case, it is possible that some small viruses may transport across the NPC intact and subsequently uncoat in the nucleus. Through indirect evidence, nuclear entry of

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intact virions has been shown to be the case for polyoma virus (Whittaker, Kann *et al.* 2000). Fluorescently labeled polyoma virus particles have been shown to enter the nucleus with no detectable loss of signal, which is argued by the authors to indicate that entry can occur without extensive prior disassembly. From this data though, the possibility cannot be excluded that what was seen in the nuclear interior was free fluorescent dye that had released from viral capsid or dye attached to contaminants of the viral preparation . Antibodies against intact CPV have been used to show that, several hours following infection, some small amount of intact CPV can be detected by an anti-capsid antibody (Vihinen-Ranta, Kalela *et al.* 1998), suggesting that this virus may also enter the nucleus as an intact virion. While these data are suggestive that some smaller viruses may enter the nucleus intact, additional data on another papovavirus suggests the opposite. It has been shown that papillomavirus in its intact form is not capable of crossing the nuclear envelope (Merle, Rose *et al.* 1999).

Another group of viruses that may be sufficiently small to cross the nuclear envelope without disassembly is that of the parvoviruses. The diameter of viruses belonging to this group is at the upper limit of NPC's size limitation of 28 nm (Chapman and Rossmann 1993). For the canine parvovirus, nuclear staining has been shown using an antibody that detects only intact capsids. However, the multiplicity of infection used for these experiments is extremely high, and may reflect an artifact of the experimental protocol (Vihinen-Ranta, Kakkola *et al.* 1997).

A.2. Viruses that uncoat at the nuclear membrane

Viruses that are known to uncoat at the nuclear membrane include the herpes viruses, adenovirus, hepatitis B virus, and the baculoviruses. These viruses are thought to bind at or near the nuclear pore complex and release their DNA cargo, leaving an empty capsid, or pieces of capsid, on the cytoplasmic side of the nuclear pore.

The best characterized of the herpes viruses include herpes simplex virus 1 (HSV-1) and cytomegalovirus. HSV-1 is too large to fit through the NPC (its internal capsid is 120 nm), so must at least partially uncoat in the cytosol. The HSV-1 capsids associate with nuclear envelopes about 2 hours after penetration of the host cell (Sodeik, Ebersold *et al.* 1997). By electron microscopy, it can be seen that the incoming HSV-1 capsids bind the nuclear envelopes at the NPC (Batterson, Furlong *et al.* 1983; Ojala, Sodeik *et al.* 2000). They attach to the cytoplasmic fibrils of the cytoplasmic ring of the NPC, approximately 50 nm from the ring, and adopt an orientation with one of the pentons facing the pore (Sodeik, Ebersold *et al.* 1997). The binding of HSV-1 to the fibrils of the cytoplasmic ring seem to be dependent on importin β and at least one of the virus' tegument proteins, however, it is unclear whether the binding requires additional importin docking molecules (Ojala, Sodeik *et al.* 2000). After attachment to the pore an intact-looking capsid devoid of DNA remains associated with the NPC for some time (Granzow, Weiland *et al.* 1997; Sodeik, Ebersold *et al.* 1997).

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Adenovirus type 2 (Ad2) seems to operate in much the same way as HSV-1. Like HSV, Ad2 is too large to enter the nucleus intact, as the diameter of its capsid is 90 nm. It has been known for several years that Ad2 DNA enters the nucleus alone, in the absence of capsid proteins (Greber, Suomalainen *et al.* 1997). More recently, however, the mechanisms through which Ad2 targets the nucleus, uncoats, and transfers its genome to the nuclear interior have been better elucidated (Trotman, Mosberger *et al.* 2001). It was demonstrated for this virus that incoming virions initially dock to the nuclear cytoplasmic ring filament component Nup214 (CAN). This was demonstrated by both *in vitro* binding studies and in tissue culture through the microinjection of antibodies against Nup214, which inhibited infection by Ad2. The final disassembly of Ad2 at the NPC is dependent upon binding of histone H1, and the transport of the Ad2 DNA genome is dependent on transport factors that are known to be important for the cytonuclear transfer of histone H1, namely importin β and importin 7.

A.3. Viral genomes that depend on a viral protein for cytonuclear transport

This third group of viruses relies upon a viral protein for piggyback access to the nucleus, without the need for the entire virion to enter the nucleus. This mechanism is used by some of the hepadnaviruses, with hepatitis B being a well described example. The genome of this virus piggybacks on the viral polymerase, to which it is attached in the intact virion, to gain transport into the nucleus, and is dependent on the classical importins, Impα and Impβ, for nuclear transport (Kann, Bischof *et al.* 1997). The current theory is that the incoming viral core attaches to the NPC via classical cellular importins, which has been

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shown to be the case in *in vitro* binding experiments. Interestingly, the binding of the hepatitis B core to the NPC is dependent upon the phosphorylation status of core proteins (Kann, Sodeik *et al.* 1999). Once bound to the NPC, the core then releases the Pol/DNA molecule for entry through the NPC lumen (Kann, Bischof *et al.* 1997).

A.4. Nuclear targeting by adeno-associated virus

AAV, like most DNA viruses, replicates in the nuclei of its host cells. After initial infection, its genome integrates into the host genome and remains latent until rescued by a helper virus. After being rescued by a helper virus, the AAV genome is replicated in the nucleus, and its progeny virions assemble in the nucleus. The dependence by AAV on the host nucleus for latency and replication requires the virus to have mechanisms for targeting the virion to the nucleus during initial viral infection, delivery of the genome to the nuclear interior, and targeting the nucleus again when transporting the newly translated capsid proteins into the nucleus for viral assembly.

Despite the importance of this critical aspect of the AAV infection, very little is known of how AAV provides for transport of its genome across the nuclear envelope and how the progeny capsid proteins gain access to the nucleus for genome encapsidation and virion assembly. It is not known whether the virus enters the nucleus intact and is subsequently uncoated in the nucleus, or whether the virus is uncoated in the cytoplasmic compartment. Nor is it known whether any components of the AAV capsid are required to escort the genome into the nucleus.

In 1999, Hogue *et al* performed a study aimed at identifying the sequence within the AAV capsid responsible for the concentration of AAV capsid proteins in the nucleus during viral assembly (Hoque, Ishizu *et al.* 1999). These authors first made a plasmid construct that expressed only VP2 of the AAV capsid, without the other capsid proteins VP1 and VP3. They then made several constructs in which N-terminus deletions were made to the VP2. These constructs were transfected into mammalian cells, and fluorescent immunohistochemistry used to localize the VP2 protein within the cell. The authors found that the VP2 protein and N-terminal deletion mutants localized to the nucleus until the residues 29-34 of VP2 were deleted (^{29}PARKRL^{34}). Once these residues were deleted, the VP2 protein was found throughout the cytoplasm and nucleus. Since this region is homologous to known nuclear localization signals, the authors concluded that this region is responsible for driving the capsid proteins into the nucleus during assembly of capsids into new virions in the nucleus of the host cell.

To further characterize the function of residues 29-34 of VP2, Hogue *et al* synthesized the residues onto the N-terminus of GFP to determine whether the region is sufficient to localize GFP to the nucleus in a transfection setting (Hoque, Ishizu *et al.* 1999). They demonstrated by fluorescence microscopy that the fusion protein localized the nuclei of the cells, but that GFP alone was localized throughout the cytoplasm and nucleus, confirming their suspicion that residues 29-34 of AAV2 VP2 acts as a nuclear localization signal for the capsid proteins.

Additional work on the mechanism of AAV cytonuclear transfer of its genome was performed by Hansen *et al* (Hansen, Qing *et al.* 2001), who

demonstrated that AAV2 was capable of infecting purified nuclei in vitro. These authors showed that when Cy-3 labeled virions were incubated with isolated nuclei, the Cy-3 entered the nucleus in a non-saturable manner, not dependent on temperature, and independent of cytosolic factors. They also showed that the trafficking of Cy-3 into the nucleus after infection of intact cells by Cy-3 AAV was not inhibited by wheat germ agglutinin (WGA), a compound that has been demonstrated to bind to and inhibit transport through the nuclear pore complex, leading the authors to conclude that the virus does not enter nuclei through the NPC. Additional evidence for a nuclear entry mechanism independent of the NPC was that antibody against p62, a nuclear pore protein, did not inhibit infection. Importantly, though, it has been shown recently that an antibody against p62 did not inhibit the transfer of Ad across the NPC, but antibodies against other nucleoporin proteins did inhibit Ad genome transfer through the NPC (Trotman, Mosberger *et al.* 2001), showing the importance of testing multiple NPC antibodies before concluding that a nuclear entry mechanism is independent of the NPC. Lastly, these authors demonstrated that the nucleus contained all of the factors necessary for uncoating and second strand synthesis of the AAV viral genome, as the viral genome replicated in the purified nuclei.

The region of AAV2 containing the putative NLS is well conserved among all of the AAV serotypes. In the table to the left, the sequence of the putative NLS of AAV2 is compared with that of homologous regions of the other serotypes, though no studies on the nuclear

AAV2:	PARKRLNF
AAV1:	PAKKRLNF
AAV3:	PARKRLNF
AAV4:	PAKKKLVF
AAV5:	PKRKKART
CPV:	PAKRARRG

targeting characteristics of these regions have been published for any of serotypes other than AAV type 2.

A possible NLS has been identified in another parvovirus, the canine parvovirus (CPV). It was found that when a basic sequence suspected of acting as an NLS in VP1 of CPV was conjugated to BSA, and the conjugate microinjected into HeLa cells, the BSA localized to the nucleus (Vihinen-Ranta, Wang *et al.* 2002). It was further demonstrated that microinjection of antibodies against the basic region into the cytoplasmic compartment of cells was sufficient to prevent subsequent infection of the cells by CPV. The sequence identified as the NLS of CPV was PAKRARRG, which is very similar to that of AAV2, PARKRLNF (Vihinen-Ranta, Kakkola *et al.* 1997). The putative NLS of AAV2 is also somewhat similar in sequence to that of the protein Myc, PAAKRVKLD (Dang and Lee 1988). Myc binds with high affinity to Impα/Impβ heterodimers employing either Impα1 or Impα2.

Some of the work in this thesis is modeled after work on the parvovirus minute virus of mice (MVM). MVM has been shown to contain multiple NLSs in its capsid that act at different times during the virus' infectious cycle (Lombardo, Ramirez *et al.* 2000; Lombardo, Ramirez *et al.* 2002). MVM contains two major capsid proteins, VP1 and VP2. Lombardo *et al* identified several basic regions within the MVM capsid that, by sequence homology to known NLSs, they thought may act to target the incoming virion to the nucleus during initial infection and to localize the capsid proteins to the nucleus during viral assembly. They found that the sequence ^{528}KGKLTMRAKLR^{538} is necessary for the localization of the major

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capsid protein to the nucleus and that VP1 relied upon VP2 for cotransport across the nuclear envelope. Once establishing the sequence necessary for the nuclear uptake of viral capsid proteins during viral assembly, Lombardo *et al* found other basic regions within the MVM capsid that conferred infectivity upon the virus, possibly by targeting the incoming virion to the nucleus of the target host. Interestingly, all of the basic regions identified were on the inside of the virion, indicating that the virus must undergo some conformational change once inside the cell in order to complete the infection.

III.B. Results

B.1. There are four basic regions of the AAV2 capsid that resemble classical nuclear localization signals

We used P-SORT to search the AAV2 capsid sequence for areas concentrated in basic residues. We found 4 regions, which we term BR1 through BR4 (Figure 9). To establish whether each basic region is critical to the life cycle of AAV2, we mutated two of the basic residues in each basic region to asparagines. The mutations were carried out by site-specific mutagenesis to the Cap gene in the pACG2 plasmid (Li, Samulski *et al.* 1997) (Figure 9), and the plasmids named pACG2-BR1Δ through pACG2-BR4Δ. As an initial step in characterizing the mutants, we attempted to make recombinant virus with the mutated capsid proteins by the normal triple transfection protocol for manufacturing recombinant adeno-associated virus. Either normal pACG2 or pACG2 containing the above basic region mutations was transfected into AAV producer cells along with pXX6-80, which contains helper functions from Ad, and pTRUFeGFP, which contains the DNA molecule to be packaged into the recombinant AAV flanked by the AAV2 inverted terminal repeats (green fluorescent protein being packaged). Cell lysates were then centrifuged through an iodixonol step gradient in order to separate assembled virions from unassembled capsid proteins and other cellular debris. The fraction of iodixonol that would normally contain the recombinant virus produced was resolved by SDS-PAGE and viral capsid proteins detected by Western blot analysis with the mAb B1, which detects unassembled and denatured AAV2 capsid proteins.

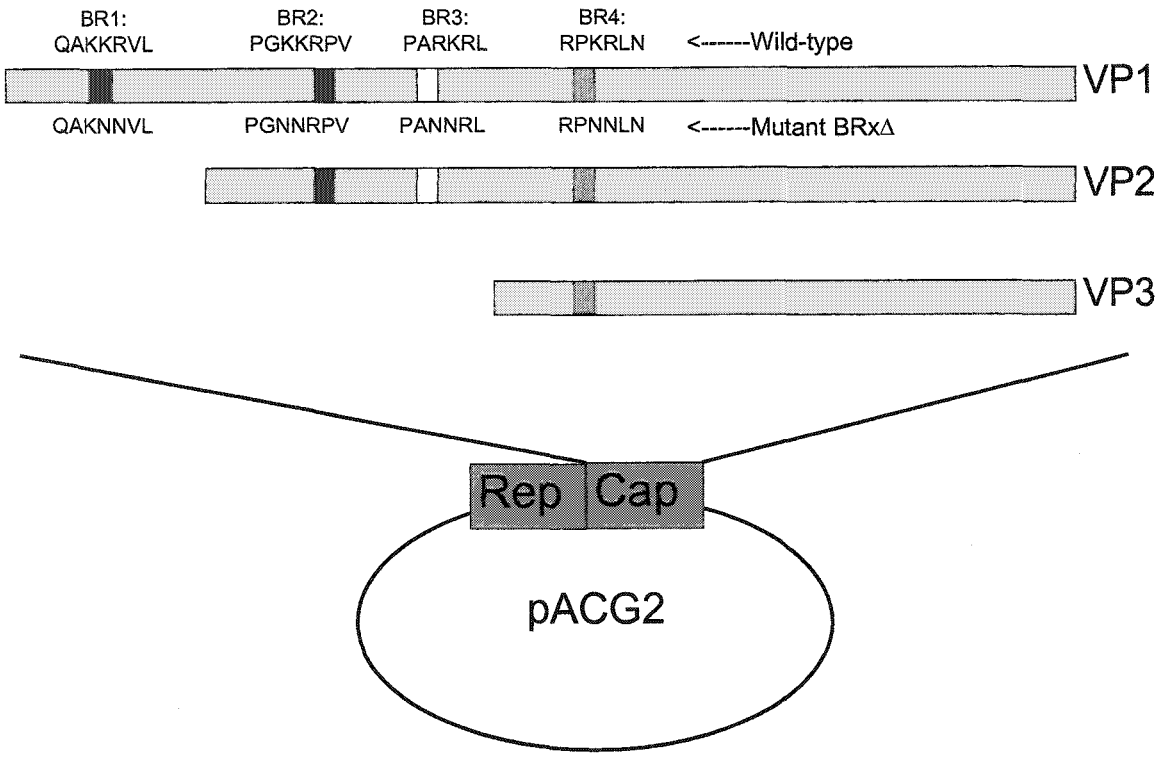

Figure 9. Diagram of basic regions (BR) in the AAV capsid, and scheme used to mutate the basic regions for analysis of function. PCR was used to introduce one of the four basic region mutations, BR1Δ-BR4Δ, resulting in four versions of the pACG2 plasmid, named pACG2-BR1D through pACG2-BR4D.

Resulting virus was then assayed for titer, ability to protect its viral genome from DNAse digestion (a test of integrity of the viral capsid), and for the virus' abilities to deliver the marker gene (green fluorescent protein) packaged during the viral assembly (a test of infectivity of the virus).

All of the BR mutants resulted in virus that protected DNA. The virus produced from the pACG2-BR1Δ construct was composed of only VP3, and neither VP1 nor VP2 could be detected in these virions (Figure 10, lane 1). Subsequent experiments shown in another section demonstrated that it is likely that VP1 and VP2 were not produced (immunoprecipitation of capsid proteins from producer cell lysates resulted in no VP1 or VP2 being detected). Despite being composed of only VP3, this virus protected its DNA genome from DNAse digestion. Though the VP3-only virus protected DNA, it was unable to infect cultured HeLa cells, as measured by its inability to convert the cells to green fluorescence (Figure 10).

Virus produced from the pACG2-BR2Δ mutant contained all three capsid proteins, and in the appropriate ratio to one another (Figure 10, lane 2). The virus was produced at levels similar to that of recombinant AAV assembled from wild-type capsids, and protected its DNA cargo from DNAse digestion. This virus infected cultured HeLa cells with efficiency similar to that of recombinant AAV made from wild-type capsids.

Mutation of AAV2 BR3 resulted in the production of AAV at normal titer (relative to recombinant virus made from wild-type capsid proteins). AAV2-

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BR3Δ-GFP protected DNA, but was non-infectious on cultured HeLa cells (Figure 10, lane 3).

AAV2-BR4Δ-GFP protected DNA, but was produced at very low titer (Figure 10, lane 4). Mutations to AAV2 BR3 and BR4 had interesting and profound effects on the virus' abilities to deliver its genome to the nuclear compartment (BR3) and the capsid proteins' abilities to localize to the nuclei of the host cell during viral assembly (BR4). These two mutants, AAV2-BR3Δ and AAV2-BR4Δ, were investigated in much greater detail and are presented in sections B.2., B.3., and B.4.

B.2. The putative NLS of AAV is not necessary for nuclear sequestering of viral capsid proteins during viral assembly, or the production of DNA-protecting virions, but is necessary for infectivity of AAV2.

Hogue *et al* identified a basic region of the AAV2 capsid that possessed high homology to known NLSs. They fused the region to the N-terminus of GFP, and characterized the region's ability to localize the GFP fusion protein to the nuclei of transfected cells. They then established the importance of the region in localizing singly expressed VP2 (in the absence of VP1 or VP3) to the nucleus (Hoque, Ishizu *et al.* 1999). The authors concluded, through N-terminus mutant studies of singly expressed VP2, that the region was critical for concentrating the 3 capsid proteins in the nucleus during viral assembly. The region they identified was VP2 residues 29-34, PARKRLN (residues 166-172 of VP1). Though

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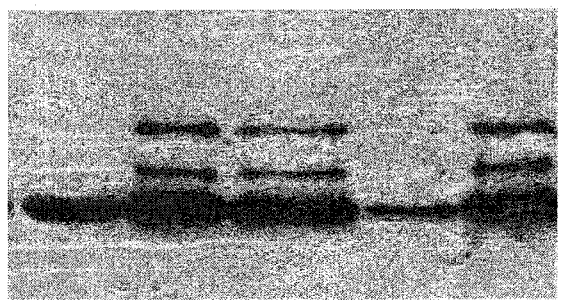
valuable, their work left open the question of whether the region is important in the context of a productive infection with all three capsid proteins present,



	BR1	BR2	BR3	BR4	wt
DNA protection	+	+	+	+	+
Physical titer, fold wt	~1	~1	~1	.005	1
Infectivity	--	+	--	?	+

Figure 12. Initial characterization of AAV2 basic region mutants. Cells were transfected with AAV producer plasmids (see text), and the lysates centrifuged through an iodixonol step gradient to remove cell debris and unassembled AAV capsid proteins. The iodixonol fraction containing potential AAV virions was assayed for the ability to protect the viral DNA from DNAse digestion (DNA protection), level of viral production (Physical titer), and the ability to infect cultured HeLa cells (Infectivity). BR1-BR4 stand for the basic region that was mutated (see figure 9).

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assembling into actual virions, and if the region is important, whether it operates from the context of VP1 or VP2.

We sought to determine whether the putative NLS proposed by Hogue *et al*, ^{166}PARKRLN^{173}, is important in localizing the AAV capsid proteins to the nucleus during an actual viral assembly. It has been shown previously that mutation of basic residues to asparagines abrogates the function of NLSs without interfering with the secondary structure of surrounding regions. We used PCR to make the point mutations K168N and R169N in the putative AAV2 NLS. These mutations were carried out in the AAV helper plasmid pACG2 (Li, Samulski *et al.* 1997), which contains the AAV Cap and Rep genes (Figure 9), were sequenced, and subcloned back into ACG2, henceforth called pACG2-BR3Δ. We then transfected the pACG2-BR3Δ construct into HEK293 AAV producer cells, along with the Ad helper construct XX6-80 and another plasmid, pTRUFeGFP (Haberman, McCown *et al.* 2000). XX6-80 contains Ad genes that are necessary for the replication of the Rep and Cap genes and viral packaging, while pTRUFeGFP contains the gene for enhanced green fluorescent protein situated between the AAV2 terminal repeats. Though we did not expect the mutated capsid proteins to traffic normally to the nucleus for viral assembly, if the capsid proteins did traffic and assemble normally, this transfection scheme would have resulted in the terminal repeats and the eGFP gene being packaged into AAV particles in the nucleus (see section II) that could then be used to test infectiousness of the particles in tissue culture experiments.

To our surprise, capsid proteins with the putative NLS of AAV2 mutated from ^{166}PARKRLN^{173} to ^{166}PANNRLN^{173} made recombinant virus at wild-type levels (Figure 10), with all 3 capsid proteins of the correct size and ratio of incorporation. Production of virus was determined by assay of producer cell lysates for DNA-protecting particles. Cell lysates were treated with DNAse, and the viral genomes (GFP gene and terminal repeats) released by treatment with protease and measured quantitatively by Southern blot against the GFP gene.

We next sought to determine whether AAV2 capsid proteins containing the R168N and K169N mutations concentrated in the nuclei of the producer cells to the same extent as do the wild-type capsid proteins during viral assembly. HEK293 cells were transfected as above with pXX6-80, pTRUFeGFP, and either pACG2 or pACG2 BR3Δ. 24 hours later, cells were fractioned into nuclear and cytoplasmic fractions, and the fractions assayed by Western blot to determine the location of the capsid proteins. As shown in figure 11, the preponderance of the wild-type capsid protein was found in the nuclear fraction. Surprisingly, the same pattern of distribution was observed for the capsid proteins that had the putative NLS mutated, i.e. the capsid proteins generated from the pACG2-BR3Δ plasmid construct localized to the nucleus.

The next step in characterizing the AAV2-BR3Δ virus was to determine whether the virus made from the mutated capsid proteins was infectious. Virus was produced as described above, with the resultant viruses being called AAV2-GFP and AAV2-BR3Δ-GFP. HeLa cells were infected with either AAV2-GFP or AAV2-BR3Δ-GFP and assayed 24 hours later by direct fluorescent microscopy

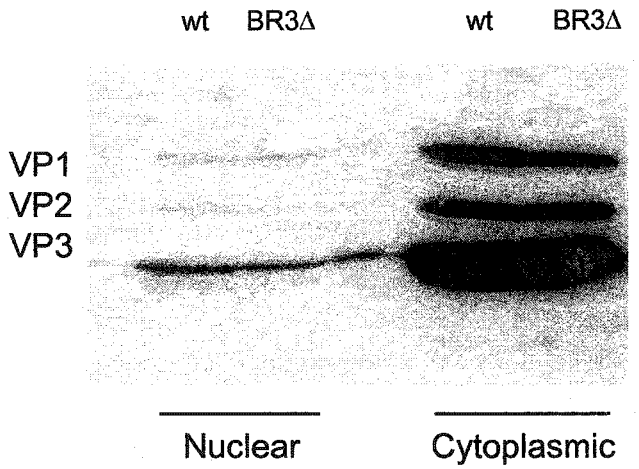
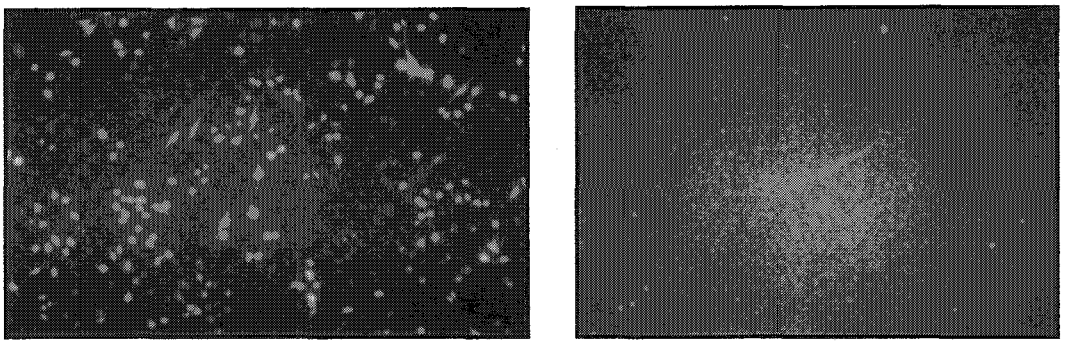


Figure 12. Subcellular localization of AAV2 capsid proteins after mutation of basic region 3. AAV producer cells were transfected with pACG2-BR3D and XX6-80. 24 hours later, cells were fractioned into cytoplasmic and nuclear fractions, and the fractions assayed by Western blot to establish the location of the capsid proteins

(the GFP gene was packaged into the virion during the viral assembly process so that infectivity could be assayed directly). Though the virus protected its DNA from DNAse digestion, and though the virus assembled at titers comparable to virus made from wild-type capsid, the AAV2-BR3Δ virus was not successful at transducing mammalian cells in tissue culture (Figure 12).



AAV2-GFP AAV2-BR3Δ-GFP

Figure 12. AAV2 BR3 mutant fails to transduce cultured HeLa cells. Cultured HeLa cells were infected with either AAV2-GFP or AAV2-BR3Δ-GFP. 24 hours following infection, cells were imaged by fluorescence microscopy.

B.3. The AAV2-BR3Δ mutant's poor transduction ability is unrelated to heparin binding delivery of its DNA genome to the nuclear periphery

After determining that the putative NLS of AAV2 is not necessary for the trafficking of the AAV capsid proteins to the nucleus of the producing cell line during viral assembly, or necessary for the formation of DNA protecting virions, we sought to further characterize the nature of the defect of the AAV2-BR3Δ virus. This characterization would include determining whether the virus is structurally correct, whether it is capable of binding the AAV2 receptor, heparin proteoglycan, and whether the virus is capable of normal AAV2 trafficking to the nuclear periphery.

As a first step in characterizing the AAV2-BR3Δ mutant, we wanted to ensure that the virus formed normal virions as assayed by scanning electron microscopy. Figure 13 shows an electron micrograph (EM) of the AAV2-BR3Δ-GFP virus produced for the studies below. The AAV2-BR3Δ-GFP virus was the appropriate size (~25 nm), and appeared normal in all regards in the EM.

We next examined the AAV2-BR3Δ virus' ability to bind the AAV2 receptor, heparin proteoglycan (Summerford and Samulski 1998). This task was performed by subjecting the iodixonol-purified virus to affinity column chromatography, with the column being immobilized heparin sulfate (Summerford and Samulski 1999). As shown in figure 14, the AAV2-BR3Δ virus bound to heparin in a manner identical to that of virus made with wild-type capsid proteins,

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and released and eluded from the heparin column at the same salt concentration as has been shown for virions with wild-type capsid proteins Summerford, 1999 #79}.

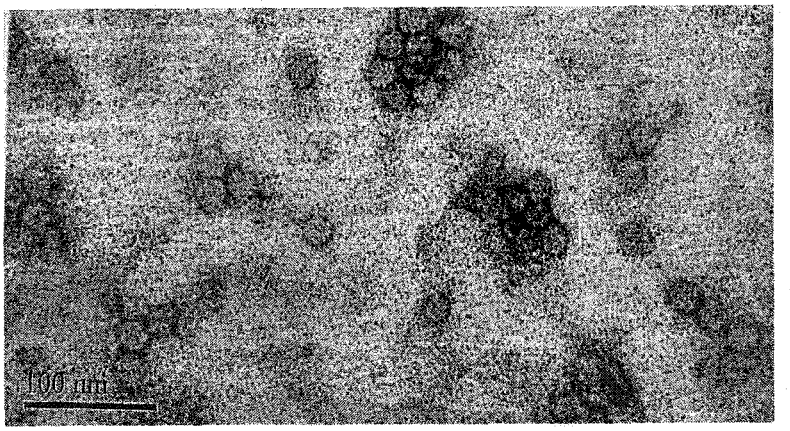

Figure 13. Electron micrograph of AAV2-BR3Δ virions. Calibration bar represents 100 nm. Intact wtAAV2 virions are approximately 25 nm in diameter.

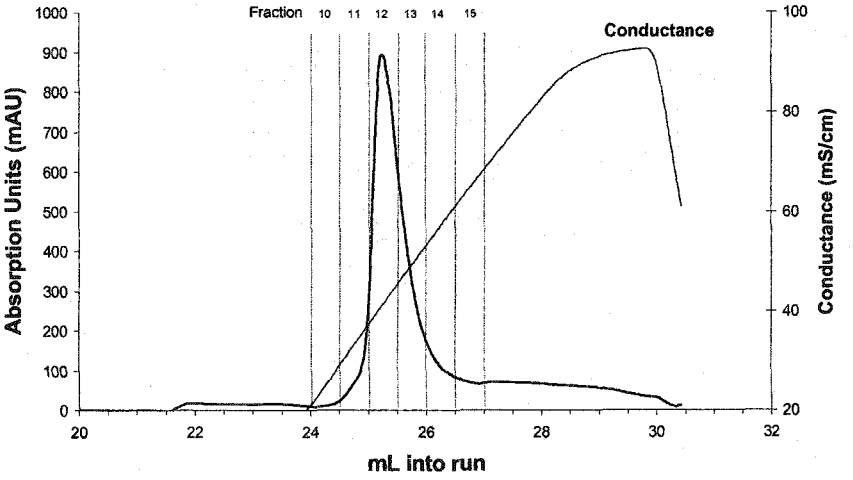

Figure 14. Binding of the AAV2-BR3Δ mutant to heparin

The next step in our characterization of the AAV2-BR3Δ virus was to determine whether the virus could compete with wild-type capsids for trafficking events subsequent to initial viral binding to the cell surface. Wild-type capsids with a transgene encoding for luciferase, AAV2-LUC, were allowed to bind HeLa cells at 4°C, which allows for binding of the virus to the cell surface receptor, but not internalization of the virus into the cell. AAV2-GFP or AAV2-BR3Δ-GFP was then added to the cell supernatant and the cells warmed to 37 degrees. Luciferase activity was measured 12 hours later to determine whether the AAV2-BR3Δ-GFP was able to compete with the trafficking of the AAV2-LUC. Figure 15 shows that wild-type AAV2 capsids, i.e. AAV2-GFP, competed with the AAV2-LUC at a step subsequent to receptor binding and reduced the expression of the AAV2-LUC by more than 50%. Surprisingly, the AAV2-BR3Δ-GFP also competed with the AAV2-LUC, and reduced the luciferase activity by 50%.

We next wanted to know whether wild-type capsids were capable of rescuing the AAV2-BR3Δ phenotype. AAV2 is thought to traffic through early and late endosomes, and possible through some interaction with proteosomes (Bartlett, Wilcher *et al.* 2000; Hansen, Qing *et al.* 2000; Douar, Poulard *et al.* 2001). It has been suggested that AAV is internalized into endosomes at a ratio of 1 viral particle per endosome (Seisenberger, Ried *et al.* 2001). However, it is unlikely that the endosomes containing AAV traffic all the way to the nucleus without merging with other AAV-containing endosomes, as enodosomal merging is part of the normal endosome trafficking mechanism in mammalian cells (Lemmon and Traub 2000). We reasoned that if transduction by the AAV2-BR3Δ

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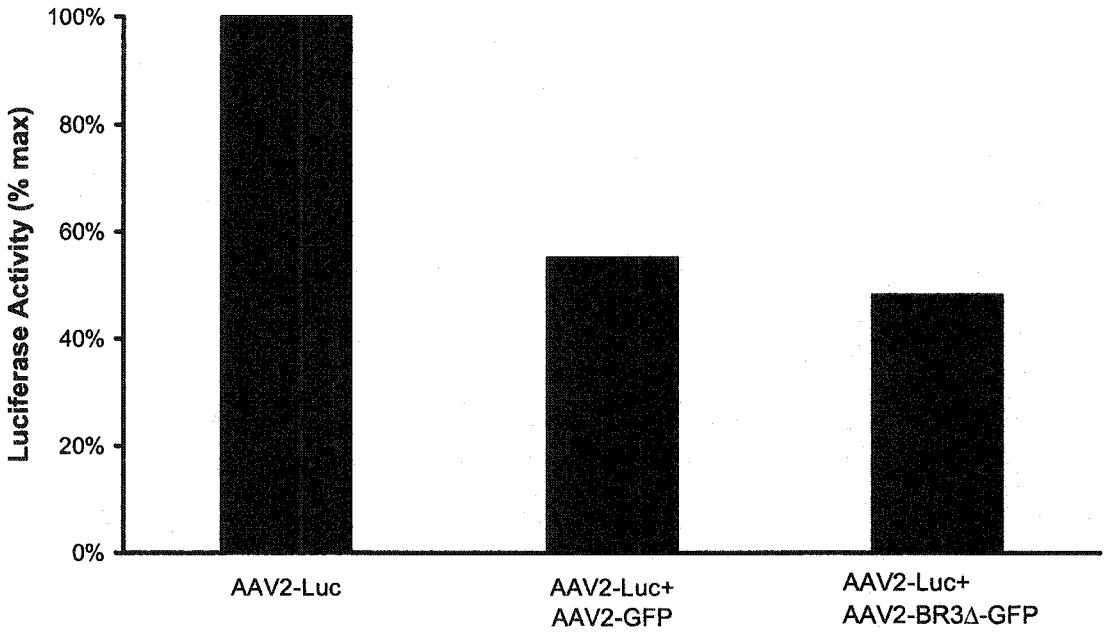

Figure 15. AAV2-BR3Δ-GFP competes with wild-type AAV2 capsids for intracellular trafficking. AAV2-Luc was allowed to bind HeLa cells at 4°C and then rinsed in cold PBS. AAV2-Luc or AAV2-BR3Δ-Luc was added to the cells and the cells warmed to 37°C. Cells were assayed 24 hours later for luciferase activity.

virus was being blocked by an inability of the virus to escape from the endosomal compartment, perhaps a coinfection with non-defective virions would allow the AAV2-BR3Δ-GFP to transduce cells by piggy backing on the endosomal escape by the wild-type capsids. HeLa cells in culture were infected simultaneously with AAV2-LUC and AAV2-BR3Δ-GFP and assayed 24 hours later by fluorescence microscopy to score for GFP expression. The left panel in figure 16 shows infection of HeLa cells by AAV2-GFP, with a high level GFP visible. The center panel shows HeLa cells infected with AAV2-BR3Δ-GFP, and no green cells are visible. The right panel shows HeLa cells infected with both AAV2-BR3Δ-GFP and AAV2-LUC, and once again no green cells are visible. In this experiment, wild-type AAV2 capsids were unable to rescue the AAV2-BR3Δ phenotype.

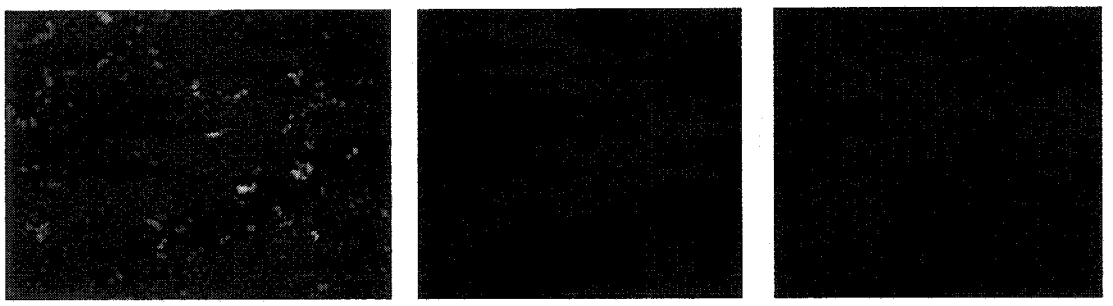

Figure 16. Coinfection of HeLa cells with AAV2-BR3Δ-GFP and AAV2-Luc. To determine whether the AAV2-BR3Δ phenotype could be rescued by wild type capsids, cells were infected simultaneously with AAV2-BR3Δ-GFP and AAV2-Luc. Positive and negative controls are provided by AAV2-GFP (left panel) and AAV2-BR3Δ-GFP (middle panel), respectively.

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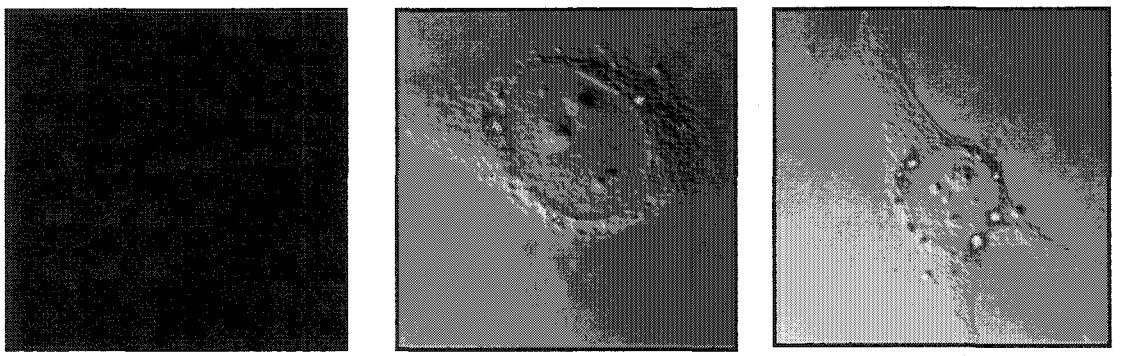
Since the AAV2-BR3Δ virus bound heparin and competed with wild-type capsids for trafficking, we next determined to what extent the virus was capable of trafficking normally through the cell. This determination was performed at two levels; that of the viral capsid proteins and that of the viral DNA.

As previous methods of detecting AAV capsid proteins in tissue culture cells required multiplicities of infection of 1 million virions per cell (Bartlett and Samulski 1998), we first established a protocol through which only 1000-5000 particles are needed per cell to image the capsid proteins by immunohistochemistry, as 1 million particles per cell (and perhaps even 5000 particles per cell) is probably well in excess of physiological levels. We optimized the tyramide system amplification (TSA, Biorad) protocol for detecting AAV capsid proteins in HeLa cells cultured in chambered glass slides (see Methods), and viewed the capsid proteins by confocal microscopy. HeLa cells in chambered glass slides were infected for 24 hours with AAV2 or AAV2-BR3Δ, processed for TSA-based immunohistochemistry, and assayed by confocal fluorescent microscopy. Figure 17 shows AAV capsid proteins for both AAV2 and AAV2-BR3Δ (red staining). The far left panel is a negative control experiment in which the cells were not infected with any virus, but the TSA and immunohistochemistry protocols were carried out as normal. The blue staining is nuclear staining by DAPI. The middle image shows AAV2 capsid proteins in the perinuclear region after labeling of the capsid proteins with the anti-VP3 antibody B1, and TSA processing. The fluorescent image was overlaid on a DIC image of the same cell. The far right image shows the capsid proteins of the AAV2-BR3Δ-

GFP virus under the same conditions and processed in the same manner. The image shows that the AAV2-BR3Δ-GFP was not only internalized by the HeLa cells, but that the viral capsid proteins accumulated at the nuclear periphery, just as did the viral capsid proteins not containing the BR3Δ mutation.



(-) AAV AAV2-GFP AAV2-BR3Δ-GFP

Figure 17. Subcellular localization of AAV2-BR3Δ capsid proteins following infection of HeLa cells. HeLa cells were grown in chambered glass slides and infected with either AAV2-GFP or AAV2-BR3Δ-GFP. Cells were processed for immunohistochemistry with the mAb B1, and then processed for tyramide system amplification of a Cy-3 coupled secondary antibody. Cells in left panel were mock infected. Blue staining in left panel is DAPI staining of nuclei.

Once establishing that the AAV2-BR3Δ virion capsid proteins trafficked to the nuclear periphery, we sought to determine whether the associated DNA crossed the nuclear membrane into the nucleus. This was important because before concluding that the AAV2-BR3Δ is defective for mediating transfer of its genome across the NE, we had to establish that the virus was not crossing the NE, but was then unable to uncoat once inside the nucleus. HeLa cells in chambered glass slides were infected with either AAV2-GFP or AAV2-BR3Δ-GFP and processed 24 hours later for fluorescent *in situ* histochemistry (FISH). Cells were then examined by confocal microscopy to determine the location of the GFP gene delivered by the incoming virions. The far left panel in Figure 18 shows the results for mock infected cells. The center panel shows the results for AAV2-GFP. Clear punctate staining can be seen inside the nucleus, representing the presence of the GFP gene delivered to the cell by the virus, though the preponderance of the GFP gene is located in a rim around the nucleus. The far right panel shows the results for AAV2-BR3Δ-GFP. For this mutant, no staining can be seen inside the nucleus; all of the staining of GFP gene can be seen in a ring around the perimeter of the nucleus.

(-) AAV2 AAV2-GFP AAV2-BR3Δ-GFP

Figure 18. Subcellular localization of DNA delivered by the AAV2-BR3Δ mutant. HeLa cells were grown in chambered slides and infected with either AAV2-GFP, or AAV2-BR3Δ-GFP. 24 hours later, cells were processed for fluorescent *in situ* hybridization against the GFP gene. Red color represents location of GFP gene.

The next experiment had two purposes. One was to make sure that the plasmid construct used to make the BR3Δ virus had no other defects other than the mutation to the putative NLS of AAV2. The second was to determine whether the homologous nuclear localization motif (NLM) identified within canine parvovirus (Vihinen-Ranta, Kakkola *et al.* 1997; Vihinen-Ranta, Wang *et al.* 2002) was capable of providing the same function provided by the AAV2 putative NLS. PCR site-directed mutagenesis was used to replace the BR3Δ mutation in pACG2-BR3Δ with the NLM identified for CPV, PAKRARR. Figure 19 shows HeLa cells 24 hours after infection with, AAV2-GFP (left panel), AAV2-BR3Δ-GFP (center panel), or AAV2-CPVNLM-GFP (right panel). Substitution of the NLM of CPV restored function to the AAV2-BR3Δ-GFP mutant as shown by the green cells in the right panel.

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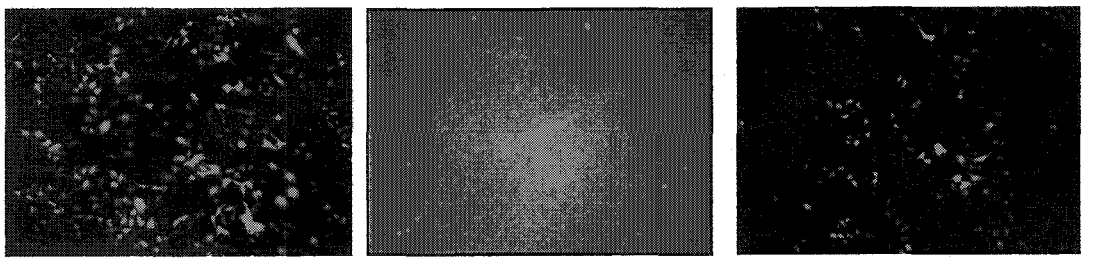

AAV2-GFP AAV2-BR3Δ-GFP AAV2-CPVNLM-GFP

Figure 19. Substitution of the canine parvovirus nuclear localization motif for the putative AAV2 NLS rescues the BR3Δ phenotype. The same plasmid that was used to generate AAV2-BR3Δ virions was mutated to replace the BR3Δ region with the nuclear localization motif of canine parvovirus, resulting in virus termed AAV2-CVP NLM-GFP, and the virus used to infect cultured HeLa cells to determine infectivity

B.4. The function provided by AAV2 BR3 resides in the VP1 capsid protein

AAV2 is composed of three capsid proteins, VP1, VP2, and VP3, which result from transcripts of the Cap gene of the AAV genome. The genetic structure of the Cap gene is such that VP3 is the major capsid protein, making up over 80% of the final AAV virion. VP1 and VP2 are minor components of the virion, each contributing about 10% to the makeup of the AAV virion structure. Also resulting from the genetic structure of the Cap gene, VP3 is an in-frame subset of VP2, and VP2 is an in-frame subset of VP1.

Since AAV2 BR3 is in both VP2 and in VP3, and since these two capsid proteins are both present in the AAV2 virion, we sought to determine whether the function provided by BR3 comes from the VP1 or VP2 capsid protein. Toward this end, we altered pACG2 so that either VP1 or VP2 would be produced during transfection of the plasmid into producer cells, but not both of the proteins simultaneously. In this way, we hoped to assemble virions composed of VP3 and either VP1 or VP2.

VP2 protein is produced from the same transcript as VP1, but its translation is started from an alternative start site downstream of the VP1 start site. VP2 translation is started from an ACG start, which greatly reduces the amount of the VP2 protein available for virion assembly.

To determine whether BR3 of VP2 is necessary for AAV2 to be infectious, we attempted to assemble AAV2 virions without the incorporation of VP2. This was accomplished by using site-specific mutagenesis to mutate the ACG start site of VP2 to ACA. This mutation was carried out in the pACG2 plasmid and the

resulting mutant plasmid was termed pACG2-ACA. pACG2-ACA was co-transfected into AAV producer cells along with pXX6-80 and pTRUFeGFP. The resulting virus was assayed by Western blot for the presence of the capsid proteins, and assayed by direct fluoroscopy for its ability to deliver the GFP gene to cultured HeLa cells.

Figure 20 shows the Western blot of lysates from producer cell transfected as described in the proceeding paragraph. Note that in the right lane, the VP2 protein is missing, resulting in virus composed only of VP1 and VP3. VP1 and VP3 formed DNA protecting particles at levels similar to those of production runs carried out with all three capsid proteins present. For this virus, the transgene packaged was luciferase. The AAV2-LUC composed of only VP1 and VP3, termed AAV2-VP2Δ-LUC bound heparin normally, and transduced HeLa cells with efficiency similar to that of virus made with all three capsid proteins, i.e. similar to that of AAV2-LUC (Figure 21). For figure 21, luciferase activity of the normal AAV2-LUC was set at 100% and the AAV2-VP2Δ-LUC compared to the normal AAV2-LUC. We used as a negative control the AAV2-Hep(-)-LUC virus, which we know to be noninfectious due to its inability to bind heparin.

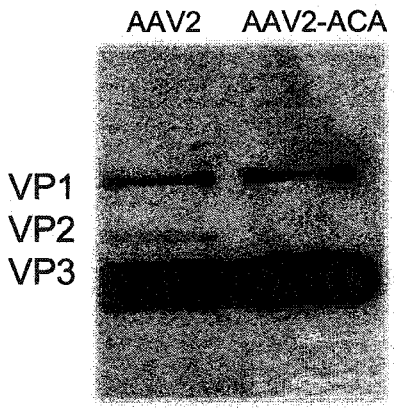

Figure 20. Production of AAV2 virions without the presence of VP2. The VP2 start site of the Cap gene of AAV2 was mutated to ACA in order to generate AAV2 without the protein. Virus was produced via the normal triple transfection protocol and Western blotted with monoclonal antibody B1 to generate the figure

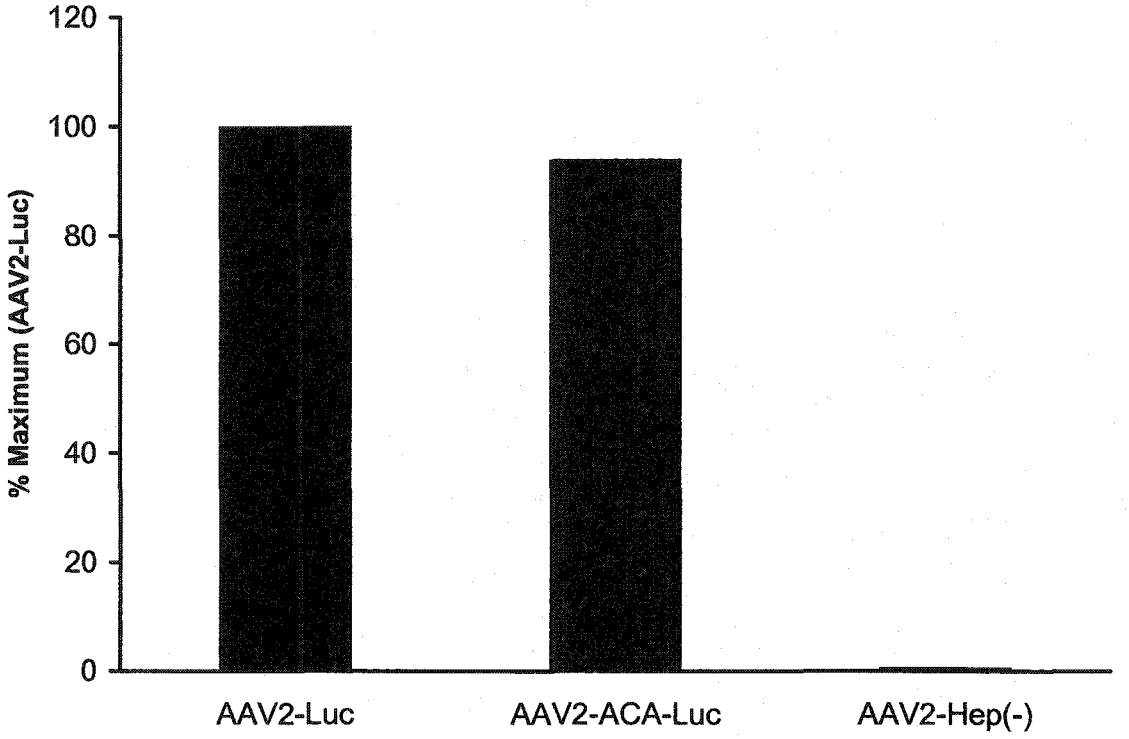

Figure 21. Infection of HeLa cells by AAV2 virions missing VP2. HeLa cells were infected with either AAV2 carrying the luciferase gene (AAV2-Luc), AAV2 missing VP2 but carrying the luciferase gene (AAV2-ACA-Luc), or a noninfectious version of AAV2 carrying the gene for luciferase (AAV2-Hep(-)). AAV2-Hep(-) has a mutation in VP3 that prevents it from binding the AAV2 receptor, heparin proteoglycan.

B.5. AAV2 BR4 contributes to localizing capsid proteins to the nucleus for assembly of progeny virions

It is clear from the data presented from experiments with the AAV2-BR3Δ mutant that the previously identified nuclear localization signal of AAV2 (Hoque, Ishizu *et al.* 1999) is not responsible for the localization of the viral capsid proteins in the nuclei of its host cells during viral assembly (Figure 11) as hypothesized by Hogue *et al.* We sought to determine whether one or more of the other basic regions we identified within the AAV2 capsid is responsible for conferring nuclear localization properties on the capsid proteins as they assemble into new virions in the nucleus of the producing cell. Since mutation of BR2 and BR3 did not affect the titer of virus produced in initial characterization of the basic regions of AAV2, we limited our study to BR1 and BR4. AAV2 producer cells were transfected with pXX6-80, pTRUFeGFP, and either pACG2, pACG2-BR1Δ, or pACG2-BR4Δ. After 24 hours, the producer cells were fractioned into nuclear and cytoplasmic fractions. Intact virions were immunoprecipitated from the fractions with the monoclonal antibody A20, which binds only to intact virions. Unassembled capsid proteins were then immunoprecipitated from the same fractions with the monoclonal antibody B1, which binds only to unassembled AAV2 capsid proteins. Immunoprecipitates were resolved by SDS-PAGE electrophoresis, and detected by Western blot with B1 antibody.

The top panel of figure 22 (end of this section) shows the results from the immunoprecipitation by mAb A20 of intact virions from the cytoplasmic and

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nuclear fractions of the producer cells. The intact wild-type AAV2 virions immunoprecipitated from the cell fractions were detected predominantly in the nuclei of the producer cells (Lanes 1 and 5, top blot). Intact AAV2-BR1Δ capsids were detected mostly in the nuclear compartment of the producer cells, though some capsids were detected in the cytoplasm (Lanes 2 and 6, top blot). The same was true for the capsid proteins produced by the AAV2-BR1Δ, i.e. the majority of assembled AAV2-BR1Δ capsids were found in the nuclear fraction. No VP1 or VP2 was detected in the A20-immunoprecipitated virions containing the AAV2-BR1Δ mutation, or in directly assayed cell lysates (not shown). Similarly to the wild-type capsids, the assembled virions of the AAV2-BR4Δ mutant were found predominantly in the nuclear fraction of the virus producing cells (Figure 22, lanes 3 and 7, top blot). Importantly, though the assembled AAV2-BR4Δ virions were found exclusively in the nuclear fraction, they were produced at a level far below that of the wild-type virus and that of the AAV2-BR3Δ virus (since A20 immunoprecipitates only assembled virions, the amount of capsid protein in the Western blot is reflective of the amount of virus produced, as long as the primary antibody is not saturated).

The bottom panel of Figure 22 shows the results of immunoprecipitation and Western blot of unassembled capsid proteins (monoclonal antibody B1) from the fractioned producer cells described in the previous paragraph. Capsid proteins generated from the pACG2 construct (all basic regions intact) were found almost exclusively in the nuclear fraction of the producer cells (bottom blot, lanes 1 and 5). Capsid proteins from the pACG2-BR1Δ construct seemed to be

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missing in VP1 and VP2 (center lane both fractions), as these capsid proteins were undetectable even in the nuclear fraction. We did not attempt to establish why these proteins were not detectable in the fractions from the transfected producer cells. However, the VP3 protein of this construct was produced normally and detected by Western blot. We found the VP3 produced from the pACG2-BR1Δ construct was predominately located in the nuclear fraction of the producer cells (Figure 22, bottom blot, lanes 2 and 6), similar to that produced from the wild-type Cap gene in pACG2.

As can be seen in lanes 3 and 7 of the bottom blot, the capsid proteins generated from the pACG2-BR4Δ construct behaved very differently from those of the wild-type construct. The pACG2-BR4Δ capsid proteins were present in the expected ratios between VP1, VP2, and VP3, but were distributed evenly between the cytoplasmic and nuclear fractions. This non preferential distribution of the three capsid proteins between the nuclear and cytoplasmic cell fractions was in contrast to that of wild-type capsid proteins, which were found almost exclusively in the nuclear fraction of the producer cells.

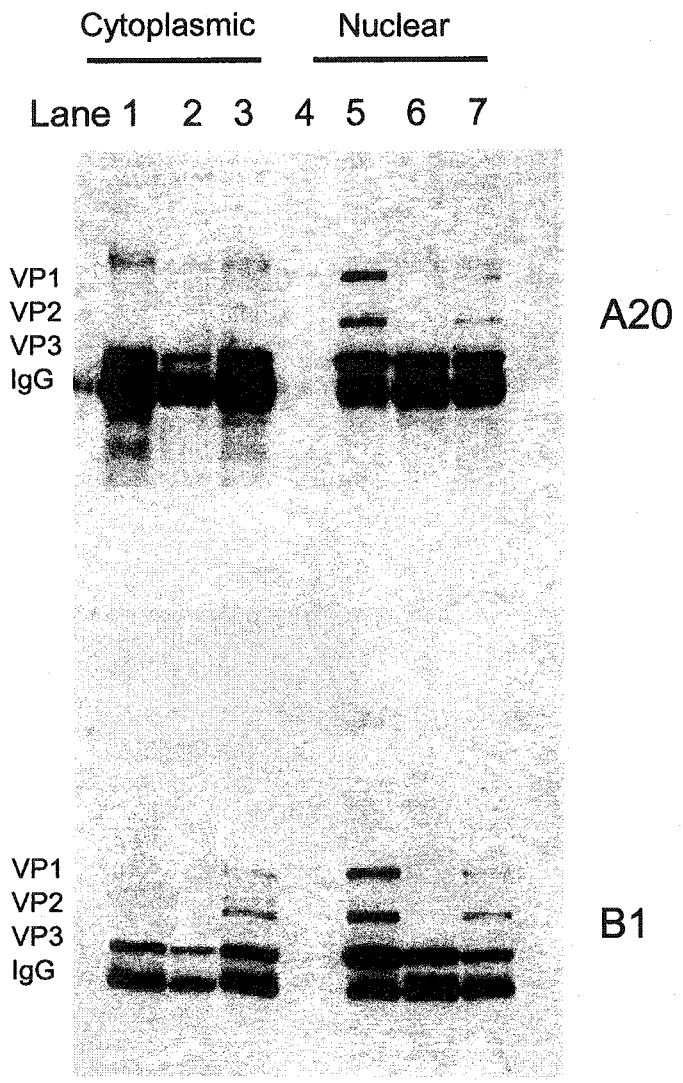

Figure 22. Subcellular distribution of AAV2 capsid proteins during viral assembly. Capsid proteins were immunoprecipitated with an antibody against intact capsids (A20), or an antibody against unassembled capsids (B1) and Western blotted. The procedure was performed for wild-type capsid proteins (lanes 1 and 5), AAV2-BR1Δ (lanes 2 and 6), and AAV2-BR4Δ (lanes 3 and 7).

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III.C. Discussion

Adeno-associated virus is gaining popularity as a gene delivery vehicle for both research and gene therapy applications. As the virus gains popularity in these applications, it will be increasingly important to understand the molecular biology of the virus. To contribute to the understanding of AAV biology, we have worked on elucidating aspects of the AAV trafficking problem, i.e. how the virus moves through its host cells to initiate an infection. We are particularly interested in the mechanisms by which incoming AAV virions target the nucleus for infection, and the mechanisms through which AAV capsid proteins target the nucleus during the process of viral assembly.

AAV capsid proteins target the nuclei of its host cells twice during a productive infection; once when the incoming virion delivers its genome to the nucleus, and again when newly formed capsid proteins enter the nucleus to form the progeny virions. While it has been known for some time that AAV takes up residence in the nuclei of its host cells, until 1999 virtually nothing was known about how AAV virions and capsid proteins targeted the nuclei of their host during initial viral entry and during subsequent production of progeny virions during viral assembly. In 1999, Hogue *et al* published the first description of a possible nuclear localization signal in the AAV2 capsid. The sequence, PARKRL, was similar to NLSs described already in the literature, such as that of SV40 large tumor antigen, PKKKRKV (Kalderon, Richardson *et al.* 1984), and human *c-myc*, PAAKRVKLD (Dang and Lee 1988). Hogue's work demonstrated that the AAV2 VP1 sequence PARKRL was important for localization of singly

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expressed VP2, and concluded that the region must be responsible for trafficking the capsid proteins to the nucleus during progeny viral assembly, but the work did not explore the function of this sequence in the course of a more physiological infection.

We first characterized the function of the putative nuclear localization signal (P-NLS) of AAV2, which corresponds to our nomenclature AAV2 BR3. When we mutated the AAV2 P-NLS by site directed mutagenesis, the mutated capsid proteins accumulated in the nuclei of the producer cells in a manner identical to that of wild-type capsid proteins (Figure 11). By electron microscopy, we found that the P-NLS mutant capsid proteins assembled into virions that looked identical to those composed of wild-type capsid proteins (Figure 13). The virions that assembled from the P-NLS mutant capsids bound to immobilized heparin, released from the heparin at the same salt concentration as does wild-type AAV2, and protected their DNA cargo from DNAse activity (Figure 14). We then established that the capsid proteins of the P-NLS mutants trafficked to the nuclear periphery during initial infection, as did the viral DNA (Figures 17 and 18). Despite the capsid proteins and viral DNA traveling to the nuclear periphery, the P-NLS mutant virions are non-infectious (Figure 12). Since it was clear that putative NLS of AAV2 was not responsible for localizing capsid proteins to the nuclear interior for assembly of AAV virions, we then characterized the other basic regions of the AAV2 capsid (BR1, BR2, and BR4) to determine whether any of these regions is important in AAV2 capsid protein trafficking, and found that AAV2 BR4 may play such a role.

Initial characterization of four basic regions in the AAV2 capsid

We used the P-SORT software to search the AAV2 capsid for regions that may confer nuclear localization characteristics on the AAV2 capsid proteins or virions. This software searches a protein sequence for domains with high homology to known cellular sorting signals, such as nuclear localization signals (NLSs). Four regions of high basic residue content (often a marker for nuclear localization signals) were identified that may act as nuclear localization signals in the AAV capsid, including the region identified by Hogue *et al* as the nuclear localization signal. Several in vitro and tissue culture techniques were used to characterize these basic regions in the AAV2 capsid identified by P-SORT. We used site-directed mutagenesis to mutate basic residues in these regions to asparagines, and as a first level of analysis, assayed the effects of the mutations on the virus' ability to form virions that protect their DNA cargo from DNAse digestion and the virus' ability to infect cultured HeLa cells.

The mutations to the basic regions were carried out using as a backbone the pACG2 plasmid. Once sequenced, the mutated pACG2 plasmids were termed AAV2-BR1Δ through AAV2-BR4Δ. One of these constructs was transfected into AAV producer cells, and 48 hours later, the cells harvested for viral production. As a quick diagnostic of whether the mutation resulted in an inability to form virions, the whole cell lysates were centrifuged over an iodixonol step gradient, which separates denser intact virions from less dense unassembled capsid proteins (Zolotukhin, Byrne et al. 1999). The iodixonol fraction that would have contained intact AAV virions was Western blotted for the

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presence of AAV capsid proteins. The iodixonol fractions were then assayed for virus that was capable of protecting its DNA from DNAse digestion, and then for virus capable of delivering its DNA transgene, GFP ((Haberman, Kroner-Lux et al. 1999) standard recombinant AAV production methods were used to package GFP into the mutant virions). The results are summarized in Figure 10.

Only one of the mutations carried out to the basic regions of the AAV2 capsid had no impact on the formation of DNA-protecting virions or the formation of infectious virions. Mutation of AAV2-BR2Δ resulted in a virus that was produced in normal titer, capable of protecting its DNA cargo, and as infectious as virus made from wild-type capsid proteins. The other 3 mutations had interesting and profound effects.Our initial characterizations of mutations to the basic regions were consistent with work performed coincidentally by another group. Wu *et al* searched the AAV2 genome for groups of charged residues (Wu, Xiao et al. 2000). For each group of charged residues, of which there were 60, they mutated several of the charged residues to alanines and assayed for the ability of the mutated capsid proteins to form infectious virions. Their mutations included alterations to BR1, BR2, BR3, and BR4. Though they used different mutations, i.e. alanine substitutions, results between our studies and theirs were largely identical for BR2 and BR3, but differed for BR1 and BR4. Our mutations are compared to their mutations in the table below. Wu *et al* found that the mutation to BR1 resulted in virions that bound heparin, but were not infectious. It is possible that VP1 and VP2 were missing from their viruses in which alanine substitutions were made in BR1, but no Western blot data is provided in the

paper, so direct comparison to our mutants of the same region is difficult to make. Results for mutations to BR2 were the same between the Wu study and our own, i.e. neither mutation to BR2 caused disruption of viral assembly or viral infectivity. Likewise, the two studies had identical results for mutations to BR3, i.e. both mutations caused a disruption to infectivity, without disrupting the capsid proteins' ability to form virions at titers similar to that of wild-type capsid proteins. Beyond its failure to infect, characterization of the BR3 mutant was not carried out by Wu *et al.*

Wild type sequence	Our mutations	Wu's corresponding mutations
^{120}QAKKRVL (BR1)	^{120}QAKNNVL	^{120}QAAAAVL
^{140}PGKKRPV (BR2)	^{140}PGKKRPV	^{140}PGAAAPV
^{166}PARKRLN (BR3)	^{166}PANNRLN	^{166}PAAAALN
^{307}RPKRLN (BR4)	^{307}RPNNLN	^{307}APAALN

Results of the two studies may or may not diverge for the BR4 mutant. Wu *et al* reported that they were unable to form virions from capsid proteins that contained the alanine insertions into BR4. We have found that virus was formed from capsid proteins containing our mutation to BR4, albeit at very low levels (Figure 22). The difference between the studies could be due to differing detection limits (Wu's Southern blot versus our immunoprecipitation and Western blot), or could be due to differences in the capsid protein structure and function given the markedly different mutations that each study made to BR4.

The putative AAV2 nuclear localization signal is not necessary for nuclear assembly of intact AAV2 virions, but is necessary for infectivity of AAV2

We next wanted to confirm that the sequence identified by Hogue *et al* was responsible for trafficking AAV capsid proteins to the nucleus for assembly into new virions. To this end, we point mutated two basic residues within the putative NLS to asparagine (i.e. PARKRL to PANNRL). These mutations were made to the Cap gene within the pACG2 plasmid, which was then termed pACG2-BR3Δ. We triple transfected AAV producer cells (HEK293) with pACG2-BR3Δ, which contains the AAV2 Rep and mutated Cap genes, pXX6-80, which contains Adenovirus helper functions necessary for AAV replication, and pTRUFeGFP, which contains the eGFP gene flanked by AAV2 terminal repeats. Viral production was determined by Southern blot against the eGFP gene. We were surprised to find that DNA-protecting AAV2 particles were produced at wild-type levels, despite the mutation to AAV2's putative NLS. Since AAV particles assemble in the nucleus, we expected that if the putative NLS was responsible for driving capsid proteins into the nucleus for assembly, there would be little virus produced. We then fractioned the producer cells into nuclear and cytoplasmic fractions 24 hours after the transfection protocol, and assayed the fractions by Western blot to determine the location of the capsid proteins as virus was being produced. We found all three capsid proteins to be predominately located in the nucleus, for both the wild type capsid proteins, and for proteins with the putative NLS mutated (Figure 11). Our findings are not necessarily in contrast with those of Hogue *et al*, as these authors demonstrated that the region

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^{166}PARKRL^{171} is necessary for the localization of singly expressed VP2 with the N-terminus deleted (expressed from transfected plasmid) to the nuclei of HeLa cells. In their experiments, Hogue *et al* made N-terminus deletions deeper and deeper into VP2. The N-terminus deleted VP2 continued to localize to the nucleus until the region ^{166}PARKRL^{171} was deleted, after which the protein was distributed between the nucleus and cytoplasm. It is possible that singly expressed VP2 with 35 residues deleted from the N-terminus folds, or is processed, in a way that masks the function of another basic region in the VP2 that is downstream from the putative NLS. Our laboratory has found that singly expressed VP3, which does not contain the ^{166}PARKRL^{171} region, is capable of localizing to the nucleus and assembling into DNA protecting virions, indicating that the region identified by Hogue *et al* may not be important for concentrating capsid proteins in the nucleus during virion assembly (Rabinowitz, Xiao et al. 1999).

Another possibility to explain the difference in findings between our work and that of Hogue *et al* is that our mutation was insufficient to abrogate the function of the ^{166}PARKRLN^{173} region. We consider the latter possibility unlikely, as the mutation abrogated other functions of the region affecting the virus' ability to infect its host cells, which is shown in a subsequent section.

Once establishing that BR3 of AAV2 is not necessary for the localization of the assembling capsid proteins to the nucleus as previously thought, we sought to further characterize the virus in which this region was mutated, AAV2-BR3Δ, by packaging the gene for eGFP into the mutated virion, resulting in a

virus we term AAV2-BR3Δ-GFP, and attempting infection of HeLa cells followed by assay of gene delivery by direct fluorescent microscopy. We found through attempted infection of HeLa cells in tissue culture that the AAV2-BR3Δ-GFP virus was non-infectious (Figure 12), despite the virus appearing normal by electron microscopy (Figure 13), so our next steps were aimed at establishing at which point of infection this AAV2 mutant fails.

The AAV2-BR3Δ mutant binds heparin normally, and delivers its genome to the nuclear periphery just as do wild-type capsids

DNA viruses must successfully navigate several steps in order to replicate in the nuclei of their host's cells. The virus must first bind to a cellular receptor and internalize into the cytoplasm. At some point during the virus' trafficking towards the nucleus, the virus must escape from the endosome. The virus must then migrate to the nuclear envelope, where it must somehow mediate transfer of the viral genome across the nuclear envelope. Our characterization of the trafficking of the AAV2-BR3Δ-GFP virus started with ensuring that the virus was capable of binding the AAV2 receptor, heparin proteoglycan. One would not expect mutations in AAV2 BR3 to affect heparin binding, as this function has been shown to exist solely in VP3 (Rabinowitz, Xiao *et al.* 1999). Figure 14 demonstrates that the AAV2-BR3Δ-GFP virus does indeed bind to heparin, as it bound to and eluted from an immobilized heparin column in a manner identical to that of wild-type capsids. It is, therefore, unlikely that the inability of the AAV2-BR3Δ-GFP virus to infect is due to inhibited binding to its primary receptor.

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AAV2 has been shown to be dependent upon endosomal events for its successful infection of cells in tissue culture, and one would assume that the virus must escape from the endosome at some point during its trafficking. It remained a possibility that the AAV2-BR3Δ-GFP virus was not infectious because it was unable to escape the endosomal compartment. Since the mechanism of endosomal escape is unknown even for wild-type capsids, establishing weather the AAV2-BR3Δ-GFP virus is defective in this regard is challenging. We decided on an indirect approach in which cells were coinfected with AAV2-BR3Δ-GFP and wild-type AAV2 capsids carrying the gene for Luciferase (AAV2-LUC). We reasoned that if the AAV2-BR3Δ-GFP virions were stuck in the endosomal compartment, then the mutant virions may be able to piggyback on the endosomal escape of wild-type capsids. It is possible, though, that AAV virions are endocytosed as a single particle per endosome (Seisenberger, Ried *et al.* 2001), however, it is unlikely that this state persists, as early endosomes are known to merge as they traffic towards the nucleus and transition to late endosomes (for review of endocytosis-mediated viral entry, see (Sieczkarski and Whittaker 2002)).

HeLa cells were infected simultaneously with AAV2-BR3Δ-GFP and AAV2-LUC. The cells were then assayed for expression of GFP. The wild-type capsids coding for luciferase were unable to rescue the AAV2-BR3Δ-GFP phenotype (Figure 16). We interpret this finding as evidence that the AAV2-BR3Δ-GFP virus' inability to transduce cells is not due to an inability to escape from the endosome, though more investigation is needed to completely rule out

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this possibility. It is possible that the AAV2-BR3Δ-GFP mutant is unable to escape the endosome, and that wild-type AAV capsids are unable to rescue the phenotype due to the mechanism of escape from the endosome. For example, if the mechanism of AAV escape from the endosomal compartment is similar to the proposed mechanism of initial virus internalization, i.e. a single virion binding a single receptor on the inner membrane of the endosomal compartment, followed by budding off, then it is possible that the wild-type capsid would not rescue the AAV2-BR3Δ-GFP if it were stuck in the endosomal compartment. An experimental approach that may address this issue is the use of an endosomolytic agent (Wattiaux, Laurent *et al.* 2000) during infection with the AAV2-BR3Δ-GFP virus; if the virus were stuck in the endosome compartment, lysis of the endosome would release the virus and allow transduction (assuming this were the only defect of the virus and that the virion had already undergone all endosome-mediated conformational changes necessary for subsequent trafficking events).

For Figure 15, we performed the converse of the experiment above, with the aim of establishing whether the AAV2-BR3Δ-GFP virus will inhibit the trafficking of wild-type capsids. AAV2-LUC was allowed to bind HeLa cells at 4°C for 1 hour (the virus will not internalize at this temperature). AAV2-BR3Δ-GFP was then added to the culture media and the cells warmed to 37°C. By allowing the AAV2-LUC to bind to cell surface receptors before addition of the AAV2-BR3Δ-GFP, we can be assured that any changes in the transduction of the cells by AAV2-LUC in the presence and absence of the AAV2-BR3Δ-GFP is due

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to competition in trafficking events downstream of receptor binding, as we had already established that the AAV2-BR3Δ-GFP binds to heparin. As shown in Figure 15, AAV2-BR3Δ-GFP competed in transduction with the wild-type capsids, AAV2-LUC. Steps in the trafficking process at which the AAV2-BR3Δ-GFP may compete with the transduction by wild-type capsids include, dynamin-dependent internalization (the availability of dynamin may have been rate limiting), endosomal escape, and binding to the nuclear import factors or to the NPC itself. It is not known whether AAV binds to receptors on the inner membranes of endosomes, but if so, this would represent one possible site of competition. Data that follows allowed us to be certain that the inhibition was not at the transcription or translational levels.

To better understand the trafficking limitations of the AAV2-BR3Δ mutant, we visualized incoming AAV2-BR3Δ-GFP virus at both the protein and DNA levels and compared it to that of recombinant AAV2 particles assembled from wild-type capsid proteins (Figures 17 and 18). For both sets of data, tyramide system amplification (TSA) was used to allow for reduced multiplicities of infection (MOI) compared to previous studies using Cy3 labeled virions (Bartlett and Samulski 1998) (see Methods). For the images shown in Figure 17, either AAV2-GFP or AAV2-BR3Δ-GFP was allowed to bind HeLa cells in chambered glass slides for 1 hour at 4°C. Cells were washed with PBS, warmed to 37°C, and incubated for 24 hours before being processed for immunohistochemistry (IHC) with an antibody against VP3 (B1 antibody, which binds to all 3 capsid proteins). As can be seen in Figure 17, the capsid proteins of AAV2-BR3Δ-GFP

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accumulated at the nuclear periphery in a manner similar to that of virus assembled from wild-type capsids (AAV2-GFP). This surprising result does not necessarily rule out a nuclear targeting role for the ^{166}PARKRL^{171} region of AAV2. Since AAV2 relies on late endosomes and proteosomes for trafficking, and since these two organelles traffic to the nuclear periphery, it would not be surprising if AAV2 were able to traffic as far as the nuclear periphery without the need for a nuclear targeting signal. Under this scenario, AAV2 would take advantage of the trafficking of the endosome or proteosome toward the nuclear periphery, escape from the endosome or proteosome at the nuclear periphery, and then target the nucleus by way of sequences in the capsid proteins. In this case, our assay would not detect an inability of the AAV2-BR3Δ to target the nucleus since the virus would already be in very close vicinity to the nucleus when released from the organelle in which it traffics. Another possibility is that the ^{166}PARKRL^{171} region is not necessary to target the incoming virion to the nucleus, but is necessary for some downstream trafficking or uncoating event. Yet another way to explain this data is that the capsid proteins seen in the assay represent spent capsids that have already released their DNA cargo, and have been trafficked into proteosomes for eventual degradation. The nature of the punctate staining may point towards this being the case, but it is difficult to distinguish between the punctate staining that would occur from trafficking in late endosomes versus the punctate staining that would occur with the capsid proteins being placed into proteosomes for degradation.

We next wanted to address the possibility that the AAV2-BR3Δ-GFP mutant enters the nucleus intact but is unable to uncoat once inside the nucleus. To address this question, we imaged the viral DNA (GFP gene in this case) by fluorescent *in situ* hybridization (FISH) of DNA probe against the GFP gene, reasoning that if the AAV2-BR3Δ-GFP virus is a nuclear entry mutant, the viral DNA would be found exclusively in the cytoplasm, whereas viral DNA delivered by wild-type capsids would be found at least partially in the nuclear compartment. If the mutant is an uncoating mutant and the virus normally enters the nucleus intact, we would have expected to find the viral DNA cargo in the nuclei of the target cells, despite their being no gene expression from the transgene. HeLa cells were infected as above and processed for FISH analysis instead of IHC analysis. DNA probe was biotinylated, and hybridized to *in situ* GFP gene (the DNA cargo carried by the incoming virions). Detection of hybridized probe was performed by binding streptavidin-HRP to the biotinylated DNA probe, followed by treatment with tyramide-Cy3 (this is a biochemical amplification method; see Methods). Figure 18 shows that the virions assembled from wild-type capsid proteins deliver at least some copies of the GFP gene to the nucleus, albeit most of the genes remain at the nuclear periphery. In contrast, no GFP genes delivered by the AAV2-BR3Δ-GFP virus could be detected in the nuclear compartments of the target cells, consistent with the lack of GFP expression following infection with this mutant. These data indicate that the ^{166}PARKRL^{171} region is important in some regard for mediating movement of the AAV2 DNA cargo from the cytoplasmic compartment to the nuclear compartment.

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The function of AAV2 BR3 resides in VP1

We took as our next step an attempt to establish whether the function provided by ^{166}PARKRL^{171} is provided from the context of VP1 or the VP2 capsid protein. Since no part of VP2 is unique from VP1, and since ^{166}PARKRL^{171} is an element of both of these proteins, it is possible that whatever function is abrogated through the mutation of BR3 could be provided by either VP1 or VP2, or that the function is redundant between the two capsid proteins. Towards finding from which capsid protein this function is provided, we attempted to manufacture virus with either VP1 or VP2 missing from the final assembled virus. This was accomplished by making a point mutation to the translation start site for either VP1 or VP2. The VP2 protein is made from the VP1 transcript from an alternative translation start site, ACG, resulting in a VP2 protein that is missing the first 139 N-terminal residues of VP1. As shown in Figure 20, mutation of the VP2 start site to ACA resulted in the absence of VP2 from producer cell lysates following transfection with pACG2. Surprisingly, this virus is normal in every measurable regard; it forms DNA-protecting virions that bind heparin and transduces cells identically to normal recombinant virus (Figure 21). This led us to conclude that the function that is provided by the ^{166}PARKRL^{171} region can be supplied by VP1 alone. This result does not preclude, however, that the function could also be provided by the VP2 protein if VP1 were absent. We made an attempt to produce virus that was composed of only VP2 and VP3 by mutating the start site for VP1. Unfortunately though, some VP1 protein is still being produced, despite the loss of the VP1 start site, possibly due to other start sites

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down stream of the VP1 start site. We are currently working on making a VP1-free virus, and once obtained, we will be able to establish whether the ^{166}PARKRL^{171} region in VP2 is capable of redundancy with the same region in VP1.

Possible functions of AAV2 BR3

The function provided by ^{166}PARKRL^{171} could be at several places in the AAV infection, including endosomal escape, targeting the virion to the nuclear envelope, or mediating transfer of the genome across the nuclear envelope, presumably through the nuclear pore complex. Each of these possible sites of disruption is discussed below.

Endosomal escape is an important aspect of an endocytosed virus' ability to infect its target cell. It is unknown how AAV2 escapes from the endosome, so it is difficult to hypothesize what functions are abrogated by the ^{166}PARKRL^{171} to ^{166}PANNRL^{171} mutation that would cause a defect in the virus' ability to escape from the endosome. One possibility is that the mutation causes a failure of the virus to change confirmation during endosome acidification. The N-terminus of canine parvovirus (CPV) undergoes a pH-dependent conformational change in the endosome that exposes a portion of VP1 that resembles classical nuclear localization signals. Without the conformational change, the region is unexposed and the virus non-infectious (Vihinen-Ranta, Wang *et al.* 2002). Another possibility to explain the defect of the AAV2-BR3Δ virus is that the virus uses the ^{166}PARKRL^{171} region to bind a membrane receptor exposed on the inner membrane of the endosome that allows for exocytotic escape of the virus from

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the endosome. It is hypothesized that the parvovirus minute virus of mice undergoes similar conformational changes (Cotmore, D'Abramo A *et al.* 1999). Since the virus probably escapes from late endosomes or proteosomes, which traffic to the nuclear periphery, our assays that show both viral capsid proteins and viral DNA at the nuclear periphery are not sufficient to determine whether the virus has escaped from its trafficking vesicle.

Another possible site of disruption by the ^{166}PARKRL^{171} to ^{166}PANNRL^{171} mutation is the targeting by the virus of the nuclear envelope prior to genome transfer. This could be via binding to a nuclear import receptor such as Importin α or Importin β, or through direct interaction with the nuclear pore complex filament proteins, as is the case for adenovirus (Trotman, Mosberger *et al.* 2001). Adenovirus has been shown to bind directly to the cytoplasmic filaments of the NPC, in particular the filament protein Nup214, independent of cytosolic nuclear import factors. The virus then relies on interaction with cellular histone H1, and its nuclear import factors, to uncoat at the face of the cytoplasmic ring of the NPC, and for transport of its genome into the nuclear compartment.

The elucidation of function of the ^{166}PARKRL^{171} in AAV2 will require additional work. It is premature to call this region a nuclear localization signal in the classical sense, at least until it can be established that the region interacts with nuclear transport components, such as the nuclear transport receptors (karyopherins, or importins), or with the nuclear pore complexes. The results presented thus far in this body of work refute the hypothesis that the ^{166}PARKRL^{171} region is responsible for localizing capsid proteins to the nuclear

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compartment during viral assembly. With this region mutated, the capsid proteins continued to accumulate in the nucleus and assembled normal, DNA-protecting virions. These virions were capable of binding heparin, and of subsequent trafficking to the nuclear periphery, just as wild-type capsids traffic. The virus was, however, defective in mediating the transport of its genome across the nuclear envelope to initiate infection. The AAV2-BR3Δ virus is the first AAV phenotype that is positive for virion formation, heparin binding, and cytoplasmic trafficking, yet defective for infection, and will provide a tool for future study of adeno-associated virus.

AAV2 BR4 contributes to localizing capsid proteins to the nucleus for assembly of progeny virions

Since ^{166}PARKRL^{171} is clearly not responsible for localizing AAV2 capsid proteins to the nuclei of host cells for assembly of progeny virions, we sought to determine whether any of the other basic regions we identified in the AAV2 capsid play a role in localizing the capsid proteins to the nucleus during viral assembly (Figure 22). We examined the cellular localization of AAV2 capsid proteins with mutations to BR1 and BR4. This was accomplished by transfecting either pACG2-BR1Δ or pACG2-BR4Δ into AAV producer cells (along with pXX6-80 and pTRUFeGFP), immunoprecipitating the capsid proteins from the cytoplasmic and nuclear fractions of the cells, and Western blotting the immunoprecipitate for AAV2 capsid proteins. The immunoprecipitations of capsid proteins from cytoplasmic and nuclear fractions were carried out with

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either mAb B1 or mAb A20. mAb B1 binds only to unassembled capsid proteins, while mAb A20 binds only to intact AAV particles (Wistuba, Kern *et al.* 1997).

Intact virions made from wild-type capsid proteins (top gel, lanes 1 and 5) were found only in the nuclear fraction. This was expected, as Wistuba *et al* have previously shown by immunohistochemistry with the A20 antibody that AAV assembles in the nuclei of its hosts (Wistuba, Kern *et al.* 1997). We also immunoprecipitated unassembled capsid proteins from the producer cell fractions and Western blotted the immunoprecipitates to determine the cellular localization of the unassembled capsid proteins (Figure 22, bottom blot, lanes 1 and 5). Again, consistent with immunohistochemistry data presented by Wistuba *et al* (who also used the B1 antibody), unassembled capsid proteins were immunopreipitated by the B1 antibody predominantly from the nuclear fraction of the producer cells. Very low levels of unassembled capsid proteins were detected in the cytoplasmic fraction of the producer cells, indicating that upon translation, AAV2 capsid proteins are rapidly taken up by the nuclear compartment during assembly of the AAV2 virion. As described above, similar experiments were performed for the AAV2-BR3Δ mutant and it was shown that, identical to wild-type capsid proteins, AAV2 capsid proteins containing the BR3Δ mutation localized predominately to the nuclear fraction of producer cells during virion assembly.

The results for the AAV2-BR1Δ mutant are shown in Figure 22, lanes 2 and 6 in both the upper and lower blots. This mutant did not make VP1 or VP2 that was detectable by the Western blot. This lack of VP1 or VP2 on the Western

blot could be from several causes. The simplest explanation is that the construct pACG2-BR1Δ possesses a mutation in addition to the intended mutation that has eliminated the VP1 and VP2, such as a frame shift mutation, which could be tested by sequencing the entire plasmid for mutations other than the one intended, or by testing other clones of the BR1Δ construct. It is also possible that the BR1Δ mutation has caused the proteins to become unstable, resulting in rapid degradation of VP1 and VP2 in the cytoplasm. Testing such a possibility would require pulse/chase experiments to determine the half life of the proteins in the cytoplasm. Regardless of the cause, the absence of VP1 and VP2 from cells transfected with our pACG2-BR1Δ construct gives rise to an interesting opportunity to investigate the importance of these capsid proteins in cytonuclear trafficking of AAV components during viral assembly. Hogue *et al* have suggested that VP2 is required for the accumulation of VP3 in the nucleus during viral assembly. If this were the case, one would expect unassembled VP3 proteins to be preferentially found in the cytoplasmic fraction of cells transfected with the pACG2-BR1Δ construct since no VP2 or VP1 would be present to facilitate the transfer of VP3 across the nuclear envelope. As shown in figure 22, singly expressed VP3 immunoprecipitated from producer cells that were transfected with pACG2-BR1Δ were found predominantly in the nuclear fraction of the cells, indicating that VP3 possesses at least some of the elements necessary to localize the protein to the nuclear interior. This data, together with other data presented in this thesis, is the first to refute the assumption that the

^{166}PARKRL^{171} region is responsible for localizing AAV capsid proteins to the nucleus for assembly into progeny virions.

Lanes 3 and 7 in the upper and lower gels of figure 22 show the cellular localization of capsid proteins and virions containing the BR4Δ mutation following transfection with the pACG2-BR4Δ construct. Intact virions were detected in the nuclear fraction (upper gel, lane 7), but not the cytoplasmic fraction (upper gel, lane 3), of producer cells transfected with the pACG-BR4Δ construct. Though virus was produced from the capsid proteins carrying the pACG-BR4Δ mutation, the virus was produced at levels far below virus produced from wild-type (lane 5, top gel).

We hypothesize that the reason virions were produced at such low levels when the capsids contained a mutation to AAV2 BR4 can be seen in the lower gel, lanes 3 and 7. Unassembled capsid proteins were found distributed in both the cytoplasmic and nuclear fractions of the producer cells, indicating that the BR4 mutation has caused a defect in the ability of the newly translated capsid proteins to localize to the nucleus for assembly into new virions. Some would expect that if the capsid proteins were distributed equally between the nuclear and cytoplasmic compartments, intact virions would be produced at a level of similar order of magnitude as virions produced from wild-type capsid proteins, or perhaps at half the normal titer. It is possible, instead, that a critical concentration of capsid proteins must be attained in the nuclear compartment in order for efficient assembly of virions to occur. Since individual capsid proteins would have to be in close proximity to one another to form virions, too few capsid

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proteins in the nuclear compartment may leave the proteins too far apart to interact during the viral assembly process.

IV. PHOSPHORYLATION CONSENSUS SITES MAY BE IMPORTANT IN AAV2 TRAFFICKING

IV.A. Introduction

The activity of nuclear localization signals often depends on the status of nearby phosphorylation sites. For example, phosphorylation is the main mechanism of controlling the nuclear transport of many transcription factors, including NFκB, c-*rel*, dorsal, and SW15 from yeast, and viral proteins that include the prototypical simian virus large tumor antigen (SV40 T-ag, (Jans and Hubner 1996)).

The activity of the SV40 T-ag nuclear localization signal is regulated by what has been termed the CcN motif. The motif is comprised of the NLS itself, represented by the "N", and two kinase consensus sequences nearby the NLS. The first "C" represents a casein kinase II site (CKII) 13 residues N-terminus to the NLS. The "c" represents a cyclin-dependent kinase (cdc) site adjacent to the NLS. Mutation of the casein kinase II serine target to the nonphosphorylatable residue alanine resulted in nuclear accumulation of SV40 T-ag at a level 4% of the wild-type protein. Conversely, mutation of the serine target within the casein kinase II consensus site to aspartic acid, which mimics phosphorylation, resulted in nuclear accumulation of T-ag at 45% of the wild-type level of nuclear accumulation (Rihs, Jans et al. 1991; Jans and Jans 1994). The p34cdc2 site regulates nuclear transport of T-ag in exactly the opposite manner of the CKII

site. Phosphorylation of the threonine within the p34cdc2 consensus site adjacent to the NLS of T-ag has been shown to abrogate the ability of T-ag to localize to the nuclei of cells microinjected with the purified protein (Jans, Ackermann et al. 1991).

IV.B. Results

We used NetPhos 2.0 software to search the AAV2 capsid for high probability phosphorylation kinase consensus sequences and their cognate phosphorylation sites surrounding the putative NLS. We found 3 kinase consensus sites in the vicinity of the putative NLS with greater than 90% probability of being active phosphorylation sites.

The three sites that were to receive further characterization (shown below with their corresponding kinases) were serine 148, threonine 159, and serine 181

MAPGKKRPVEHS^{CKII}PVEPDSSSGT^{PKC}GKAGQQ**PARKRLN**FGQTGDADS^{CKII}VPDPQPLG

(the numbers correspond to residue position in VP1). These sites were mutated in a manner consistent with the mutations of Jans *et al* (Jans, Ackermann et al. 1991; Jans and Jans 1994; Jans and Hubner 1996), i.e. we mutated each of them to either a nonphosphorylatable alanine, to simulate an unphosphorylated serine, or to an aspartic acid to simulate a constitutively phosphorylated serine, resulting in six mutants, S148A, S148D, T159A, T159D, S181A, and S181D. The mutations were carried out by PCR in the same context as mutations of basic regions in the previous section, i.e. the mutations were placed into the pACG2 plasmid and the mutated plasmid transfected, along with AAV helper plasmids pXX6-80 and pTRUFeGFP, into AAV producer cells. As in the previous section, the eGFP gene was packaged into the mutated virions so that their infectivity could be easily assayed.

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All of the mutants were capable of forming virus at normal titers. We infected cultured HeLa cells with 500 particles/cell of each of the 6 mutants to establish whether the potential phosphorylation sites are important in the virus' ability to deliver its genome to the target cell during an infection. Infected cells were assayed for green fluorescence by fluorescent microscopy and the results are shown in figure 23. AAV2-eGFP made from wild-type capsids was used as a positive control. The S148A, S148D, T159A, and T159D mutations had no effect, or little effect, on the virus' ability to infect the HeLa cells and to deliver the eGFP gene carried as their cargo. However, the S148 and S148D had large effects on the infectivity of the virus. The same number of particles containing S181A mutation infected far more cells than did the wild-type, and the same number of particles containing the S181D mutation infected almost no cells at all (Figure 23).

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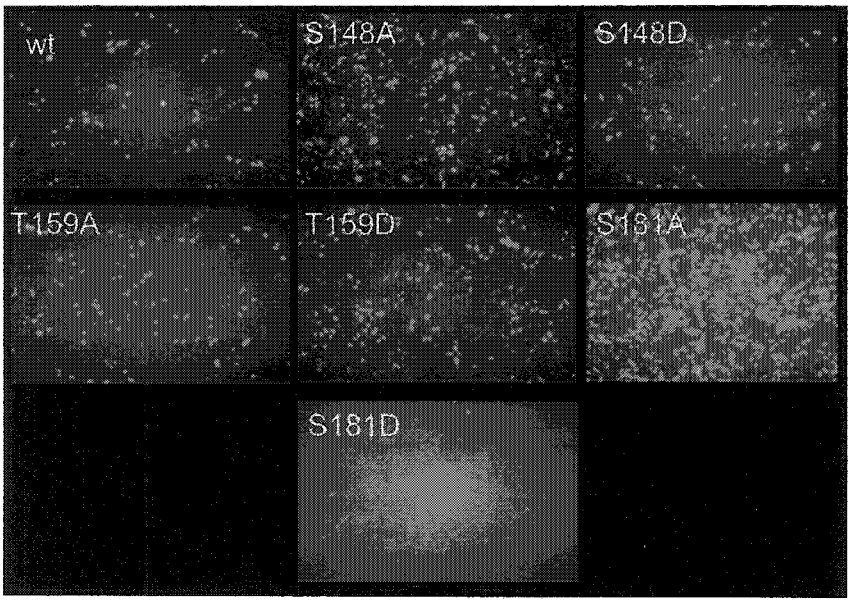

Figure 23. Infection of HeLa cells by AAV2 virions carrying mutations to possible phosphorylation sites flanking basic region 3. Numbers represent the number of residues from the beginning of VP1. S represents serine, while D represents aspartic acid.

IV.C. Discussion

In this section, we examined the importance of several possible phosphorylation sites nearby the putative nuclear localization signal (NLS) of AAV2. The possible phosphorylation sites were determined by search with software that scans for known kinase consensus sites (NetPhos). We found three kinase consensus sequences within then vicinity of the AAV2 putative NLS, two for casein kinase II (CKII) with phosphorylatable serines targets, and one for protein kinase C (PKC) containing a phosphorylatable threonine. We mutated each of the phosphorylatable residues to either alanine, to simulate a nonphosphorylated residue, or aspartic acid, to simulate a constitutively phosphorylated residue.

We present preliminary evidence that one of the possible phosphorylation sites, serine 181, may play some role in AAV2's ability to infect its target cell. When S181 was mutated to a nonphosphorylatable alanine, the result was a virus that transduces target cells with greater efficiency than did the wild-type virus. When S181 was mutated to a residue that simulates phosphorylation, the result was a virus that transduced the target cells very poorly. Based on Jans' data for T-ag discussed above, we expected the serine to alanine mutations to result in lowered transduction efficiency if the residue is important for regulating the putative NLS. Likewise, we expected the serine to aspartic acid mutation to suffer, at most, a slight decrease in transduction efficiency versus the total loss of transduction ability that is shown in figure 23. It may be premature in the field's

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development to assume that a given phosphorylation status will have the same effect on all NLSs, as there is insufficient evidence in the literature to support this assumption. This issue will be better understood with future studies on other nuclear localization signals.

Until we establish unequivocally that basic region 3 of AAV2 is an NLS, it is premature to assign the cause of the changes in transduction efficiency resulting from mutations to S181 as interfering with the function of an NLS. However, if for the sake of discussion we assume that S181 regulates the function of an NLS, with the NLS being contained in BR3, there are a couple of mechanisms that would be possible as mechanisms of NLS regulation by S181. One is that the phosphorylation status of S181 controls the folding of the capsid protein in such a way that results in masking and unmasking of the NLS. If the NLS were masked through folding, it would be unable to recognize nuclear import factors, or the nuclear pore complex, depending on where the NLS must bind for its nuclear transport. This possibility would explain the effect of the S181D mutation, which abrogated the virus' transduction ability.

Another possibility is that the phosphorylation status of S181 is important in the binding of some cytoplasmic factor that when bound to the capsid protein, causes cytoplasmic retention of the capsid, or interference with the capsid's binding to a nuclear transport factor or the nuclear pore complex. This mechanism is responsible for the cell cycle dependence of cellular localization of the S. *cerevisiae* protein TF SWI5. The NLS of this protein is masked by the phosphorylation of a CDC28 site nearby the NLS. At anaphase, the site is

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dephosphorylated, the NLS revealed, and the protein moves into the nuclear compartment.

Establishing whether S181 plays a role in regulating AAV2 BR3 will have to wait for further description of the role of BR3 in AAV infection. If further study of AAV2 BR3 reveals that the region acts as a nuclear localization signal by binding to cytoplasmic nuclear transport factors or by interacting directly with the nuclear pore complex, then S181 will be an obvious choice of possible regulators of the NLS.

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V. FUTURE DIRECTIONS

AAV production

The problem of producing adeno-associated virus (AAV) in commercial quantity and of purity sufficient for clinical use will be one of the greatest challenges facing AAV as a gene therapy vector. The method presented in this dissertation, i.e. a method for the recovery of recombinant AAV from purified nuclei, represents a substantial improvement towards obtaining high titer, high purity recombinant AAV. However, impediments remain. One aspect of AAV production that remains inefficient and non-scaleable is the necessity of transfecting the AAV producer cells with the three AAV producer plasmids. Transfection is a fickle and labor intensive technique that must be replaced as AAV moves towards commercial applications. Current and future research into AAV production will focus on the use of cell lines that inducibly express AAV production helper proteins and the use of cell factories, both of which will contribute to large-scale efficiency of AAV production. The other half of large-scale AAV production, the purification of the recombinant virus product, will also require additional research and development. The current method of iodixonol step gradient purification of AAV is time consuming and labor intensive. Methods must be developed that allow for cell lysate of AAV producer cells to be directly applied to an immobile matrix that can be used to purify the AAV away from cellular contanimants.

Mechanisms of nuclear targeting by AAV virions and capsid proteins

A great deal of work remains in establishing the mechanisms through which AAV virions and capsid proteins traffic through their host cells. Very little of the pathway from receptor binding to gene expression has been unequivocally determined for the virus. For AAV2, it is widely accepted that the virus depends on heparin proteoglycans for initial attachment to the target cell and depends on $\alpha_v\beta_5$ integrin as a coreceptor that enhances the virus' internalization, while for AAV4 and AAV5 sialic acid seems to be important for viral attachment. It is unknown what receptor mediates infection by AAV1, AAV3, AAV7, and AAV8. Researchers are also fairly confident that AAV virions are internalized into endosomes, and that the endosomes play an important role in trafficking AAV virions to the nuclei of its host cells.

Trafficking events subsequent to viral internalization remain to be clearly determined. Some confusion remains as to whether AAV2 escapes from early or late endosomes, though evidence is starting to accumulate that point towards a late endosomal escape for the virus. The mechanism through which AAV virions escape from the endosomal compartment is completely unknown. Also unknown are how the incoming AAV virus targets the nucleus, and how it mediates transfer of its genome across the nuclear envelope.

The slow progress in establishing AAV trafficking steps can be partially attributed to a paucity of techniques that allow for visualization of low numbers of AAV virions and viral genomes. Classical methods of visualizing proteins and

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nucleic acids, such as immunohistochemistry, and *in situ* hybridization, do not provide levels of sensitivity low enough to visualize virions and viral DNA transfer at physiological viral loads. The determination of AAV's trafficking events will be dependent on the development of new techniques of visualizing the virus during physiological infections. Progress towards improved and more physiological visualization of AAV infection was recently made by Seisenberger *et al* who are developing a method of tracing single AAV particles in real time during AAV infection (Seisenberger, Ried et al. 2001). Such a technique will aid in answering the question of whether AAV capsid proteins carry the AAV genome into the nucleus of its target cells, or whether the genome enters the nucleus unaided by the AAV capsid proteins. With more time and refinement, this technique may allow investigators to answer some of the difficult questions of AAV trafficking.

In the meantime, however, existing techniques could be used to extrapolate on data presented in this dissertation. The technique of microinjection of proteins into cultured cells represents an exciting opportunity for future research on AAV trafficking. Microinjection of antibodies against several possible AAV trafficking mediators would help identify cytoplasmic factors involved in AAV trafficking, in turn shedding light on the mechanisms the virus employs to mediate its transcytoplasmic travel and its cytonuclear transfer. For example, antibodies against a particular karyopherin (Importin) α or β molecule could be injected into HeLa cells immediately prior to infection by AAV to determine whether the virus uses the importin for docking to the nuclear pore complex. Additionally, antibodies against epitopes on the nuclear pore complex

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cytoplasmic filaments could be microinjected immediately prior to AAV infection to rule in or rule out the nuclear pore complex as a conduit for the transfer of the AAV genome across the nuclear envelope. This same set of experiments employing microinjection of antibodies against cellular nuclear import elements could be performed during the viral assembly process in order to determine whether the viral capsid proteins use the nuclear pore complex and cellular nuclear import machinery to transfer capsid proteins into the nucleus for viral assembly of the capsid proteins following production of the capsid proteins on the cytoplasmic ribosomes.

Microinjection of antibodies could also be used to answer the question of whether the virus escapes from early or late endosomes. There are specific cytoplasmic factors that are known to be critical for the transition of early endosomes to late endosomes. One such factor is MAB7. If microinjected antibodies against MAB7 interfere with infection by AAV2, then this would be quite clear evidence that the virus is dependent upon the late endosome for its trafficking to the nuclear periphery.

Other questions that could be answered using conventional techniques are those that surround the functions of the basic regions characterized in this dissertation. One of the questions is whether basic region 3 and/or basic region 4, which are both clearly important in some element of AAV trafficking, interact with cytoplasmic importin proteins. This information could be gathered by far-Western blots in which AAV capsid proteins are transferred to nitrocellulose following SDS-PAGE resolution, and purified importin proteins assayed for

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binding to the immobilized AAV capsid proteins. Unfortunately, though, determining whether importins interact with one of these basic regions on an intact AAV virion, such as in a filter binding assay, may not be helpful. It is possible that AAV undergoes conformational changes in the endosomal compartment that expose BR3 (Cotmore, D'Abramo A et al. 1999), so determining whether the incoming virion interacts with cytoplasmic nuclear import factors may require a more sophisticated approach. One such approach is the one taken by Trotman *et al* in which the authors identified cytoplasmic proteins that interact with adenovirus in the context of its nuclear import (Trotman, Mosberger et al. 2001). The procedure they used allows for transfer of a biotin molecule between crosslinked proteins as an indicator of interaction, and led to their discovery that Adenovirus interacts with a nuclear pore complex cytoplasmic protein called Nup214. This approach could be used by modifying AAV with SSBED, infecting cells with the modified virions, UV cross-linking the AAV to its interactive proteins in the cytoplasm, and then looking for cytoplasmic proteins to which the SSBED transfers a biotin molecule.

Once the functions of AAV2's basic region 3 have been elucidated, one may determine whether the possible phosphorylation sites identified in this dissertation play a role in regulating the functions of basic region 3. However, establishing whether these sites are actually used by a kinase or phosphatase will be challenging. It is likely that the region of the AAV capsid that contains this region is folded inside the virion (the antibody A69, whose epitope is near the putative AAV2 NLS does not immunoprecipitate AAV virions), and is exposed

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through some modification within the host cell endosome when the virion is incoming. Therefore, *in vitro* studies to determine whether serine 148 or serine 181 can be phosphorylated by various kinases or dephosphorylated by phosphotases will not be productive. Some information could be gleaned by infecting cells with AAV, interrupting the infection immediately (AAV reaches the nucleus just minutes after internalization), and analyzing the capsid proteins (and analyzing those of the serine 148 and 181 mutants) by 2-dimensional gel electrophoresis to determine phosphorylation status.

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VI. METHODS

Cell culture

HEK293 cells and HeLa cells (American Type Tissue Culture) were maintained at 37°C in DMEM (Sigma) supplemented with 5% CO_2, 10% fetal bovine serum (Sigma), 100 U/mL penicillin, and 100 U/mL streptomycin.

Viral production

Confluent 293 cells were split 1:3 24 hours prior to transfection. Triple transfections with pACG2 (Li, Samulski *et al.* 1997), pTrufEGFP (Haberman, McCown *et al.* 2000), and pXX6-80 (adenovirus helper plasmid) were carried out by calcium phosphate precipitation as described (Haberman, Kroner-Lux *et al.* 1999). Forty-eight hours post-transfection, cells were scraped, pelleted, and resuspended in phosphate-buffered saline (PBS). For studies involving the comparison between nuclear extraction and detergent lysis as methods of viral harvest, the cells were then split into two fractions; one fraction each for the deoxycholate lysis method and the nuclear extraction method.

Nuclear extraction for viral harvest of rAAV2

Cells suspended in PBS (from above step) were pelleted by centrifugation at 1,500xg. The cell pellet was gently resuspended in a volume of hypotonic buffer

(10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM spermine, pH 7.9 at 4°C) equal to 5 times the packed cell volume (PCV). The cells were swelled on ice for 10 minutes. 0.11 volumes of 10x restore buffer (67.5% sucrose, w/v, in hypotonic buffer) was added to the swollen cells. The swollen cells were then transferred to a Kontes dounce homogenizer and homogenized by 15 strokes with a type B pestle. Cellular lysis was verified by trypan blue staining of nuclei. Nuclei were pelleted by centrifugation at 500xg for 15 minutes in a Sorvall H1000B swinging bucket rotor (the resulting nuclear pellet was usually one half the volume of the PCV). The cytoplasmic fraction was then aspirated and the pelleted nuclei washed twice by resuspension in10 mL of 1x restore buffer (hypotonic buffer with 6.75% sucrose) and then re-pelleted by centrifugation at 500xg for 10 minutes. To prevent clumping during the nuclear lysis step, after the second wash, the nuclei were resuspended and removed from the sides of the tube in 0.5 mL of low-salt buffer (20 mM HEPES, 20 mM KCl, 1.5 mM $MgCl_2$, pH 7.9 at 4°C). The nuclei were then lysed by osmotic shock by suspension of the nuclei in high-salt buffer (20 mM HEPES, 1.0 M KCl, 1.5 mM $MgCl_2$, 25% v/v glycerol, pH 7.9 at 4°C). For virus preparations that were to be purified by cesium chloride instead of iodixonol and heparin affinity, the nuclei were lysed by the addition of the cesium chloride solution in which the virus was to be centrifuged instead of the high salt solution. The viscous nuclear lysate was sonicated until the nuclear lysate no longer adhered to the side of its container (approximately 20 seconds). Sonication was carried out by a Branson sonicator (Danbury, CT), set for 50% duty cycle and 50% maximum output. The nuclear lysate was cleared by

centrifugation at 25,000xg for 30 min. After clearing, a thin white layer of debris was present on the top of the supernatant and was aspirated before proceeding. The supernatant was loaded onto an iodixonol gradient (described below). Virus in the peak virus-containing iodixonol fraction was isolated by heparin affinity purification (below). For virus preparations that would be purified by cesium chloride, the pellet of nuclei were resuspended directly into the cesium chloride solution and sonicated before centrifugation.

Deoxycholate lysis for viral harvest of rAAV2

Cells from 10 15-cm plates were resuspended in 7 mL of PBS with 0.35 mL of 10% (w/v) deoxycholic acid (Sigma) and 375 units of Benzonase (Novagen), and then incubated at 37°C for 30 min. After incubation, the lysate was centrifuged at 8,400xg and the supernatant transferred to a new tube. The supernatant was then frozen and thawed 2 times between a dry ice/ethanol bath and a warm water bath. The lysate was then cleared again at 8,400xg and the supernatant subjected to iodixonol and heparin sulfate purification.

Iodixonol and heparin sulfate purification of rAAV2

Cell lysate was loaded onto an iodixonol step gradient and centrifuged essentially as described (Zolotukhin, Byrne *et al.* 1999). A Biorad peristaltic pump connected to a glass sampling capillary was used to underlay the sample with the iodixonol steps in a 12.5 mL ultracentrifuge tube (Beckman). The iodixonol step gradients were then centrifuged in an NVT65 fixed-angle rotor at 65,000 rpm

(402,000xg) for 1 hour. The iodixonol step gradient was fractionated from the bottom of the tube. Fraction sizes were 0.7 mL, 0.7 mL, 0.75 mL, 0.5 mL, 0.5 mL for fractions 1 through 5, respectively. Peak concentrations of virus (usually fraction 3) were found by dot blot hybridization (below). Heparin sepharose affinity chromatography of the peak iodixonol fraction was carried out by FPLC as described (Haberman, Kroner-Lux *et al.* 1999). Briefly, a 1 mL Hitrap heparin sepharose column was equilibrated (Amersham Pharmacia Biotech) with 10 mL of PBS-MK (PBS with 1 mM Mg^{2+} and 2.5 mM K^{+}). The peak iodixonol fraction was then injected into the FPLC injection loop and the virus allowed to bind to the heparin sepharose column. Virus was eluted from the heparin sepharose column into 0.5 mL fractions by a linearly increasing gradient of PBS-MK with 1 M NaCl.

SDS-Page, silver staining, and Western blotting

Purity of the rAAV was determined by silver stain. Equal volumes (lysate and iodixonol fractions, 10 uL) or equal number of particles (heparin sulfate purified virus, ~10^9 particles) were loaded onto a sodium dodecyl sulfate polyacrylamide gel (10%). Proteins were resolved by electrophoresis at 90 V for 20 minutes (stacking), then 180 V for 60 minutes. Gels were silver stained by using a Biorad silver stain kit in accordance with manufacture's instructions. For Western blot, 3 ul each of the nuclear fraction and of the cytoplasmic fraction were electrophoresed as above. Separated proteins were transferred to nitrocellulose membranes and probed with the monoclonal capsid antibody B1 (diliuted 1:100),

and visualized by probing with HRP-conjugated secondary antibody diluted 1:5000 (Santa Cruz Biotechnologies).

Virus titration

Recovery of virus in terms of genome copies was determined by DNA hybridization. 10, 3.3, 1.1, and 0.38 uL of each sample was incubated in 50 uL DNA digestion buffer (10 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 50U/mL DNase I) for 1 hr at 37 °C. DNA digestion was stopped by the addition of 10 uL of 0.1 M EDTA per sample. Virus-protected DNA was released by the addition of 60 uL of proteinase solution (1 M NaCl, 1% Sarkosyl, 10 g/mL proteinase K) and incubation at 50 °C for 30 min. Viral DNA was then denatured by the addition of 120 uL of 0.5 M NaOH and a 10-min incubation at room temperature. The denatured viral DNA was then blotted onto a nylon membrane and probed with a P^{32}-labelled probe against EGFP as described by Haberman (Haberman, Kroner-Lux *et al.* 1999). Viral titer was also measured by infectious titer. For infectious titer, HeLa cells were plated on a 24-well plate at a density of 1.5×10^5 cells/well. 24 hours later, the cells were coinfected with adenovirus and serial dilutions of the recombinant AAV-GFP (five-fold dilutions starting with 0.1 uL). Green cells were scored by fluorescence microscopy 24 hours after infection.

Electron microscopy

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Nuclei were isolated from 293T cells that 72 hours prior had been transfected with pXX-680, pACG2, and pTrufEGFP. The pelleted nuclei were immersion fixed for 24 hrs at 4°C. The fixative consisted of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 M PBS, pH 7.4. The nuclei were decalcified by 0.1 M EDTA in PBS and post-fixed in potassium ferrocyanide-reduced osmium tetroxide for 1 hr. The nuclei were then dehydreated through a graded series of ethanol washes, and embedded in Spurr's resin. A diamond knife was used to cut 70 nm-thick sections of the embedded nuclei. The sections were mounted on 200-mesh grids and stained with 4% aqueous uranyl acetate and Reynold's lead citrate and examined with a Zeiss EM 910 transmission electron microscope at an accelerating voltage of 80 kV. The same procedure was used for the visualization of purified AAV virions.

Site-specific mutagenesis

Mutations to pACG2 were carried out by PCR using the Stratagene Quick Change kit according to manufacturer's instructions. The sequences of primer pairs used for the mutations are shown below. Cycling parameters were as follows: 95°C x 1 min (1 cycle), 95°C x 1 min (15 cycles), 55°C x 2 min (15 cycles), and 68 ° C x 16 min (15 cycles). PCR products were digested with DpnI and used to transform electrocompetent XL1-Blue cells (Stratagene), which were then selected for plasmid uptake by growth on ampicillin selection plates. Colonies were picked and grown overnight in LB growth media, and plasmids isolated by Qiagen Maxiprep kits. Mutations were verified by sequencing of the

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plasmids (sequencing primer shown in table with mutational primers). DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a 3100 Genetic Analyzer (Applied Biosystems). The sequencing reaction was done using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase (Applied Biosystems).

Immunoprecipitations

10-cm plates were transfected as above for viral production. Cells were lysed 24 hours later by hypotonic swelling and dounce homogenization in 500 ul of hypotonic buffer, and the nuclei separated from the cytoplasmic supernatant by centrifugation. The cytoplasmic fraction was made isotonic with .11 volumes of 1 M TBS and centrifuged at 10,000 x g to clear the sample of cytoplasmic debris. The nuclear pellet was resuspended in 100 ul high salt buffer, drawn several times through a small-bore needle and centrifuged at 10,000 x g to clear the sample of nuclear debris. The sample was brought to 500 ul by the addition of 1X TBS. 40 ul of either B1 or A20 hybridoma supernatant was added to the samples, and the samples tumbled overnight at 4°C. 40 ul of protein A/G beads was then added to the samples and the beads collected by centrifugation at 500 x g. Beads were washed 3x in RIPA buffer, and subjected to SDS-PAGE and Western blotting as described above for the analysis of capsid proteins.

Immunohistochemistry by tyramide system amplification

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Immunohistochemistry was performed by tyramide system amplification (TSA, Perkin-Elmer) according to manufacturer's instructions with modifications. HeLa cells were seeded onto two-chamber glass slides at $0.5x10^5$ cells/well. 24 hours later, cells were infected with ~3000 physical particles per cell at 4°C for 1 hour, washed, and placed at 37°C. After the indicated time, cells were fixed and permeabalized in 100% methanol at -20°C for 10 minutes, then washed 3x5 min in room temperature PBS. Cells were blocked in TSA blocking buffer for 1 hour at room temperature. Cells were then incubated with mAb B1 diluted 1:200 in PBS-Tween (PBS-T, PBS with 0.05% Tween). Cells were washed in PBS-T 3x5 min and incubated for 1 hour with the secondary antibody (HRP-conjugated rabbit anti-mouse, Sigma) diluted 1:5000 in PBS-T. Cells were again washed 3x5 min in PBS-T, incubated for 10 minutes in Tyramide-Cy3 labeling solution, and washed 3x5 min in PBS-T (in light-protected Coplin jar). Two drops of antifade mounting media containing DAPI (Vecta Shield) was placed on the slide, and a slide cover sealed onto the slide with nail polish. Slides were imaged immediately by confocal microscopy on a Leica SP2 scanning confocal microscope capable of DIC overlay.

Fluorescent in situ hybridization by tyramide system amplification

Fluorescent *in situ* hybridization was carried out by tyramide system amplification (TSA) in accordance with manufacturer's instructions with modifications (Perkin-Elmer). HeLa cells were seeded onto two-chambered slides at a density of $0.5x10^5$ cells/well. The cells were infected 24 hours later by ~3000 AAV2

particles/cell (or mutants of AAV2) as described above (4°C for 1 hour, followed by washing with PBS, and placement at 37°C). Following the indicated incubation time, cells were washed 4X with PBS, and fixed in cold methanol/acetic acid (3:1) for 5 min at -20°C. Cells were then washed 3x5 min in PBS. Dehydration of the cells was carried out by 2 minute incubations in each of 70% ethanol at -20°C, 90% ethanol at room temperature, and 100% ethanol at room temperature. Slides were then treated with protease at100 ug/mL in PBS for 60 minutes at 37°C, and again dehydrated as above. Cells were then incubated in 50% formamide/2x SSC for 5 min at 72°C. *Preparation of DNA probe.* GFP gene DNA was labeled with biotin (Boerhinger Biotin NT) according to manufacturer's instructions. Probe solution was made with 10 ng/ul of biotinylated DNA, 15 ng/ul salmon sperm DNA, 50% formamide, 7% dextran sulfate, and 0.75X SSC. Probe solution was boiled for 10 min, placed onto the cells, the cells covered with slide covers, and then sealed with rubber cement. Incubation of the cells with the biotinylated probe was carried out overnight at 37°C. After overnight incubation, cells were washed 3x5 min in 50% formamide/2xSSC at 42°C, then washed in PBS-T 3x5 min. Streptaviden-HRP was diluted 1:100 in PBS-T and placed on the cells. Cells were then washed 3x5 min in PBS-T, and incubated for 30 min in Tyramide-Cy3 labeling solution. Cells were washed 3x5 min in PBS-T. Two drops of Vecta Shield antifade mounting media was placed on each slide, the cells covered with a cover slip, and the cover slip sealed to the slide with rubber cement. Visualization of the sample was carried out on a Leica SP2 scanning confocal microscope.

Luciferase assay

Assays of luciferase activity were carried out in accordance with manufacurer's instructions, without modification (Promega). Luciferase activity was measured by a Tropix TR717 automated plate reader.

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