Insertional Mutagenesis of AAV2 Capsid and the Production of Recombinant Virus

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Received August 2, 1999; returned to author for revision August 30, 1999; accepted October 11, 1999

The structural genes of adeno-associated virus serotype 2 (AAV2) have been altered by linker insertion mutagenesis in order to define critical components of virion assembly and infectivity. An in-frame restriction site linker was inserted across the capsid coding domain of a recombinant plasmid. After complementation in vivo, recombinant AAV2 viruses were generated and assayed for capsid production, packaging, transduction, heparin agarose binding, and morphology. Three classes of capsid mutants where identified. Class I mutants expressed structural proteins but were defective in virion assembly. Class II mutants generated intact virions that protected the viral genome from DNase, but failed to infect target cells. The majority of these mutants bound the heparin affinity matrix, suggesting that attachment to the AAV primary receptor was not rate limiting. One class II mutant, H2634, assembled virions and bound heparin using only Vp3, indicating that this subunit is responsible for mediating AAV receptor attachment. Finally, class III mutants assembled virions, encapsidated DNA, and infected target cells. Infectivity of these mutants ranged from 5 to 100% of that of the wild-type, demonstrating for the first time the ability to alter capsid proteins without interfering with infectivity. These AAV virions with altered capsid subunits will provide critical templates for manipulating AAV vectors for cell-specific gene delivery in vivo. In summary, the AAV capsid variants described here will facilitate further study of virus assembly, entry, and infection, as well as advance the development of this versatile vector system.

INTRODUCTION

Adeno-associated virus serotype 2 (AAV2) is a small nonenveloped virus in the parvoviridae family that requires a helper virus, adenovirus or herpesvirus, for efficient productive infection (Casto et al., 1967; McPherson et al., 1985). AAV2 packages a 4861-nucleotide (Srivastava et al., 1983) genome of both positive and negative polarity (Berns and Adler, 1972; Berns and Rose, 1970; Rose et al., 1969) into a 20- to 25-nm virion with icosahedral symmetry (Atchison et al., 1965; Hoggan et al., 1966). The viral DNA consists of two open reading frames flanked by inverted terminal repeats. The right open reading frame, which is expressed from the p40 promoter, codes for three structural proteins Vp1, Vp2, and Vp3. The regions that encode the three capsid genes share overlapping reading frames. The capsid genes are regulated by alternative splicing (Vp1) and translational initiation (ACG for Vp2 and AUG for Vp3) during a productive infection (Becerra et al., 1988; Muralidhar et al., 1994; Su et al., 1996; Trempe and Carter, 1988). After translation, the capsid proteins migrate to the nucleus and assemble into progeny virions. The virions are composed of 60 subunits with a stoichiometry of 1:1:20 for Vp1, Vp2, and Vp3, respectively (Rose et al., 1971). The ratio of virion capsid subunits correlates with in vivo expression levels seen during productive infection.

Early studies of AAV2 support the idea that all three capsid subunits are required to extract single-stranded genomes from the pool of replicating double-stranded DNA. These genomes are then sequestered into preformed immature particles that mature to infectious particles. These particles have a density between 1.32 and 1.41 g/mL in cesium chloride and sediment between 60 and 125S in sucrose (Myers and Carter, 1981; Myers et al., 1980). Mutagenesis studies of AAV2 capsids have shown that insertions and deletions in the Vp3 domain completely inhibit the accumulation of single-stranded virions and production of infectious particles (Hermonat et al., 1984; Ruffing et al., 1994). Missence mutagenesis of Vp1, Vp2, and Vp3 initiation codons resulted in no accumulation of single-stranded template or production of infectious particles (Muralidhar et al., 1994), suggesting an essential role for the individual capsid subunits during a productive infection. In contrast, it has been shown that a 186-bp Xhol deletion in the unique coding region of Vp1 does not inhibit accumulation of single-stranded template, but results in low infectious particles (Hermonat et al., 1984; Janik et al., 1984; Tratschin et al., 1984). The use of an amber mutation, which decreased production of Vp1 to 5% of wild-type levels in an amber suppressor cell line, was still sufficient for the accumulation of single-stranded DNA and production of infec-
tious particles (Smuda and Carter, 1991). In fact 45% of the Vp1 unique region can be deleted or >90% of the protein depleted using the amber suppressor mutation and low infectious particles still form (Hermonat et al., 1984; Janik et al., 1984; Smuda and Carter, 1991; Tratschin et al., 1984). These studies suggest that accumulation of single-stranded DNA requires the presence of Vp1 but the levels may be variable and insertions and deletions can be tolerated. While mutant analysis of the Rep coding region has been more extensive than that of the capsid coding region, the above analyses have been informative about the role of structural proteins in AAV assembly.

In fact, now several studies have demonstrated that parvovirus capsid proteins can be mutated and virion assembly studied. In one study, the coding region for 147 amino acids of the hen egg white lysozyme was substituted for the B19 Vp1 unique coding sequence. This modification resulted in purified empty particles that retained lysozyme enzymatic activity (Miyamura et al., 1994). In addition, expression of peptides (9 and 13 residues) in B19 Vp2 resulted in empty particles that were immunogenic in mice supporting surface presentation of the insertions (Brown et al., 1994). In a more recent study, the CD8 + CTL epitope (residues 118–132) against lymphocytic choriomeningitis virus (LCMV) nucleoprotein was inserted into the Vp2 capsid protein of porcine parvovirus (ppv) (Sedlik et al., 1997). This capsid protein with the epitope cloned at the N-terminus self-assembled when expressed in a baculovirus system. This chimeric virus-like particle was then used to immunize mice against a lethal challenge from LCMV (Sedlik et al., 1997). These studies establish the flexibility of the B19 and ppv capsids when foreign sequences are inserted into domains outside the predicted core β-barrel motif when using empty particles.

The identification of domains that are responsible for AAV2 host range, binding to the major cell surface receptor, and domains that can accommodate insertions without loss of function is important for a virus that is being considered as a candidate vector for human gene therapy. Mutagenesis along with a crystal structure should provide sufficient details to characterize the role of the AAV structural subunits in virion assembly and infectivity. However, in the absence of a crystal structure, we have used linker insertion mutagenesis to isolate a collection of mutants throughout the capsid coding region. Using Western blot, electron microscopy (EM) analysis, DNase protection, and virion transduction assays, we have identified three classes of insertion mutants. Class I mutants are defective for virions assembly and/or stability. Class II mutants protect viral DNA from DNase digestion but are not infectious, while class III mutants express foreign peptides and maintain morphology, stability, and infectivity of the wild-type virion. This unique assortment of viable insertion mutants will be critical reagents for studying AAV assembly and infectivity as well as serve as templates for the development of targeting AAV vectors for human gene therapy.

RESULTS

Construction of insertional mutations in rAAV2

In order to evaluate the role of AAV structural proteins in assembly and infectivity, we generated a collection of capsid linker insertion mutants. A 2.8-kb HindIII fragment of pAAV/Ad (Samulski et al., 1989) containing the sequences coding for the capsid domain of AAV2 was subcloned into pBS +. This plasmid, pAV2Cap, was used for partial digestion with HaelI, NlaIV, and RsaI to generate a substrate for capsid-specific insertions (Fig. 1). These three DNA restriction enzymes constitute 43 sites that span the AAV-2 capsid coding sequence, of which only 4 overlap. To efficiently identify clones that contain insertions, a kanamycin resistance gene (Kanr) flanked by a novel oligo (Nael/EcorV) was ligated with partially digested, full-length, linearized pAV2Cap (see Materials and Methods for details and Fig. 1). Using ampicillin and kanamycin selection in Escherichia coli, insertion mutants were identified and the Kanr gene was shuttled out of the capsid coding region by digesting and religation with the nested pair of EcoRV sites (see Materials and Methods). This resulted in a specific linker insertion of 12-bp carrying a single copy of the unique EcoRV site in the capsid coding sequences. The exact positions of the linker insertion were further refined by restriction enzyme digestions and, in six cases, sequencing (data not shown). The position of insertion mutants are identified by the first letter of the enzyme used in the partial digestion followed by the nucleotide position of the restriction site in the AAV2 genome, for example, NlaIV 4160 would be N4160.

The capsid coding sequence from these mapped insertion mutants were subcloned into the helper vectors pACG2 or pAAV/Ad for biological characterization in vivo (Fig. 1) (Li et al., 1997; Samulski et al., 1989). Sequence analysis predicts that this 12-bp insertion cannot result in a termination codon for any of the 43 insertion sites (Table 1). Owing to the random nature of the cut site for the enzymes (HaelI, NlaIV, and RsaI) with respect to codon frame usage and the degeneracy of the NlaIV recognition sequence, the 12-bp linker resulted in the insertion of the amino acids GDIA in frame 1 and AISP in frame 3 (for all three enzymes, while insertions in frame 2 resulted in WRYPH for RsaI, GRYRP for HaelI, and both GRYRP and GRYRH for NlaIV. The bold-faced amino acids in these examples represent missense mutations (Table 1). The mutant helper constructs, pACG2 +, were individually transfected into 293 cells along with an AAV reporter vector, containing the β-galactosidase gene in adenovirus d309 (M.O.I. = 5)-infected cells (Li et al., 1997). The transfected cells were then assayed for cap-
The expression and recombinant virus production (see Materials and Methods) (Li et al., 1997).

Analysis of capsid proteins

Before assaying for vector production using mutant capsid constructs in complementation assays, each insertion mutant was tested for expression of capsid subunits in 293 cells after transfection. The ability to produce Vp1, Vp2, and Vp3 at normal stoichiometry would suggest that linker insertions did not alter capsid protein expression or stability. Since the linker did not introduce stop codons, it was expected that each insert would produce all three capsids. Forty-eight hours after transfection, cell lysates were analyzed by Western blot for AAV capsids. The Western blot analysis in Fig. 2 is a representation of insertion mutant capsid expression in cell lysates. With the exception of H2634 (Fig. 2, lane 2), the stoichiometry of the three capsid subunits does not appear significantly different than that of wild-type controls (Fig. 2 compare lanes 1, 3–7 to lane 8). By this assay, insertion mutant H2634 appears to produce only Vp3 subunits (Fig. 2, lane 2). In longer exposures, the minor capsid subunits in Fig. 4, lanes 4 and 5, were apparent (data not shown).

Mutant capsid ability to produce stable virions

To test for the production of stable virions that protect a vector genome from DNase digestion, we subjected the cell lysates to cesium chloride (CsCl) gradient centrifugation. Virus densities were measured by refractometry, and aliquots from appropriate fractions were subjected to dot blot hybridization (Fig. 3A). Based on this analysis, particles that package intact recombinant genomes should display a buoyant density similar to that of the wild-type and be resistant to DNase treatment, with the exception of H2944, which has a buoyant density slightly higher than that of the wild-type. Results for this assay separated insertion mutants into two classes. Class I mutants were negative for protecting the viral genome, while class II mutants appeared normal for packaging and protecting the vector substrate (Table 1).

Infectivity of class II insertion mutants

Virions generated by insertion mutants in the complementation assay were tested for infectivity by monitoring FIG. 1. Insertional mutagenesis strategy for AAV2 capsid. A cassette containing the kan’ gene flanked by EcoRV and Nael sites were cloned into the plasmid pAV2Cap, pAV2Cap, which contains the open reading frame of AAV2 capsid, was partially digested with HaeIII, NlaIV, and RsaI separately so that unit-length products were isolated. The 43 positions of restriction sites for these enzymes are shown above the diagram of the capsid open reading frame. The position of the Kan’ insert was mapped by restriction enzyme digestion and in some cases sequenced. Once the position was determined the Kan’ gene was removed by EcoRV digestion, and the capsid domain subcloned into pACG. This strategy resulted in inserting a 12-bp fragment, with half the Nael sites flanking a unique EcoRV site, into the respective HaeIII, NlaIV, and RsaI sites. The 12 bp code for four amino acids, one of which is shown above the diagram of pACG2.
transduction of LacZ reporter gene in human cells. Using viral titers derived from dot blot hybridization, HeLa cells were infected with mutant virus stocks at equivalent particle numbers. Twenty-four hours postinfection, expression of the transgene was detected by X-gal staining. A representative figure of this analysis is shown (Fig. 3B) and all mutants assayed are presented in Table 1. In this assay, wild-type virions transduced 5.6 \times 10^5 HeLa cells/1.75 \times 10^8 protected particles (Fig. 3B and Materials and Methods). Based on the sensitivity of this assay, the range of infection efficiency for class II insertion mutant viruses was from 0 to 1.6 \times 10^6 transducing units/1.75 \times 10^8 protected particles. Results from this analysis further subdivided the capsid insertion mutants from class II (normal for packaging and protecting the vector substrate) into a class III phenotype (normal for packaging and protecting the vector substrate and infectious virions). Two insertion mutants negative for infectivity and initially identified as class II mutants (N2944, H3595) based on CsCl purification and DNase protection tested positive for viral transduction after purification using an iodixanol step gradient (Table 1). This virus

![FIG. 2. Expression of capsid proteins in cells transfected with wild-type and insertion mutant helper plasmids of pACG2. Cell lysates from 293 cells transfected with 1, H2285; 2, H2634; 3, H2690; 4, N2944; 5, H2944; 6, H3595; 7, H4047; 8, wild-type were analyzed by acrylamide gel electrophoresis and immunoblotting with the B1 monoclonal antibody and detected by peroxidase-conjugated secondary antibody. On the left of the Western blot are the positions of the molecular weight standards and on the right are the positions of the major capsid protein, VP3, and the minor capsid proteins, VP2 and VP1.](#)
The purification technique is not as harsh as CsCl and has been shown to increase virus recovery by 10-fold (Zolotukhin et al., 1999). However, other class II mutants remained noninfectious after purification using an iodixanol step gradient (data not shown). Although we determined that insertion mutant viruses N2944 and H3595 were infectious using the LacZ transduction assay, it should be noted that these mutants resulted in low infectious titers ($1 \times 10^2$ transducing units/1.75 x $10^8$ particles), similar to those of previously published low infectious particle (lip) mutants (Hermonat et al., 1984).

**Electron microscopy of class II and class III mutants**

To further characterize class II and III rAAV2 insertion mutants for biological differences, we visualized mutant particles by EM. The EM analysis revealed only gross morphology of the infectious class III viruses, which were indistinguishable from wild-type virions (compare Figs. 4A with 4B and 4C). Whereas distinct differences were observed between class II/III mutant virus H3595 when compared to wild-type virions (Figs. 4A and 4F, bottom four panels), EM images of H3595 revealed a slightly larger roughly pentagonal outline, while wild-type virus appeared uniformed in size and was hexagonal. Interestingly, class II mutant H2634, which was negative for Vp1 or Vp2 by Western blot (Fig. 2, lane 2), appeared normal in morphology by EM analysis (Fig. 4D). Based on this analysis, virion morphology alone is not sufficient to distinguish class II mutants from class III since small insertions within the capsids can result in either nondetectable (Figs. 4B–4E) or noticeable alterations in virion structure (Fig. 4F, bottom four panels). However, this approach was able to provide additional data to our characterization of these linker insertion mutants (Fig. 4, compare A to F).
Capsid ratio of class II and class III virions

Rose et al. (1971) established that AAV2 particles are composed of Vp1, Vp2, and Vp3 at a 1:1:20 ratio (Rose et al., 1971). In an effort to determine if class II and class III mutant virions maintained this ratio, Western blots were performed on the cesium chloride-purified virus. Purified viruses analyzed by Western blot showed similar amounts of Vp3 in all mutants sampled (Fig. 5, Vp3 arrow); between $1 \times 10^9$ and $2.5 \times 10^9$ viral particles were used for each sample. The amounts of Vp2 and Vp1 are also nearly equivalent in all test samples, except H2634, where no minor capsid components were ob-

FIG. 4. Electron microscopy of insertion mutants. Samples (200 μl) of each virus from the peak fraction of gradient were dialyzed against 1× PBS + 1 mM MgCl₂, speed-vac desiccated, and then resuspended in 20 μl of distilled H₂O. Samples were negatively stained with 2% phosphotungstic acid. (A) rAAV2 with wild-type virion. Infectious insertion viruses (B) H2690 and (C) H2591. Noninfectious viruses (D) H2285, (E) H2634, and (F) H3595 (bottom four panels). The black bar in A is 100 nm; the magnification is equivalent in each panel.

FIG. 5. Analysis of virion composition from wild-type and various insertion mutant viruses isolated from cell lysates by cesium chloride gradient centrifugation. Peak fractions of virus were determined by dot blot hybridization and dialyzed against 1× PBS + 1 mM MgCl₂. For each, viral sample between $1.0 \times 10^8$ and $2.5 \times 10^8$ particles were used. Virions from (1) wild-type rAAV2; (2) H2285; (3) R2349; (4) H2591; (5) H2634; (6) H2690; (7) H3766; and (8) N4160 were analyzed by acrylamide gel electrophoresis and immunoblotting with the B1 monoclonal antibody and detected by peroxidase-conjugated secondary antibody. On the right of the Western blot are the positions of the molecular weight standards and on the left are the positions of the major capsid protein, VP3, and the minor capsid proteins, VP2 and VP1.
served (Fig. 5, lane 5). The lack of minor capsid components for H2634 is consistent with the Western results from cell lysates (Fig. 2). At the limit of detection in this assay, the class II insertion mutant H2634 appears to assemble AAV virions without Vp1 and Vp2, even though EM analysis suggests that this mutant has normal morphology (Fig. 4D).

Heparin binding of class II and class III mutants

Recently our lab established that AAV-2 uses a heparan sulfate proteoglycan as a primary receptor for infectivity (Summerford and Samulski, 1998). To determine what role heparin binding may have in class II particles’ inability to infect cells as well as the ability of class III virus to bind heparin agarose, heparin batch binding experiments were performed. Not surprisingly, all class III mutants were positive for heparin binding, with the majority of virus eluting in the 1 M NaCl2 step (data not shown). To determine if loss of infectivity of class II mutant viruses was related to a lack of heparin binding, batch binding experiments were analyzed by dot blot hybridization (Fig. 6). For each of the viral samples tested, an internal control to determine 100% bound was spotted on the filter independently of heparin binding (Fig. 6, 100% bound). This allowed us to determine the percentage of virus retained, at each step of heparin purification. After binding to heparin agarose, samples were washed and then eluted using increasing salt concentrations (see Materials and Methods). Recombinant AAV2 with wild-type virion shells demonstrated 90% binding, with 10% released in the wash, followed by 60% recovered in the elution buffer, and 20% remaining bound to heparin agarose (Fig. 6, lane 1). Class II mutants H2285, H2416, and H2634 demonstrated similar binding and elution profiles (Fig. 6, lanes 2–4). However, class II mutant H3761 was distinct in its heparin agarose binding profile, with the majority of the virion in the binding buffer and the washes (Fig. 6, lane 5). Further analysis is required to determine the reason for lack of heparin binding in this batch assay.

Interestingly, H2634 binds heparin agarose under these conditions, which, by Western blot does not carry detectable Vp1 or Vp2 subunits (Fig. 5, lane 4). The lack of Vp1 and Vp2 in H2634 and its ability to bind heparin agarose suggest that the heparin binding domain may be located in Vp3 capsid proteins.

DISCUSSION

We have used insertional mutagenesis of the structural coding sequence of AAV serotype 2 in order to determine critical capsid domains and positions capable of tolerating insertions without loss of function. The linker insertions isolated were identified in each of the three AAV capsid subunits. Based on Western blot, cesium chloride isopycnic centrifugation, DNase protection, EM, as well as virus transduction analysis, we separated the capsid insertion mutants into three classes. The classes were distinguished based on whether the mutations resulted in inability to assemble virions (class I), ability to package and protect virion DNA (class II), and finally ability of assembled virions that protect viral DNA to infect target cells (class III).

To carry out this study, we used a cloning strategy that employed partial digestion of the capsid coding sequences to create a collection of in-frame linker insertion mutants. The restriction enzymes we utilized (HaeII, NlaIV, and Rsal) have 43 sites within this region of AAV2. Although this strategy appeared straightforward, of the enzymes chosen, only HaeII did not show a site preference. The restriction enzyme NlaIV showed a 25% site preference for position 4160, while Rsal displayed a 45% site preference for position 3391. These site preferences hampered the inclusion of all 43 potential insertion sites in this study (Table 1). Regardless of these limitations, we were able to generate 22 insertion mutants that spanned all three capsid coding domains (Fig. 1). A critical aspect of this approach was the choice of the EcoRV restriction
site as part of the linker insertion. The sequence of this insert was chosen for two reasons: (1) EcoRV is not present in the AAV genome and (2) the 12-bp insertion would not produce a stop codon within any frame using these restriction enzymes. Incorporation of Kan’ gene between two EcoRV restriction sites allowed for easy isolation of plasmids carrying novel insertions (drug selection in E. coli). The Kan’ coding sequences were removed by EcoRV digestion and reforming a single 12-bp insertion site containing a unique EcoRV site within the capsid gene (Fig. 1). Following detailed mapping of linker insertion sites, the capsid coding sequences were subcloned into AAV helper plasmids previously established in our lab and assayed for AAV2 vector production. Utilizing the linker-modified helper plasmids, we were able to test rAAV vectors for virion assembly, capsid composition, stability, heparin binding, and infectivity.

Class I insertion mutants

Of the 22 capsid insertion mutants isolated, 7 were identified with a class I phenotype. Class I mutants did not protect the viral genome as assayed by DNase and dot blot hybridization of cesium chloride gradient fractions (data not shown). This class of capsid mutants may include both packaging and assembly defects. Additionally, some class I mutants may have reduced stability during purification through cesium chloride or temperature-dependent assembly. While we did not characterize these capsid variants further, these mutants are similar to cap mutants first described by Hermonat et al. (1984). The mutants described by Hermonat et al. (1984) were also defective for virus production (Hermonat et al., 1984). Although defective for production of stable AAV virions, these mutants may be important reagents for studying AAV maturation.

For example, studies examining the subcellular location of wild-type AAV2 capsid subunits resulted in Vp3 dispersed throughout the cell, while Vp1 and Vp2 accumulate in the nucleus (Hunter and Samulski, 1992; Wistuba et al., 1997). Using a conformation-specific antibody against assembled AAV2, nucleolar localization was shown (Wistuba et al., 1997), similar to previous fluorescent antibody assays against AAV virions (Hunter and Samulski, 1992). Furthermore, when individual Vp1 and Vp2 capsid subunits were expressed in HeLa cells, wild-type patterns of distribution were observed. However, only when Vp3 was expressed in the presence of Vp1 or Vp2 did this protein also accumulate in the nucleus as previously described (Ruffing et al., 1992). These results suggest that interactions between the subunits are important for the assembly of AAV2 particles even in the absence of terminal repeats or rep protein functions. Similar analysis of class I capsid subunit distribution may help demonstrate the presence of domains that are responsible for proper nuclear localization and assembly.

Class II insertion mutants

The class II insertion mutants isolated in this study protect the viral genome in a DNase assay, but are negative for virus infection. By Western blot analysis these mutants appear to assemble the virion with the same stoichiometry as wild-type virions (Fig. 2), with the exception of H2634. The linker insertions for this class were also distributed across all three capsid subunits (Table 1). The class II mutants described in this study appear distinct from the cap mutant category described previously (Hermonat et al., 1998), in that although defective for infectious virions, these mutants accumulate and protect ssDNA. In addition, the majority of these mutants were positive for retention to a heparin column, suggesting that binding to the AAV primary receptor (heparan sulfate) was not a rate-limiting step (Summerford and Samulski, 1998). Surprisingly, one mutant, H2634, did not appear to express Vp1 and Vp2 in either cell lysates (Fig. 2) or purified virions (Fig. 5) but bound heparin sulfate similarly to the wild-type (Fig. 6). Although Vp1 and Vp2 could not be detected, EM analysis did not reveal any gross alteration in morphology (Fig. 4D). The absence of Vp1 and Vp2 is difficult to explain since it contradicts the requirement of these proteins to sequester Vp3 in the nucleus, as previously described (Hunter and Samulski, 1992; Ruffing et al., 1992; Wistuba et al., 1997). Although H2634 inserts an ADIG and the aspartic acid results in a net negative charge, the position of the ADIG is directly downstream of two lysines. The insertion of ADIG may cause a local misfolding of the capsid protein, exposing these or other lysines to ubiquitin-conjugating enzymes, thereby altering the half-life of Vp1 and Vp2 but not Vp3 (Hershko and Ciechanover, 1992). It is plausible that Vp1 and Vp2 are still able to sequester Vp3 to the nucleus for assembly but their shortened half-life reduces their incorporation in the assembled virion to levels undetectable by Western blots.

Since the class II mutants bound heparin, the loss of productive infection to HeLa and 293 cells appears unrelated to receptor binding. The viral entry pathway of AAV2 has not been extensively studied. Recent work has demonstrated that AAV2 enters the cell through an endosome and is released in a pH-dependent manner. This is followed by migration to the perinuclear space and uncoating in productive infections (Barlett, personal communication). A similar pathway is used by canine parvovirus, suggesting conservation in entry mechanism among these parvoviruses (Vihinen-Ranta et al., 1998). The ability of class II mutants to bind heparin sulfate but fail to transduce target cells suggests a blocked in infection that may occur at any step from viral entry to
uncoating. Recently, we demonstrated the ability to assay early steps in AAV entry using fluorescent virus infus ed in rat brain (Bartlett and Samulski, 1998). These studies determined that the virus bound to target cells within 10 min, followed by transport to the nucleus within the first hour postinfection. Similar analysis using these mutants should be extremely helpful in better defining the defects of these mutants in early steps of AAV infection.

Class III insertion mutations

The class III mutants were unique in that they protected the viral genome and retained infectivity at levels near that of wild-type virus (Fig. 3B). Similar to class II, class III mutants protected viral DNA, bound heparin (data not shown), had normal morphologies by EM (Fig. 4B), and were derived from linker insertions positioned in each of the three capsid subunits (Table 1). The characterization of these capsid mutants represents the first description of AAV virions that can tolerate amino acid inserts without interfering with normal viral infectivity. In addition, these mutants validate the concept of expressing foreign epitopes on the surface of AAV capsids that may influence normal infectivity, such as retargeting. Previous modifications with B19 Vp1 and egg white lysozyme fusion proteins resulted in virus-like particles that retain lysozyme enzyme activity. While these data supported the ability to form capsid fusion proteins, the particles generated were empty and not infectious (Miyamura et al., 1994). The viable class III mutants isolated in this study ranged from 5% (H2690) to 100% (N4160) of wild-type infectivity. Understanding the distinction between low infectious particles and high transducing particles should help determine critical steps in AAV infection. In addition, the wild-type viable insertion mutants provide a unique opportunity to test insert size limitation as well as ligand-specific retargeting of modified virions (Rabinowitz and Samulski, manuscript in preparation). Recently, the sequence and viral tropism of AAV serotypes 4 and 5 have been characterized (Chiorini et al., 1999, 1997). These viruses do not appear to utilize heparan sulfate as a receptor for infection, and therefore domain swapping between serotypes should provide a unique opportunity to identify amino acids specific for viral binding to target cells (Rabinowitz and Samulski, manuscript in preparation). Detailed analysis of these mutants should provide a better understanding of both wild-type and vector-modified infection.

Two mutants (H/N2944 and H3595), which were first identified as class II after CsCl gradient purification, demonstrated a class III phenotype when isolated using an iodixanol step gradient. Interestingly, mutants of this type have been described as lip (Hermonat et al., 1984). However, unlike the lip mutants of Hermonat et al. (1984), which were positioned solely within the Vp1 unique coding region (Hermonat et al., 1984), H/N2944 and H3595 were localized to Vp3. Interestingly, mutant H3595 displayed morphological differences by EM when compared to wild-type virions. This mutant had a pentagonal appearance and was slightly larger than the wild-type (Fig. 4D). Because the H3595 insertion is within a putative loop domain of Vp3 coding sequences, the alteration is present in all 60 subunits and results in the insertion of amino acids GRYRP (Table 1). A comparison of CPV and FPV crystal structures has determined that 5 of the 10 amino acid differences in Vp2 are on the surface of the virion (Llamas-Saiz et al., 1996). One of these changes, 375 N-D, causes a displacement of 7 Å in the domain between loops 3 and 4 (Agbandje et al., 1993). These examples demonstrate the significance of both genetic and crystal analysis when studying virion structure and function. The alteration seen with this virion by EM and lip phenotype observed after infection maybe due to the amino acid insertion interfering with capsid domain folding, number of assembled subunits, and or assembly symmetry. The exact explanation of this phenotype will require information obtained from AAV crystal structure analysis.

The availability of crystal structures for some of the parvoviruses has complemented genetic studies and provided sufficient detail to test critical domains involved in entry and infectivity. The parvovirus B19 crystal structure has been determined to 8 Å (Chapman and Rossmann, 1993). Although there is only a 15% overall identity between B19 and the feline panleukopenia virus (FPV) virion, the core antiparallel β-barrel of B19 is similar with respect to the overall symmetry of the antiparallel β-barrel in FPV (Agbandje-McKenna et al., 1994). The eight antiparallel β-strands form a barrel domain that is well conserved not only among parvoviruses but also among many types of viruses (Chapman and Rossmann, 1993; Rossmann, 1989). The crystal structure of CPV has been determined at 2.8 Å (Tsao et al., 1991). This information has allowed the precise positioning of amino acid residues with respect to the surface domains of the viral subunit. Amino acids 93 and 323 of CPV are required for maintenance of host range infectivity (Chang et al., 1992), and from the crystal structure, these regions have been determined to be part of loops 1 and 3 (Tsao et al., 1991). The crystal structure of FPV has been determined at 3.3 Å (Agbandje et al., 1993).

In summary, we have generated a collection of AAV capsid mutants that fall into three phenotypes; class I mutants, which are defective for producing stable virions, class II mutants, which assemble noninfectious DNA intact virions, and class III mutant virions, which are infectious. We believe that these capsid mutants will be important reagents for analyzing basic questions of AAV infections that focus on virion assembly, receptor-mediated entry, uncoating, and viral transgene expression. The importance of understanding these fundamental
steps in AAV entry is best illustrated by recent studies using AAV vectors for cystic fibrosis gene therapy. These vectors have met with limited success due to the inability to infect target cells (Bartlett et al., in preparation). The availability of these AAV class II and III capsid mutants will be valuable substrates for engineering rAAV vectors with novel targeting capabilities. In addition to deriving targeting vectors, further characterization of mutant capsids as well as the elucidation of the AAV crystal structure should provide a detail paradigm for AAV viral assembly and entry.

MATERIALS AND METHODS

Cells and viruses

Human 293 and HeLa cells were maintained at 37°C with 5% CO₂ in 10% fetal bovine serum (HyClone) in Dulbecco’s modified Eagle’s medium (Gibco BRL), with streptomycin and penicillin (Linberger Comprehensive Cancer Center, Chapel Hill, NC). Four × 10⁶ 293 cells were plated the day before transfection onto a 10-cm plate. Cells were transfected by both calcium phosphate (Gibco BRL) and Superfection (Qiagen) according to manufacturers’ specifications. The insertional mutant packaging plasmids, described below, were transfected along with pAB11, containing the CMV-driven LacZ gene with a nuclear localization signal. For each transfection the same amounts of packaging plasmid (12 µg) and pAB11 (8 µg) were used for each 10-cm plate.

For each transfection an additional plate was used containing the transgene plasmid only to assess transfection efficiencies. After transfection the cells were infected with helper virus Ad5 d5/309 at an M.O.I. of 5, and 48 h later the cells were lysed and the virus was purified.

Recombinant virus was purified using cesium chloride isopycnic or iodixanol gradients. In both cases cells were centrifuged at 1500 rpm (Sorvall RT 6000B) for 10 min at 4°C. Proteins were precipitated from the supernatant using ammonium sulfate (30% w/v) and resuspended in 1 × phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 7H₂O, 1.4 mM KH₂PO₄). The cell pellet was resuspended in 1 × PBS containing 0.1 mg/ml DNase I (Boehringer-Mannheim) lysed by three freeze–thaw cycles, combined with the protein portion of the supernatant, and incubated at 37°C for 30 min. This material was subjected to sonication (Branson Sonifier 250, VWR Scientific), 25 bursts at 50% duty, output control 2. Cell debris was removed by centrifugation (Sorvall RT 6000B). To each milliliter of supernatant 0.6 g of CsCl was added and the solution was centrifuged for 12–18 h (Beckman Optima TLX ultracentrifuge) in a TLS 55 rotor at 55,000 rpm. Alternatively, the supernatant was layered on top of an iodixanol (Opti-Prep-Nycomed Pharma As, Oslo, Norway) gradient of 60, 45, 30, and 15. This gradient was centrifuged in a Beckman Optima TLX ultracentrifuge using a TLN 100 rotor at 100,000 rpm for 1 h. Fractions were recovered from these gradients and 10 µl from each fraction was utilized for dot blot hybridization to determine which fraction contained the peak protected virion (see Titration of recombinant virus).

Construction of AAV packaging plasmids

The capsid domain of pAAV/Ad was cloned into pBS⁺ (Stratagene) using HindIII, resulting in pAV2Cap. Partial digestion of pAV2Cap using the restriction enzymes HaellI, NlaIV, and Rsal and gel purification of the unit-length DNA fragment resulted in the isolation of the starting material for cloning. The aminoglycoside 3’-phosphotransferase gene, conferring kanamycin resistance (Kan’), from pUC4K (Pharmacia) digested with SaI was flanked by linkers containing Nael and EcoRV sites, a SaI overhang at one end, and an EcoRI overhang at the other end (top 5’-AATTGCGGCCGATATC-3’, bottom 5’-TCGAGATATCGCCGGC-3’). This fragment was cloned into the EcoRI site of pBluescript (SK⁺) (Stratagene). Digestion with Nael released the Kan’ gene, and this fragment was ligated into the pAV2Cap partials. The resulting plasmids were screened for insertion into the capsid domain and then digested with EcoRV to remove the kan’ gene, leaving the 12-bp insertion 5’-GGC-GATATCGCCG-3’ within the capsid domain. Multiple enzyme digests and DNA sequencing were used to determine the position of the 12-bp insertion within the capsid coding domain. The enzyme digests included EcoRV/BanII, EcoRV/BstNI, EcoRV/PstI, and EcoRV/HindIII.

The capsid domains of the resulting plasmids were digested with Asp718 and subcloned into the pACG2 packaging plasmid, with the exception of one NlaIV clone that overlapped the 3’-Asp718 site. This insertion mutant was cloned into pAAV/Ad using a HindIII/NsiI digestion.

Western blotting

Cell lysates after freeze–thaw lysis and sonication was centrifuged to remove large cell debris. Twenty microliters of supernatant was immediately added to 20 µl of 2× SDS gel loading buffer containing dithiothreitol and boiled for 5 min. Proteins were analyzed by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose electrophoretically. The nitrocellulose membranes were immunoblotted using the anti-Vp3 monoclonal antibody B1 (a generous gift from Jurgen A. Kleinschmidt). Each of the insertion mutants was tested at least twice by Western blot analysis. The secondary anti-mouse horseradish peroxidase IgG was used to indirectly visualize the protein by enhanced chemiluminescence (ECL, Amersham). The Western blots were scanned from enhanced chemiluminescence-exposed BioMax film (Kodak) into Adobe Photoshop and analyzed by ImageQuaNT software (Molecular Dynamics Inc.).

Viral proteins were visualized by Western blotting fol-
lowed by immunoblotting as described above. Between 1.0 and 2.5 × 109 viral particles were used for each sample. The virus was isolated from the peak cesium gradient fraction as determined by dot blot and dialyzed against 0.5× PBS containing 0.5 mM MgCl₂ prior to polyacrylamide gel electrophoresis.

**Titration of recombinant virus**

Fractions from CsCl gradients were obtained by needle aspiration. The refractive index was obtained using a refractometer (Leica Mark II), and the index was used to determine the density of fractions. Aliquots of 10 μl from fractions between 1.36 and 1.45 g/ml were tested for the presence of protected particles by dot blot hybridization. The aliquots were diluted 1:40 in viral dilution buffer (50 mM Tris–HCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 μg/ml RNase, 10 μg/ml DNase) and incubated at 37°C for 30 min. To the samples sarcosine (final concentration 0.5%) and EDTA (final 10 mM) were added and incubated at 70°C for 10 min. Proteinase K (Boehringer-Mannheim) was added to a final concentration of 1 mg/ml and the samples were incubated at 37°C for 2 h. Following this incubation the samples were denatured in NaOH (350 mM final) and EDTA (25 mM final). The samples were applied to equilibrated Nytran (Gene Screen Plus, NEN Life Science Products) using a dot blot manifold (Minifold I, Schleicher & Schuell). The membrane was probed with a random-primed (Boehringer-Mannheim) [35S]dCTP-labeled LacZ DNA fragment. The membranes were exposed to film (BioMax MR, Kodak) or to phosphor imaging screens (Molecular Dynamics) and intensity estimates were done using ImageQuant software (Molecular Dynamics). Peak fraction of virus were then dialyzed in 1× PBS for transducing titer.

Transductions titers were determined by histochemical staining for LacZ activity. HeLa cells had been infected with Ad d309 at a m.o.i. of 5 for 1 h. The cells were then washed with 1× PBS and fresh medium was added. Aliquots of virus from peak fractions, equivalent to 1.75 × 10⁹ particles, were used to infect HeLa cells. Twenty to twenty-four hours later cells were washed with 1× PBS, fixed (2% formaldehyde, 0.2% glacial acetic acid in 1× PBS), washed, and stained with 5'-bromo-4-chloro-3-indoly-β-D-galactopyranoside (Gold Bio Technology) dissolved in N,N-dimethyl formamide (Sigma) diluted to 1 mg/ml in 1× PBS, pH 7.8, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂ at 37°C for 12–24 h. Stained HeLa cells were counted in ten 400× microscope fields. The transducing number was determined by averaging the number of stained cells in 10 fields, multiplying by the number of fields on the plate, and dividing that number by the number of nanograms of protected template.

**Electron microscopy**

Peak fractions of rAAV with wild-type virion or mutagenized virions were dialyzed in 0.5× PBS containing 0.5 mM MgCl₂. The virus was placed on a 400-mesh glow-discharged carbon grid by inverting on a 10-μl drop of virus for 10 min at room temperature, followed by three 1× PBS washes for 1 minute each. The virus was stained in 2% phosphotungstic acid for 1 min. Specimens were visualized using a Zeiss EM 910 electron microscope.

**Heparin agarose binding assay**

Recombinant virus containing wild-type capsids or insertion in the capsids were dialyzed against 0.5× PBS containing 0.5 mM MgCl₂. One hundred microliters of each virus was bound to 100 μl of heparin agarose type 1 (H-6508, Sigma, pre-equilibrated in 20 vol of 0.5× PBS containing 0.5 mM MgCl₂) at room temperature for 1 h in a 1.5-ml microfuge tube. After each step, binding washes and elutions samples were centrifuged at 2000 rpm (Sorvall MC 12V) for 2 min to collect supernatant. Samples were washed six times with 0.5 ml of 0.5× PBS containing 0.5 mM MgCl₂, and the supernatants collected. Samples were eluted in three steps of 100-μl volumes containing 0.5, 1.0, and 1.5 M NaCl in 0.5× PBS containing 0.5 mM MgCl₂ and the supernatants collected. For each sample 20 μl of supernatant from each step was used for dot blot hybridization. The 100% bound control was an internal standard equivalent to one fifth of each input virus used in the dot blot. The heparin agarose viral mixtures were washed six times with 0.5× PBS, 0.5 mM MgCl₂ in volumes that resulted in a 1:15,625 dilution.

**ACKNOWLEDGMENTS**

We acknowledge Shamasia Shafi, Mitch Sally, and Lisa Hanson for technical help and Terry Van Dyke for critical reading of the manuscript. Joseph Rabinowitz was supported by a grant from the Muscular Dystrophy Association. In addition, this work was supported by NIH Grants DK53423-01 and HL51818-06.

**REFERENCES**


Berns, K. L., and Adler, S. (1972). Separation of two types of adeno-


