Molecular Cloning of Adeno-associated Virus Variant Genomes and
Generation of Infectious Virus by Recombination in Mammalian Cells*

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Continued passage of the human parvovirus, adeno-associated virus (AAV), at high multiplicity of infection in human cells results in the accumulation of AAV particles containing variant genomes. We have analyzed the structure of individual variant AAV genomes by molecular cloning in the Escherichia coli plasmid, pBR328. Each of the AAV inserts in six individual recombinant plasmids contained a single internal deletion but in contrast to a previous model, the locations of the deletions were nonrandom. The molecular cloning protocol also generated recombinant plasmids containing the entire AAV DNA sequence which yielded infectious AAV particles when transfected into human KB cells in the presence of helper adenovirus using a DEAE-transfection procedure. Infectious AAV genomes were also generated by recombination when cells were jointly transfected with a mixture of plasmids containing two different mutant AAV genomes. The efficiency of this recombination appear to be influenced by the degree of homology between the mutant AAV genomes.

AAV1 is a defective human parvovirus that grows only in cells in which certain helper functions are provided by expression of adenovirus (1, 2) or herpes virus genes (3). The genome of AAV type 2 is a single linear DNA strand 4675 nucleotides in length (4-7). AAV DNA strands of both “plus” and “minus” polarity are encapsidated with equal frequency into individual virions (4). Upon extraction from virus particles in the presence of phenol, the plus and minus strands reassociate to form AAV DNA duplexes (4).

When lysates of infected cells are banded to equilibrium in CsCl buoyant density gradients several populations of AAV particles are observed (2, 8-10). The majority of infectious (standard) AAV particles, (AAV (1.41)), band at a density of approximately 1.41 g/cm³, and empty AAV capsids containing no DNA band at 1.31–1.32 g/cm³ (11, 12). In addition, in amounts which vary with the passage history of the particular AAV stock used, a heterogeneous population of variant AAV particles with densities ranging from 1.41 to 1.32 g/cm³ is observed.

The structure and properties of populations of variant particles of parvoviruses have recently been reviewed (13). Briefly, analysis of the biological properties of the variant AAV particles showed that they were noninfectious but exhibited the properties of DI particles (10, 14).

The physical structure of the AAV DNA present in the populations of variant particles was examined, and a general model for their structure was proposed (15). According to this model the variant DNAs differed in length due to internal deletions in the genome sequence, and it was proposed that both the size and the location of the deletions were random. Each variant genome retained the terminal palindromic sequences which comprise part of the AAV DNA replication origins (5, 6, 16-18). Many variant genomes were spontaneously renaturing molecules (15) in which the cross-link was the terminal covalent hairpin structure analogous to that found in intracellular AAV RF DNA molecules (16, 18, 19).

These variant AAV DNA molecules offer a source of naturally occurring mutations. We report here the molecular cloning of individual variant AAV genomes by insertion at the Ball restriction endonuclease cleavage site in the Escherichia coli plasmid, pBR328 (20). Analysis of a limited set of such molecular clones shows that, in contrast to the previously proposed model (15), AAV variant genomes possess deletions at nonrandom locations. In parallel with these studies we obtained analogous clones of standard AAV2 genomes inserted in pBR328 which yielded infectious AAV particles when transfected into human cells together with helper adenovirus. We also demonstrate production of infectious AAV by recombination between mutant AAV plasmids following transfection into human cells.

MATERIALS AND METHODS

Virus Growth and Purification of DNA—A spinner culture (12L) of KB cells was grown at 37 °C in Eagle’s medium supplemented with 5% horse serum and infected with AAV2 (15 infectious units/cell) and adenovirus type 2 (10 plaque forming units/cell) and harvested at 40 h after infection. The AAV stock used was a crude lysate preparation which had been obtained by 5 crude lysate passages in human KB spinner cells at multiplicities of about 10 infectious units/cell. Under these conditions significant amounts of AAV variant DI particles accumulate (10, 14, 15). The infected cells were lysed using the trypsin-deoxycholate procedure, and the lysate was banded in a CsCl equilibrium buoyant density gradient (15). To obtain infectious (standard) AAV the visible band of particles at 1.41 g/cm³ was collected. Variant particles were collected by pooling the entire region of the CsCl gradient above 1.41 g/cm³ up to and including the visible band of adenovirus at 1.35 g/cm³ (14, 15). Each pool of virus particles was banded once more in CsCl and then dialyzed at 4 °C for 1 h against 0.3 M NaCl, 50 mM Tris-HCl, pH 8.0. The dialyzed particle preparations were purified by velocity sedimentation in sucrose gradients to remove contaminating adenovirus particles and cell or adenovirus DNA (14). Virus particles were recovered from the sucrose gradient pools by ethanol precipitation and resuspension in 10 mM Tris-HCl, 1 mM Na2-EDTA, 0.5% sodium dodecyl sulfate. AAV DNA was extracted from the virus particles using proteinase K and phenol and dialyzed extensively against TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Construction of Recombinant Plasmids—The plasmid pBR328 (20)

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*The abbreviations used are: AAV, adeno-associated virus; DI, defective-interfering; RF, replicative form.
and recombinant plasmids derived from it were grown in E. coli LE392 (23). AAV2 DNA was cloned by cleavage with restriction endonuclease BalI and insertion at the BalI site of pBR328. Because the BalI preparations (obtained from New England Biolabs, Beverly, MA) had some exonuclease activity, preparations of AAV2 or pBR328 DNA were digested for 4 h at 37 °C using 0.2 unit of enzyme/µg of DNA (1 µg of pBR328 DNA in 60 µl of saline containing EDTA in a volume of 50 µl) in 10 mM Tris-HCl, pH 7.5, 12 mM MgCl₂, 10 mM 2-mercaptoethanol, and 100 µg/ml of bovine serum albumin. These digestion conditions yielded cleavage of about 80% of the available BalI sites with little or no exonuclease digestion as judged by agarose gel electrophoresis. After BalI digestion, pBR328 DNA (15 µg) and AAV2 DNA (5 µg) were incubated together in the conditions for blunt end ligation in a final volume of 250 µl containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 100 mM EDTA, 0.5 mM ATP, 200 µg/ml of bovine serum albumin, and 50 units of T4 DNA ligase (Boehringer Mannheim) for 16 h at 22 °C followed by 2 h at 4 °C. The ligated DNA mixture was then used to transform E. coli LE392 according to a high efficiency protocol employing RbCl (24). Transformed bacteria were plated on LB agar containing ampicillin (20 µg/ml). Ampicillin-resistant colonies were picked and tested for resistance to tetracycline and sensitivity to chloramphenicol. Appropriate colonies were picked and tested for the presence of AAV DNA sequences by growth on nitrocellulose filters and colony hybridization (25) with an in vitro nick-translated AAV2 [³²P]DNA probe. Bulk preparations of plasmids were grown in M9 medium supplemented with casamino acids (0.2%), thiamine (1 µg/ml), and ampicillin (20 µg/ml) and purified according to the procedure of Humphreys et al. (26) and Kwan et al. (27). Small scale plasmid preparations were grown in LB medium and purified according to the rapid boiling procedure (28). All manipulations with recombinant DNA were performed in accordance with the National Institutes of Health guidelines.

Analysis of DNA—DNA preparations were cleaved with restriction endonucleases obtained from New England Biolabs or Bethesda Research Laboratories and used according to the supplier's specifications. DNA was electrophoresed in horizontal 1% agarose gels in 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 8.0, or vertical 7.5% polyacrylamide gels in 60 mM Tris, 9 mM boric acid, 3 mM Na₂EDTA, pH 8.0. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV illumination. DNA was extracted from gels using the crude and soak procedure (33).

Infectivity of AAV Plasmids in Human Cells—Approximately 10⁶ human 293-31 cells grown at 37 °C in a 35-mm dish were transfected with supercoiled recombinant AAV plasmid DNA using the DRAExtran procedure (34) as described before (21). Each 35-mm dish received 4 µg of pPS11 or pPS12, 3 µg of pPS21, pPS23, pPS24, or pPS30 DNA or AAV2 particles (20 infectious units/cell) together with Ad2 particles (10 plaque forming units/cell). After 48 h transfection, cultures were frozen and thawed three times and heated (60 °C, 15 min) to inactivate adenovirus. This lysate (2.5 ml) was designated P1, and portions (0.3 ml) were passed 2 or 3 more times by infection of 293 cells with helper adenovirus added at each passage (P2, P3, or P4). The infectious AAV particles present in each passage were frozen and thawed three times and heated (60 °C, 15 min) to inactivate adenovirus. This lysate (2.5 ml) was designated P1, and portions (0.3 ml) were passed 2 or 3 more times by infection of 293 cells with helper adenovirus added at each passage (P2, P3, or P4). The infectious AAV particles present in each passage were measured in the standard AAV infectivity assay on KB cells (10) which detect infected cells by indirect immunofluorescence of AAV capsid protein. This assay does not detect infectivity levels below 5 x 10⁶ infectious units/ml.

RESULTS

Sequence Content of the AAV Variant DNA Population—The AAV variant DNA preparation used for the cloning represented particles ranging in density from 1.35 to 1.41 g/cm³ which should include all variant molecules longer than about 600 nucleotides. The restriction endonuclease maps for AAV2 DNA shown in Fig. 1 were used to analyze the structure of the variant molecules prior to cloning. The variant molecules were a heterogeneous collection ranging in size from 0.6 to 2.0 kilobases (Fig. 2A). Cleavage with the endonucleases HpaII and HaeIII and I (27) showed that fragments located between AAV map positions 0 and 12 and between 63 and 100 were present in abundance whereas fragments between 12 and 63 map units were present, if at all, at very low abundance. Thus, in the variant DNA population there was a clear bias toward retention of more AAV2 sequence from the right half of the genome than from the left.

Rationale of Cloning Procedure—The cloning protocol is shown schematically in Fig. 3. The general model for the AAV variant genome is that proposed previously (15). We took advantage of the cleavage sites for endonuclease BalI just inside either extremity of the AAV2 terminal palindromic sequences which comprise 125 nucleotides at both AAV2 DNA termini. This allowed excision of the unique region of AAV DNA and removed the terminal covalent RF hairpin structure present in many of the variant molecules (15). The BalI-cleaved AAV DNA was inserted into the BalI site of the plasmid pBR328 by blunt-end ligation. In parallel with the variant AAV2 DNA we constructed similar plasmid clones from standard AAV2(1.41) DNA.

Selection of Individual Plasmid Clones Containing AAV2 DNA—Thirty-six chloramphenicol-sensitive colonies derived from standard AAV(1.41) DNA were examined, and 26 contained AAV DNA sequences. Of these 26 colonies, 9 gave a very strong hybridization signal and were subsequently shown to contain either the entire AAV2 Bal-I fragment or the entire AAV2 genome. Three of these clones, pPS11, pPS12, and pPS14, are described here. The other 17 positive colonies gave a uniform weak hybridization signal and contained only the AAV2 Bal-I fragment. One such clone, pPS30, is described here.

Of 108 chloramphenicol-sensitive colonies derived from the AAV variant DNA preparation, 43 contained AAV sequences as determined by colony hybridization. 34 of these AAV-positive colonies gave a uniformly weak hybridization signal and contained only the AAV2 Bal-I fragment. The remaining 9 AAV-positive colonies gave hybridization signals of intermediate strength. Six of these colonies contained plasmids pPS21, pPS22, pPS23, pPS24, pPS25, and pPS26, respectively, and were selected for further analysis.

Structural Analysis of the Plasmid AAV Recombinants—Purified plasmid DNA preparations were cleaved with BalI, and the AAV inserts were purified by electrophoresis in agarose gels (Fig. 4). In each case the BalI cleavage produced an AAV DNA unique sequence region fragment which for pPS11, -12, and -14 was the BalI-A fragment but for pPS21-26 was a smaller fragment containing a deletion. In some cases one or two BalI-B fragments were also released. Because the BalI digestion was again performed under conditions of incomplete cleavage the data in Fig. 4 provided an initial estimate of whether one or both terminal palindromes (i.e. BalI-B fragments) were present in the AAV insert. For pPS21, pPS22, and pPS25 the AAV-unique region fragment appears as a doublet which implies the presence of only one BalI-B fragment. For pPS24, the unique region fragment was a triplet which implies the presence of two BalI-B fragments (i.e. both AAV genome termini). For pPS30 only the BalI-B fragment was released.

The AAV inserts purified from individual plasmids were then cleaved with endonucleases HpaII or HaeIII and electrophoresed in polyacrylamide gels (Fig. 5). This provided a general map of each AAV insert. The mapping was extended with additional restriction enzymes including AluI, HhaI, and AvaI (data not shown). In each case the extremities of the deletion were mapped to within a small region of the AAV genome map as summarized below.

Orientation of AAV Inserts and Location of AAV Terminal Palindromes within the Recombinant Plasmids—To determine the orientation of the AAV insert within the plasmid and to confirm the presence and location of the BalI-B fragments the experiment shown in Fig. 6 was performed.
Molecular Cloning and Recombination of Variant AAV DNA

FIG. 1. Restriction endonuclease map of AAV2 DNA. The locations of restriction endonuclease cleavage sites, shown by vertical bars, are indicated according to map units (above the line) and nucleotide number (below the line). One map unit is approximately 47 nucleotides. The nucleotide numbers are based upon the AAV2 DNA sequence (7). The location of the cleavage sites is as determined previously (29, 30) with minor revisions based upon the nucleotide sequence (7).

FIG. 2. Analysis of AAV2 variant DNA. A. The variant AAV2 DNA preparation was electrophoresed in a 1.0% agarose gel (track 2) together with λ phage DNA fragments (track 1). The sizes of the λ DNA fragments are 23.5, 21.6, 9.6, 7.5, 6.6, 5.9, 5.7, 4.8, 4.4, 3.6, 2.3, 1.95, and 0.59 kilobase pairs, respectively. B. Electrophoresis of DNA in a 7.5% polyacrylamide gel. Track 3, standard AAV2 DNA cleaved with HaeIII. Track 4, variant AAV2 DNA cleaved with HaeIII. Track 5, variant AAV2 DNA. Track 6, variant AAV2 DNA cleaved with HpaII. Track 7, standard AAV2 DNA cleaved with HpaII. The HaeIII fragments of standard AAV2 DNA are indicated at the left, and the HpaII fragments are indicated at the right. The designation of the fragments is according to the maps in Fig. 1.

The plasmids were each cleaved successively with the endonucleases EcoRI, HindIII, and KpnI. The only KpnI sites present are those in AAV and for the clones pPS21-26 the only KpnI site is that derived from AAV2 map position 91.0. For the clones pPS21-26 the only EcoRI and HindIII sites present are those in the plasmid on either side of the AAV insert (Fig. 3).

Following the multiple cleavage with EcoRI, HindIII, and KpnI, one-half of each plasmid preparation was cleaved further with endonuclease SmaI. The only SmaI cleavage sites present are those derived from the AAV2 terminal palindrome sequence (i.e. the AAV2 BalI-B fragment). Thus, SmaI cleavage confirms the presence of an AAV2 BalI-B fragment and allows the location of this fragment to be determined relative to the AAV2 insert and the plasmid sequence. In all cases except one, the presence of SmaI cleavage sites (Fig. 6) corresponded with the presence of an excisable BalI-B frag.
Molecular Cloning and Recombination of Variant AAV DNA

**Fig. 4.** Preparative acrylamide gel electrophoresis of AAV inserts from recombinant plasmids. Tracks 11, 12, 14, 21, 22, 23, 24, 25, and 26 contain 30 µg of pPS11, pPS12, pPS14, pPS21, pPS22, pPS23, pPS24, and pPS26, respectively, digested with *Bal*I. Track *m* contains *αX174* RF DNA *HaeIII* fragments with the size (in nucleotide base pairs) indicated at the right. *A* shows tracks from a single gel. *B* shows tracks derived from 4 separate gels, run in parallel, in which the *αX174* DNA markers included in each were superimposable. The *arrows* indicate the positions of the AAV-specific fragments; the 117-base pair fragment is *Bal*I-(*in*). In *A* the upper *arrow* is the AAV *Bal*I-A fragment. As noted in the text (see under “Results”) the *Bal*I digestion was incomplete. The change in position of the upper plasmid *pBR328* DNA band for tracks 12 and 26 reveals the deletion which occurs in the plasmid sequence as described in Fig. 6.

**Fig. 5.** Analysis of AAV2 DNA inserts from individual recombinant plasmids. The AAV inserts from individual recombinant pPS plasmids were excised by *Bal*I cleavage and purified as described in Fig. 4 and then digested with *Hpa*II (*A*) or *Hae*III (*B*) and electrophoresed in an acrylamide gel and stained with ethidium bromide. Tracks 21, 22, 23, 25, and 26 contain AAV2 inserts from pPS21, pPS22, pPS23, and pPS26, respectively. Markers for individual AAV2 DNA fragments were provided by digestion of purified duplex AAV2 DNA with *Hpa*II (tracks *Hp*) or *Hae*III (track *Ha*) followed by additional digestion with *Bal*I (tracks *HpB* and *HaB*, respectively). The AAV2 *Hpa*II or *Hae*III fragments are designated by letters at the right of *A* and *B*, respectively, according to the map shown in Fig. 1. Note that *Hpa*II G and C, which show heterogeneity because of the internal flip-flop in AAV DNA between map positions 1 to 2 and 98 to 99 (7, 29, 30), are cleaved by *Bal*I. Track *m* contains *αX174* RF DNA cleaved with *Hae*III as described in Fig. 4.

ment. The exception was pPS26 (Fig. 6) which has a *Sma*I cleavage site located within the position expected for the AAV2 *Bal*I-B fragment, but no *Bal*I-B fragment was excised from pPS26 (Fig. 4). It is likely that in pPS26 the *Bal*I site at 100 map units was lost during cloning since removal of 5 nucleotides from the AAV2 terminus would be sufficient to destroy the *Bal*I sites at map positions 100 or 0. The orientation of each AAV insert relative to the plasmid sequence was determined as described more fully in the legend to Fig. 6.

**Fig. 6.** Analysis of the orientation of the AAV insert in plasmids and confirmation of presence or absence of the terminal palindrom sequence (*i.e.* the AAV2 *Bal*-B fragment). Pairs of tracks labeled 11, 12, 14, 21, 22, 23, 24, 25, and 26 contain, respectively, pPS11, pPS12, pPS14, pPS21, pPS22, pPS23, pPS24, pPS25, and pPS26 DNA jointly digested with *Kpn*I, *Hind*III, and *Eco*RI. One-half of each digested DNA preparation was (+) or was not (-) also digested with *Sma*I. Tracks *m* contain a mixture of fragments of *αX1857 Sam7* DNA derived by individually digesting with *Hind*III and *Eco*RI as described in Fig. 2. For each construction the orientation of the AAV insert relative to the pBR328 sequence was deduced by reference to the maps in Figs. 1, 2, and 7. The AAV inserts have either the same or opposite sense such that the same sense means that the left to right orientation of the AAV reads in the same sense as clockwise on the pBR328 genome as diagrammed in Fig. 1. The designation of certain fragments is indicated. The identity of the remaining fragments can be deduced by reference to the maps in Figs. 1, 2, and 7. *HR* is the 3.65-kb *Hind*III/*Eco*RI fragment from pBR328. *HRΔ* results from a deletion of results from a deletion of about 1.6 kilobases of pBR328 sequence (in the vicinity of the tetracycline-resistance gene and spanning the *Hind*III site) which occurs in some constructions (e.g. pPS12 and pPS26) as a consequence of the 482-base pair inverted duplication present in pBR328 (31, 32). For the same sense AAV inserts, the diagnostic fragment is the *KH* (*Kpn*I/*Hind*III) fragment generated by fusion of the AAV *Kpn*I fragment (map positions 89.9 to 100) fused to the pBR328 *Bal*-*Hind*III fragment. *KHΔ* is obtained when no AAV2 *Bal*-B fragment is present. *KH*, but not *KHΔ*, is cleaved by *Sma*I. For AAV inserts in the opposite sense the diagnostic fragment is the *RK* (*Eco*RI/*Kpn*I) fragment generated by fusion of the pBR328 *Eco*RI/*Bal*I fragment to the AAV *Kpn*I fragment from map position 100 to 88.9. If no AAV2 *Bal*-B fragment is present *RKα* is obtained. *RK* but not *RKΔ*, is cleaved by *Sma*I. *SK* is the AAV2 fragment from the *Sma*I site at about map position 99.0 to the *Kpn*I site at 88.9. *RS* is the pBR328 *Eco*RI/*Bal*I fragment fused to the AAV fragment from one or other genome terminus to the first *Sma*I site in the terminal palindrome. *SK* and *RS* can be derived from either insertion orientation if an AAV2 *Bal*-B fragment is present.

The structural maps derived for each AAV recombinant plasmid are shown in Fig. 7. Each of the clones pPS21-26 contains a single internal deletion within the unique sequence region of AAV2, but the location of these deletions appears to be nonrandom. For the six clones described, the left-hand ends of all the deletions were clustered near the extreme left-hand genome terminus. There may be some preferred locations for the right-hand end of the deletions also since in three of the six clones (pPS22, pPS24, and pPS25) this is located between 74.2 and 76.9 map units.

**Infectivity of AAV Plasmid DNA**—When recombinant plasmids containing the entire AAV genome were transfected into human cells together with adenovirus particles, normal infectious AAV particles containing AAV genomes free of plasmid DNA were produced (21, 22). We tested the infectivity of the AAV mutants. Human 293 cells were transfected with AAV plasmid DNA and adenovirus particles, and 48 h later the cultures were lysed. This lysate (P1) was passaged twice in succession (P2, P3) on 293 cells. Samples of each passage
were then assayed for AAV infectivity (Table I). As expected, pPS11 yielded infectious AAV in the first passage but at a significantly lower level than the maximum titer obtained in P1 by infection with AAV2 particles. None of the AAV mutants (pPS12, pPS21, pPS23, pPS24, pPS30) yielded infectious AAV in any passage. These results are consistent with the AAV genome structure (7) which contains two major open reading frames: one between map units 58 and 90 and the other between map units 22 and 24, respectively, the left to right orientation of the AAV insert within the plasmid was determined from the data of Fig. 6. Thus for pPS12, 22, 23, 24, 25, and 26, respectively, the left to right orientation of the AAV insert is clockwise (i.e. same) with respect to the pBR328 map shown in Fig. 1. The orientation of the AAV inserts in pPS11, 14, and 21, respectively, is counterclockwise (i.e. opposite) with respect to the plasmid sequences.

AAV genomes in productive crosses was confirmed (data not shown) by infecting 293 cells with P3 lysates and analyzing viral DNA following Hirt extraction and gel electrophoresis exactly as described before (21). In the nonhomologous cross, pPS12 × pPS30, the amount of infectious AAV detected in P2 was 100- to 300-fold lower than in the other three productive (homologous) crosses. In this cross the only AAV sequence homology between the two mutants is the 6-nucleotide combination frequency between these AAV mutants is influenced by the amount of AAV sequence homology. The particular variant AAV genomes described here may have arisen by intermolecular or intramolecular recombination, since the deletion near the right-hand end offers some other selective advantage. In any case it has not been determined whether the AAV variants arose by intermolecular or intramolecular recombination events.

Our experiments demonstrate intermolecular recombination in human cells between mutant AAV genomes to produce infectious progeny virus. Thus genetic analysis of AAV is now

![Diagram of AAV2 DNA inserts in recombinant plasmids](Image)

Fig. 7. Map of AAV2 DNA inserts in recombinant plasmids derived from mapping experiments such as those shown in Figs. 4, 5, and 6. The genome scale in map units is shown at the top. The AAV2 genome is represented by diagonal shading for the unique sequence (nucleotides 146 to 4528). The stippled areas (nucleotides 1 to 125 and 4549 to 4674) indicate the terminal palindromes. The solid vertical bars indicate the 20-nucleotide inverted repeat sequences, i.e. the sequence from nucleotide 4529 to 4548 is an inverted repeat of that between nucleotides 126 to 146 (7). The gaps indicate the internal deletion or the absence of one or other BalI-B fragment. The solid horizontal bars at either end of the internal deletions denote the regions within which either end of the deleted sequence is located. Note that at the right-hand terminus of pPS26 the AAV2 Bal site at nucleotide 4672 is not present. The map of each insert is shown with the same left to right orientation as the genome map. The orientation of the AAV insert within the plasmid was determined from the data of Fig. 6. Thus for pPS12, 22, 23, 24, 25, and 26, respectively, the left to right orientation of the AAV insert is clockwise (i.e. same) with respect to the pBR328 map shown in Fig. 1. The orientation of the AAV inserts in pPS11, 14, and 21, respectively, is counterclockwise (i.e. opposite) with respect to the plasmid sequences.

![Table I](Image)

Table I

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possible. Recombination has not been demonstrated previously for paroviruses either following infection with particles or after introduction of recombinant DNA molecules into cells. The AAV recombination system offers an alternative approach to study recombination in eukaryotic cells and in particular in cells infected by adenoviruses or herpes viruses. Intermolecular recombination between AAV mutants having extensive regions of homology (homologous recombination) occurred much more efficiently than between mutants having no homology (nonhomologous recombination) other than the 6 nucleotides at the BalI site. This latter AAV recombination may resemble other nonhomologous recombination events which often occur within very short stretches of homology.

Analysis of recombination in higher eukaryotic cells is facilitated by introduction of defined genes. It has been well documented that for DNA-containing viruses such as papovaviruses, adenoviruses, and herpesviruses recombination occurs by efficient end-to-end joining and lapping subgenomic fragments of adenoviruses (43). In these studies it was suggested that nonhomologous or indiscriminate recombination occurred by introduction of defined genes. It has been well documented that for DNA-containing viruses such as papovaviruses, adenoviruses, and herpesviruses recombination occurs by efficient end-to-end joining and lapping subgenomic fragments of adenoviruses (43).

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