

## Cloning of Adeno-Associated Virus Type 4 (AAV4) and Generation of Recombinant AAV4 Particles

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**We have cloned and characterized the full-length genome of adeno-associated virus type 4 (AAV4). The genome of AAV4 is 4,767 nucleotides in length and contains an expanded p5 promoter region compared to AAV2 and AAV3. Within the inverted terminal repeat (ITR), several base changes were identified with respect to AAV2. However, these changes did not affect the ability of this region to fold into a hairpin structure. Within the ITR, the terminal resolution site and Rep binding sites were conserved; however, the Rep binding site was expanded from three GAGC repeats to four. The Rep gene product of AAV4 shows greater than 90% homology to the Rep products of serotypes 2 and 3, with none of the changes occurring in regions which had previously been shown to affect the known functions of Rep68 or Rep78. Most of the differences in the capsid proteins lie in regions which are thought to be on the exterior surface of the viral capsid. It is these unique regions which are most likely to be responsible for the lack of cross-reacting antibodies and the altered tissue tropism compared to AAV2. The results of our studies, performed with a recombinant version of AAV4 carrying a *lacZ* reporter gene, suggest that AAV4 can transduce human, monkey, and rat cells. Furthermore, comparison of transduction efficiencies in a number of cell lines, competition cotransduction experiments, and the effect of trypsin on transduction efficiency all suggest that the cellular receptor for AAV4 is distinct from that of AAV2.**

Adeno-associated virus (AAV) is a small nonpathogenic virus of the *Parvoviridae* family (for a review, see reference 6). AAV is distinct from the other members of this family by its dependence upon a helper virus for replication. To date, five distinct AAV serotypes have been isolated from humans or primates (1). The most extensively studied of these isolates is AAV type 2 (AAV2). The genome of AAV2 is 4,680 nucleotides in length and consists of single-stranded DNA of either plus or minus polarity (41, 59). Two large open reading frames (ORFs) have been identified within the genome. The left ORF encodes the nonstructural Rep proteins which are involved in the regulation of replication and transcription and are necessary for the production of single-stranded progeny genomes (13–16, 19, 23, 28, 30, 34, 38–40, 43, 54, 57, 60–62). Furthermore, two of the Rep proteins have been associated with the preferential integration of AAV genomes into a region of the q arm of human chromosome 19 (37, 56, 64). Rep68/78 have also been shown to possess nucleoside triphosphate binding activity as well as DNA and RNA helicase activities (31, 32, 36, 68). The Rep proteins possess a nuclear localization signal (36, 70), as well as several potential phosphorylation sites. Mutation of one of these kinase sites resulted in a loss of replication activity (36).

The ends of the genome are short inverted terminal repeats (ITR) which have the potential to fold into T-shaped hairpin structures that serve as the origin of viral DNA replication. Within the ITR region, two elements have been described which are central to the function of the ITR, a GAGC repeat motif and the terminal resolution site (TRS). The repeat motif has been shown to bind Rep when the ITR is in either a linear or a hairpin conformation (15, 16, 44). This binding serves to position Rep68/78 for cleavage at the TRS, which occurs in a site- and strand-specific manner. In addition to their role in

replication, these two elements appear to be central to viral integration. Contained within the chromosome 19 integration locus is a Rep binding site with an adjacent TRS (64, 66). These elements are functional and necessary for locus-specific integration (26).

The AAV2 virion is a nonenveloped icosohedral particle approximately 25 nm in diameter, consisting of three related proteins referred to as VP1, VP2, and VP3. These proteins are found in a ratio of 1:1:10 (10) and are all derived from the right-hand ORF. The capsid proteins differ from each other by the use of alternative splicing and an unusual start codon. Deletion analysis has shown that removal or alteration of VP1 which is translated from an alternatively spliced mRNA results in a reduced yield of infectious particles (28, 60). Mutations within the VP3 coding region result in the failure to produce any single-stranded progeny DNA or infectious particles (28, 60).

The following features of AAV have made it an attractive vector for gene transfer (29). AAV vectors have been shown in vitro to integrate stably into the cellular genome, possess a broad host range, transduce both dividing and nondividing cells in vitro and in vivo (24, 35, 48, 52), and maintain high levels of expression of the transduced genes (65). Viral particles are heat stable; are resistant to solvents, detergents, changes in pH, and elevated temperature; and can be concentrated on CsCl gradients (1, 2). Integration of AAV provirus is not associated with any long-term negative effects on cell growth or differentiation (3, 67). The ITRs are the only *cis* elements required for rescue, replication, packaging, and integration (55) and may contain some promoter activities (25).

While other serotypes of AAV have been isolated from both humans and monkeys, antibodies and viral particles to AAV4 have been identified only in monkeys (7, 20). DNA hybridization studies have indicated a similar level of homology for AAV1 through AAV4 (4, 50). However, mRNA hybridization studies suggest that AAV4 may be organized differently from AAV2, since only one size class of mRNA has been fractionated from infectious cells (46). Therefore, to understand the

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nature of AAV4 and to determine its usefulness as a vector for gene transfer, the viral genome was cloned and sequenced and recombinant virus was produced with the AAV4 Rep and Cap proteins.

#### MATERIALS AND METHODS

**Cell culture and virus propagation.** Cos and HeLa cells were maintained as monolayer cultures in D10 medium (Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 1 × amphotericin B as recommended by the manufacturer [GIBCO, Gaithersburg, Md.]). All other cell types were grown under standard conditions which have been previously reported. AAV4 stocks were obtained from the American Type Culture Collection (no. VR-646).

Recombinant virus was produced and subjected to titer determination as previously described (17). The helper plasmid for production of the recombinant virus has been modified to remove any homologous sequence between the helper and vector plasmids. This step was taken to minimize the potential for wild-type (wt) particle formation by homologous recombination.

**DNA cloning and sequencing.** The DNA sequence was determined by using an ABI 373A automated sequencer and the FS dye terminator chemistry. Both strands of the plasmids were sequenced, and the sequence was confirmed by sequencing of a second clone. As further confirmation of the authenticity of the sequence, bases 91 to 600 were PCR amplified from the original seed material and directly sequenced. The sequence of this region, which contains a 56-base insertion compared to AAV2 and AAV3, was identical to that derived from the cloned material. The ITR was cloned with Deep Vent Polymerase (New England Biolabs), as specified by the manufacturer, and the following primers: primer 1, 5'TCTAGTCTAGACTTGGCCACTCCCTCTCTGCGCGC; primer 2, 5'AGG CCTTAAGAGCAGTCGTCACCACCTTGTTCC. The cycling conditions were 97°C for 20 s, 65°C for 30 s, and 75°C for 1 min for 35 rounds. Following the PCR, the mixture was treated with *Xba*I and *Eco*RI and the amplified band was purified by agarose gel electrophoresis. The recovered DNA fragment was ligated into Bluescript SKII+ (Stratagene) and transformed into competent Sure strain bacteria (Stratagene). The helper plasmid (pSV4oriAAV<sub>4,2</sub>) used for the production of recombinant virus, which contains the *rep* and *cap* genes of AAV4, was produced by PCR with *Pfu* polymerase (Stratagene) as specified by the manufacturer. The amplified sequence, nucleotides 216 to 4440, was ligated into a plasmid that contains the simian virus 40 origin of replication described previously (17). The cycling conditions were 95°C for 30 s, 55°C for 30 s, and 72°C for 3 min for 20 rounds. The final clone was confirmed by sequencing. The β-galactosidase reporter vector has been described previously (17).

**HA assays.** Hemagglutination (HA) was measured essentially as described previously (33). Serial twofold dilutions of virus in EDTA-buffered saline were mixed with an equal volume of 0.4% human erythrocytes (RBC) (type O) in plastic U-bottom 96-well plates. The reaction was complete after a 2-h incubation at 8°C. HA units (HAU) are defined as the reciprocal of the dilution causing 50% HA.

**Transduction of cells.** Exponentially growing cells ( $2 \times 10^4$  cells) were plated in each well of a 12-well plate and transduced with serial dilutions of virus in 200 µl of medium for 1 h. After this period, an additional 800 µl of medium was added and the mixture was incubated for 48 h. The cells were then fixed and stained for β-galactosidase activity overnight with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (ICN Biomedicals) (71). No endogenous β-galactosidase activity was visible after a 24-h incubation in X-Gal solution. Infectious titers were determined by counting the number of positive cells in the different dilutions with a calibrated microscope ocular (diameter, 3.1 mm) and then multiplying by the area of the well and the dilution of the virus. Titters were determined by counting the average number of cells in a minimum of 10 fields/well.

**Competition assay.** Cos cells were plated at  $2 \times 10^4$ /well in 12-well plates 12 to 24 h prior to transduction. The cells were transduced with  $0.5 \times 10^7$  particles of recombinant AAV2 (rAAV2) or rAAV4 (containing the *lacZ* gene) in 200 µl of Dulbecco's minimal essential medium and increasing amounts of rAAV2 containing the gene for the human coagulation factor IX. Prior to transduction, the CsCl was removed from the virus by dialysis against isotonic saline. After a 1-h incubation with the recombinant virus, the culture medium was supplemented with complete medium and allowed to incubate for 48 to 60 h. The cells were then stained and counted as described above.

**Trypsinization of cells.** An 80% confluent monolayer of  $10^7$  Cos cells was treated with 0.05% trypsin-0.02% EDTA solution (Biofluids) for 3 to 5 min at 37°C. Following detachment, the trypsin was inactivated by the addition of an

equal volume of medium containing 10% fetal calf serum. The cells were then further diluted to a final concentration of  $10^4$ /ml. Then 1 ml of cells was plated in a 12-well dish and incubated with virus at a multiplicity of infection of 260 for 1 to 2 h. Following attachment of the cells, the medium containing the virus was removed, the cells were washed, and fresh medium was added. Control cells were plated at the same time but were not transduced until the next day. The transduction conditions were as described above for the trypsinized cell group. The number of transduced cells was determined by staining 48 to 60 h posttransduction and counting as described above.

**Nucleotide sequence accession number.** The viral nucleotide sequence determined in this study is available through GenBank under accession no. U89790.

#### RESULTS

To obtain AAV4 genomic DNA for cloning, a stock of AAV4 was amplified by coinfection with wt adenovirus type 5 in Cos cells and the lysate was used to infect HeLa S3 cells. The resulting viral particles were isolated by CsCl banding and assayed by dot blot hybridization with an AAV2 DNA probe. The DNA dot blots of the gradient fractions indicated that peak genomes were contained in fractions with a density of 1.41 to 1.45 g/cm<sup>3</sup>. This is very similar to the buoyant density previously reported for AAV4 of 1.44 g/cm<sup>3</sup> (47). Analysis of annealed DNA obtained from these fractions indicated a major species of 4.8 kb which upon restriction analysis gave bands similar in size to those previously reported (46) (data not shown).

Additional restriction analysis indicated the presence of *Bss*HII sites near the ends of the DNA. Digestion with *Bss*HII yielded a 4.5-kb fragment which was then cloned into Bluescript SKII+ (Stratagene), and two independent clones were sequenced. The entire AAV4 genome sequence is presented in Fig. 1 and is also available through GenBank. In addition to the previously identified capsid ORF on the right-hand side of the genome (46), a second ORF is present on the left-hand side. As with AAV2 and AAV3, there appears to be a single polyadenylation signal after the second ORF. The first ORF, which could encode a protein of 623 amino acids, has a high degree of homology to the *rep* ORF of AAV2 and AAV3. At the amino acid level, the ORF is 90 and 92% identical to those of AAV2 and AAV3, respectively, with approximately 5% of the changes being conservative (data not shown). None of the changes occurred in regions which had previously been shown to affect the known functions of Rep68 or Rep78. The region surrounding the p19 promoter was also conserved, suggesting that proteins corresponding to AAV2 Rep52 and Rep40 could be expressed.

Inspection of the p7 promoter region of AAV4 showed a high degree of conservation of known functional elements which have been identified within the p5 promoter of AAV2. Two YY1 binding sites at -60 and +1 and a TATA box at -30, which are important for regulation of gene expression, have previously been identified in AAV2 (11, 58). These sites are conserved between AAV2 and AAV4. A binding site for Rep has been identified in the AAV2 p5 promoter at -17 and is also conserved in AAV4 (42). The only divergence between the two viruses in this region appears to be in the sequence surrounding these transcriptional regulatory elements. AAV4 contains an additional 56 bases inserted between the AAV2 YY1 element at -60 and the adjacent E-box/USF element at

FIG. 1. Sequence of the AAV4 genome. The genomes of AAV2, AAV3, and AAV4 were aligned with the Align program (Scientific and Educational Software). The sequences of the ITRs are presented in lowercase type, and the rest of the genome is shown in capitals. TRSs are indicated by vertical arrows. Proposed transcription factor binding sites are indicated by underlining and overlining, with the name of the site above. E-box elements are indicated by highlighting, and the unique region of AAV4 in the p5 promoter is shown in boldface type. Promoters are indicated by horizontal arrows, with their corresponding map positions indicated above. Initiation and termination codons are boxed, as is the single polyadenylation site. Splice donor and acceptor sites are indicated by solid and open triangles, respectively.



AAV2 GAGTCGGCCAAAGCCATCTCGGAGGAAGCAAGGTGCGCGTGGACCAGAAATGCAAGTCC TCGGCCAGATAGACCCGACTCCCGTGATCGTCACCTCCCAACCAACATGTGCGCCGTG 1601  
 AAV3 ...AGC...G...G...C...G...C...G...G...G...G...A...A...G...A...C...C...A...C...C...A...C...C...A...C...C...A...G 1598  
 AAV4 ...AGC...G...G...C...G...C...G...G...G...G...A...A...G...A...C...C...A...C...C...A...C...C...A...C...C...A...G 1652

AAV2 ATTGACGGGAATCAACGACCTTCGAACACCAGCAGCCGTGCAAGACCCGGATGTTCAAATTTGAACTCACCCGCGCTCTGGATCATGACTTTGGGAAGGTCACCAAGCAGGAAGTCAAA 1721  
 AAV3 ...AGC...C...G...T...G...C...G...G...T...G...G...G...G...T...G...T...C...C...C...A...A...G 1718  
 AAV4 ...C...A...A...G...C...G...C...G 1772

mtf p40 TATA

AAV2 GACTTTTCCGGTGGCCAAAGGATCACGTGGTTGAGGTGGAGCATGAAATTCACGTCAAAGGTTGGAGCCAAAGAAAGACCCGCCCGAGTGACGCAGATATAAGTGAGCCCAACCG 1841  
 AAV3 ...TTCC...AC...C...CT...G...G...G...G...T...C...C...T...A...G...C...A...C...G...G...G...G...G...G...G...G...G 1838  
 AAV4 ...GTCA...ACC...ACT...C...G...T...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G 1892

AAV2 GTGCGGAGTCAGTTGCGCAGCATCGACGTACAGCAGCGGAAGCTTCGATCACTACGACAGCAGGTACCAAAACAAATGTTCTCGTCAAGTGGCCATGAATCTGATGCTGTTCCTCCCTG 1961  
 AAV3 .A.T..AC...C...GA.A...AC..GCGG...G...T...T...T...T...A.G...-A.ACT...TCCA...G.CA.C.C.GATT.- 1958  
 AAV4 .CCT.TCC...C...G.GG...G...T...T...T...T...A.G...-A.ACT...TCCA...G.CA.C.C.GATT.- 2012

AAV2 AGACAATGCGAGAGAATGAATCAGAATCAAAATATCTGCCTCACTCACGGACAGAAAGAC-CG---TATCAGAACTGTGTACATTCATCATATCATGGGAAAGGTCAGAC---G-- 2127  
 AAV3 .A.AC...T...T...C...G...T...T...G...T...T...A.G...-A.ACT...TCCA...G.CA.C.C.GATT.- 2133  
 AAV4 C.G...G...T...G 2183

Rep78 stop

AAV2 -CG---TATCAGAACTGTGCTACATTCATCATATCATGGGAAAGGTCAGAC---G---CTTGCACCTGCCTGCGATCTGGTCAATGTGGATTGGATGACTGCATCTTGAACAATTA 2186  
 AAV3 -A.ACT...TCCA...C...C...G...CA...C...GATT...-C...TT.G...T...C...C...C...T...G...T...C...G... 2192  
 AAV4 A...-...TCCG...C...G...G...C...C...G...-T.G...C...T.G...A...C...C...C...TGA.A.G... 2243

VP1 Rep68 stop

AAV2 ATGATTTAAATCAGGTAATGCTGCGGATGGTTATCTCCAGATTGGCTCGGAGACACTCTCTGAGGAAATAAGACAGTGGTGGAAAGCTCAAACCTGGCCCACCACCAAAAGCCCGC 2306  
 AAV3 ...C...C...C...T...C...G...CA...C...GATT...-C...TT.G...T...C...C...C...T...G...T...C...G... 2312  
 AAV4 ...C...C...C...A...A...-...C...C...C...A...AC...AC...CG.TC.G...GC...GC...AG.C...TAA...C...G.AAA 2360

AAV2 AGAGCGGCATAAGGACGACAGCGGGGTCTTGCTTCCTGGGTACAAGTACCTCGGACCTTCAACGGACTCGACAAAGGAGAGCGGTCAACGAGCCAGCCCGCCCTCGAGCA 2426  
 AAV3 CC.A.AA...CC...A.C.TC...G...T...A...GGT...A...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G 2432  
 AAV4 TC.A.AA...C...A.GCTC...G...T...A...GG...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G 2480

AAV2 CGACAAAGCCTACGACCCGAGCTCGACAGCGGAGACAACCCGTACCTCAAGTACAACACCGCCAGCGGGAGTTCAGGAGCGCCTTAAAGAAGATACGTCTTTGGGGCAACCTCGG 2546  
 AAV3 ...T...T...A...GGC...T...G...A.C...A...GGT...A...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G 2552  
 AAV4 ...G...A...A...GGC...T...C...C...G...C...G...C...C...A...G...G...G...G...G...G...G...G...G...G...G...G...G...G 2600

VP2

AAV2 ACGAGCAGTCTCCAGCGGAAAGAGGGTCTTGAACCTCTGGGCTGGTTGAGGAACCTGTAAAGACCGCTCCGGGAAAAGAGCGCGGTAGAGCACTCTCCTGTGGGCCAGACTC 2666  
 AAV3 CA...C...G...T...T...G...A.C...A...GGT...A...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G 2672  
 AAV4 CA...C...T...T...C...G...G...G...G...T...G...G...A...T.GATTG.A.C.CCA.C...C... 2720

VP3

AAV2 CTCTGGTCTGGGAACTAATACCATGCTACAGGCAGTGGCCACCAAT---GGCAGACAATAACGAGGGCCGACGGAGTGGGTAATTCCTCGGAAATGGCATTGCGCATTCCACAT 2902  
 AAV3 .A.AA...T...T...T...T...T...G 2908  
 AAV4 G...A...-AC.G.G.G.T.CCT..AG...CTG.C.A.TG..G.CGAG..CG.....T...T...G...G...G...G...G...G...G...G...G...G...G...G 2941

AAV2 GGATGGGCGACAGATCATCACCAACCGACCCGCAACCTGGGCGCTGCCACCTFACAACAACCCCTCTACAACAATTTCCAGCCAATCAG---GAGCCTCGAACGACAATCACTACT 3019  
 AAV3 .C...A...A...A...G...G...T...T...G 3025  
 AAV4 .TCT.AG.G.CAC...CG...A...T.T...C...C...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G...G 3049

AAV2 TTTGGCTACAGCACCCCTTGGGGGTATTTTGACTTCAACAGATTCCACTGCCACTTTTACACCGTACTGGCAAAGACTCATCAACAACCTGGGGATTCCGACCCCAAGAGACTCAACT 3139  
 AAV3 ...T...T...T...T...T...G 3145  
 AAV4 AC.A.T.TC...C...A.C...C 3169

FIG. 1—Continued.

AAV2 TCAAGCTCTTTAATCAATCAAGTCAAAGAGGTCACGCAGAAATGACGGTACGACGACGATTGGCAATAACCTTACCAGCAGGTTCCAGGTGTTTACTGACTCGGAGTACCAGCTCCCGTACG 3259  
 AAV3 .....C.....C.....T.G..G.....C.T..C.....T.....A.....G.....T..... 3265  
 AAV4 .....AA.....C.....C.G.....G.....AC.TCGA.....CGA.....A.....G.G.T.....A.C.....G.G.....TC.....G.A..G..... 3289

AAV2 TCCTCGGCTCGGCGCATCAAGGATGCCTCCCGCGTTCCGACGAGACGCTTTCATGGTGCCACAGTATGGATACCTCACCTGAACAACGGGAGTCAG---GCAGTAGGACGCTC---- 3371  
 AAV3 .G.....G.....C.....C.....T.....G.....C.....T.....A.....A---.G..G..... 3377  
 AAV4 .GA.G.ATG...GT..AG.G..CA...G..T..T..CAAC.....T.....C.....C.....TGTGGA...GTG.C...C..-A..CTTC...C.AC.GA..GACAGA 3408

AAV2 --TTCATTTTACTGCCTGGAGTACTTTCCTTCTCAGATGCTGCTACCGGAAACAACCTTTACCTTACAGTACACTTTTGAGGAGCTTCCTTTCCACAGCAGCTACGCTCACAGCCAGAT 3489  
 AAV3 --A..C.....C.....G.....AA.G..T...T...CCAA...T..C..C...T..A...T..... 3495  
 AAV4 AA.G.C..C.....G.....G.....G..T..C.....GAAA.T.CG...G.....A.G..G.....TCG.TG...G.....C 3528

AAV2 CTGGACCGTCTCATGAATCCTCTCATCGACAGTACCTGTATTACTTGAGCAGAAACAAC-ACTCCAAG---TGGAAACCACCAGCAGTCAAGGCTT-----CAGTTTTCAGGCC 3597  
 AAV3 T...T..CT.G.....T..T..T.....C.....C.....G..A-GGAA...CCTC.....A.....AC..A...C...G-----TT...AGC.....T 3606  
 AAV4 .....G..G.....C.....G.G.CTG...T.G..CA.C.C--C.....CT.A.TG.CG..AC.GCCACCAC..AC..AC..A..T 3638

AAV2 GGAGCG-ACFTGACATTCGGGACCAGTCTAGGAACCTGGCTTCCTGGACCTGTTACCGCCAGCAGGAGTATCAAAGACATCTGCGGATAACAACAACAGTGAATACTCGTGGACTGGAGC 3716  
 AAV3 .GC.T-CAGTCT..GTCTTTG...G.C..A..T.....A.....C.....G..A..A..A..C.T.....TG..AAC..C.....A.C.TTC.T...A.CG... 3725  
 AAV4 .CG..CT.CCA..T..TCCA..TTTAAA.A.....G..C..G..T.CAAT.AAG.....G.CT.C.....TG.CAATC.A...T...G.TCCCTGC.A.CG...T.A.ACAG 3758

AAV2 T---ACCAAGTACCAC-----CTCAATGGCAGAGACTCTCTGGTGAATCCGGGCCCGCCATGGCAAGCCACAAGGACGATGAAGAAAAGTTTTTCTCAGACGGGGTTCT 3821  
 AAV3 C---.G..A..T..T-----C.....C.....G.....A..A..A..T...C..T.....A.....C...AT.CA...CAA... 3830  
 AAV4 .CTC.T..A..G.GACGCACAGCACT..GG.C..A...TGGAG.GCCC..CC..C..A..TC.A...C--GGCT...-C..CG..C.GCAAG..CAGCA.C...CA... 3872

AAV2 CATCTTTGGGAAGCAAGGCTCAGAGAAAACA--AATGTGGACATTGAAAAGG-----TCATGATTACAGCAGCAAGGAAATCAGGACAACCAATCCCGTGGCTACGGAGCAGTATGGT 3933  
 AAV3 A..A.....C..AG...GA..AC.GC..GT--.C.CA..AT.A..T..T-----A.....G..T...A..G..TC.T..C.....T.....A..A.....A 3942  
 AAV4 .....-----GG..CT.A.C.G..GGC..CAC..C..CC.T.CCC.GGACTC.G..CT.C..CTCT..G...GC.GGCAG.C...CG..ACC.A.....CAT..GG... 3986

AAV2 TCTGTATCTACCAACCTCCAGAGAGGCAAC-AGACAAGCAGTACC-GCAGAT-GTCAACACAC--AAGGCGTTC-----TCCAGGCATGGTCTGGCAGGACAGAGATGTGTACTCT 4041  
 AAV3 A...GG.A.AT..T.G...CTCA..T-.C.GCTC.CA.G..T-.G.AC.-...TCAT--.G..G.CCT-----A..T.....G...A..TC.T..C..... 4050  
 AAV4 -----A.CTG.C..TG..C...GC.A...A..T..C..CC..GG...G..TG.CA..C..GGGAGCCG.G..T..A.....AA.....CA.T...TAC 4095

AAV2 CAGGGGCCCATCTGGGCAAGATTCCACACACGGACGGACATTTTACCCTCTCCCTCATGGTGGATTCCGACTTAAACACCTCTCCACAGATTTCTCATCAAGAACACCCCGGTA 4161  
 AAV3 .A..A..T.....T.....T.....C.....T..T.....T..G...A..C..T.....G...T..G...T..A..CA.G...A..T..T..... 4170  
 AAV4 .....T.....T.....C.....T..T..C..T.....C.....A..G..G..T...G..T..G.....G.....T..A..T..T..... 4215

AAV2 CCTCGCAATCCTTCGACCCTTCAGTGCGGCAAGTTTGCTTCCCTTATCACACAGTACTCCACGGGACAGGTCAGCGTGGAGATCGAGTGGGAGCTGCAGAAGGAAAACAGCAAACGC 4281  
 AAV3 .G..A.....C.....G..T.....CC.....C.....T.....A.....T.....A.....A.....A.....C.G..T..C...T 4290  
 AAV4 .....G.A..G.....CT.TA.TCC.G.AAAC.....T..T.....AG..T..C.....GTGC...C.....T..C.....A.C.....GCGTCT... 4335

AAV2 TGGARTCCCGAAATTCAGTACACTTCCAACTAC---AACCACTCTGT [E]TGTGGACTTTACTGTGGACACTAATGGCGTGTATTTCAGAGCCTCGCCCAATGGCACCAGATACCTGA 4396  
 AAV3 .....A..G.....[E].....A.....T.....T.....AGT..A.....T.....A.....C.G..T..C...T 4405  
 AAV4 .....C.....GG.C...TT..C.....GGAC.G..AA..C.C.GT.....---G..CCC..TG.GGC...GAAA..CA.T...A.GG.T..C..T..C.C...C. 4450

AAV2 CTCGTAATCTGTAATTTGCTTGTAAAT [A]ATAA [C]CGTTTAAATTCGTTTTCAGTTGAACCTTTGCTCTCGCTATTTCCTT-TCT---TATCTAGTTTCCATGGCTAC---GTAGATAAGTAG 4509  
 AAV3 .A..A..CT..G.A.C..G.....[A]ATAA [C]CTCT..T.C.C...-A.CTT...T.....TGC.....C... 4524  
 AAV4 .C.ACC.C... [C]AA.C.....[A]ATAA [C]G..T.....C.T..CC...A.....C.....TGC...C.....C... 4567

AAV2 C-----ATGGCGGGTAAATCAATTAACATAAgga-accctagtgtatggagttggccactcctctctctgctcctcctcactgaggccg 4597  
 AAV3 .GGCTGGCGCGCTTGGCGCTTCGCGGTTTACA.CT..T.....AT...CTCgct.t.t.....a.....a.....gg..g...t 4644  
 AAV4 .GGCTGGCGCGCTTGGCGCTTCGCGGTTTACA.CT..C.....G.....T.Tg.c.-.a.ag.....attag..a.....a.....cg.c.t 4685

AAV2 ggcgaccaaaagtcgcccagcggggcttggccggcgccctcagtgagcagcagcagcgcgagagagggagtgccaa 4685  
 AAV3 .....a...ga..t.....a..tc...c..cc.....t.....t..... 4685  
 AAV4 .a.....t..a..tg...c.c.g...ca..g.cg.....t.....t..... 4685

FIG. 1—Continued.



FIG. 2. AAV4 ITR. The sequence of the ITR is shown in the hairpin conformation. The putative Rep binding site is boxed. The cleavage site in the TRS is indicated by an arrow. Bases which differ from the ITR of AAV2 are outlined.

–77. Based on its position in the viral genome and efficient use of limited sequence, it is reasonable to expect that this sequence may be involved in regulation of virus gene expression. Computer analysis of this region indicated the presence of an additional E-box element within this region. Comparison to

AAV2	MAADGYLPDWLEDTLSEGIQRQWKLKPPPPKPAERHKDDSRGLVLPQYKYLGP	55
AAV3	.....N.....E..A.....V..Q.....ANQQ..Q..NR.....	55
AAV4	..T.....N.....V..E..A..Q.....A..K.....ANQQ..Q..NA.....	54
AAV2	FNGLDKGEPVNEADAALAEHDKAYDRQLDSGDNPFLKYNHADAEFQERLKEEDTSF	110
AAV3	G.....D.....K.....Q.....KA.....	110
AAV4	G.....D.....A.....Q.....KA.....	109
AAV2	GGNLGRAVFAKRRVLEPLGLVEEPVKTAPGKKRPVHSPVPEPSSSGTKAGAQQ	165
AAV3	.....I.....AA.....GA..DQ..Q.....V..S..K.....	165
AAV4	.....LIE..QQ.....T..I..K..K.....	164
AAV2	PARKRLNFQGTGDADSVDPDQPLGPPAAPSGLGTMTAGTSGSAPMADNNEGADG	220
AAV3	..E.....SE.....E.....TS..S.....S..G.....	220
AAV4	..K..K..V..EDET.GDG..P----EGSTSGMSDDSE..RAAA..GAAVEGGQ...	214
AAV2	VGNSSGNWHCDSTWMDRVTITSTRTWALEPTYNHLYKQLSSOSGASNHNHYFGY	275
AAV3	.....Q..L.....	275
AAV4	..A..D.....SEGH.T.....V.....R.....ESLQS.T.N.F	266
AAV2	STPWGYFDNRFHCFHSPRDWQRLINNNWGFPRKRLNFKLFNIQVKEVTQNDGTT	330
AAV3	.....K..S.....RG.....	330
AAV4	.....M.....AMRV..I.....TSH..E	321
AAV2	TEANNLTSTVQVFTDSEYQLPVLVLSAHQGLPFPFADVFMVPOGYLTLNNGS	384
AAV3	.....E.....S.....S.....CG.VT..NT	384
AAV4	.....I..A..S.E.....MDAQOE.S.....N.....CG.VT..NT	376
AAV2	QAVGRSSFYCLEYFPQMLRTGNFTFSYTFEDVPFHSSYAHSQSLDRMLNPL	437
AAV3	.....Q.....	437
AAV4	SQ..QTD.NA.....EIT..S..K.....M.....	431
AAV2	IDQVLYLRSRTNTPSGTTQSRQLQFSQAGASDIRDQSRNWLPGPCYRQRVSKT	491
AAV3	.....P..N..QG..T.....N.....L.....PQSMSE..A.....L.....	492
AAV4	.....RG..QS..TTCGTTINACTATTN..TKLRPTNFSNFKK.....SIK..GF...	486
AAV2	SADNNN-SEYSWTGATKYH----LNGRDSLUNPVPAMASHKDDREKFFPQSGVLI	541
AAV3	AN.....NFP..A.S.....MHGN..	542
AAV4	ANQ.YKIPATGSDSLI..ETHST.D..W..ALT..P..TACPADS..SN..QLIF	540
AAV2	FGKQGEKTNVDLEKVMITDEEIRITNPVATEQVGSVSTNLRQGNRQAAATADVN	596
AAV3	.....E..TTAS..AELDN.....T..AN.....SSNAPT..GT.....M.....	597
AAV4	A..PRQNGN..ATVPGTLIF..S.....LAA..ATD..DMW..NLPFGD..SNSNLPVDRIT	595
AAV2	TCQVLPGMVWQDRDVLQCPWIKAPHTDGHFSPMLGGFGLKHPPIQILKNT	651
AAV3	H..FA.....	652
AAV4	ALAV.....N..I..Y.....I.....F.....	650
AAV2	PVPANPSTTFSAAKFASITQYSTGQVSEIWELEKENSKRWNPEIQSTGNLYNK	706
AAV3	.....P.....P.....	707
AAV4	.....A.....STEPVN.....Q..D..I.....R.....V..F.....GQ	705
AAV2	SUNVDPTVDTNGVYSEPRPICTRNLN	735
AAV3	.....	736
AAV4	QNSLLWAP..AA..K..T..A.....HH.....	734

FIG. 3. Comparison of the capsid ORFs. The capsid ORFs of AAV2, AAV3, and AAV4 were compared by using the Palgin program of Pcgene. Identical amino acids are indicated by a single dot. Dashes indicate gaps in the sequence added by the alignment program. The theoretical initiator codons of VP2 and VP3 are underlined and indicated above the sequence. Regions which have been proposed to be on the surface of AAV (12) are highlighted.

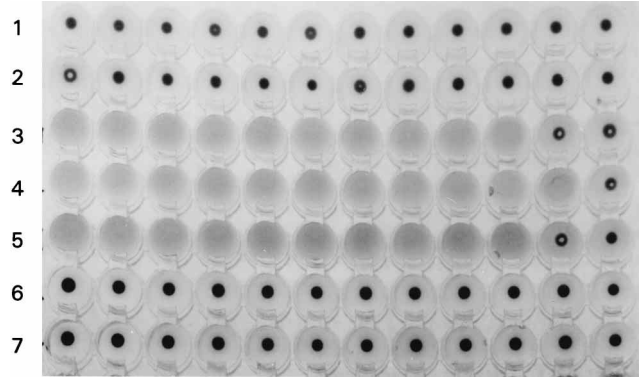


FIG. 4. HA. Human type O RBCs were incubated with twofold serial dilutions of viruses. Row 1, RBCs only; row 2, AAV3; row 3, wtAAV4; row 4, wtAAV4; row 5, rAAV4; row 6, rAAV2; row 7, adenovirus.

other E-box elements suggests that it may bind Myc with a higher affinity than it binds USF (27). In contrast to AAV3, the TATAA box present in AAV2 at nt 1823 in the P40 promoter is conserved in AAV4.

The ITRs were PCR amplified with a primer derived from the complementary sequence of the TRS contained within the *Bss*HIII fragment and a primer in the *rep* ORF. The resulting fragments were cloned and found to contain a number of sequence changes compared to AAV2. However, all the changes were accompanied by complementary changes and thus would not affect the ability of this region to fold into a hairpin structure (Fig. 2). While the TRS motif was conserved between AAV2 and AAV4, the Rep binding site contained two alterations which expand the binding site from three (GAGC) repeats to four (16). Each of the first two repeats in AAV4 contain a T instead of a C in the fourth position. This type of repeat is present in the p5 promoter as well as in the consensus sequence which has been proposed for Rep binding (18). Gel shift experiments with random synthetic oligonucleotide probes and AAV2 Rep78 or Rep68 showed enrichment for probes containing multiple repeats of the GAGC motif. Thus, expansion of this element in the ITR of AAV4 should affect its affinity for Rep. Methylation interference data have suggested the importance of the CTITG motif found at the tip of one palindromic in Rep binding, with the underlined T residues affecting Rep binding to both the flip and flop forms (53). While most of this motif is conserved in AAV4, the middle T residue is changed to a C and may indicate the importance of the structure of the palindrome and not the particular DNA sequence (8).

In contrast to the high degree of homology between the Rep ORF of AAV2, AAV3, and AAV4, the capsid ORF of AAV4, which encodes a protein of 734 amino acids, is only 62 and 63% identical to the corrected AAV2 and AAV3 sequences, respectively (45, 51) (Fig. 3). Comparison to Moscow duck and goose parvoviruses showed 53% homology, and little homology to other autonomous parvoviruses was observed. While the N terminus of VP1 (amino acids 44 to 133) and the start site of VP2 are conserved among AAV2, AAV3, and AAV4, the start site for VP3 is in the middle of a divergent region. By using the three-dimensional structural analysis of the canine parvovirus and computer-aided sequence comparisons (12), the regions of the AAV2 capsid that are predicted to be exposed on the exterior surface of the particle could be inferred. Comparison of the AAV2 and AAV4 sequences indicates that these regions are not well conserved between the viruses and suggests that

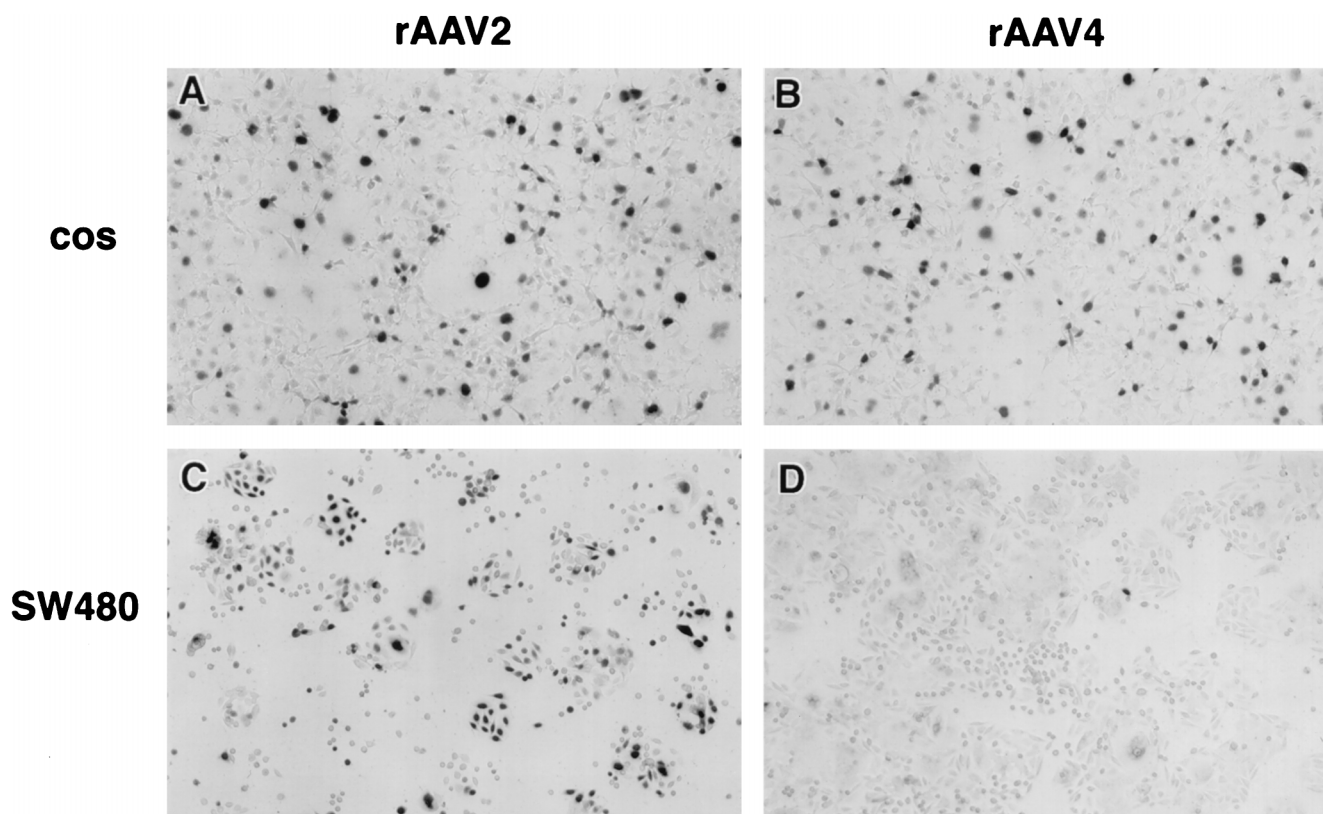


FIG. 5. Transduction of Cos and SW480 cells. Cos and SW480 cells were transduced with either rAAV2 or rAAV4 expressing *lacZ* at a multiplicity of infection of 500 and stained 60 h posttransduction. (A) Cos cells transduced with rAAV2. (B) Cos cells transduced with rAAV4. (C) SW480 cells transduced with rAAV2. (D) SW480 cells transduced with rAAV4.

they may exhibit altered tissue tropism. Very little is known about the tissue tropism of any dependovirus. While AAV4 had been shown to hemagglutinate human, guinea pig, and sheep erythrocytes (33), it is thought to be exclusively a simian virus, since humans but not captive African green monkeys lack neutralizing antibodies (7). Additional work has involved the isolation of virus from the monkey kidney and intestine, with AAV4 antigen being detected in the kidneys, tonsils, lungs, liver, and blood following experimental infection (20). Therefore, to test AAV4 tissue tropism, we constructed recom-

binant AAV4 particles which contained the gene for nucleus-localized  $\beta$ -galactosidase. Because of the structural similarities of the AAV2 and AAV4 ITRs, a genome containing AAV2 ITRs which had been previously described was used for packaging into AAV4 capsids.

Virus was produced as previously described for AAV2, using a  $\beta$ -galactosidase vector plasmid and a helper plasmid containing the AAV4 *rep* and *cap* genes (17). The helper plasmid was constructed to eliminate homologous sequence between the helper and vector plasmids. This step was taken to minimize the potential for wt particle formation by homologous recombination.

Virus was isolated from Cos cell lysates by CsCl banding, and the distribution of *lacZ* sequences across the gradient was determined by DNA dot blot analysis of gradient fractions. The majority of packaged genomes were found in fractions with a density of 1.43 g/cm<sup>3</sup>, which is similar to that obtained for wt AAV4. Typical yields of rAAV4 particles per producer cell were three- to fivefold greater than those of rAAV2 particles.

To determine if recombinant AAV4 particles were capable of hemagglutination, purified recombinant AAV4 particles were incubated with human RBC (type O) for 2 to 3 h at 8°C. As shown in Fig. 4, both wt AAV4 and rAAV4 can hemagglutinate human RBCs with HA titers of approximately 1,024 and 512 HAU/ $\mu$ l, respectively. No HA activity was detected with AAV3, recombinant AAV2, or the helper adenovirus (Fig. 4). If the temperature was raised to 22°C, HA activity decreased 32-fold (data not shown). Comparison of the viral particle number per RBC at the end point dilution indicated that

TABLE 1. Titers of rAAV2LacZ and rAAV4LacZ<sup>a</sup>

Cell type	Titer of:	
	AAV2	AAV4
Primary rat brain	1	4.3 $\pm$ 0.7
Cos	4.2 $\times$ 10 <sup>7</sup> $\pm$ 0.46 $\times$ 10 <sup>7</sup>	2.2 $\times$ 10 <sup>7</sup> $\pm$ 0.25 $\times$ 10 <sup>7</sup>
SW 480	7.75 $\times$ 10 <sup>6</sup> $\pm$ 1.7 $\times$ 10 <sup>6</sup>	1.3 $\times$ 10 <sup>5</sup> $\pm$ 0.68 $\times$ 10 <sup>5</sup>
HeLa	2.1 $\times$ 10 <sup>7</sup> $\pm$ 0.1 $\times$ 10 <sup>7</sup>	1.3 $\times$ 10 <sup>6</sup> $\pm$ 0.1 $\times$ 10 <sup>6</sup>
SW620	1.2 $\times$ 10 <sup>5</sup> $\pm$ 0.39 $\times$ 10 <sup>5</sup>	4 $\times$ 10 <sup>4</sup>
KLEB	1.2 $\times$ 10 <sup>5</sup> $\pm$ 0.35 $\times$ 10 <sup>5</sup>	9 $\times$ 10 <sup>4</sup> $\pm$ 1.4 $\times$ 10 <sup>4</sup>
HB	5.6 $\times$ 10 <sup>5</sup> $\pm$ 2 $\times$ 10 <sup>5</sup>	3.8 $\times$ 10 <sup>4</sup> $\pm$ 1.8 $\times$ 10 <sup>4</sup>
SW1116	5.2 $\times$ 10 <sup>4</sup>	8 $\times$ 10 <sup>3</sup>
SW1463	8.8 $\times$ 10 <sup>4</sup>	8 $\times$ 10 <sup>3</sup>
NIH 3T3	3 $\times$ 10 <sup>3</sup>	2 $\times$ 10 <sup>3</sup>

<sup>a</sup> The different cell lines were transduced with an equal number of particles in serial dilutions of either rAAV2 or rAAV4 expressing *lacZ* as described in Materials and Methods. The results are expressed as transducing units per milliliter. Because of the heterogeneous nature of the cell population in the rat brain cultures, only relative transduction efficiencies are reported.

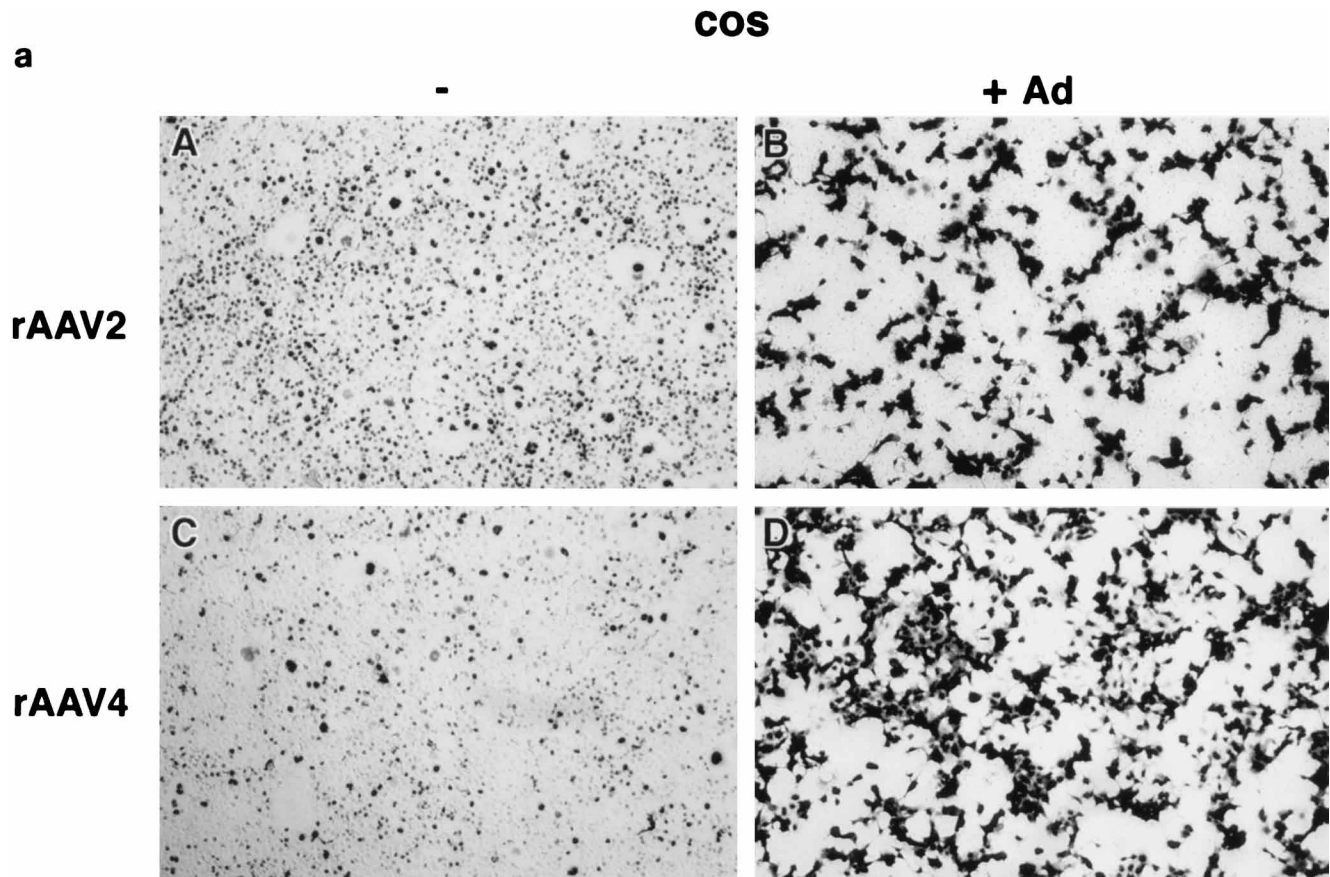


FIG. 6. Adenovirus coinfection. (a) Cos cells were transduced with  $4 \times 10^7$  particles of either rAAV2 (A and B) or rAAV4 (C and D) in the presence (B and D) or absence (A and C) of  $2 \times 10^8$  PFU of wt adenovirus. (b) HeLa cells were transduced with  $4 \times 10^7$  particles of either rAAV2 (A and B) or rAAV4 (C and D) in the presence (B and D) or absence (A and C) of  $2 \times 10^8$  PFU of wt adenovirus.

approximately 1 to 10 particles per RBC were required for HA. This value is similar to that previously reported (33).

Tissue tropism was assessed by transduction of a variety of cell lines with serially diluted rAAV4 or rAAV2. As shown in Fig. 5 and Table 1, Cos cells transduced with equivalent amounts of rAAV2 and rAAV4 particles resulted in similar transduction levels (Fig. 5A and B). However, other cell lines exhibited differential transducibility. Transduction of the human colon adenocarcinoma cell line SW480 with rAAV2 was 60 times higher than that obtained with rAAV4 (Fig. 5C and D). HeLa and SW620 cells were also transduced more efficiently with rAAV2 than with rAAV4. In contrast, transduction of primary rat brain cultures exhibited a greater transduction of glial and neuronal cells with rAAV4 than with rAAV2. Because of the heterogeneous nature of the cell population in the rat brain cultures, only relative transduction efficiencies are reported (Table 1). Previous work has shown that adenovirus can increase the transduction efficiency of AAV2 (21, 22). As a control for adenovirus contamination of the viral preparations, Cos and HeLa cells were coinfecting with rAAV and adenovirus and stained after 24 h. While the titer of rAAV2 increased in the presence of adenovirus in both Cos and HeLa cells (Fig. 6a and b, panels B), adenovirus increased the titer only in the Cos cells transduced with rAAV4 and not in the HeLa cells (Fig. 6a and b, panels D), suggesting that the difference in transduction efficiencies is not the result of adenovirus contamination. Furthermore, both vectors transduced SW1116, SW1463, NIH 3T3, and monkey fibroblast FL2 cells

very poorly. These data suggest that AAV4 may use a cellular receptor distinct from that used by AAV2.

This hypothesis was tested by cotransduction experiments with rAAV2 and rAAV4. Cos cells were transduced with an equal number of rAAV2 or rAAV4 particles containing the *lacZ* gene and increasing amounts of rAAV2 particles containing the human coagulation factor IX gene (rAAV2FIX). At a 72:1 ratio of rAAV2FIX to rAAV4LacZ, only a twofold effect on the level of rAAV4LacZ transduction was obtained (Fig. 7). However, the same ratio of rAAV2FIX to rAAV2LacZ reduced the transduction efficiency of rAAV2LacZ approximately 10-fold. Comparison of the 50% inhibition points for the two viruses indicated a sevenfold difference in sensitivity.

Previous research had shown that binding and infection of AAV2 are inhibited by trypsin treatment of cells (44). Transduction of Cos cells with the rAAV2LacZ gene was also inhibited by trypsin treatment prior to transduction (Fig. 8). In contrast, trypsin treatment had a minimal effect on rAAV4LacZ transduction. This result and the result of the previous competition experiment are both consistent with the utilization of distinct cellular receptors for AAV2 and AAV4.

## DISCUSSION

AAV4 is a distinct virus based on sequence analysis, physical properties of the virion, HA activity, and tissue tropism. Similar to AAV2 and AAV3, AAV4 contains two ORFs which code for Rep proteins or capsid proteins. The genome of



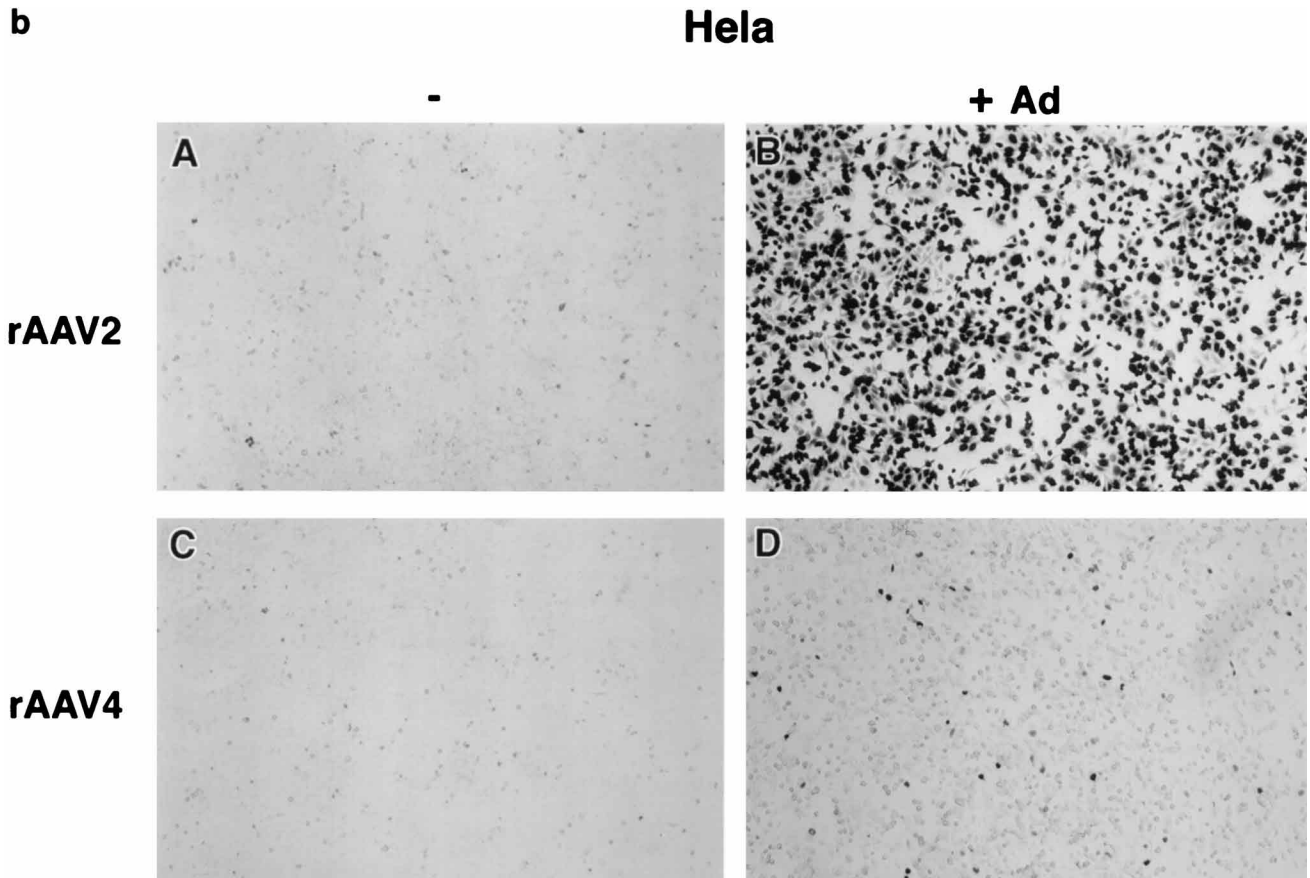


FIG. 6—Continued.

AAV4 is 4,767 nt in length and contains additional sequence upstream of the p7 promoter which may affect promoter activity. An additional E-box element is contained within this region; thus, AAV4 has two of these activator elements compared to the one of AAV2 and AAV3. Furthermore, this additional element differs in its flanking sequence from the

conserved E box and therefore is more similar to a Myc binding site than to a USF site. It is interesting that the position of the new E box is still in the same place relative to the one in AAV2 and AAV3, suggesting the importance of this element in relation to the other promoter elements.

AAV4 contains an expanded Rep binding site within the

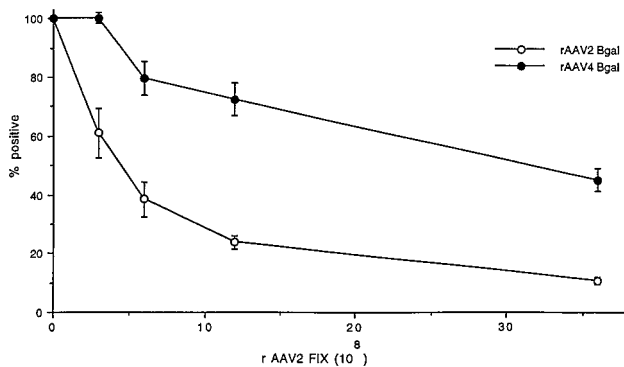


FIG. 7. Cotransduction of rAAV2 and rAAV4. Cos cells were transduced with a constant amount of rAAV2 or rAAV4 expressing  $\beta$ -galactosidase and increasing amounts of rAAV2 expressing human factor IX (rAAV2FIX). For the competition, the number of positive cells detected in the cotransduced wells was divided by the number of positive cells in the control wells (cells transduced with only rAAV2LacZ or rAAV4LacZ) and expressed as a percentage of the control. This value was plotted against the number of particles of rAAV2FIX.

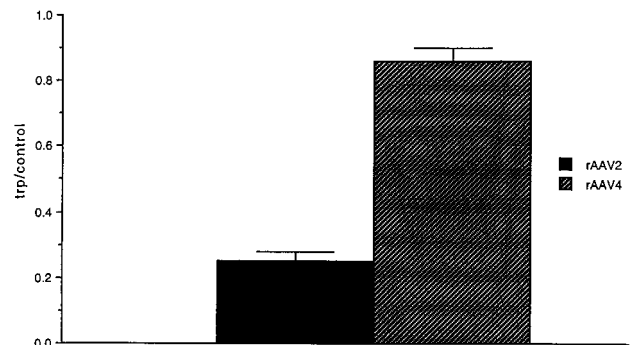


FIG. 8. Effect of trypsin treatment on Cos cell transduction. Cos cell monolayers were trypsinized and diluted in complete medium. The cells were incubated with virus at a multiplicity of infection of 260, and the virus was removed following cell attachment. As a control, an equal number of Cos cells were plated and allowed to attach overnight before transduction with virus for the same time. The number of positive cells was determined by staining 50 h posttransduction. The data are presented as a ratio of the number of positive cells seen with the trypsinized group to the number seen with the control group.

ITR. The Rep binding site consensus sequence suggests that multiple repeats of the GAGC motif create a higher-affinity binding site (18). This alteration could affect the ability of the ITR to function as an origin of replication or as a promoter or its ability to participate in Rep-mediated locus-specific integration. However, the strong conservation of this element, as well as the TRS and Rep binding site in the p5 promoter, underscores the importance of these elements in AAV biology.

The majority of the differences in the capsid proteins lie in regions which have been proposed to be on the exterior surface of the virus. These changes are most probably responsible for the lack of cross-reacting antibodies, the HA activity, and the altered tissue tropism compared to AAV2. However, not all of the changes are confined to the proposed exterior regions, and they may also be important for the other unique properties of AAV4 versus AAV2. The conservation of sequence in the N terminus of VP1 is in agreement with the results of mutational studies, which demonstrated that alteration of this region decreases the yield of particles. Work by Prasad and Trempe has suggested that Rep may interact with this region and facilitate packaging (49).

The high degree of sequence conservation in the Rep ORF stresses the importance of this group of proteins in the life cycle of the virus. The data suggest that while the target cells of the two viruses may have diverged over time, the basic functions required for rescue, replication, and packaging carried out by the Rep proteins have been conserved. The high degree of homology among AAV4, AAV2, and AAV3 clearly places AAV4 in the family of dependoviruses, but it also possesses a high degree of homology to the goose and duck parvoviruses. The primate AAV Rep proteins are more than 90% homologous but are only 40% homologous to the Rep proteins of the goose and duck parvoviruses. While AAV2, AAV3, and AAV5 have been isolated from humans, no clinical isolates of AAV4 have been reported. Our studies, using a recombinant version of the virus, suggest that it can transduce cells of human origin as well as those of monkey and rat origin. Furthermore, comparison of transduction efficiencies in a number of cell lines suggests that the cellular receptor for AAV4 is distinct from that of AAV2. Cotransduction of Cos cells with either rAAV2 or rAAV4 expressing *lacZ* and with rAAV2 expressing factor IX showed strong competition between the two rAAV2 vectors compared to that of rAAV4 and rAAV2 cotransduction. Furthermore, AAV2 transduction was shown to be more trypsin sensitive than that of AAV4. These results again suggest a distinct cellular receptor for AAV2 and AAV4. Unique receptors have also been suggested for AAV1 and AAV3 (44). While most of the autonomous parvoviruses have been shown to be hemagglutinating, AAV4 is the only dependovirus with this property. HA, which has been extensively studied with canine parvovirus, has been found to be independent of receptor binding and uptake (5, 63). However, in parvovirus B19, the P antigen which mediates HA is also thought to be the receptor for infectivity (9). By sequence comparisons, it is unclear to which of these two groups AAV4 belongs, and further experimental determination is required. The use of the ORFs of AAV4 to package a recombinant genome suggests that the cloned genome is functional. While the difference in transduction efficiencies obtained with the cell lines emphasizes the individuality of this virus, the interchangeability of the ITRs and the conservation of the Rep ORF highlight the importance of these elements in viral replication and packaging.

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