VIROLOGY 221, 208–217 (1996)
ARTICLE NO. 0367



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*Received February 9, 1996; accepted April 26, 1996*

We have determined the complete nucleotide sequences of adeno-associated virus 3 (AAV-3) and generated an infectious clone. The single-stranded DNA genome of AAV-3 is 4726 nucleotides in length. The positive strand contains two large open reading frames; the left open reading frame encodes the nonstructural proteins and the right open reading frame encodes the structural proteins. The coding regions are flanked by identical inverted terminal repeat sequences containing palindromes. AAV-3 has little homology with the autonomous parvoviruses or erythroviruses but has 82% overall sequence homology with AAV-2. At the amino acid level there was 88% homology with AAV-2 nonstructural (Rep) proteins and 87% homology with AAV-2 capsid proteins. In addition, AAV-3 differed importantly from AAV-2 in the lack of a typical promoter sequence (TATA box) at p40 and the presence of the consensus sequence for adenovirus-related transcription factor E4F binding within the upstream region of the p5 promoter. These results suggest that AAV-3 not only consists of serologically distinct structural proteins but that viral propagation also may be controlled by different gene regulatory elements at the transcription level. The infectious clone confirmed the sequence and may be useful for developing new vectors for gene therapy. 

## INTRODUCTION

Adeno-associated viruses (AAVs) are small, nonenveloped, single-stranded DNA viruses, classified as dependoviruses because they were originally thought to replicate only in the presence of a co-infecting helper adeno- or herpes virus (Murphy *et al.,* 1995). AAVs were first discovered as contaminants of laboratory stocks of adenoviruses propagated in tissue culture (Atchison *et al.,* 1965; Hoggan *et al.,* 1966). AAVs have been isolated from a variety of different animal species, including primates, dogs, cows, horses, chickens, and sheep (Murphy *et al.,* 1995), where they appear to be nonpathogenic. To date, five primate AAVs have been described, distinguished by techniques that assay capsid protein variation, including complement fixation, immunodiffusion, neutralization, and fluorescent–antibody binding (Hoggan *et al.,* 1966; Parks *et al.,* 1967; Georg-Fries *et al.,* 1984). Isolates of AAV-2, AAV-3, and AAV-5 have been obtained directly from human clinical specimens, and man appears to be their natural host. In contrast, neither AAV-1 nor AAV-4 has been isolated from clinical specimens, but serum antibodies to AAV-1 and AAV-4 have been detected in African green monkeys, suggesting that they are simian viruses (Blacklow *et al.,* 1968; Parks *et al.,* 1970). AAV-5 appears more distantly related to the other primate AAVs, the only AAV-5 isolate was from a penile condylomatous lesion, and, epidemiologically, transmission appears to follow acquisition of herpesviruses rather than adenoviruses (Georg-Fries *et al.,* 1984).

Of the many dependoviruses described, only AAV-2 has been characterized at the genomic organization and nucleotide sequence levels (Srivastava *et al.,* 1983; Cassinotti *et al.,* 1988; Ruffing *et al.,* 1994). The virus has a single-stranded DNA genome, with identical double-stranded hairpin structures at each end. The AAV-2 genome contains three transcriptionally active promoters, from which mRNAs for four nonstructural (Rep) proteins and three capsid (Cap) proteins are transcribed by alternative splicing (Carter *et al.,* 1990). Binding of the Rep protein to the "Rep-binding" motif in the hairpin structure appears necessary for preferential targeted integration of the AAV-2 genome into host cell chromosomal DNA (Weitzman *et al.,* 1994). The ability to integrate into human chromosome in the absence of helper viruses and lack of apparent pathogenicity have made AAV-2 a popular candidate as a viral vector for gene therapy (for review, Flotte and Carter, 1995).

Apart from serological studies on the antigenic differences between AAV-2 and AAV-3 performed in the 1960s, very little is known about AAV-3. In contrast to AAV-4, not even a restriction enzyme map for the viral genome has been determined, and there is some question as to whether AAV-3 is a serotype of AAV-2 or a distinctly different virus. DNA–RNA cross-hybridization studies suggest there is only 37% homology between AAV-2 and AAV-3 (Rose *et al.,* 1968); more recent DNA cross-hybridization studies with goose parvovirus DNA also sug-

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0042-6822/96 $18.00

gested that there was a distinct difference between the DNA sequences of AAV-2 and AAV-3 (Brown *et al.,* 1995b). In addition, polyacrylamide gel electrophoresis of virions indicated that the capsid proteins were of different sizes (Rose *et al.,* 1971), and binding studies of the two viruses have suggested that they bind to different receptors on cellular membranes (Mizukami *et al.,* 1996).

We were interested in confirming that AAV-3 was a distinctly different virus and not a serotype of AAV-2. We have therefore cloned and sequenced the entire genome of AAV-3. Sequence analysis showed similar genomic organization and high homology to AAV-2 at both the DNA and the amino acid level, but significant divergence in the structural proteins and especially in the p5 and p40 promoter organization. Production of AAV-3 infectious clone confirmed that the sequence was correct.

## MATERIALS AND METHODS

### Cell culture and preparation of viral DNA

Seed viruses (AAV-3, AAV-2, and adenoviruses types 2 and 5) and cells (KB, 293-31, and Detroit 6 cells) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and penicillin–streptomycin. Either KB or Detroit 6 cells were infected with AAV-3 or AAV-2 at an input multiplicity of infection (m.o.i.) of 0.3 with adenovirus helper (m.o.i. 10). Cells were incubated for 40 hr before harvesting. Virions were obtained by lysis of cells and banding in a cesium chloride gradient as previously described (Mizukami *et al.,* 1996). Replicative viral DNA was obtained by extraction of infected cells and collection of the low molecular weight DNA fraction (Hirt, 1967). Virion-associated DNA was obtained by lysis of virus particles with 0.2 mg/ml proteinase K and 0.5% SDS in 50 m*M* NaCl at 50° for 16 hr.

### Restriction enzyme analysis

To obtain a rough restriction enzyme map of the AAV-3 genome for cloning purposes, DNA was extracted from the supernatant of infected cell cultures and single-stranded viral DNA allowed to anneal by incubation in 50 m*M* NaCl at 50° for 16 hr. DNA was incubated with restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I), and the fragments were analyzed by Southern hybridization using a $^{32}$P-labeled AAV-2 probe excised from pAV2 (ATCC).

### Cloning and sequencing of coding sequence

Replicative AAV-3 virion DNA was digested with pairs of restriction enzymes (double digest), and the products were cloned into pUC19. Clones were screened for AAV-3 inserts by Southern hybridization using a $^{32}$P-labeled AAV-2 probe, as described above.

Clones containing AAV-3 were amplified and sequenced by the dideoxynucleotide chain termination method using either Sequenase II (Amersham, UK) for manual sequencing or Ampli*Taq* DNA polymerase (Perkin Elmer, CA) for automated DNA sequencing (Applied Biosystems, Inc.). Initially, "universal" M13 forward and reverse primers were used; subsequently primers were designed from previously obtained sequence. Inserts were completely sequenced in both directions.

Additional sequence into the right termini was obtained by PCR amplification. PCR primers were designed based on the termini sequences of AAV-2 and the obtained AAV-3 sequence. The products were cloned into a cloning vector (TA cloning; Invitrogen) and sequenced as above.

### Cloning and sequencing of the termini

AAV-3 virion-associated DNA was digested with the restriction enzyme *Sac*I and the products were analyzed on agarose gels. After the appropriate bands were excised from the gels, DNA was extracted using a gel extraction kit (Qiagen, CA). Two of the fragments were ligated into the *Sac*I/*Sma*I site of pUC18. Clones containing AAV-3 terminal sequence were amplified and sequenced by a PCR-based cycle sequencing method using Ampli*Taq* DNA polymerase (Perkin Elmer).

### Cloning of the entire viral genome

To obtain the sequence of the entire genome, AAV-3 virion-associated DNA was digested with one or both restriction enzymes *Pst*I and *Sac*I. The products were cloned into pUC18 and then subcloned into pBluescript II (KS$^+$). The sequences of the junction regions were confirmed by direct PCR-based cycle sequencing of extracted viral DNA.

### Sequence analysis

All sequences obtained were analyzed using DNAStar (DNAStar, Inc.) and GeneWorks (IntelliGenetics, Inc.). Transcription factor recognition sequences were searched with the GeneWorks program based on the Transcription Factor database of the National Center for Biotechnology Information.

### Transfection of cells with AAV plasmid

293-31 cells were transfected with 10 $\mu$g of plasmid DNA per 10-cm dish by using lipofectin (Gibco BRL) according to manufacturer's instructions. At 24 hr, the lipofectin medium was replaced with DMEM supplemented with 2% fetal calf serum and containing the helper adenovirus type 5 (Ad5; m.o.i. 10). At 40 hr, DNA was extracted (Tissue kit; Qiagen, Inc.) and digested with *Eco*RI. The

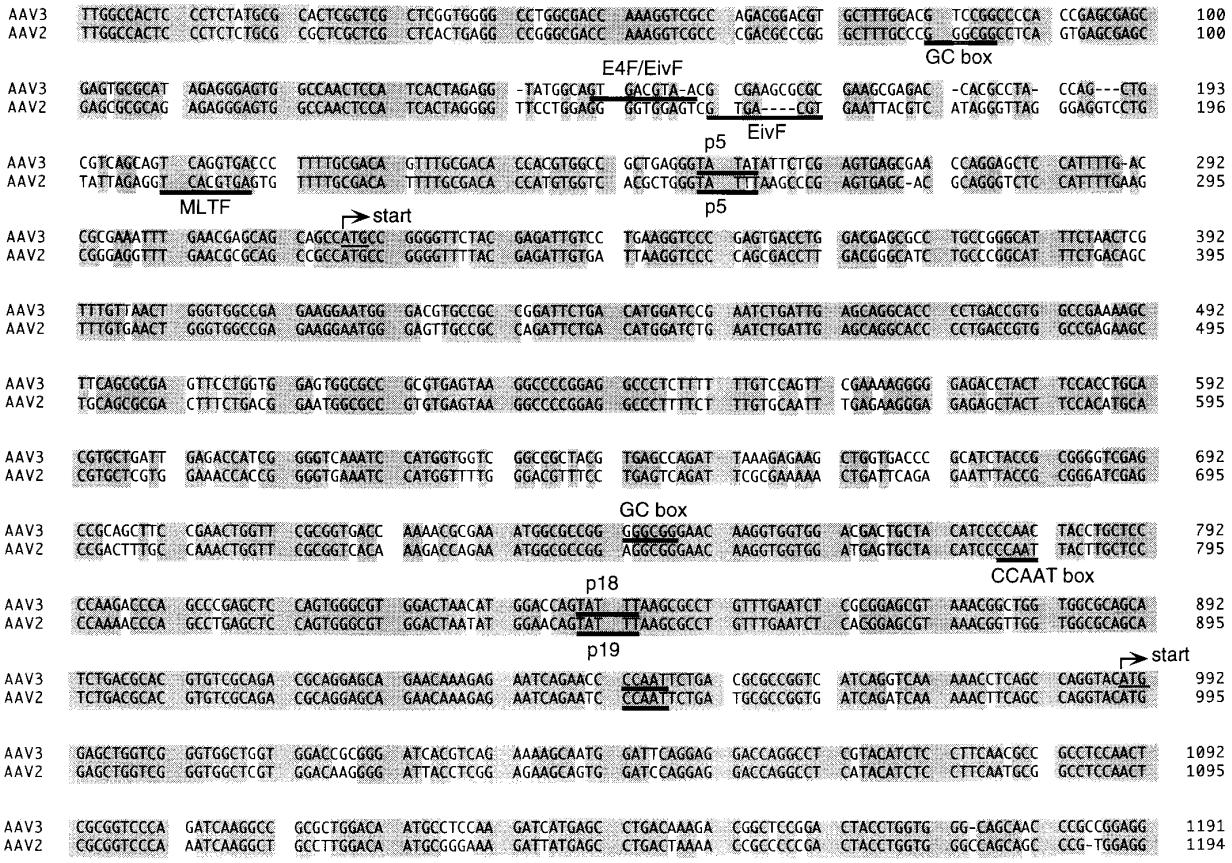


FIG. 1. Complete nucleotide sequences of AAV-3 and AAV-2 (Srivastava *et al.,* 1983; Cassinotti *et al.,* 1988; Ruffing *et al.,* 1994). The sequences correspond to the complementary (plus) strands. Darkened bases are matches. Dashes (-) indicate gaps introduced into the sequence to increase homology. Start and stop codons for nonstructural proteins (Rep) and capsid proteins (VP) are underlined. Transcription factor binding sites or promoters (TATA box, MLTF, E4F, EivF, GC box, CCAAT box) are double underlined. Polyadenylation and splice donor and acceptor sites are also shown (*).

DNA was then fractionated by electrophoresis on 1% agarose gel, transferred to nylon membrane, and hybridized to $^{32}$P-labeled AAV-3 full-length DNA that was excised from the AAV-3 plasmid with *Eco*RI and *Xba*I. From one dish, cells and supernatant were frozen and thawed three times, heated at 60° for 15 min to inactivate excess adenovirus, and used to infect additional cultures. AAV-3 antigen expression was analyzed by the indirect immunofluorescence technique. The first antibody was an antiserum against AAV-3 virion purified by banding in a cesium chloride gradient (Braton Biotech, Inc., Rockville, MD) and the second antibody was fluorescein-conjugated goat anti-guinea pig immunoglobulin G (Zymed, San Francisco, CA).

## RESULTS

### Restriction map

Southern analysis of DNA extracted from AAV-3 virions produced two bands of approximately 1.7 and 4.7 kb (data not shown). After incubation with multiple restriction enzymes, the 1.7-kb band remained undigested, a result that implied a single-stranded DNA structure. The 4.7-kb band was incised with restriction enzymes *Bam*HI, *Hin*dIII, and *Pst*I, indicating self-annealed double-stranded DNA and that, similar to AAV-2, AAV-3 virions encapsidated both positive and negative strands of DNA. AAV-3 DNA was not affected by treatment with the restriction enzyme *Eco*RI.

### Cloning and sequencing of viral DNA

Self-annealed double-stranded viral DNA was incubated with pairs of restriction enzymes *Pst*I/*Hin*dIII and *Pst*I/*Bam*HI, and the products were ligated into the appropriate restriction enzyme sites of pUC19. Terminal sequences were obtained by excision of virion DNA with *Sac*I, and the two small *Sac*I fragments were blunt-ligated into the *Sac*I/*Sma*I site of pUC18. The PCR fragment was cloned into a pCRII vector.

To obtain the entire genomic sequence, AAV-3 virion-associated DNA was digested with one or both restriction enzymes *Pst*I and *Sac*I. The products were cloned into pUC18 and then subcloned into pBluescript II (KS$^+$). As previously, the sequences of the junction regions

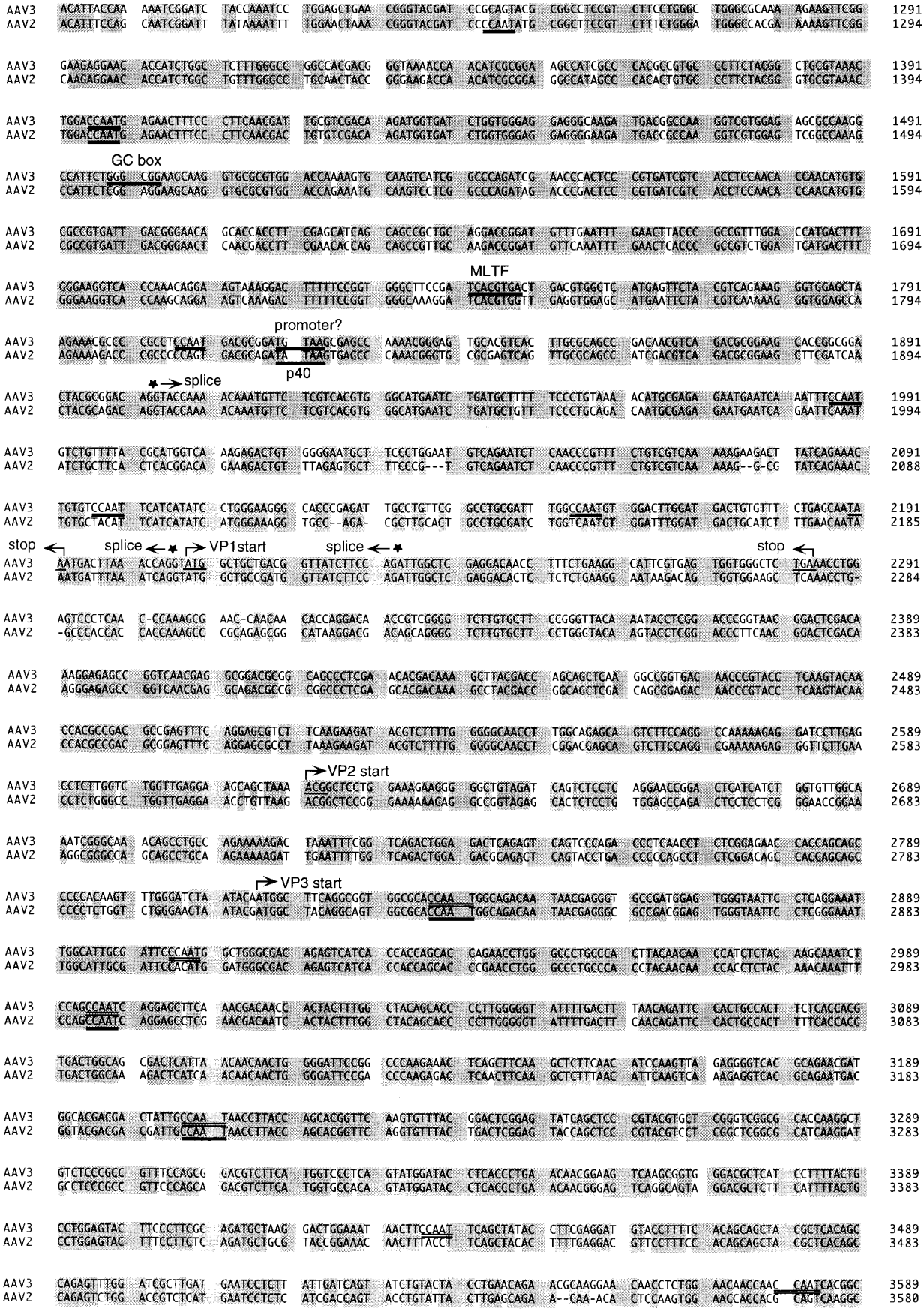


FIG. 1—*Continued*

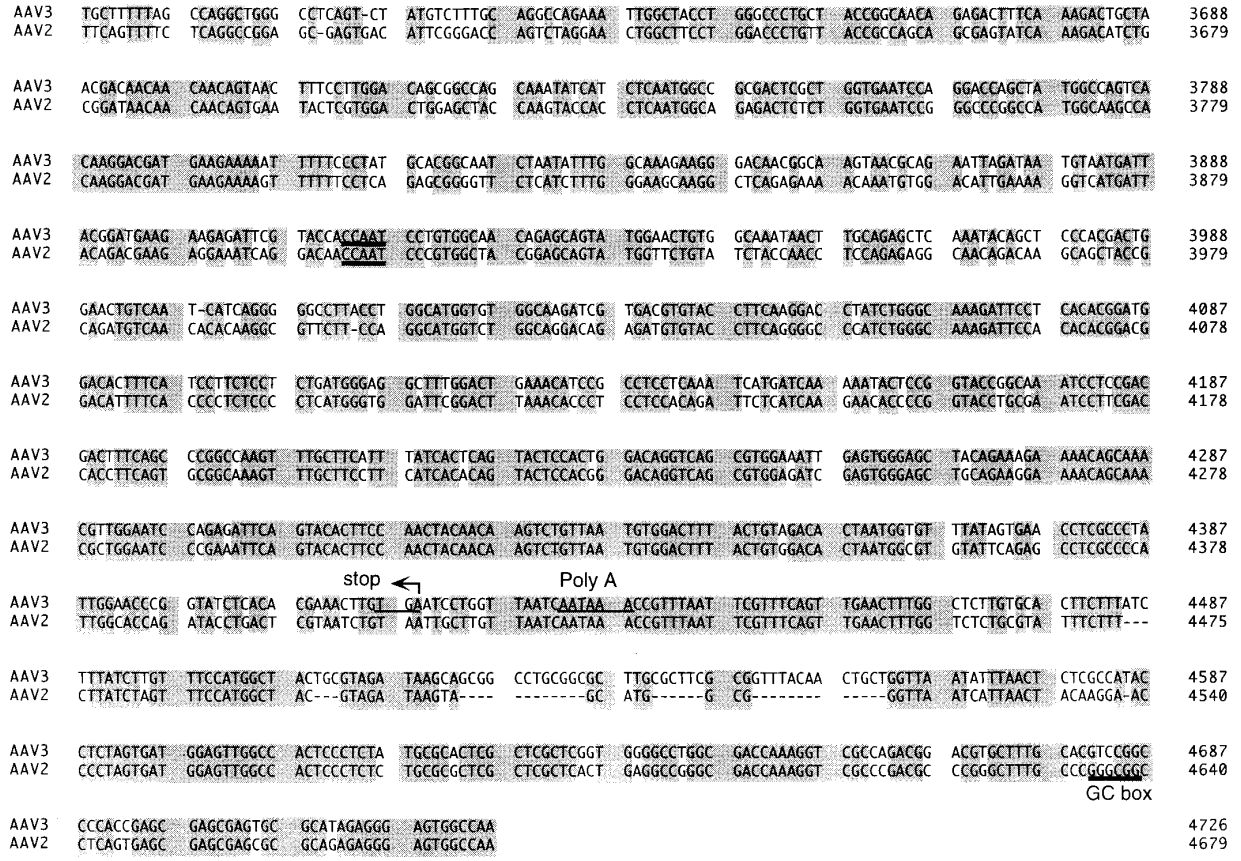


FIG. 1—*Continued*

were confirmed directly by PCR-based cycle sequencing of viral DNA.

## Nucleotide sequence

The genome of AAV-3 was 4726 nucleotides in length. Comparison with the genome of AAV-2 showed 82% overall sequence homology (Fig. 1). Among other parvoviruses, the closest relative of AAV-3 appeared to be the goose parvovirus which showed 54% sequence homology. There was $<42\%$ homology with the autonomous parvoviruses, including erythroviruses.

As with AAV-2, both ends of the AAV-3 genome consisted of identical inverted repeats (ITRs) containing palindromes of 146 bp and consistent with the formation of T-shaped hairpin structures. The AAV-2 nonstructural Rep protein binding motif (Weitzman *et al.,* 1994) and terminal resolution site (Snyder *et al.,* 1993) in the hairpin appeared highly conserved, with only a single base-pair substitution between the two AAVs (Fig. 2).

## Genomic organization

The location of ATG start and stop codons indicated that AAV-3 had a genomic organization similar to those of other members of the Parvoviridae, with one large open reading frame (ORF) in the left half of the genome and a second long ORF in the right. There were no large open reading frames in the complementary strand, suggesting that it did not encode proteins.

A search for the consensus promoter sequence (TATA boxes) in the AAV-3 genome revealed two promoter regions starting at nucleotides 252 and 840, which we designated p5 and p18, corresponding to p5 and p19 of AAV-2. However, in contrast to AAV-2, there was no typical midgenome promoter sequence, with the sequence TGTAA at nucleotide 1820 in the analogous position to the TATAA sequence of the AAV-2 p40 promotor.

As with AAV-2, there was a single polyadenylation site (AATAAA) at the far right side (starting at nt 4433).

For transcription factor binding sites, there were 2 GC boxes (GGGCGG; nt 743 and 1499) and 13 CCAAT boxes as well as binding sites for transcription factors E4F (TGACGTAAC; nt 149), EivF (GTGACGT; nt 148), and MLTF (TCACGTGA; nt 1742). In AAV-2, GC boxes exist in ITRs (nt 80 and 4634) and EivF and MLTF binding sites are located upstream of the p5 promoter (nt 160, 206).

## Nonstructural (Rep) coding region

By analogy with other parvoviruses, the left-sided open reading frame (nt 318–2189) encoded the nonstructural or Rep protein(s). The AAV-3 left open reading frame

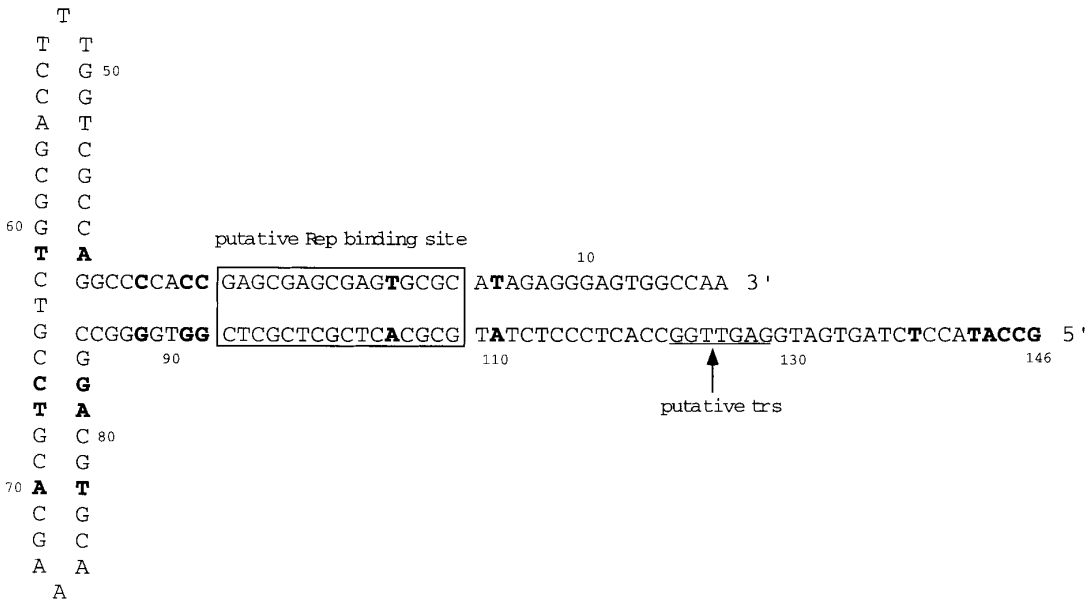


FIG. 2. Hairpin structure of the left ITR of AAV-3. The sequences homologous to the Rep binding motif and terminal resolution site (trs) of AAV-2 are outlined. Bases different from AAV-2 are in bold.

encoded a putative nonspliced nonstructural protein of 624 amino acids and of a predicted molecular weight of 71 kDa. The putative Rep protein showed homology to the nonstructural proteins of other parvoviruses, with highest relationship to the unspliced Rep78 protein of AAV-2 (88%) and lower homologies for nonstructural proteins of goose and duck parvoviruses (44%). Nonstructural proteins of other autonomous parvoviruses showed little homology to AAV-3 (B19 24%, simian 26%, bovine 19%). As with AAV-2 Rep78, the amino acid sequence included an ATP/GTP binding site motif (amino acids 334–341 of Rep 78) (Saraste *et al.,* 1990) and a putative zinc binding motif at the carboxyl terminus (Carter *et al.,* 1990) (Fig. 3a).

AAV-2 encodes four nonstructural proteins by alternative splicing. The acceptor/donor sequences for this alternative splicing also were conserved in AAV-3 (donor at nt 1904, acceptor at 2134) and if used would encode proteins corresponding to Rep68 and Rep40 of AAV-2.

## Capsid protein coding region

The right-sided open reading frame (nt 2209–4416) encoded a capsid protein of 736 amino acids, with a predicted molecular weight of 82 kDa. The putative capsid protein showed 87% homology to the VP1 capsid protein of AAV-2 and 54% homology to goose and duck parvoviruses' VP1, but little homology to other autonomous parvoviral capsid proteins (B19 19%, simian 18%, bovine 24%).

The AAV-2 VP2 capsid protein is believed to initiate from an ACG triplet, and there was an identical triplet at nucleotide 2620 in AAV-3. The spliced VP2 and VP3 proteins would contain 600 and 535 amino acids, respectively, with predicted molecular weights of 66 and 60 kDa. All three AAV-3 capsid proteins showed high similarity to AAV-2 proteins (87% for VP1, 88% for VP2, and 89% for VP3), with the largest dissimilarity between them located in the middle of VP3, where two possible N-linked glycosylation sites exist in AAV-3, but not in AAV-2 (Fig. 3b).

## Biological activity of AAV-3 plasmid in human cells

Plasmid containing the entire AAV-3 sequence was transfected into cells, and the progeny DNA was compared with that produced by standard AAV-3 virus infection by Southern blot hybridization (Fig. 4). In the cells transfected with AAV-3 plasmid alone, only one band was detected (lane 3). Two bands were detected in cells subjected to AAV-3 plasmid transfection and Ad5 coinfection (lane 4). In this experiment, the upper band was at the position corresponding to the plasmid containing AAV-3 and the lower band was characteristic of normal AAV-3 infection (lane 1); in addition, the signal intensity of the lower band was stronger than that of the upper band, suggesting amplification of the viral sequence. Under our extraction conditions, only double-stranded viral DNA (either reannealed or replicative form) was detected in both the infection and the transfection lanes. No band was detected in cells infected by Ad5 alone (lane 2).

AAV-3-coded proteins after transfection were detected using an indirect immunofluorescence assay: an intranuclear fluorescence pattern indistinguishable from that seen in virus infected cells was observed (Fig. 5).

To confirm that infectious AAV-3 was produced, a virus stock was prepared from cultures transfected by AAV-3 plasmid by freezing and thawing the cells and supernatant of one 10-cm dish after plasmid transfection. After

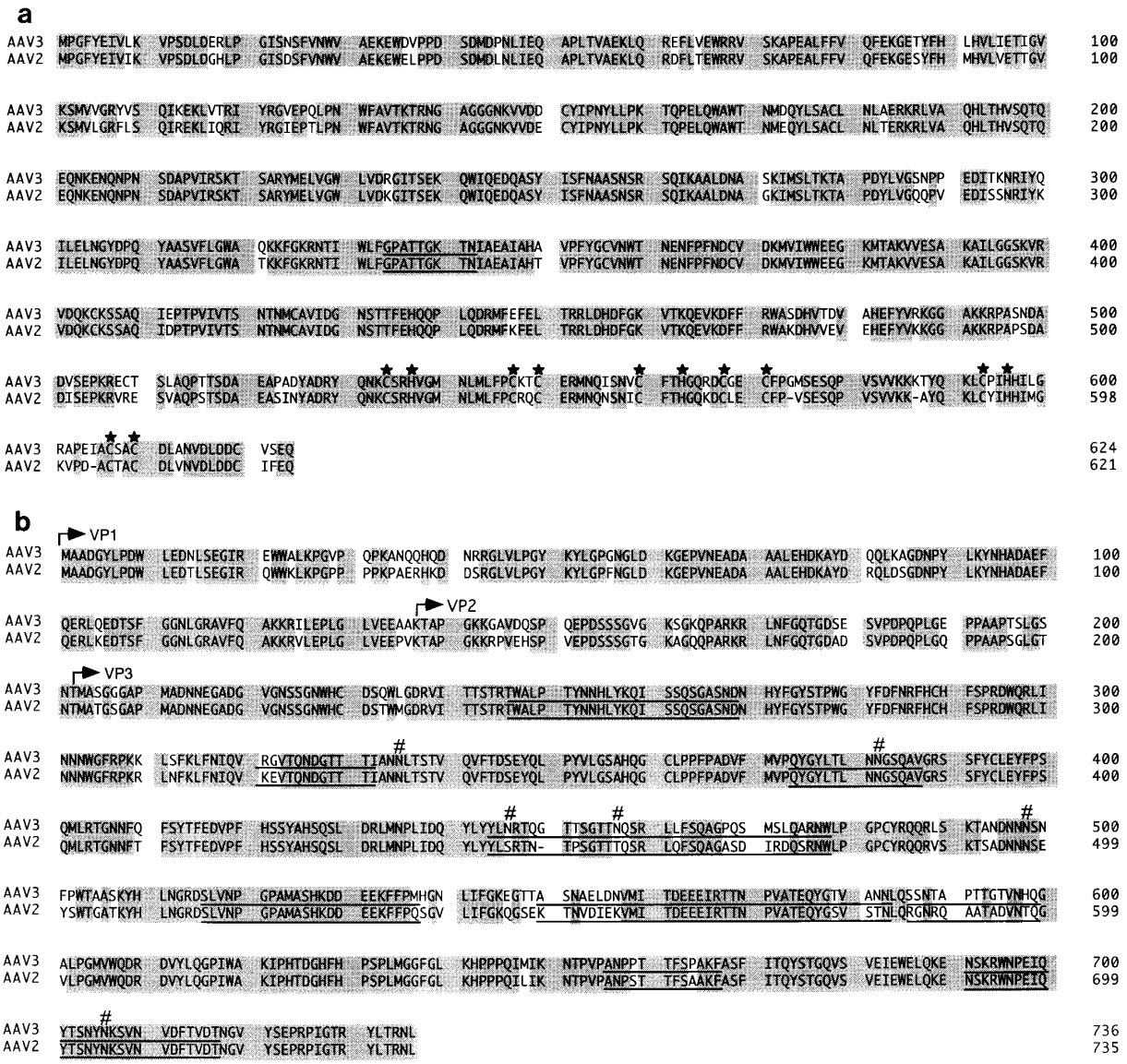


FIG. 3. Amino acid homologies of the nonstructural proteins and capsid proteins between AAV-3 and AAV-2. AAV-2 sequence as described in the legend to Fig. 1. Darkened amino acids are matches. (a) Amino acid sequence of AAV-3 nonstructural protein to AAV-2 Rep78 sequence. The ATP/GTP binding site motif is underlined. The cysteines and histidines of a putative zinc binding motif at the carboxyl terminus are indicated (*). (b) Amino acid sequence alignment of capsid proteins of AAV-3 and AAV-2. The first amino acids of VP2 and VP3 are indicated by arrows. The regions predicted to be exposed on the surface of the capsid are underlined. Possible N-linked glycosylation sites are marked (#).

heat treatment to inactivate excess adenovirus we used this preparation to infect additional 293-31 cells with fresh Ad5 helper (m.o.i. 10). At 38 hr, immunofluorescence with an anti-AAV-3 antibody showed an intranuclear fluorescence pattern typical of AAV-3 infection.

## DISCUSSION

We have cloned and sequenced the entire genome of a second human dependovirus, AAV-3. We confirmed that AAV-3 encapsidates DNA strands of both plus and minus polarity and that both strands are encapsidated with equal frequency into individual virions (Rose *et al.,* 1969), allowing double-stranded DNA to be obtained by annealing under low-salt condition. A similar approach was used for cloning of AAV-2 (Samulski *et al.,* 1982; Senapathy and Carter, 1984), bovine parvovirus (Shull *et al.,* 1988), and simian parvovirus (Brown *et al.,* 1995a).

The genome of AAV-3 (4726 bp) was longer than that of AAV-2 (4679 bp). This difference in length can be mainly attributed to short deletions in the regions between the inverted terminal repeats and open reading frames on both sides of AAV-2 genome. Cross-hybridization studies of AAV DNA–RNA interactions performed in the 1960s indicated a homology of approximately 37% between AAV-2 and AAV-3 (Rose *et al.,* 1968). Our results show that the homology between them is much greater.

AAV-3 had a genomic organization similar to the AAV-

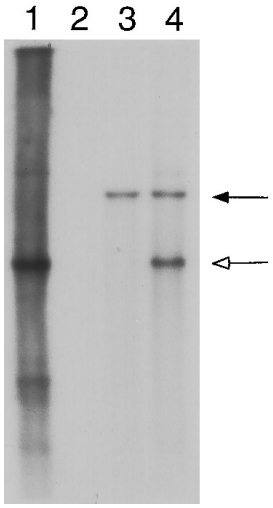


FIG. 4. Southern blot analysis of rescue and amplification of AAV-3 DNA from infectious clone. DNA was isolated from 293-31 cells that had been infected or transfected with AAV-3 virion and Ad5 (lane 1), Ad5 alone (lane 2), AAV-3 plasmid alone (lane 3), and AAV-3 plasmid and Ad5 (lane 4). The DNA was digested with *Eco*RI, fractionated on a 1% agarose gel, transferred to a nylon membrane, and hybridized to $^{32}$P-labeled AAV-3 DNA. Black arrow indicates bands corresponding to the AAV-3 plasmid, and white arrow indicates bands corresponding to AAV-3 virus replication.

2. The internal portion of the genome divided into two open reading frames that encode nonstructural and structural proteins. In AAV-2, nonstructural proteins Rep78 and Rep68 control DNA replication and also can mediate the formation of a complex between the ends of the AAV genome and the chromosome 19 integration locus. If the reduced number of GAGC repeats in the putative Rep binding motif of AAV-3 is significant for Rep binding, it might be expected to affect site-specific integration as well as DNA replication.

Whether the TGTAA sequence at nucleotide 1820 functions as a promotor remains to be elucidated. However, it is unlikely that this sequence is a cloning or sequencing artifact because five different clones, all directly derived from either replicative form or self-annealed form of virus DNA and sequenced by both manual and automated methods, showed the same results. In addition, the corresponding region of AAV-1 also lacks a TATA box (K. E. Brown *et al.,* manuscript in preparation). Upstream from this sequence is a GC box (starting at nt 1499) and binding sites for the major late transcription factor (MLTF; nt 1742). In parvovirus B19, there is no functionally active midgenome TATA box and the GC box is a major controlling sequence for *in vitro* transcription (Blundell and

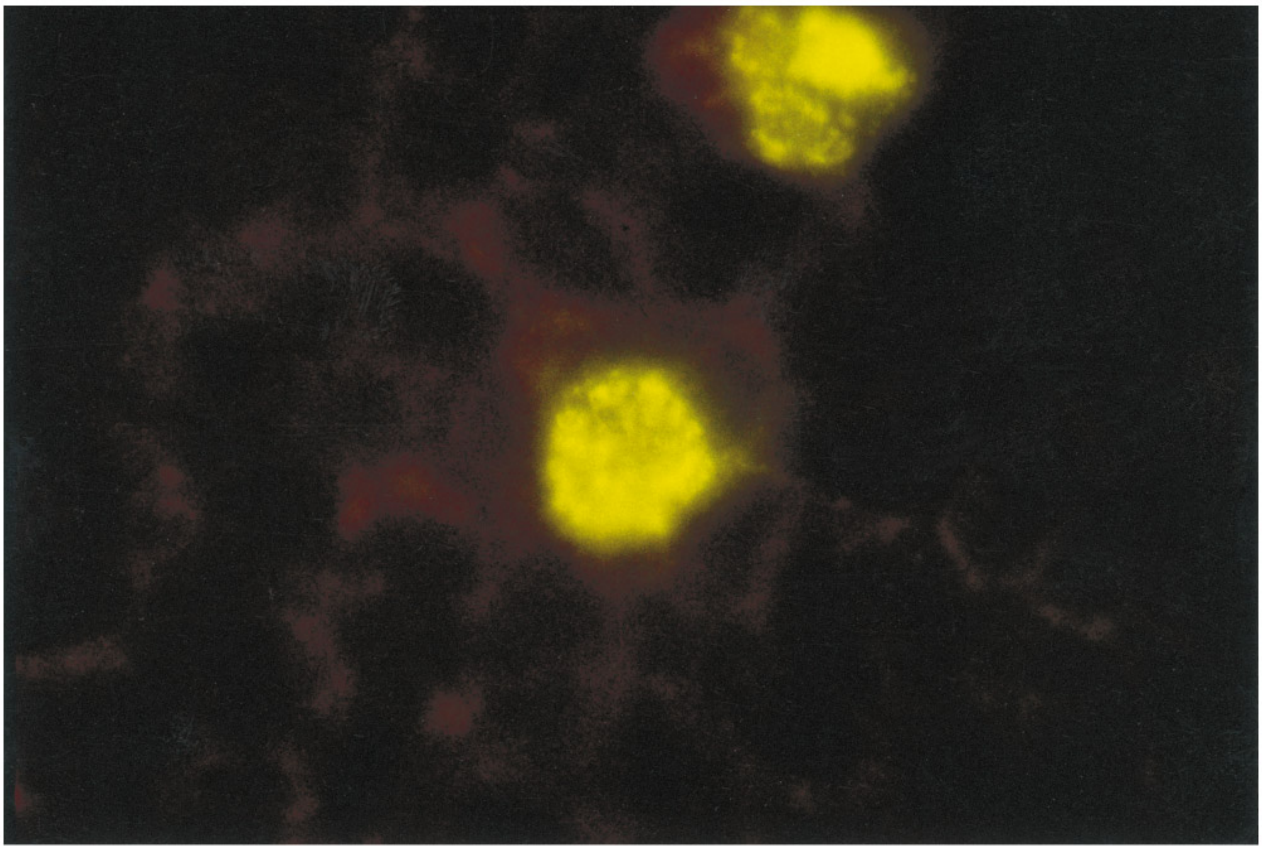

FIG. 5. Immunofluorescent staining with polyclonal guinea pig serum antibodies directed against AAV-3 capsids in 293-31 cells transfected with AAV-3 plasmid co-infected with Ad5 as a helper. Cells were prepared 3 days after transfection with AAV-3 plasmid.

Astell, 1989). In AAV-2, a MLTF binding site exists in the upstream region of p5 promoter and reduces the basal activity and enhances the E1A-induced activity of the p5 control region (Chang *et al.,* 1989). Thus, AAV-3 and AAV-2 may be controlled by different gene regulatory elements at the transcription level. We are now determining the transcription map of AAV-3.

It is important to note that within the upstream region of the AAV-3 p5 promoter there was a consensus sequence for transcription factor E4F or EivF binding. These cellular factors are involved in E1A-responsive transactivation of the adenovirus E4 promoter and function in the initiation of adenovirus replication (Raychaudhuri *et al.,* 1987; Cortes *et al.,* 1988; Rooney *et al.,* 1990). E4F binds to a sequence (TGACGTAAC) within the adenovirus E4 promotor overlapping two activating transcription factor (ATF) binding sites, and EivF binds to a sequence (GTGACGT) closely related to the ATF sequence (TGACGT). An adenovirus E4 gene is required as part of the AAV helper function during infection of human cells with adenovirus particles (Carter *et al.,* 1983), and recent studies have shown that plasmid expression of the E4 open reading frame 6 protein in cells infected with recombinant AAV increases gene transduction up to 1000-fold (Ferrari and Samulski, 1995). The EivF binding sequence overlaps the M26 motif, an enhancer element for recombination, which is also found in AAV-2 and in its integrated cellular sequence (Giraud *et al.,* 1994). The E4F binding sequence is not found in the AAV-2.

The most divergent regions for the VP3 polypeptides were located in the middle region of these proteins. Based on the atomic structure determined by X-ray crystallography of canine parvovirus, these regions may be exposed at the surface of capsid proteins (Tsao *et al.,* 1991; Chapman and Rossmann, 1993). Different capsid proteins presumably contribute to the distinct antigenicities of AAV-2 and AAV-3 and may explain the difference in binding to cell membranes of the two viruses (Mizukami *et al.,* 1996). Differences in cell binding may also reflect different cell tropisms for AAV-2 and AAV-3.

Intact AAV-3 genome can be rescued from a recombinant clone when transfected cells are superinfected with adenovirus. The rescued genome is functional in that AAV-3 protein is expressed in the nuclei of cells and infectious virus is produced. The infectious clone helps to validate the sequence that we report here. An infectious clone will allow study of AAV-3 at a molecular level comparable to AAV-2 and it may be possible to develop novel gene therapy vectors based on AAV-3.

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