

Gene Therapy Vectors Based on Adeno-Associated Virus Type 1

WEIDONG XIAO, NARENDRA CHIRMULE, SCOTT C. BERTA, BETH McCULLOUGH,
GUANGPING GAO, AND JAMES M. WILSON*

*Institute for Human Gene Therapy and Departments of Molecular and Cellular Engineering
and of Medicine, University of Pennsylvania, and The Wistar Institute, Philadelphia, Pennsylvania 19104*

Received 15 December 1998/Accepted 29 January 1999

The complete sequence of adeno-associated virus type 1 (AAV-1) was defined. Its genome of 4,718 nucleotides demonstrates high homology with those of other AAV serotypes, including AAV-6, which appears to have arisen from homologous recombination between AAV-1 and AAV-2. Analysis of sera from nonhuman and human primates for neutralizing antibodies (NAB) against AAV-1 and AAV-2 revealed the following. (i) NAB to AAV-1 are more common than NAB to AAV-2 in nonhuman primates, while the reverse is true in humans; and (ii) sera from 36% of nonhuman primates neutralized AAV-1 but not AAV-2, while sera from 8% of humans neutralized AAV-2 but not AAV-1. An infectious clone of AAV-1 was isolated from a replicated monomer form, and vectors were created with AAV-2 inverted terminal repeats and AAV-1 Rep and Cap functions. Both AAV-1- and AAV-2-based vectors transduced murine liver and muscle *in vivo*; AAV-1 was more efficient for muscle, while AAV-2 transduced liver more efficiently. Strong NAB responses were detected for each vector administered to murine skeletal muscle; these responses prevented readministration of the same serotype but did not substantially cross-neutralize the other serotype. Similar results were observed in the context of liver-directed gene transfer, except for a significant, but incomplete, neutralization of AAV-1 from a previous treatment with AAV-2. Vectors based on AAV-1 may be preferred in some applications of human gene therapy.

Adeno-associated viruses (AAV) are small, nonenveloped, single-stranded DNA viruses which require helper virus to facilitate efficient replication (3). The 4.7-kb genome of AAV is characterized by two inverted terminal repeats (ITRs) and two sets of open reading frames, which encode the Rep and Cap proteins. The Rep open reading frames encode four proteins with molecular masses of 78, 68, 52, and 40 kDa. These proteins function mainly in regulating AAV replication and integration. The Cap open reading frames encode three structural proteins with molecular masses of 85 kDa (VP1), 72 kDa (VP2), and 61 kDa (VP3) (3). The two ITRs are the only *cis* elements essential for all steps in the AAV life cycle.

AAV have been found in many animal species, including nonhuman primates, canines, fowl, and humans (18). A total of six serotypes of AAV, including AAV type 1 (AAV-1), have been isolated from primates, and two have been isolated from nonhuman primates; AAV-2, AAV-3, and AAV-5 are from humans, and AAV-6 is from a human adenovirus preparation. AAV-2 is the most characterized primate serotype, since its infectious clone was the first one made (24). The full sequences for AAV-3A, AAV-3B, AAV-4, and AAV-6 recently were determined (4, 17, 22). Generally, all primate AAV show more than 80% homology in nucleotide sequence.

A number of unique properties make AAV a very promising vector for human gene therapy (19). AAV are not associated with any known human diseases and are generally not considered pathogenic. Wild-type AAV are capable of integrating into the host chromosome in a site-specific manner (14, 26). Recombinant AAV (rAAV) vectors can integrate into tissue culture cells at chromosome 19 if the Rep proteins are supplied *in trans* (1, 29). The transduced genomes of AAV have been shown to confer long-term gene expression in a number of tissues, including muscle, liver, brain, and retina (8, 13, 27, 28,

30, 31). The development of new methods for producing high-titer rAAV has largely removed the hurdles which prevented AAV vectors from being tested in large-animal models of human diseases and in human clinical trials (5, 6, 11, 32).

Among AAV-1 to AAV-6, only AAV-1 and AAV-4 are considered to be simian viruses, since they were isolated from nonhuman primates and monospecific antibodies to the viruses have not been detected in human serum (20). They may have advantages for use in human gene therapy to replace or augment the use of AAV-2 vectors. For example, AAV-1 vectors could be used in patients who develop anti-AAV-2 neutralizing antibodies (NAB) due to a naturally acquired infection or previous treatment with AAV-2 vectors. To study the possibility of using AAV-1 as a gene therapy vector, we constructed an AAV-1 infectious clone and determined its full sequence. Vectors derived from this infected clone were evaluated in murine models of liver and muscle gene transfer.

MATERIALS AND METHODS

Murine studies. C57BL/6 mice (6- to 8-week-old males) were obtained from Jackson Laboratory. AAV vectors were administered by either intramuscular or intrasplenic injection as described before (8, 30).

Nonhuman primates. Wild-caught juvenile rhesus monkeys were purchased from Covance (Alice, Tex.) and LABS of Virginia (Yemassee, S.C.) and kept in full quarantine. The monkeys weighed approximately 3 to 4 kg. The nonhuman primates used in the Institute for Human Gene Therapy research program are purposefully bred in the United States from specific-pathogen-free closed colonies. All vendors are U.S. Department of Agriculture class A dealers. The rhesus macaques are therefore not infected with important simian pathogens, including the tuberculosis agent, major simian lentiviruses (simian immunodeficiency virus and simian retroviruses), and cercopithecine herpesvirus. The animals are also free of internal and external parasites. The excellent health status of these premium animals minimizes the potential for extraneous variables. The use of these monkeys in protocols was approved by the Infection Control Committee of the Hospital of the University of Pennsylvania and the Environmental Health and Safety Office, the Institutional Biosafety Committee, and the Institutional Animal Care and Use Committee of the University of Pennsylvania. For this study, serum was obtained from monkeys prior to initiation of any protocol.

Human subjects. Normal volunteers (*n*, 77) were analyzed for immune reactivity to AAV. Individuals were members of the University of Pennsylvania community. The study was approved by the Institutional Review Board of the

* Corresponding author. Mailing address: 204 Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104-4268. Phone: (215) 898-3000. Fax: (215) 898-6588. E-mail: wilsonjm@mail.med.upenn.edu.

University of Pennsylvania. The median age was 27 years (range, 18 to 54 years), and the age distributions in the two groups were similar.

Cell culture and virus. AAV-free 293 cells and 84-31 cells were obtained from the Human Applications Laboratory of the University of Pennsylvania. These cells were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum (Hyclone), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a moisturized environment supplied with 5% CO₂. The 84-31 cell line constitutively expresses adenovirus E4 proteins and has been described previously (7). AAV-1 (ATCC VR-645) seed stock was purchased from the American Type Culture Collection (Rockville, Md.). AAV were propagated in 293 cells with wild-type adenovirus type 5 as a helper virus.

rAAV generation. rAAV were generated by transfection with an adenovirus-free method described elsewhere (30a). Briefly, the *cis* plasmid (with AAV ITRs), the *trans* plasmid (with the AAV *Rep* gene and *Cap* gene), and a helper plasmid (pFΔ13, with an essential region from the adenovirus genome) were cotransfected into 293 cells at a ratio of 1:1:2 by calcium phosphate precipitation. The AAV vectors used in this study express murine erythropoietin or human α1-antitrypsin from a chicken β-actin promoter enhanced by sequences from cytomegalovirus. pFΔ13 has an 8-kb deletion in the adenovirus E2B region and most of the late genes. The cells were harvested 96 h posttransfection and subjected to two rounds of CsCl gradient purification. For the generation of rAAV based on AAV-2, p5E18 was used as the *trans* plasmid, since it greatly improved the rAAV yield. To make rAAV with AAV-1 virions, pAVIH or p5E18(2/1) was used as the *trans* plasmid to provide *Rep* and *Cap* functions.

DNA techniques. Hirt DNA extraction was performed as described before with minor modifications (25).

To construct AAV-1 infectious clones, the Hirt DNA from AAV-1-infected 293 cells was repaired with the Klenow enzyme (New England BioLabs) to make sure that the ends were blunt. The treated AAV-1 Hirt DNA was then digested with *Bam*HI and cloned into three vectors. The internal *Bam*HI fragment was cloned into pBluescript II-SK(+) cut with *Bam*HI to produce pAV1-BM. The left and right fragments were cloned into pBluescript II-SK(+) cut with *Bam*HI and *Eco*RV to obtain pAV1-BL and pAV1-BR, respectively. The AAV sequences in these three plasmids were subsequently assembled into the same vector to produce AAV-1 infectious clone pAAV-1.

For sequencing, an ABI 373 automatic sequencer was used to determine the sequences for all plasmids and PCR fragments used in this study by fluorescence sequencing (FS) dye chemistry. They were confirmed by sequencing both plus and minus strands. For sequencing the AAV terminal repeats, the fragment containing AAV ITRs was amplified from pAAV-1, pAV1-BR, or pAV1-BL with M13F (GTAAACGACGGCCAGT) and CAP1a (GAA CCG TGC TGG TAA GGT TAT T) or M13F and VIRepl1 (CCA TGC CGG GCT TCT ACG AGA TCG TTA TCA GGG TG). The PCR was performed with a GC-advantage kit (Clontech Laboratories, Inc.). The 50-µl reaction mixture contained 1× reaction buffer (40 mM Tricine-KOH [pH 9.02] at 25°C, 15 mM KOAc, 3.5 mM Mg(OAc)₂, 5% dimethyl sulfoxide, 3.75 µg of bovine serum albumin per ml); 1× GC melt; 10 mM dATP, dCTP, 7-deazaGTP-dGTP (3:1), and dTTP; 10 ng of template; 500 ng of each primer; and 2.5 U of polymerase. The purified PCR product was sequenced as described above.

A plasmid expressing AAV-2 *Rep* and *Cap*, p5E18(2/2), was constructed to maximize vector production. This was accomplished by relocating the p5 promoter to a position 3' to the *Cap* gene, thereby minimizing expression of *Rep*78 and *Rep*68. This strategy was initially described by Li et al. (15). p5E18(2/2) was constructed in the following way. The previously described pMNTV-*trans* vector (30a) (i.e., the mouse mammary tumor virus promoter substituted for the p5 promoter in an AAV-2-based vector) was digested with *Sma*I and *Cla*I, filled in with the Klenow enzyme, and then recircularized with DNA ligase. The resulting construct was digested with *Xba*I, filled in, and ligated to the blunt-ended *Bam*HI-*Xba*I fragment from pCR-p5, constructed in the following way. The p5 promoter of AAV was amplified by PCR with the following oligonucleotides: TGT AGT TAA TTA ACC CGC CAT GCT ACT TAT C and GGC GGC TGC GCG TTC AAA CCT CCC GCT TCA AAA TG. The amplified fragment was subsequently cloned into pCR2.1 (Invitrogen) to yield pCR-p5. The helper plasmid pAVIH was constructed by cloning the *Bfa*I fragment of pAAV-1 into pBluescript II-SK(+) at the *Bco*rV and *Sma*I sites. The 3.0-kb *Xba*I-*Kpn*I fragment from p5E18(2/2), the 2.3-kb *Xba*I-*Kpn*I fragment from pAVIH, and the 1.7-kb *Kpn*I fragment from p5E18(2/2) were incorporated into a separate plasmid, p5E18(2/1), which contains AAV-2 *Rep*, AAV-1 *Cap*, and the AAV-2 p5 promoter located 3' to the *Cap* gene.

For Southern blot analysis, Hirt DNA was digested with *Dpn*I to remove bacterium-borne plasmid and probed with the internal *Bam*HI fragment of AAV-1. The membrane was then washed under high-stringency conditions: twice for 30 min each time with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C and twice for 30 min each time with 0.1× SSC–0.1% SDS at 65°C. The membrane was then analyzed by both phosphorimager analysis and X-ray autoradiography.

Western blot analyses. Serum samples were analyzed for reactivity to various AAV-2 capsid proteins by Western blotting as described previously (8). Purified AAV-2 vectors were used as antigens for the capsid proteins, and a monoclonal antibody that recognizes shared epitopes on VP1, VP2, and VP3 (clone B1; American Research Products) was used as a positive control. AAV-2 antigens were electrophoresed by SDS–10% polyacrylamide gel electrophoresis and trans-

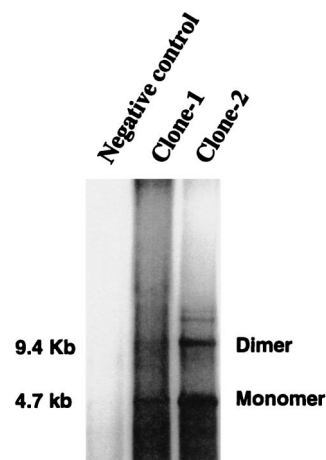


FIG. 1. DNA hybridization analysis of the rescue and replication of the AAV-1 infectious clone. Two independent clones of pAAV-1 (Clone-1 and Clone-2) were transfected into 293 cells, which were then superinfected with adenovirus type 5. Hirt DNA was extracted 48 h postinfection and digested with *Dpn*I before electrophoresis in an 0.8% agarose gel. An internal *Bam*HI fragment of pAAV-1 was used as a probe. The autoradiograph shows the locations of replicated dimers and monomers and their molecular sizes. The analysis included cells not infected (negative control) or infected with clone-1 or clone-2.

ferred to a nitrocellulose membrane (Hybond-ECL; Amersham). The reactivity of the serum was measured by incubating membrane containing AAV antigen with test serum samples, followed by peroxidase anti-human immunoglobulin G antibody. The reaction was detected with an ECL kit (Amersham).

Anti-AAV NAB. NAB titers were analyzed by assessing the ability of serum antibody to inhibit the transduction of reporter virus (AAV1-GFP or AAV2-GFP) into 84-31 cells, which are subclones of 293 cells that stably express adenovirus E4 proteins, which render them permissive for AAV transduction. Various dilutions of antibodies preincubated with reporter virus for 1 h at 37°C were added to 90% confluent cell cultures. Cells were incubated for 48 h, and the expression of green fluorescent protein (GFP) was measured by FluoroImaging (Molecular Dynamics). NAB titers were calculated as the highest dilution at which 50% of the cells stained green.

Human α1-antitrypsin assay. The concentration of human α1-antitrypsin in mouse serum was measured by an enzyme-linked immunosorbent assay (ELISA). The coating antibody was rabbit anti-human α1-antitrypsin (Sigma). We used goat anti-human α1-antitrypsin (Sigma) as the primary detection antibody (30). The sensitivity of the assay is 0.3 to 30 ng/ml.

Nucleotide sequence accession number. The AAV-1 sequence is available through GenBank under accession no. AF063497.

RESULTS

Generation of an infectious clone of AAV-1. The replicated form of AAV-1 was extracted from 293 cells infected with AAV-1 and wild-type adenovirus type 5. Analysis of Hirt DNA revealed three bands with apparent molecular sizes equivalent to a double-stranded dimer (9.4 kb), a double-stranded monomer (4.7 kb), and single-stranded DNA (1.7 kb). The monomer band was excised from the gel and digested with *Bam*HI, revealing a pattern of three fragments (1.1, 0.8, and 2.8 kb), in accordance with the original description of AAV-1 by Bantel-Schaal and zur Hausen (2). The three fragments were subcloned into one plasmid-based construct to obtain pAAV-1.

This clone was tested for its abilities to rescue AAV-1 genomes from the plasmid backbone and to replicate and package infectious virus. Plasmid pAAV-1 was transfected into 293 cells infected with adenovirus type 5 at a multiplicity of infection of 10, and the virus supernatant was used to reinfect 293 cells. DNA hybridization analysis of *Dpn*I-digested Hirt DNA from infected 293 cells demonstrated the rescue and replication of AAV-1 genomes (Fig. 1).

Genomic structure of AAV-1. The entire AAV-1 genome was sequenced from pAAV-1 (Fig. 2). PCR products derived

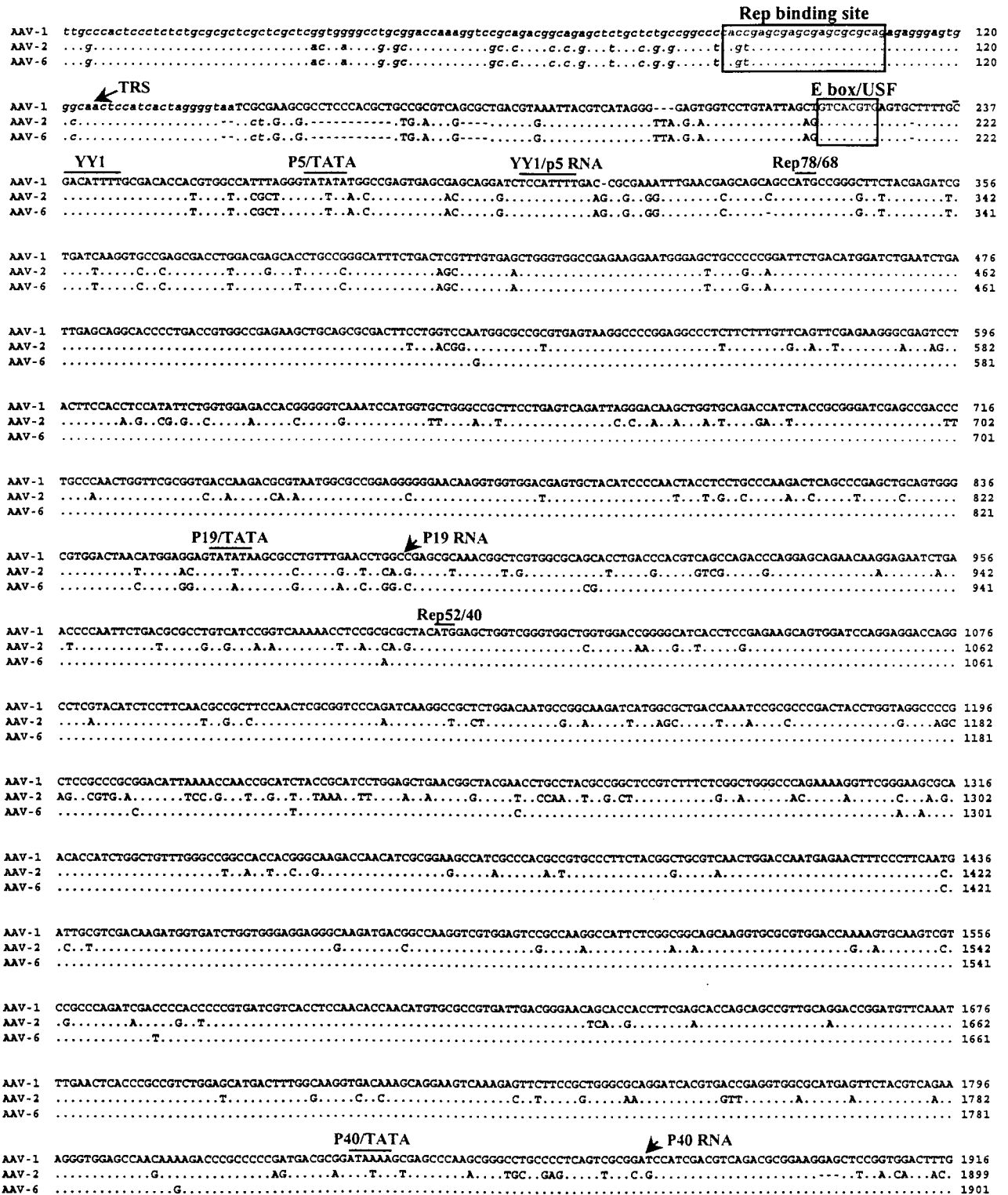


FIG. 2. Alignment of nucleotides for AAV-1, AAV-2, and AAV-6. The full sequence of AAV-1 is indicated in the top line; the AAV-2 and AAV-6 sequences are shown below with nucleotide differences. Homology is indicated by dots. Critical landmarks in the structures of the AAV genomes are shown. Gaps are indicated by dashes. The 3' ITR of AAV-1 is shown in the same configuration as in the published AAV-2 and AAV-6 sequences (22). TRS, terminal resolution site.

from the original AAV-1 seed stock were also sequenced to confirm the structure of the replicated form. No major differences were noted. The length of AAV-1 (4,718 nucleotides) is very close to that of AAV-3 (4,726 nucleotides), which is

shorter than AAV-4 (4,774 nucleotides) but longer than AAV-2 (4,681 nucleotides) and AAV-6 (4,683 nucleotides).

The AAV-1 genome shows more than 80% identity with other known AAV and contains the characteristic structural

AAV-1	CCGACAGTACCAAAA	CAAAATGTTCTCGT	CACGGGCGATGCTT	CAGATGCTGTTTCC	TGCAAGACATGCG	GAGAAATGAATCAG	AAATTTCAACATTT	GCTTACCGCACGGG	ACGAGAG	2036																					
AAV-2	.A.	.T.	.T.	.T.	.AA.	.T.	.GACA.	.CA.	.T.	.C.	2019																				
AAV-6C.	2021																				
AAV-1	ACTGTT	CAGAGT	GCTTCCCC	GGCGTGT	CAGAAT	CTCAACCGGTC	---	GTCAGAAAGAGG	ACGTATCGGAA	ACTCTGTGCCAT	TATCATCTGCTGGG	CGGGCTCCC	GAGATTGCTT	GCT	2153																
AAV-2	.A.	.T.	.T.	.T.	.T.	.TTCT.	.GTC.	.A.	.A.	.G.	.CTA.	.A.	.CA.	.AAA.	.TG.	2133															
AAV-6	.	.A.	.T.	2138															
AAV-1	CGGCCT	GCGATCT	GGTCAAC	TGGACCT	TGGACT	GTGTTCT	GAGCAATAA	TGACTTAA	ACCAGGTAT	TGGCTG	CCGATGTTAT	CTTCCAGAT	TGGCTCGG	GAGCAACCT	CTCTGAG	2273															
AAV-2	.T.	.T.	.T.	.T.	.T.	.TT.	.CA.	.C.	.T.	.A.	.T.	.T.	.	.	.	2253															
AAV-6AC.	.G	2258															
AAV-1	GGCATT	CGGAGT	GGTGGG	ACTTGA	AACCTGG	AGCCCGCAAG	CCCAAGCCAA	AGCCAA	CCAGCAA	AGCAGG	ACGACGG	CGGGGTCT	GGTCTT	CCTGGCT	CAAGTACCT	CGGACCTT	CAAC	2393													
AAV-2	.A.	.AA.	.AC.	.A.	.GC.	.C.	.CC.	.A.	.ACCA.	.A.	.GC.	.GCAG.	.GGC.	.TA.	.A.	.A.	.T.	2373													
AAV-6	2378													
AAV-1	GGACT	CGCAAG	GGGAG	CCCGT	CAACG	CGGGG	CGGACG	CAGCGG	CCCTCG	AGCACG	CAAGCC	TACGAC	CAGCTCAA	AGCGGGT	GACAAT	CCGTAC	CTGCGG	TATAAC	CGCCGAC	2513											
AAV-2	.	.A.	.G.	.A.	.A.	.C.	.A.	.A.	.A.	.G.	.G.	.CAGC.	.A.	.A.	.C.	.CAA.	.C.	.	.	2493											
AAV-6	2498										
AAV-1	GCCGAG	TTTCAG	GAGCGT	CTGCA	AGAAG	ATACGT	CTTTGG	GGGCA	CCCTCG	GGGAG	CAGTCT	TCCAG	GCCA	AGAAG	CGGGT	CTC	GAAC	CTCT	CGGT	CTGGT	TGAG	GAAG	GGCGT	AAG	2633						
AAV-2	.G.	.	.C.	.TA.G.	.A.	.A.	.T.	.	.G.	.C.	.	.CCT.	.T.	.	.	2613							
AAV-6	.	.C.	.	.T.	.GC.G.	.	.	.A.T.	.T.	.	.T.	.	.	.	2618							
VP2																															
AAV-1	ACGGCT	CCTG	GAAGAA	ACGTCC	GGTAG	GAGCAGT	CGCCACA	AGAGCC	AGACTC	CTCTCG	GGCATCG	CGCAAG	CAAGCAG	CCCGCT	AAAGAG	AGACTCA	ATTTGGT	CAGACT	GGC	2753											
AAV-2	.G.	.	.A.	.GA.	.G.	.C.	.T.	.TGTG.	.	.A.	.C.	.A.	.G.	.G.	.	.T.	.A.	.G.	.A.	.T.	.G.	.	.	A	2733						
AAV-6	.	.T.	.G.	.AC.	.T.	.	.G.	.G.	.ACAA.	2738						
VP3																															
AAV-1	GACTC	AGAGT	CAGTCC	CCGAT	CCACA	ACCTCT	CGGAG	ACCTCC	AGCA	CCCCCG	TGCTGT	GGGAC	TACTA	CAATGG	CTCAG	GGTGG	CGCAC	CAATGG	CAGACA	TAA	CGA	AGGC	2873								
AAV-2	.G.	.C.	.A.	.T.	.C.	.C.	.G.	.C.	.G.	.A.	.G.	.T.	.G.	.C.	.A.	.A.	.G.	.A.	.A.	.	.	.G.	2853								
AAV-6	.T.	.G.	.C.	.C.	.A.	.A.	.G.	.A.	.T.	.A.	.G.	2858								
AAV-1	GCCGAC	GGAGT	GGTAA	TGCTC	AGAAAT	TGGCAT	TGGAT	TCCAT	GGTGG	CGCAG	AGTCA	TCCACC	CAGAC	CCCGC	ACCTGG	GGCTT	GCCCA	CTACA	ATAAC	CACCT	TAC	2993									
AAV-2	.	.	.T.	.C.	2973									
AAV-6	2978									
AAV-1	AAGCA	AACTCC	AGTCTT	CAACGG	GGCC	CAGCA	ACGCA	CAACCA	CTACT	TCGGT	TACAG	CACCC	CTGG	GGTAT	TTTTG	ATTTCA	CACAG	ATTC	CACTG	CCACT	TTTT	CACCA	CGTGA	CTGG	3113						
AAV-2	.A.	.T.	.CCAA.	.A.	.TCG.	.T.	.T.	.T.	.T.	.C.	3090							
AAV-6	3098							
AAV-1	CAGCG	ACTCAT	CAACA	CAAT	TGGG	ATTCC	GGCC	CAAG	AGACT	CAACT	TCAA	ACTT	CAAA	CTTCA	ACAT	TCCA	AGTCA	AGG	GTCA	CGA	GAAT	TGG	CGTCA	CAAC	CCAT	CGTAA	TAA	CCTT	3233		
AAV-2	.AA.	.	.C.	.	.A.	.	.G.	.T.	.T.	.A.	.	.CA.	.	.C.	.TACG.	.G.	.G.	.T.	.C.	3210				
AAV-6	3218				
AAV-1	ACCAG	CAGCG	TTCAAG	TCTT	CTCG	ACTCGG	AGTACC	GACTT	CCGT	ACGCT	CGCT	CTGG	CTG	CGC	ACC	AGGG	GTCT	CCCT	CCGT	CCG	GGG	CACT	GTT	CAT	GATT	CCG	CAAT	ACGGC	3353		
AAV-2	.	.G.	.G.	.TA.	.T.	.	.C.	.	.G.	.T.	.A.	.A.	.G.	.	.A.	.A.	.C.	.	.G.	.G.	.A.	.G.	.T.	.A	3330						
AAV-6T.	.G.	3338				
AAV-1	TACCT	GACG	CTCA	CAAT	TGGC	AGCC	AGCG	GGGCG	TTTCA	TCTT	ACTG	CTGGA	ATTTT	CCCTT	CTCAG	ATGCT	GAGAAC	CGGCA	CAACT	TTTAC	CTT	CAGCT	TAC	ACTTT	TGAG	3473					
AAV-2	.C.	.C.	.G.	.C.	.G.	.T.	.G.	.A.	.A.	.C.	.T.	.A.	.	.G.	.C.	.T.	.	.C.	.T.	.C.	.A.	3450					
AAV-6	.	.A.	.	.	.G.	.A.	.	.G.A.	.G.	3458					
AAV-1	GAAGT	GCCTT	CCAC	AGCAG	TAC	GGCC	ACAG	CCAG	CGCT	GGAC	CGGCT	GAT	GAAT	CTCT	CTCAT	CGAC	CAATA	CCTGT	TATT	ACTG	AA	CAGAA	CTCA	AAAT	CAGT	CCG	GAAGT	GC	CAA	3593	
AAV-2	.C.	.T.	.	.T.	.	.T.	.	.T.	.C.	.	.G.	.	.T.	.G.	.	.AA.	.C.	.C.	.CAAGT.	.CCA.	.ACG	3570									
AAV-6	.	.C.	3578			
AAV-1	AACAG	GA	CTT	GTCT	TAG	CCG	TGGT	CTCC	AGTGG	CAT	GTCT	GTTC	AGCC	CAAA	AACT	GGTAC	CTGG	ACCT	GT	TAT	CGG	CAG	CGG	CTT	CTA	AAAA	CAAA	AC	GACA	CAAC	3713
AAV-2	.C.	.GTC	.AAGG	.T.	.A.	.TCT.	.AG.	.CCG	.GAG.	.GAG.	.A.	.TCGG.	.AC.	.T.	.T.	.GG.	.	.T.	.	.C.	.C.	.	.A.	.A.	.CA.	.G.	.TCTG.	.G.	.T.	3690	
AAV-6	3698			
AAV-1	AACAG	CAATTT	TAC	TGG	ACT	TGGT	CTCAA	AAAT	AATAC	CTCA	ATGG	CGTGA	ATCC	ATCAT	CAAC	CTGG	CACT	GT	TAT	CGG	CAG	CGG	CTT	CTA	AAAA	CAAA	AC	GACA	CAAC	3713	
AAV-2	.TG.	.A.	.ACT.	.G.	.	.A.	.A.	.C.	.G.	.CC.	.	.CA.	.A.	.C.	.TC.	.GG.	.G.	.T.	.G.	.GC.	.C.	.C.	.	.AAGC.	.G.	.	.T.	.A.	.T.	TCA.	3810
AAV-6	.	.C.	3818		

FIG. 2—Continued.

features. The ITRs of AAV-1 are predicted to form T-shaped hairpin structures (Fig. 3). The right and left ITRs of AAV-1 are identical and virtually the same as the right ITR of AAV-6, except for 1 nucleotide in the A and A' sequences and the

last nucleotide in the D sequence. The AAV-2 Rep binding motif (GCTCGCTCGCTCGCTG) found in the AAV-2 pre-integration region in human chromosome 19 is well conserved in AAV-1.

Downloaded from jvi.asm.org by on July 16, 2009

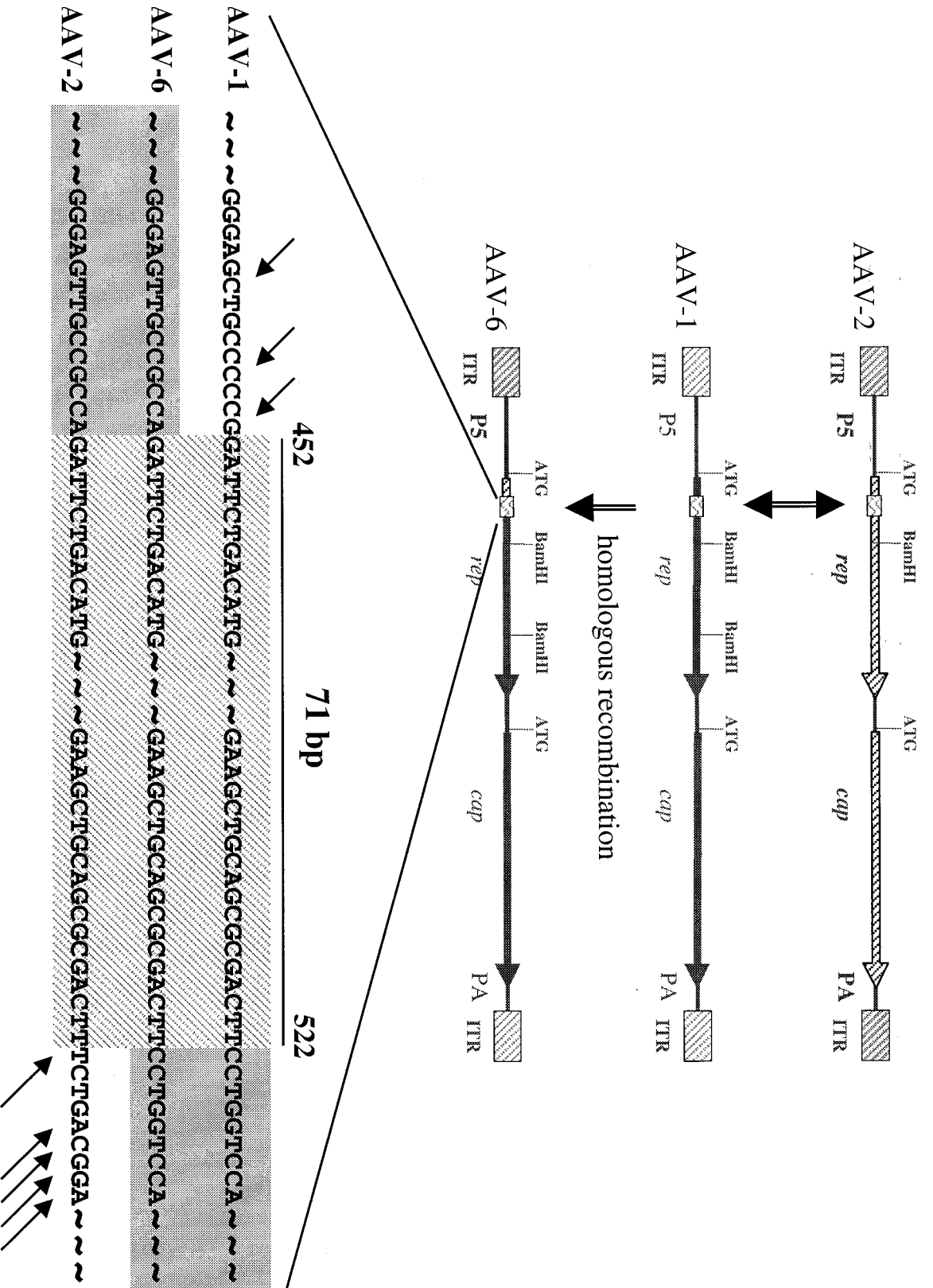


FIG. 4. Hypothesis for the creation of AAV-6 from homologous recombination between AAV-1 and AAV-2. The first line represents the AAV-2 genome, with Rep and Cap sequences demarcated by hatched arrows. The second line represents the AAV-1 genome, with the Rep and Cap sequences depicted as solid arrows. The hypothesis suggests homologous recombination between AAV-2 and AAV-1 at the region of the box containing wavy lines; this region contains a highly homologous sequence of the Rep gene shared by AAV-1 and AAV-2. PA, poly(A). A more detailed illustration of the common region of these three viruses is shown at the bottom. Arrows indicate nucleotides that differ.

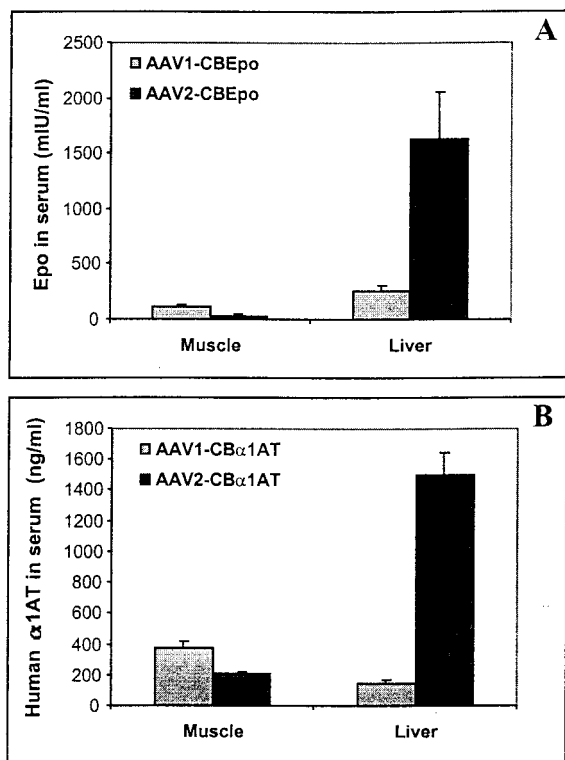


FIG. 5. In vivo activities of AAV-1 and AAV-2 vectors. Recombinant stocks of virus were generated by transfection and purified through cesium chloride gradients. (A) AAV-1 and AAV-2 vectors expressing murine erythropoietin (Epo) from a cytomegalovirus-enhanced β -actin promoter (CB). Equivalent stocks of these two vectors were injected into muscle (5×10^{10} genomes) or liver via the portal circulation (1×10^{11} genomes), and the animals (four groups) were analyzed on day 30 for the presence of erythropoietin in blood. Data for the groups were combined. Error bars show standard errors. (B) An identical experiment was performed with AAV-1 and AAV-2 vectors expressing human α 1-antitrypsin (α 1AT) from the same promoter. Serum harvested from four groups of animals 30 days after gene transfer was analyzed for the presence of α 1-antitrypsin by an ELISA. Data are reported as in panel A.

were purified on CsCl gradients, yielding low titers of the recombinant vector (i.e., 5×10^{10} genomes/50 15-cm² plates).

The AAV helper vector was revised in two ways to improve yield. First, the AAV-1 Rep gene in pAV1H was substituted for the corresponding gene in AAV-2. Second, the p5 promoter was relocated behind the Rep and Cap open reading frames to reduce the expression of the p78 and p68 forms; this modification has been used to enhance the titers of AAV-2 vectors (15,300). The resulting plasmid (p5E182/1) produced 10- to 20-fold higher quantities of the vector than pAV1H (i.e., 10^{12} genomes/50 15-cm² plates).

The performance of AAV-1 vectors was evaluated with immunodeficient mice (i.e., RAG-1 KO) injected intramuscularly or in the portal circulation to target the liver (Fig. 5). Direct comparisons were made to equivalent quantities of AAV-2 vectors containing the same vector genomes encoding secreted proteins easily measured in blood (i.e., murine erythropoietin and human α 1-antitrypsin). Early experiments indicated similar in vivo performances of AAV-1 vectors produced with pAV1H and p5E18(2/1) (data not shown). All subsequent studies used AAV-1 vectors derived from p5E18(2/1) because of the increased yield.

AAV-2 vectors consistently produced 10- to 50-fold more serum erythropoietin or α 1-antitrypsin when injected into liver compared to muscle. This result was very different from that for AAV-1 vectors, with which muscle expression was equivalent to or greater than liver expression. In fact, AAV-1 outperformed AAV-2 in muscle when equivalent titers based on genomes were administered.

NAB to AAV-1 and AAV-2 in nonhuman and human primates. Simple and quantitative assays for NAB to AAV-1 and AAV-2 were developed with recombinant vectors. A total of 33 rhesus monkeys and 77 normal human subjects were screened. Figure 6 summarizes the results. A reciprocal dilution of NAB equal to 20 is the baseline (i.e., no detectable NAB).

Analysis of NAB in rhesus monkeys showed that 61% of animals tested positive for AAV-1; a minority (24%) had NAB to AAV-2. Over one-third of animals had antibodies to AAV-

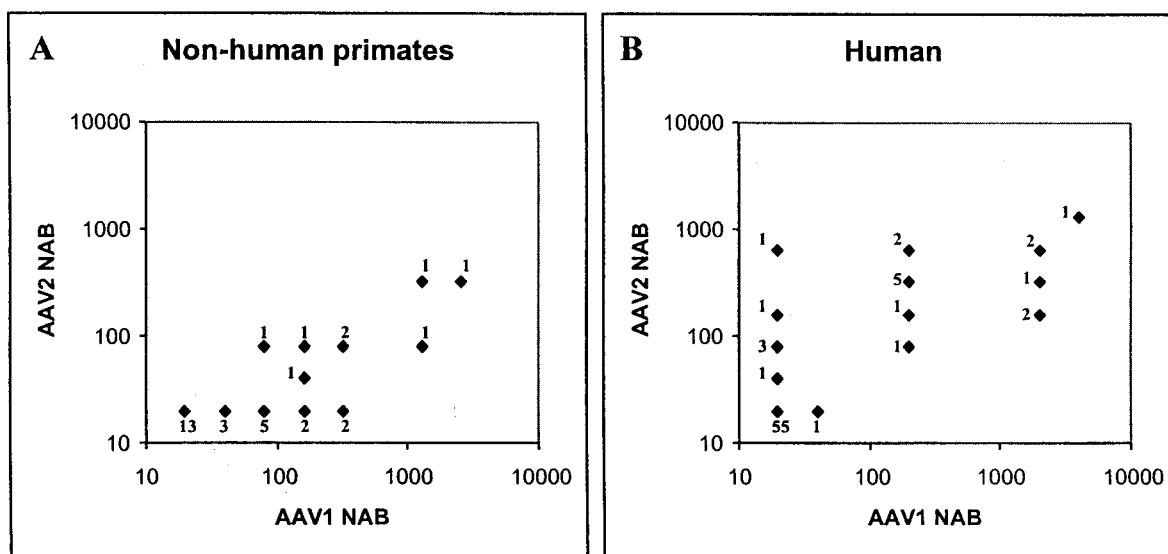


FIG. 6. NAB to AAV-1 and AAV-2 in nonhuman and human primates. Sera were analyzed for neutralizing activity against rAAV-1 and rAAV-2 as described in Materials and Methods. The reciprocal dilution of the NAB was plotted for AAV-2 versus AAV-1. Sera were titrated in sequential twofold dilutions. A titer of 1:20 represents the background (i.e., no detectable NAB). The number next to each datum point represents the total number of animals or humans with a particular NAB profile. In panel A, 33 adolescent rhesus monkeys were analyzed. In panel B, 77 normal human volunteers were evaluated.

1 but not AAV-2 (i.e., were monospecific for AAV-1), whereas no animals were positive for AAV-2 without reacting to AAV-1. These data support the hypothesis that AAV-1 is endemic in rhesus monkeys. The presence of true AAV-2 infections in this group of nonhuman primates is less clear, since we cannot rule out cross-neutralizing activity of an AAV-1 response to AAV-2. It is interesting that there is a linear relationship between AAV-2 NAB and AAV-1 NAB in animals that had both.

Analysis of sera from normal human subjects was remarkable for a relative lack of NAB to either virus, with 71% of subjects scoring negative for both AAV-1 and AAV-2. Only 1 of 77 individuals was positive for AAV-1 and not AAV-2 (the titer in this person was only twofold higher than the baseline), whereas 6 individuals (8%) were positive for AAV-2 but not AAV-1. Overall, more patients had NAB to AAV-2 (27%) than to AAV-1 (20%), and the overall titers against AAV-2 appeared to be higher. These data support the hypothesis that AAV-2 infections are present in the human population, although independent primary infections with AAV-1 cannot be ruled out. The lack of monospecific antibodies to AAV-1 argues against this virus infecting humans to any appreciable degree.

Applications of AAV-1 vectors in animal models of gene therapy. AAV-1 could have a role in human gene therapy in situations in which AAV-2 is rendered ineffective because of preexisting NAB, such as in patients with a prior history of AAV-2 infection or those who received gene therapy with an AAV-2 vector. This hypothesis presumes that there is little cross-neutralization *in vivo* between AAV-1 and AAV-2. Experiments were designed to test this hypothesis in the context of AAV-mediated gene transfer to murine liver and muscle. The basic paradigm is to inject AAV-1 or AAV-2 expressing human α 1-antitrypsin and to follow up with a second vector of the same or different serotype and expressing murine erythropoietin. The efficiency of gene transfer is quantitatively measured by ELISA analysis of erythropoietin and α 1-antitrypsin in blood.

An analysis of vector readministration in muscle is presented in Fig. 7. Administration of AAV-2 vectors led to high levels of AAV-2 NAB that blocked the readministration of AAV-2 *in vivo* (group 1). The NAB in these animals did not neutralize AAV-1 *in vitro*, although there was a modest diminution (i.e., fivefold) in the transduction of an AAV-1 vector *in vivo* (group 5) to a level below that achieved in naive animals (group 4). Similarly, AAV-1 vectors resulted in high levels of AAV-1 NAB that blocked AAV-1 readministration *in vivo* (group 2) but did not interfere with AAV-2 vector transduction *in vivo* (group 6), compared to the results for naive animals that received AAV-2 alone (group 3).

Similar studies performed with a model of liver-directed gene transfer, with vectors being injected into the portal circulation via the spleen, gave mixed results (Fig. 8). Previous exposure to AAV-1 does not interfere with AAV-2 gene transfer. However, the opposite is not true. Initial treatment with AAV-2 elicited high levels of NAB to AAV-2 and low levels of NAB to AAV-1 sufficient to suppress the gene transfer of AAV-1 20-fold.

DISCUSSION

AAV vectors have been based primarily on serotype 2, a human-derived parvovirus (19, 23). The early availability of an infectious clone of AAV-2 stimulated work on the development of replication-defective vectors. The utility of AAV-2-based vectors for achieving long-term, stable, and safe gene transfer has been demonstrated with small-animal models; a

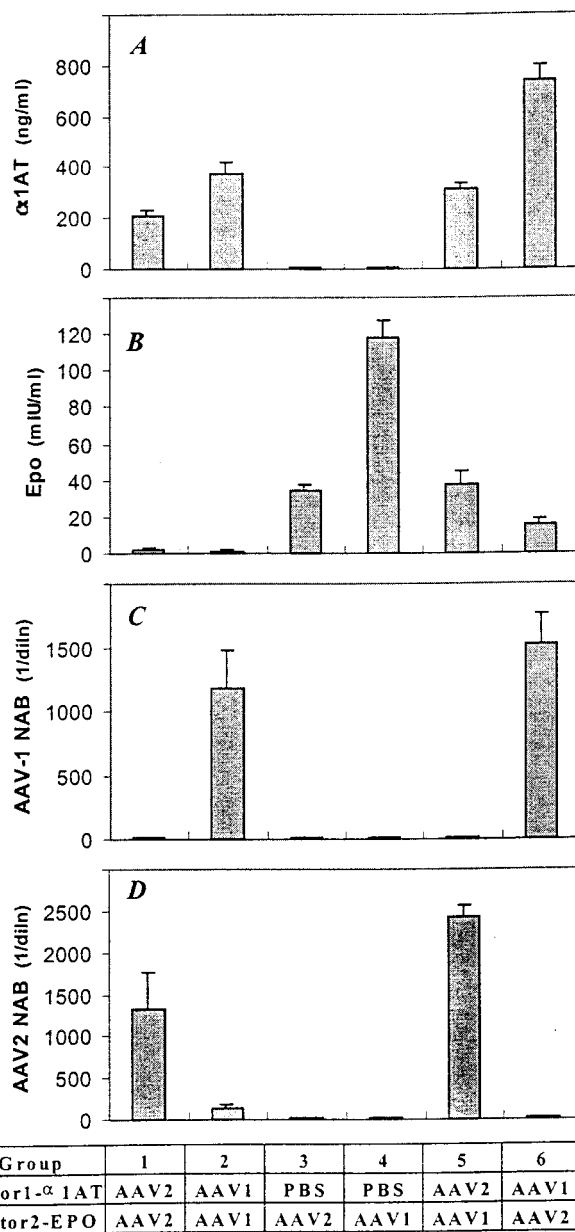


FIG. 7. Readministration of AAV vectors in muscle. C57BL/6 mice were evaluated for AAV-mediated gene transfer following introduction into naive animals as well as 30 days following the first vector administration. The different experimental groups are shown below the graphs. In each case, vector 1 expressed α 1-antitrypsin (α 1AT) from a cytomegalovirus-enhanced β -actin promoter, while vector 2 expressed murine erythropoietin (EPO) from the same promoter. Group 1, AAV-2 followed by AAV-2; group 2, AAV-1 followed by AAV-1; group 3, phosphate-buffered saline (PBS) followed by AAV-2; group 4, PBS followed by AAV-1; group 5, AAV-2 followed by AAV-1; group 6, AAV-1 followed by AAV-2. (A) Serum α 1-antitrypsin 30 days after vector 1 administration. (B) Serum erythropoietin (Epo) measured by an ELISA 30 days after vector 2 administration. (C) Reciprocal dilution (diln) of NAB to AAV-1 at day 30. (D) Reciprocal dilution of NAB to AAV-2 at day 30.

number of studies with large animals, including humans, are either planned or under way (9, 10, 21, 27). The tropism of replication-defective AAV-2 for efficient *in vivo* gene therapy is narrow.

The goal of this study was to isolate an infectious clone of AAV-1, which would be sequenced and used to develop a recombinant vector. The hypothesis was that AAV-1 vectors

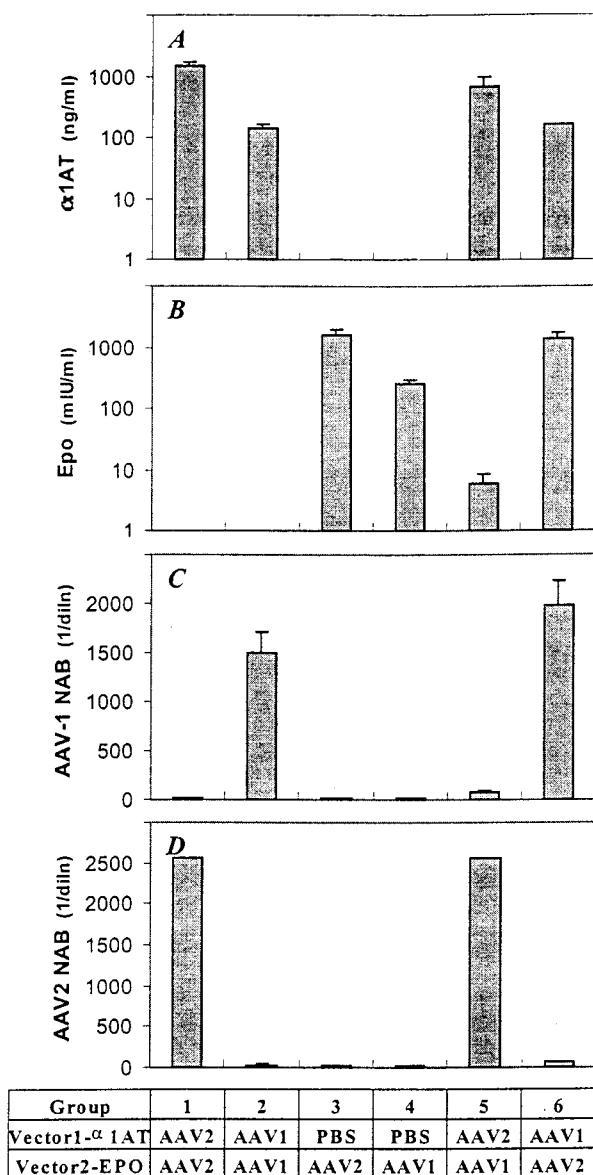


FIG. 8. Readministration of AAV vectors in the liver. These experiments are identical to those described in the legend to Fig. 7, except that animals received vectors in the portal circulation to target the liver.

would have distinct biological profiles with advantages over AAV-2 vectors in some applications. Furthermore, AAV-1 and AAV-2 vectors should not cross-neutralize, providing important advantages when used in the context of preexisting immunity due to a natural infection or prior gene therapy treatment.

An infectious clone of AAV-1 was generated from a replication intermediate. Sequencing of the AAV-1 genome confirmed the presence of the structural components that characterize the AAV family. There is approximately 80% homology in nucleotide sequence between AAV-1 and AAV-2. Delineating the entire sequence of AAV-1 has clarified a potential etiology of AAV-6, which was previously isolated as a contaminant in a human adenovirus preparation. AAV-6 appears to be a hybrid of AAV-1 and AAV-2 formed by homologous recombination between highly conserved regions spanning 452 to 552 nucleotides. There has been minimal divergence from the precursor genomes since this homologous event occurred.

The implications of this observation for gene therapy are unclear. The homologous sequences at the site of potential recombination are located at the 5' end of the Rep gene, which is deleted in the AAV vectors. Recombination of this kind may lead to additional diversity within the AAV family, although it is not clear if AAV-6 resulted from recombination *in vitro* or occurred *in vivo* during naturally acquired infections.

The initial strategy for exploiting the biology of AAV-1 for vectors was to create actual chimeras in which the vector genome was formed from AAV-2 ITRs, whereas the Rep and Cap sequences used to generate the virions were derived from AAV-1. Our goal was to retain the favorable biology of AAV-2 in terms of efficient and stable transduction while modifying the entry pathways and potentially diverting humoral immune responses (8, 31). Analysis of these vectors with murine models of liver and lung gene therapy demonstrated some important differences. When normalized for equivalent doses of administered vector genomes, AAV-2 performs better in liver than in muscle, whereas the opposite is true of AAV-1. This finding presumably is due to differences in the efficiency of entry, although postentry events that differ between the two vectors cannot be ruled out.

Another important advantage of alternative serotypes is to avoid neutralization due to preexisting humoral immunity. The existence of monospecific NAB to AAV-1 in nonhuman primates and to AAV-2 in humans supports the same serospecificity of these AAV. It should be noted, however, that seropositivity to AAV-1 and AAV-2, which is not neutralizing, is detected at higher frequencies with Western assays or ELISAs (data not shown). Administration of the vectors into murine muscle demonstrated the predicted result, in that previous treatment with AAV-2 blocks the subsequent administration of AAV-1 while having only a modest impact on the efficiency of AAV-1 treatment. The opposite is also true, in that AAV-1 administered to muscle blocks a second treatment with AAV-1 but does not interfere with gene transfer mediated by AAV-2. The murine model of AAV gene transfer to liver yielded mixed results. Clearly, AAV-2 and AAV-1 completely blocked readministration of the same serotype. Initial treatment with AAV-1 did not affect a follow-up administration of AAV-2, although the opposite was not true. Specifically, initial treatment with AAV-2 diminished retreatment with AAV-1 approximately 20-fold, a value which will likely be significant in therapeutic applications. We conclude that AAV-2 generates cross-neutralizing antibodies to AAV-1 that have an impact on AAV-1-mediated gene transfer. This effect is more significant in the context of intravascular administration. There is no evidence that antibodies to AAV-1 cross-neutralize AAV-2-mediated gene transfer to muscle or liver.

One should consider these results in the context of human applications of AAV vectors. The majority of humans do not have NAB to either AAV-1 or AAV-2. In fact, the absence of monospecific antibodies to AAV-1 in humans, together with the fact that it was isolated from monkeys, argues against it being a human virus. What is the relevance of neutralizing activity to AAV-1 in 20% of humans? Does this value reflect primary infection or cross-neutralization in the context of infection with wild-type AAV-2? Will this neutralizing activity have an impact on the efficiency of AAV-1 uptake? The data presented here suggest that the AAV-1 vector would be the preferred initial vector for muscle-directed gene therapy. It is more efficient than AAV-2 and does not preclude follow-up treatment with AAV-2. The situation for the liver is less clear. The AAV-1 vector is less efficient in the context of naive animals, although initial treatment with AAV-2 partially inhibits a second administration of AAV-1.

The impact of humoral immune responses to AAV vectors on human applications of gene therapy is an important consideration for chronic diseases. It appears that AAV expression will persist for a prolonged period of time in most target organs. What is less clear is how long the humoral immune response will persist. The use of vectors based on different serotypes should allow at least two treatments. A requirement for more frequent readministration of vectors over short time periods may require other strategies which blunt the initial humoral immune response (12, 16).

ACKNOWLEDGMENTS

We thank the Vector, Cell Morphology and Immunology Cores of the Institute for Human Gene Therapy and Wei Cao, Marcia Houston-Leslie, Rosalind Barr, Ruth Qian, George Qian, and Sarah Ehlen-Haecker for technical support.

This work was supported by grants from the NIH (P30 DK47757-06 and P01 HD32649-04), the Muscular Dystrophy Association, and Genovo, Inc., a company that J. M. Wilson founded and has equity in.

REFERENCES

- Balague, C., M. Kalla, and W. W. Zhang. 1997. Adeno-associated virus Rep78 protein and terminal repeats enhance integration of DNA sequences into the cellular genome. *J. Virol.* **71**:3299–3306.
- Bantel-Schaal, U., and H. zur Hausen. 1984. Characterization of the DNA of a defective human parvovirus isolated from a genital site. *Virology* **134**:52–63.
- Berns, K. I. 1995. Parvoviridae: the viruses and their replication, p. 1007–1041. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fundamental virology*, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
- Chiorini, J. A., L. Yang, Y. Liu, B. Safer, and R. M. Kotin. 1997. Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. *J. Virol.* **71**:6823–6833.
- Clark, K. R., F. Voulgaropoulou, D. M. Fraley, and P. R. Johnson. 1995. Cell lines for the production of recombinant adeno-associated virus. *Hum. Gene Ther.* **6**:1329–1341.
- Clark, K. R., F. Voulgaropoulou, and P. R. Johnson. 1996. A stable cell line carrying adenovirus-inducible rep and cap genes allows for infectivity titration of adeno-associated virus vectors. *Gene Ther.* **3**:1124–1132.
- Fisher, K. J., G. P. Gao, M. D. Weitzman, R. DeMatteo, J. F. Burda, and J. M. Wilson. 1996. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *J. Virol.* **70**:520–532.
- Fisher, K. J., K. Jooss, J. Alston, Y. Yang, S. E. Haecker, K. High, R. Pathak, S. E. Raper, and J. M. Wilson. 1997. Recombinant adeno-associated virus for muscle directed gene therapy. *Nat. Med.* **3**:306–312.
- Flotte, T., B. Carter, C. Conrad, W. Guggino, T. Reynolds, B. Rosenstein, G. Taylor, S. Walden, and R. Wetzl. 1996. A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. *Hum. Gene Ther.* **7**:1145–1159.
- Flotte, T. R., and B. J. Carter. 1997. In vivo gene therapy with adeno-associated virus vectors for cystic fibrosis. *Adv. Pharmacol.* **40**:85–101.
- Gao, G., G. Qu, L. Z. Faust, R. K. Engdahl, W. Xiao, J. V. Hughes, P. W. Zoltick, and J. M. Wilson. 1998. High-titer adeno-associated viral vectors from a Rep/Cap cell line and hybrid shuttle virus. *Hum. Gene Ther.* **9**:2353–2362.
- Halbert, C. L., T. A. Standaert, C. B. Wilson, and A. D. Miller. 1998. Successful readministration of adeno-associated virus vectors to the mouse lung requires transient immunosuppression during the initial exposure. *J. Virol.* **72**:9795–9805.
- Kaplitt, M. G., P. Leone, R. J. Samulski, X. Xiao, D. W. Pfaff, K. L. O'Malley, and M. J. Doring. 1994. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nat. Genet.* **8**:148–154.
- Kotin, R. M., M. Siniscalco, R. J. Samulski, X. D. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns. 1990. Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **87**:2211–2215.
- Li, J., R. J. Samulski, and X. Xiao. 1997. Role for highly regulated rep gene expression in adeno-associated virus vector production. *J. Virol.* **71**:5236–5243.
- Manning, W. C., S. Zhou, M. P. Bland, J. A. Escobedo, and V. Dwarki. 1998. Transient immunosuppression allows transgene expression following readministration of adeno-associated viral vectors. *Hum. Gene Ther.* **9**:477–485.
- Muramatsu, S., H. Mizukami, N. S. Young, and K. E. Brown. 1996. Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. *Virology* **221**:208–217.
- Murphy, F. A., C. M. Fauquet, M. A. Mayo, A. W. Jarvis, S. A. Ghabrial, M. D. Summers, G. P. Martelli, and D. H. L. Bishop. 1995. Classification and nomenclature of viruses: sixth report of the International Committee on Taxonomy of Viruses. *Arch. Virol.* **1995**:169–175.
- Muzyczka, N. 1992. Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr. Top. Microbiol. Immunol.* **158**:97–129.
- Parks, W. P., D. W. Boucher, J. L. Melnick, L. H. Taber, and M. D. Yow. 1970. Seroepidemiological and ecological studies of the adenovirus-associated satellite viruses. *J. Virol.* **2**:716–722.
- Rubenstein, R. C., U. McVeigh, T. R. Flotte, W. B. Guggino, and P. L. Zeitlin. 1997. CFTR gene transduction in neonatal rabbits using an adeno-associated virus (AAV) vector. *Gene Ther.* **4**:384–392.
- Rutledge, E. A., C. L. Halbert, and D. W. Russell. 1998. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. *J. Virol.* **72**:309–319.
- Samulski, R. J. 1993. Adeno-associated virus: integration at a specific chromosomal locus. *Curr. Opin. Genet. Dev.* **3**:74–80.
- Samulski, R. J., K. I. Berns, M. Tan, and N. Muzyczka. 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc. Natl. Acad. Sci. USA* **79**:2077–2081.
- Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka. 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. *Cell* **33**:135–143.
- Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook, D. E. Housman, N. Epstein, and L. A. Hunter. 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J.* **10**:3941–3950. (Erratum, 11:1228, 1992.)
- Snyder, R. O., C. H. Miao, G. A. Patijn, S. K. Spratt, O. Danos, D. Nagy, A. M. Gown, B. Winther, L. Meuse, L. K. Cohen, A. R. Thompson, and M. A. Kay. 1997. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat. Genet.* **16**:270–276.
- Song, S., M. Morgan, T. Ellis, A. Poirier, K. Chesnut, J. Wang, M. Brantly, N. Muzyczka, B. J. Byrne, M. Atkinson, and T. R. Flotte. 1998. Sustained secretion of human alpha-1-antitrypsin from murine muscle transduced with adeno-associated virus vectors. *Proc. Natl. Acad. Sci. USA* **95**:14384–14388.
- Surosky, R. T., M. Urabe, S. G. Godwin, S. A. McQuiston, G. J. Kurtzman, K. Ozawa, and G. Natsoulis. 1997. Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. *J. Virol.* **71**:7951–7959.
- Xiao, W., S. C. Berta, M. M. Lu, A. D. Moscioni, J. Tazelaar, and J. M. Wilson. 1998. Adeno-associated virus as a vector for liver-directed gene therapy. *J. Virol.* **72**:10222–10226.
- Xiao, W., and J. M. Wilson. Unpublished data.
- Xiao, X., J. Li, and R. J. Samulski. 1996. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J. Virol.* **70**:8098–8108.
- Xiao, X., J. Li, and R. J. Samulski. 1998. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J. Virol.* **72**:2224–2232.