

Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein

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Abstract

We demonstrated the presence of two adeno-associated viruses (AAVs), designated AAV10 and AAV11, in cynomolgus monkeys by isolating and sequencing the entire viral coding regions from the monkey DNA. AAV10 and AAV11 capsid proteins shared 84% and 65%, respectively, of amino acids with AAV2. A phylogenetic analysis of AAV capsid proteins showed that AAV10 and AAV11 resembled most AAV8 and AAV4, respectively. To characterize the capsid protein, we pseudotyped an AAV2 vector with the monkey AAV capsid proteins and examined the resulting pseudotypes AAV2/10 and AAV2/11, in comparison with the AAV2 vector, for their host ranges in cell lines and tissue tropism in mice. AAV2/10 and AAV2/11 transduced primate cells less efficiently than AAV2. Whereas AAV2 transduced undifferentiated C2C12 mouse myoblasts more efficiently than differentiated ones, AAV2/10 and AAV2/11 transduced the undifferentiated myoblasts less efficiently than differentiated ones. Three weeks after injection to the muscle of the hind legs, AAV2/10 and AAV2 induced transgene expression similarly, but AAV2/11 did not transduce the skeletal muscle. Six weeks after systemic administration, transduced vector DNA was detected by PCR in the liver and spleen of mice inoculated with AAV2, in the liver, heart, muscle, lung, kidney, and uterus of mice with AAV2/10, and the muscle, kidney, spleen, lung, heart, and stomach of mice with AAV2/11. Mouse antisera against capsid protein VP2 of the three AAVs neutralized the respective vector particles in a type-specific manner. The results indicate that AAV10 and AAV11 capsid proteins, which are antigenically distinct from each other and AAV2, are likely to determine their host ranges and tissue tropism that are different from AAV2s, suggesting that cynomolgus AAVs could provide a broader choice of pseudotype AAV vectors for gene therapy.

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Keywords: AAV vector; AAV10; AAV11

Introduction

Adeno-associated virus (AAV), a nonenveloped small DNA virus, belongs to the genus *Dependovirus* of the family Parvoviridae and usually requires coinfection with a helper virus such as adenovirus for its replication (Muzyczka and Berns, 2001). Among eight primate AAV serotypes known

to occur to date, human AAV2 has been most extensively studied for its genomic structure and gene functions. The genome, 4.7 kb single-stranded linear DNA, has two large open reading frames (ORFs); the left ORF encoding the nonstructural Rep proteins and the right ORF, the capsid proteins VP1, VP2, and VP3. At each end of the genome is a 145 base region (inverted terminal repeat, ITR) containing the viral origin of DNA replication and the packaging signal (Muzyczka and Berns, 2001). Upon infection, AAV2 was believed to specifically integrate its genome into human chromosome 19; thus, it has been studied for its potential use as a gene-transfer vector in gene therapy.

Pseudotyping the AAV2 vector with capsids from AAVs other than AAV2 has been conducted to address

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some of the problems that AAV2 inherently has, host range and antigenicity (both determined by the capsid) (Rabinowitz et al., 2002). Development of pseudotyped vectors would require more information on AAVs and preferably more new isolates. AAV1, 2, 3, 4, and 6 were initially found as contaminants in laboratory adenovirus stocks (Atchison et al., 1965; Hoggan and Blacklow, 1966; Melnick et al., 1965). The natural host of AAV1 is unclear at present. AAV2 and AAV3 were later isolated from human infants. AAV4 was isolated from a culture of a rhesus monkey kidney. AAV5 was isolated from a human penile condylomatous wart (Bantel-Schaal and zur Hausen, 1984; Georg-Fries et al., 1984). AAV6 appears to be a recombinant between AAV1 and AAV2 (Xiao et al., 1999). AAV7, AAV8, and AAV9 have been recently found in rhesus monkeys (Gao et al., 2002, 2004). A recent study suggests that many AAVs as yet to be identified are widely disseminated throughout multiple tissues of nonhuman primate species (Gao et al., 2003, 2004).

In this study, we isolated and sequenced coding regions of two novel AAV sequences, AAV10 and AAV11, from the tissues of cynomolgus monkeys. We pseudotyped an AAV vector, using capsids of these AAVs, and compared the resulting vectors with the AAV2 vector for their infectivity on cultured cell lines, transduction efficiencies in the mouse skeletal muscle, and organ tropism in mice. We prepared mouse antisera against VP2 of three AAVs and examined them for their cross-neutralization of the three vectors and vectors of AAV4 and AAV8, of which amino acid sequences resembled those of AAV10 and AAV11, respectively.

Results

Isolation of DNA fragments encoding cap genes of AAV10 and AAV11

The alignment of the genomes of known eight types of AAV shows that several regions have common nucleotide sequences. The common sequences were used to design the consensus primers, CP1 to CP5 (Fig. 1A), for PCR to amplify unidentified AAV genomes putatively contained in DNA samples that were extracted from five cynomolgus monkeys. CP1 and CP2 are primers to amplify the “signature regions (Gao et al., 2002)”, of which nucleotide sequences are highly type specific.

Two novel AAV “signature regions” were found in the DNA samples extracted from liver (animals #1, #4, and #5) and heart (#5). The DNA fragments amplified by PCR were cloned and sequenced. The nucleotide sequences of the clones did not agree with those of the signature regions of the known AAVs, indicating that they were derived from two novel AAVs; thus designated AAV 10 and AAV11. The sequence of AAV10 was found in DNA samples extracted

from the livers of #1, #4, and #5 and the heart of #5. The sequence of AAV11 was found in DNA samples extracted from the livers of #1 and #5.

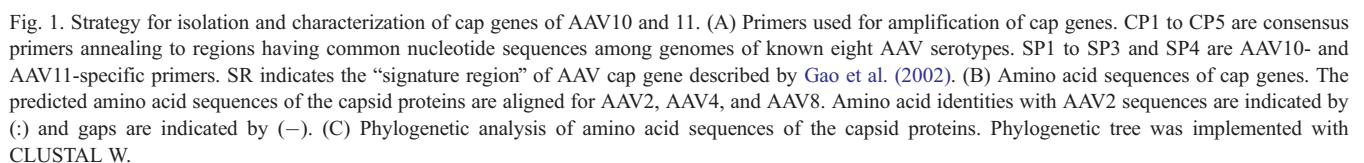
The entire coding region of AAV10 was obtained by three sets of PCR (Fig. 1A). The DNA sample extracted from the intestine of #4, which contained relatively abundant copies of AAV10 “signature region”, was used for the template to amplify the coding region of AAV10 DNA. The DNA fragment (10-A) was obtained by using CP3 and SP1 that were complementary to the central region of the AAV10 “signature region”. Based on the nucleotide sequences of 10-A, two AAV10-specific primers (SP2 and SP3) were designed. The 5′ region of the coding region (10-B) was obtained by using SP2 and CP4 and the 3′ region of the coding region (10-C) was obtained by using SP3 and CP5. The nucleotide sequences of 3′ region of 10-B were identical with those of 5′ region of 10-A and the nucleotide sequences of 3′ region of 10-A were identical with those of 5′ region of 10-C. 10-A and 10-C were connected at *Bsu*36I site (Fig. 1A), and the resultant DNA fragment was used to generate AAV2/10 (GenBank accession number for AAV10: AY631965).

The entire coding region of AAV11 was obtained by two sets of PCR using the DNA sample extracted from the liver of #5 (Fig. 1A). The 5′ region of the coding region (11-A) was amplified by using CP4 and SP4 that were complementary to the central region of the AAV11 “signature region”. The 3′ region of the coding region (11-B) was obtained by using CP3 and CP5. The nucleotide sequences of 3′ region of 11-A were identical with those of 5′ region of 11-B (GenBank accession number for AAV11: AY631966).

The amino acid sequences of capsid protein of AAV10 and AAV11 were deduced from the nucleotide sequences (Fig. 1B). AAV10 and AAV11 capsid proteins shared 84% and 65%, respectively, of amino acids with AAV2. Phylogenetic analysis showed that AAV10 and AAV11 were closely related to AAV8 and AAV4, respectively (Fig. 1C).

Production of pseudotype vectors AAV2/10 and AAV2/11

Pseudotype AAV vectors AAV2/10 and AAV2/11 were generated by the cross-packagings of the AAV2 vector genome expressing a reporter gene, the β -galactosidase (β -gal) gene or a gene encoding the enhanced green fluorescence protein (EGFP) fused with alpha-tubulin, into capsids of AAV10 and AAV11. The helper/packaging plasmids for AAV2/10 and AAV2/11 were constructed by replacement of the 3′ region of AAV2 genome with those of AAV10 or AAV11 genome (Fig. 2). The pseudotyped vectors were purified by CsCl-equilibrium density gradient centrifugation. The density of AAV2/11 particles was slightly higher than those of the AAV2 vector and AAV2/10 particles. Although the AAV2 vector bound to heparin-agarose as previously reported, AAV2/10 and AAV2/11 did not bind. AAV2/10 and AAV2/11 bound to anion-exchange resin in a buffer with 0.1 M NaCl. AAV2/



AAV2/10 and AAV2/11 were capable of transducing various human and monkey cells. Among the conventional

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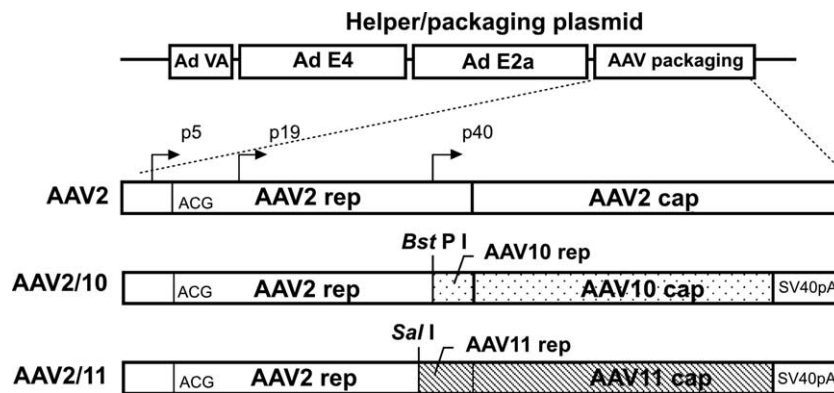


Fig. 2. Schematic representation of the helper and packaging plasmids used for producing the pseudotyped vectors. The helper/packaging plasmid for the production of AAV2 vector was constructed by subcloning of E2a, E4, and VA genes of adenovirus type 5, rep and cap genes, and polyadenylation signal of AAV2 into pSP72 (Promega). To enhance the efficiency of vector production, the first ATG of rep gene was mutated to ACG (Xiao et al., 1998). To produce AAV2/10 and AAV2/11 pseudotype vectors, the entire cap and 3' end of rep genes of AAV2 were replaced with those of AAV10 and AAV11, respectively. The *Bst*PI or *Sal*I sites of the AAV2 rep gene was used for the replacement of AAV2 sequence with AAV10 and AAV11 sequence, respectively. The polyA signal of SV40, indicated as SV40pA, was located downstream of the cap gene.

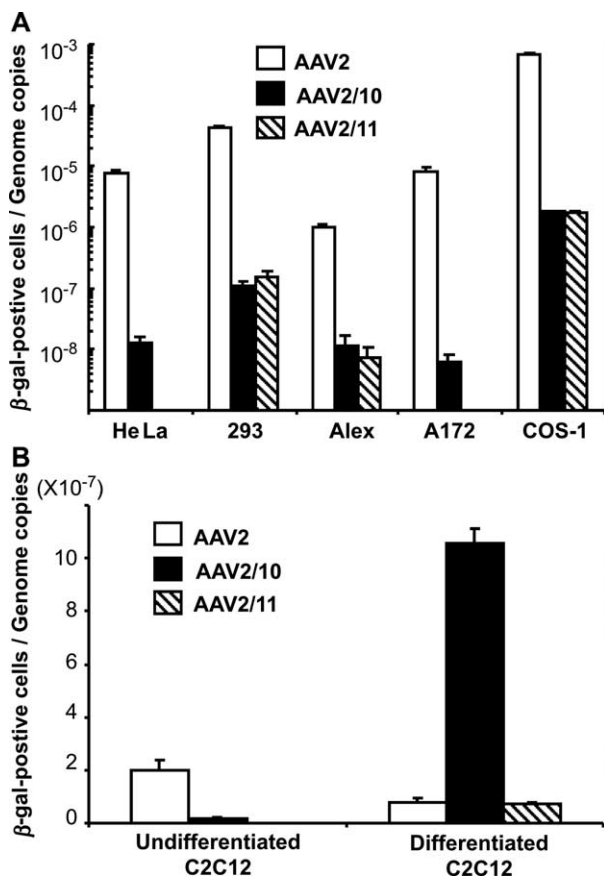


Fig. 3. Transduction efficiency of AAV2 and the pseudotyped vectors. Cells were inoculated with the serially diluted vectors capable of expressing β -galactosidase (β -gal). Two days later, cells positive for β -gal were counted. Each bar represents the average of three separate inoculations, with the standard deviation indicated by an error bar. (A) Cells cultured with the conventional method. (B) C2C12 mouse myoblasts were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum as undifferentiated cells. When cells became confluent, the medium was changed into DMEM with 2% horse serum and maintained for 10 days to obtain differentiated cells.

C2C12 cells, mouse myoblast cells, remain in an undifferentiated state when cultured in medium containing 10% fetal bovine serum (FBS). When the confluent culture of the cells is maintained in medium containing 2% horse serum for several days, the cells differentiate (Blau et al., 1983). The AAV2 vector transduced the undifferentiated C2C12 cells more efficiently than the differentiated C2C12 (Fig. 3B). AAV2/10 transduced the undifferentiated cells only marginally but transduced the differentiated cells more efficiently than the AAV2 vector (Fig. 3B). AAV2/11 did not transduce the undifferentiated cells but transduced the differentiated cells as efficiently as the AAV2 vector did (Fig. 3B).

Injection of the vectors into the hind leg muscle of BALB/c mice

The result of the transduction of C2C12 led us to examine the transduction efficiencies of the skeletal muscle with the pseudotyped vectors. The AAV vectors capable of expressing β -gal (5×10^8 genome copies) were injected into the tibialis anterior of 4-week-old BALB/c mice. Three weeks later, the levels of β -gal were measured (Fig. 4). Extracts from the muscles injected with AAV2/10 showed β -gal activities comparable to that shown with the AAV2 vector. AAV2/11 did not transduce the skeletal muscle.

Systemic administration of the vectors to BALB/c mice

Tissue distribution of the vector genome significantly varied with the vector in mice systematically administered with the AAV2 vector, AAV2/10, and AAV2/11. BALB/c mice were injected with the vectors (10^9 genome copies per animal) via tail vein. One and six weeks later, the tissues were harvested and analyzed for the presence of vector genomes by semi-quantitative PCR. Fig. 5 shows the

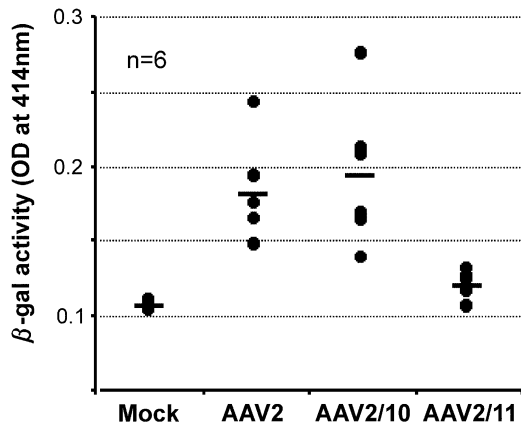


Fig. 4. Transduction of mouse skeletal muscle injected with AAV2, AAV2/10, and AAV2/11 vectors capable of expressing β -galactosidase. AAV vectors of 5×10^8 genome copies or physiological saline (for a mock-inocula) was injected into the tibialis anterior of 4-week-old BALB/c mice (three animals: six legs). Three weeks after the injection, the β -gal activities were measured.

presence of vector genomes in 500 ng DNA extracted from the tissues.

In mice injected with the AAV2 vector, the vector genomes were found in the liver, spleen, kidney, and muscle at 1 week. At 6 weeks, the vector genomes in the kidney and muscle disappeared and the genomes were mainly present in the liver and spleen. Low levels of the genome were found in the lung, muscle, and heart. In mice injected with AAV2/10, the vector genomes were found in all the tissues examined at 1 week. At 6 weeks, the genomes were found in the liver, heart, muscle, lung, kidney, and uterus. In

mice injected with AAV2/11, the vector genomes were found in all the tissues examined at 1 week. At 6 weeks, the genomes were found in the muscle, kidney, spleen, lung, heart, and stomach. Noteworthy is that the genome in the liver was marginal.

Neutralization of the pseudotyped vectors with antibodies against VP2 of AAV2, AAV10, and AAV11

Mouse anti-AAV2, AAV10, and AAV11 were raised by immunizing BALB/c mice with purified VP2 of AAV2, AAV10, and AAV11. The amino acid sequence of VP2 includes the entire VP3 (the major capsid protein) sequence and the great majority of VP1 sequence. Since amino acid sequences of VP2s of AAV10 and AAV11 resemble those of AAV8 and AAV4, respectively (Fig. 1C), similarly produced AAV2/8 (Gao et al., 2004) and AAV2/4 (Rabinowitz et al., 2002) were included in neutralization tests. Neutralizing activities of the antisera were examined by testing the ability to inhibit transduction of COS-1 cells by these vectors. Anti-AAV2 and anti-AAV10 showed type-specific neutralization. Anti-AAV11 did not neutralized AAV2 and AAV10 but showed low level of cross-neutralizing activities for AAV4.

Discussion

Previously described “signature regions (Gao et al., 2002)”, which have highly type-specific nucleotide sequences and are flanked by nucleotide sequences common to the

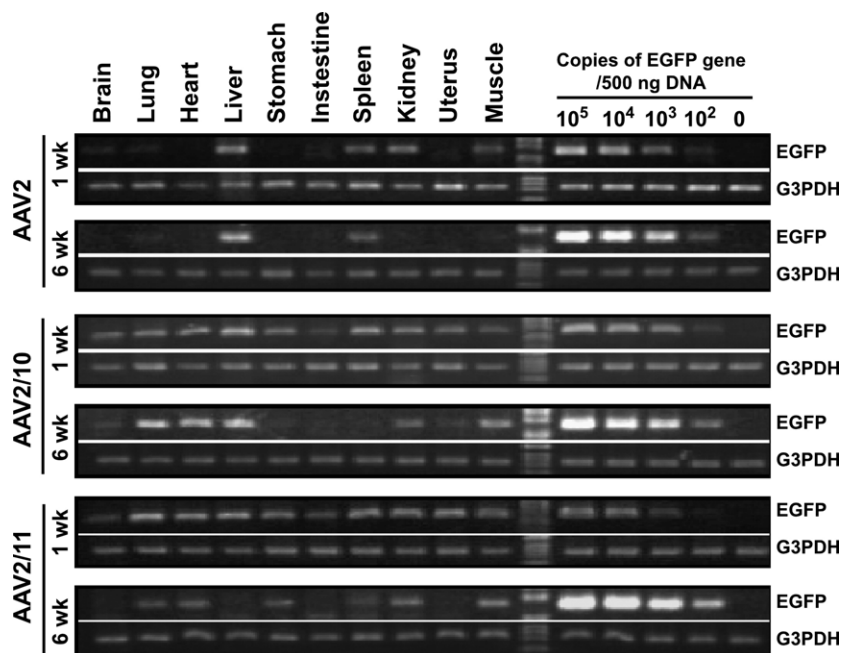


Fig. 5. Distribution of AAV vector genomes in murine tissues. BALB/c mice were inoculated with vectors having enhanced green fluorescence protein (EGFP) gene via tail vein. One and six weeks later, mice were sacrificed and the tissues were harvested. Genomic DNA was isolated from tissues indicated and AAV genomes in the DNA samples were amplified by semi-quantitative PCR using primers for EGFP gene. The amplified DNA fragment was analyzed by agarose gel electrophoresis. G3PDH gene was amplified for references. Results obtained by two animals were very similar.

known AAV serotypes, enabled us to search for unidentified AAV sequences in cynomolgus monkeys. By amplifying the signature regions, we found two novel AAV sequences, designated AAV10 and AAV11, using the DNA samples obtained from five adult monkeys; two had AAV10, two had both AAV10 and AAV11, and one had neither AAV10 nor AAV11. From the available records on breeding and maintenance of the monkeys, it was not clear how the animals had been infected with these AAVs.

Sequencing the cloned viral coding regions, particularly the capsid gene, revealed the similarity and difference between AAV2 and the cynomolgus AAVs, which turned out to resemble most of the rhesus AAVs (Fig. 1). Capsids play important roles in several steps of infection; binding with a cellular receptor, trafficking to the nucleus, and uncoating. Capsid is also the target of neutralizing antibodies. It is interesting to know whether the structural difference of capsid protein affects the functions of a capsid. To characterize AAV10 and AAV11 capsid proteins, we pseudotyped the AAV2 vector with the cynomolgus AAV capsids (Fig. 2) and compared the resulting pseudotyped vectors AAV2/10 and AAV2/11 with the AAV2 vector for their biological functions (Figs. 3–5). Studies of such pseudotyped AAV2 vectors having capsids of AAV1 and 3–8 show that they have tissue tropism different from that of the AAV2 vector and that they are not neutralized by anti-AAV2 sera (Grimm and Kay, 2003).

In this study, newly produced pseudotypes AAV2/10 and AAV2/11 showed that their tissue tropism is different from and apparently broader than that of the AAV2 vector in mice. AAV2/10 transduced the differentiated C2C12, a mouse myoblast line, more efficiently than undifferentiated C2C12, although the AAV2 vector transduced differentiated C2C12 much less efficiently than undifferentiated C2C12 (Fig. 3). Similarly, AAV2/11 transduced the differentiated cells more efficiently than undifferentiated cells, although the level of the transduction was low (Fig. 3). Probably, the capabilities of AAV2/10 and AAV2/11 to transduce the differentiated C2C12 are related with the expression of the transgene in the skeletal muscle injected with AAV2/10 and AAV2/11 (Fig. 4). The tropism to the muscle was also observed with AAV2/10 and AAV2/11 after systemic administration of vectors (Fig. 5). Previous studies indicate that the pseudotyped AAV2 vectors having capsids of AAV1, AAV5, and AAV7 transduce the mouse skeletal muscle more efficiently than the AAV2 vector (Chao et al., 2000; Duan et al., 2001; Gao et al., 2002; Hildinger et al., 2001).

The mouse antibodies against AAV2, AAV10, and AAV11 were not cross-neutralizing one another (Table 1). This finding indicates that AAV2/10 and AAV2/11 apparently have the advantage of not being neutralized with the antibody against AAV2 when used as a gene-transfer vector for humans. Although capsid amino acid sequences of AAV10 and AAV11 resemble those of AAV8 and AAV4, respectively, anti-AAV10 did not cross-neutralized AAV8

Table 1

Cross reactivity of anti-VP2 antisera

Antiserum	Vector				
	AAV2	AAV2/4	AAV2/8	AAV2/10	AAV2/11
AAV2	160	<10	<10	<10	<10
AAV10	<10	<10	40	2560	10
AAV11	<10	160	<10	<10	2560

Antibody titer was presented as the reciprocal of the highest dilution that repressed number of β -gal positive cells to half of the number obtained with the sample mixed with the similarly diluted serum from nonimmunized mice.

(1:40 in Table 1) and anti-AAV11 showed a very low level of cross-neutralization for AAV4 (1:160 in Table 1).

These results, taken together with their characteristic tissue tropism, suggest that AAV10 and AAV11 could provide a broader choice of pseudotyped AAV vectors for trials of gene therapy.

Materials and methods

Animals and tissues

Tissues of cynomolgus macaques were obtained from the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases. BALB/c mice were purchased from Nihon SLC Co. Ltd. (Shizuoka, Japan). All animal studies were performed in accordance with the guidelines of the National Institute of Infectious Diseases.

Cells

HEK293 (human embryonic kidney cells transformed by adenovirus type 5), HeLa (human cervical cancer cells), Alexander (human hepatoma cells), C2C12 cells (mouse myoblast), COS-1 (CV1 cells positive for SV40 T), and A172 (human glioblastoma) were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and cultured in 5% CO₂ at 37 °C. Sf9 insect cells were grown in SFM medium supplemented with 3% FBS at 27 °C.

Extraction of DNA from tissues

Animal tissues were harvested at necropsy and stored at –80 °C until use. DNA was extracted from approximately 25 mg of each frozen tissue by using QIAamp DNA extraction kit (Qiagen GmbH, Hilden, Germany).

PCR to amplify DNA fragments of AAV10 and AAV11

The consensus primers CP1 and CP2 used for amplification of the “signature region” of AAV cap gene were described by Gao et al. (2002). Other consensus primers used in this study were the following: CP3, 5'-

GGTGC GTAACTGGACCAATGAGAAC; CP4, 5'-TTG-AACGAGCAGCAGCCATG; CP5, 5'-CGCAGAGACCA-AAGTTCAACTGAAACGA. AAV10-specific primers were the following: SP1, 5'-GTGATGACTCTGTGCGCCAG; SP2, 5'-AGGACTTGCACTTTTGGTCG; SP3, 5'-ATGGC-TGCAGGCGGTGGCGCT. AAV11-specific primer SP4 was 5'-TGGTTGTTGTAGGTGGGCAAGA.

PCR consisted of the initial heating at 94 °C for 5 min, 40 cycles of incubation at 94 °C for 30 s plus at 68 °C for 2 or 5 min, and the final incubation at 68 °C for 7 min. PCR products were purified by gel filtration and cloned into pGEM-T Easy vector (Promega corporation, Madison, WI). Nucleotide sequence was analyzed with an ABI-PRISM 310 sequencer (PE Applied Biosystems, Foster City, CA). Consensus sequences of at least five clones obtained by independent PCR amplifications were selected as the sequences of AAV10 and 11.

Production and purification of pseudotyped AAV vectors

AAV vectors were produced by cotransfection of vector plasmid (pAAV β -gal or pAAVEGFP_{tub}) and helper/packaging plasmid (pEEV2 for AAV2 vector, pEEV2/10 for pseudotyped AAV10 vector, pEEV2/11 for pseudotyped AAV11 vector, pEEV2/4 for pseudotyped AAV4 vector, and pEEV2/8 for pseudotyped AAV8 vector). pAAV β -gal had a DNA region composed of the inverted terminal repeats (ITR) of AAV2, the expression unit composed of cytomegalovirus immediate early promoter, β -gal gene, and another ITR. pAAVEGFP_{tub} had a DNA region composed of ITR, the human elongation factor I α promoter, a fusion gene of enhanced green fluorescence protein gene and α -tubulin gene, and another ITR. pEEV2 was constructed by subcloning of E2a, E4, and VA genes of adenovirus and rep and cap genes and polyadenylation signal of AAV2 into pSP72 (Promega). pEEV2/10 had a 2/10-chimeric rep gene (5' end to the *Bst*PI site of AAV2 rep gene plus the *Bst*PI site to 3' end of AAV10 rep gene), AAV10 cap gene, and SV40 polyadenylation signal. pEEV2/11 had a 2/11-chimeric rep gene (5' end to the *Sal*I site of AAV2 rep gene plus the *Sal*I site to 3' end of AAV11 rep gene), AAV11 cap gene, and the SV40 polyadenylation signal. pEEV2/4 had a 2/4-chimeric rep gene (5' end to the *Sal*I site of AAV2 rep gene plus the *Sal*I site to 3' end of AAV4 rep gene), AAV4 cap gene, and the SV40 polyadenylation signal. The AAV4 region was obtained from AAV4 DNA (ATCC No. VR-646). pEEV2/8, which had AAV2 rep gene and AAV8 cap gene and polyadenylation signal, was constructed from pAAV2/8 (Gao et al., 2002).

Subconfluent cultures of HEK293 cells in 15-cm dish (10 dishes) were transfected with a mixture of 250 mg of vector plasmid and 250 mg of helper/packaging plasmid by the calcium phosphate coprecipitation method. Three days later, the cells were collected by a low-speed centrifugation and resuspended with phosphate-buffered saline (PBS) contain-

ing 10 mM MgCl₂. The suspension was frozen and thawed three times, followed by a brief sonication, and centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was incubated with Benzonase (50 U/ml) for 30 min at 37 °C, then mixed with CsCl to a final density of 1.41 g/ml, and centrifuged in a SW50.1 swinging bucket rotor (Beckman Coulter Inc., Fullerton, CA) at 36000 rpm for 42 h at 20 °C. Fractions containing the vectors were collected, dialyzed against PBS or physiological saline at 4 °C to remove CsCl, and stored at 4 °C as a vector stock. Genome copy numbers of the vector stocks were measured by real-time PCR using TaqMan probes (Perkin-Elmer Biosystems, Foster City, CA).

Transduction of cultured cells with the AAV vectors

Cells, except for C2C12 cells, were seeded in 48-well plates at a density of 1×10^4 cells/well. Next day, cells were inoculated with AAV vectors diluted serially (from 2×10^8 to 2×10^4 genome copies). Cells were incubated for 2 h with occasional rocking at 37 °C, and then fresh medium was added to each well. Two days after the inoculation, the cells were fixed and stained for β -gal expression by In Situ β -Galactosidase Staining Kit (Stratagene, La Jolla, CA). The cells positive for β -gal were counted under light microscopy.

In the case of C2C12 cells, undifferentiated cells (2×10^4 cells/well) and differentiated cells (confluent cells were cultured in DMEM containing 2% horse serum for 10 days) in 48-well plate were inoculated with AAV vectors diluted serially (from 2×10^8 to 2×10^4 genome copies). Three days after inoculation, β -gal-positive cells were counted similarly.

Transduction of the skeletal muscle with the AAV vectors

For muscle-directed transduction, 5×10^8 genome copies of AAV vectors having β -gal gene were injected into the tibialis anterior of 4-week-old BALB/c mice. Three weeks after injection, mice were sacrificed and lower legs were collected. The lower leg was homogenized in 500 μ l of lysis buffer (0.25 M Tris pH 8.0) by Mixer Mill MM300 (Qiagen) at 25 Hz for 2 min. After centrifugation at 12000 rpm for 5 min at 4 °C, 30 μ l of the supernatant was used to measure β -gal activity by using β -Gal Assay kit (Invitrogen) according to the manufacture's protocol. Briefly, the sample was mixed with 200 μ l of cleavage buffer and 70 μ l of *o*-Nitrophenyl- β -D-Galactopyranoside and incubated at 37 °C for 16 h. To stop the reaction, 500 μ l of stop buffer was added, and the absorbance of the reaction mixture was measured at 414 nm.

Tissue distribution of the AAV vectors in mice

Eight-week-old female BALB/c mice (four animals per group) were injected with the AAV vectors (1×10^9 genome

copies) having EGFP-tubulin fusion gene from tail vein. At 1 and 6 weeks after injection, two animals in a group were sacrificed. At necropsy, organs were collected, frozen immediately in a dry ice, and stored at -80°C . DNA (500 ng) was extracted from the organ and used for the template of PCR to amplify EGFP gene with primers (the forward primer: 5'-TTCATCTGCACCACCGGCAA, the reverse primer: 5'-CCGTCCTCCTTGAAGTCGAT). For the internal control, the G3PDH gene was amplified by using the primer set described previously (Mori et al., 2002). PCR consisted of the initial heating at 94°C for 5 min, 34 cycles (for amplification of EGFP gene) or 20 cycles (for amplification of G3PDH gene) of incubation at 94°C for 30 s plus at 68°C for 1 min, and incubation at 68°C for 5 min.

Production of antiserum against VP2 of AAV2, AAV10, and AAV11

The VP2 protein was expressed in insect Sf9 cells by using recombinant baculovirus system (Bac system, Invitrogen). The VP2 coding region was inserted downstream of the polyhedrin promoter of plasmid pFastBac1 (Invitrogen). The plasmid was introduced into *Escherichia coli* DH10Bac-competent cells (Invitrogen) that contain Bacmid DNA and the helper plasmid to obtain recombinant Bacmid DNA. The recombinant baculovirus was produced in Sf9 cells transfected with the recombinant Bacmid DNA. For large-scale expression of VP2 protein, Sf9 cells (five bottles of 175-cm^2 culture flask) were infected with the recombinant baculovirus at multiplicity of infection (MOI) of 10 and incubated for 4 days at 27°C . The cells were collected and suspended in 5 ml PBS containing 0.5% NP-40. After incubation on ice for 15 min, the cells were centrifuged at $10000 \times g$ for 15 min at 4°C to precipitate nuclei. The nuclei were washed with PBS containing 0.5% NP-40, resuspended with 2 ml PBS containing the protease inhibitor cocktail (Roche Diagnostics Co. Ltd., Mannheim, Germany), and lysed with brief sonication. The lysate was layered onto sucrose cushions of 30% and 5% sucrose (wt/vol) in PBS and centrifuged at $120000 \times g$ for 2.5 h at 4°C in a SW50.1 rotor (Beckman Coulter Inc.). The precipitate, which contained majority of VP2, was resuspended in PBS containing CsCl (1.32 g/ml) and the protease inhibitor cocktail (Roche Diagnostics Co. Ltd.) and lysed with brief sonication. The lysate was centrifuged at 35000 rpm for 20 h at 20°C with an SW50.1 rotor (Beckman Coulter Inc.). The fractions with a visible protein band was collected and dialyzed against physiological saline at 4°C to remove CsCl.

Eight-week-old female BALB/c mice (five animals) were immunized with VP2 protein (20 $\mu\text{g}/\text{animal}$) emulsified with TiterMax Gold (TiterMax USA INC., Norcross, GA) twice at an interval of 2 weeks subcutaneously. At 2 weeks after the second immunization, mice were boosted with 5 μg of VP2 protein by intravenous injection. Serum was

collected from five animals at 4 days after the booster injection and pooled.

Neutralizing activity of antiserum

Neutralizing activities of the mouse anti-VP2 serum were measured by testing inhibition of transduction of COS-1 cells by the AAV vectors expressing β -gal. COS-1 cells (1×10^5 cells/well) were seeded in 24-well plate 1 day before inoculation. One hundred and ten microliters of vector solution (2×10^3 transducing unit/ml) was mixed with 110 μl of the serum that had been serially diluted from 1:10 to 1:40960 with PBS and incubated for 1 h at 37°C . Then, the sample was mixed with 200 μl of DMEM containing 10% FBS and inoculated to the cells in two wells (200 $\mu\text{l}/\text{well}$). After incubation with occasional rocking for 2 h at 37°C , 500 μl of fresh DMEM containing 10% FBS was added to each well. Two days later, cells positive for β -gal were detected as described above. Neutralizing titer of the antiserum was expressed as the reciprocal of the highest dilution that repressed number of β -gal positive cells to half of the number obtained with the samples mixed with the similarly diluted serum from nonimmunized mouse.

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