

Efficient Production of Adeno-associated Virus Vectors Using Split-type Helper Plasmids

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Adeno-associated virus (AAV) vectors are potentially useful vehicles for the delivery of therapeutic genes into human cells. To determine the optimal expression pattern of AAV proteins (Rep78, Rep68, Rep52, Rep40, and Cap proteins) for packaging the recombinant AAV genome, helper plasmids were split into two portions. In this study, two sets of split-type helper plasmids were prepared; i.e., 1) a Rep expression plasmid (pRep) and Cap expression plasmid (pCap), and 2) a large Rep expression plasmid (pR78/68) and small Rep plus Cap expression plasmid (pR52/40Cap). When AAV vectors were produced using these sets of split-type helper plasmids at various ratios, the optimal ratio of (large) Rep expression plasmid and Cap expression plasmid was 1 to 9 for both sets. More importantly, the titers were comparable to or even higher than that of a conventional helper plasmid (pIM45) ($4.9 \pm 2.1 \times 10^{11}$ vector particles/10 cm dish for pRep and pCap; $2.9 \pm 1.6 \times 10^{11}$ vector particles/10 cm dish for pR78/68 and pR52/40Cap; and $1.8 \pm 0.16 \times 10^{11}$ particles/10 cm dish for pIM45). Western analysis of AAV proteins suggests that the expression of a relatively small amount of large Rep and a large amount of Cap is important for optimal vector production. The present study shows that the AAV helper plasmid can be split without losing the ability to package the recombinant AAV genome, and provides us with valuable basic information for the development of efficient AAV packaging cell lines.

Key words: AAV vector — Helper plasmid — Rep — Cap — Gene therapy

Adeno-associated virus (AAV) has received increased attention because of its potential as a vector for human gene therapy. AAV is naturally defective, requiring coinfection with a helper virus such as adenovirus or herpes simplex virus to establish a productive infection,¹⁻³ and no human disease has been found to be associated with AAV infection.⁴ This safety seems to be the most important feature of AAV-based vectors. In addition, AAV vectors are capable of transducing both dividing and nondividing cells. The vector genome can stably integrate into the genome of target cells, facilitating long-term expression of transgenes.⁵⁻⁸

The AAV genome consists of 4681 nucleotides (nt) of single-stranded DNA (ssDNA), including two inverted terminal repeats (ITRs) of 145 nt long, which serve as an origin of replication and are also essential for packaging and rescue.⁹ The left half of the genome encodes four overlapping nonstructural proteins, Rep78, Rep68, Rep52, and Rep40, and the right half codes for three overlapping

structural proteins, VP1, VP2, and VP3 (Cap proteins). Transcription from the p5 promoter generates spliced and unspliced transcripts which encode Rep68 and Rep78, respectively. Rep78/68 are required to regulate transcription from all three promoters and to replicate the viral DNA.^{10, 11} Furthermore, Rep78/68 are also involved in the preferential integration of AAV genomes into the AAVS1 locus.¹² Spliced and unspliced transcripts from the p19 promoter encode Rep40 and Rep52, respectively. Rep52/40 are required for the accumulation of ssDNA forms,¹³ and it has been reported that Rep52/40 attenuate the repression of the p5 promoter by Rep78/68 in the presence of helper virus.¹⁴ Transcription from the p40 promoter generates two alternatively spliced forms, the minor transcript encoding VP1 and the major transcript for VP2 and VP3.^{15, 16}

One of the limiting factors for AAV vector-mediated gene therapy is the difficulty in producing high-titer AAV vector stocks. The conventional method for AAV vector production is based on cotransfection of a helper plasmid expressing the viral genes, *rep* and *cap*, without ITRs and a vector plasmid consisting of a foreign gene flanked by ITRs into 293 cells infected with adenoviruses.¹⁷ To

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improve AAV vector production, several methods have been developed, such as amplification of the copy number of the helper plasmid by introducing a simian virus 40 (SV40) replicon.¹⁸ In recent years, an adenovirus-free system in which the AAV vector is produced by using a plasmid expressing adenovirus genes with helper function (E2A, E4, and VA) in place of the adenovirus itself, has been developed as a more efficient and safer method.^{19,20} However, if plasmid transfection is required each time AAV vectors are produced, it is difficult to prepare clinical-grade AAV vectors on a large scale. Therefore, it is important for the widespread clinical use of AAV vectors to establish efficient AAV packaging cell lines.^{21,22} So far, no efficient packaging cell line which constitutively expresses the *rep* and *cap* genes has yet been established because of the cytostatic effect of Rep78/68. In addition, Rep52/40 and Cap also seem to be rather cytotoxic, because it is difficult to obtain cell lines which constitutively express them in large quantities (our preliminary observation). It has also been reported that Rep52/40 may have a negative effect on the growth of 293 cells.²³ Accordingly, for an ideal packaging cell line, all AAV proteins should be expressed only when the AAV vectors are produced. To this end, large Rep (Rep78/68), small Rep (Rep52/40), and Cap expression have to be regulated independently.

Since it is technically difficult to regulate each AAV protein expression in a single plasmid, it is an important step to examine whether it is possible to express each AAV protein in a separate plasmid to produce AAV vectors efficiently. In order to examine whether the AAV helper plasmid can be physically split upstream of the p19 promoter (for small Rep) or the p40 promoter (for Cap) without losing the ability to package the recombinant AAV genome, we constructed two sets of split-type helper plasmids; 1) a Rep expression plasmid (pRep) and Cap expression plasmid (pCap), and 2) a large Rep expression plasmid (pR78/68) and small Rep plus Cap expression plasmid (pR52/40Cap). AAV vectors were produced using these split-type helper plasmids at various ratios, and the efficiency was compared with that of a conventional helper plasmid. Furthermore, we systematically determined the optimal expression pattern of AAV proteins for packaging the recombinant AAV genome by changing the Rep and Cap expression pattern gradually in proportion to the amounts of the split-type helper plasmids.

MATERIALS AND METHODS

Cell line and plasmids The 293 cells, a human embryonic kidney cell line transformed by adenovirus type 5,²⁴ were propagated in Dulbecco's modified Eagle's medium plus F-12 nutrient mixture (DMEM/F-12; GibcoBRL, Tokyo) supplemented with 10% fetal bovine serum, 2 mM

L-glutamine (GibcoBRL), 100 U/ml penicillin (GibcoBRL), and 100 µg/ml streptomycin (GibcoBRL) at 37°C and 5% CO₂.

As shown in Fig. 1, pIM45 contains the wild-type *rep* and *cap* genes at nt 145 to 4493 in a Bluescript M13+ vector.²⁵ To construct the Rep expression plasmid (pRep), a polylinker containing recognition sites for the enzymes 5'-*SacI*-*ClaI*-*EcoRI*-*SmaI*-*BamHI*-*XbaI*-*HincII*-*PstI*-*EcoRV*-*HindIII*-*XhoI*-*KpnI*-3' was cloned into the *Bss*HIII site of pUC-MCS,⁸ and then the following fragments were cloned into the plasmid; an AAV fragment containing the *rep* gene (nt 145–2252 in the AAV genome) into *SpeI*-*PstI* and the 135-bp *HpaI*-*BamHI* blunt-end modified SV40 early polyadenylation signal from pCMV-β plasmid (CLONETECH Laboratories, Palo Alto, CA) into the *HpaI* site. The Rep78/68 expression plasmid (pR78/68) was derived from pRep with a mutation of the first ATG of Rep52/40 to GGA as described previously.^{12,13}

Since it has been demonstrated that the p5 promoter sequence has the ability to enhance both the p19 and p40 promoter activities during productive infection,¹⁴ we inserted the p5 promoter sequence at the 3' end of the polyadenylation sequence of pCap and pR52/40Cap. To construct the Cap expression plasmid (pCap) and Rep52/40 plus Cap expression plasmid (pR52/40Cap), a *BamHI*-*XbaI* fragment (nt 1498–4488) and *SfiI*-*XbaI* fragment (nt 537–4488) of pRepCap were cloned into pUC18, respectively.¹² Subsequently, the *XbaI*-*PstI* fragment (nt 188–500) of pRepCap containing the p5 promoter sequence was inserted into the 3' end of the polyadenylation signal of pCap and pR52/40Cap.

The AAV vector plasmid (pAAV-LacZ) contains a *LacZ* gene under the control of the CMV-IE promoter between ITRs.²⁶ The pladeno plasmid has been constructed to express *E2A*, *E4*, and *VA* genes of adenovirus type 5.¹⁹

Plasmid transfection and western analysis of AAV proteins On the day before transfection, 293 cells were seeded at a density of 2×10⁶ cells per 10 cm dish. Cells were transfected with 10 µg of AAV helper plasmids (pIM45 alone or split-type plasmids), 5 µg of pAAV-LacZ, and 5 µg of pladeno by the calcium phosphate procedure as described elsewhere.²⁷ For western analysis, the transfected cells were harvested at 48 h post-transfection and resuspended in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 100 IU/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysates (10 µg) were mixed with an equal volume of 2× sample loading buffer containing 120 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.004% bromophenol blue, and 10% 2-mercaptoethanol, and then boiled for 5 min. The samples were loaded onto a 10% polyacrylamide gel, electrophoresed, and transferred to Immobilon membranes

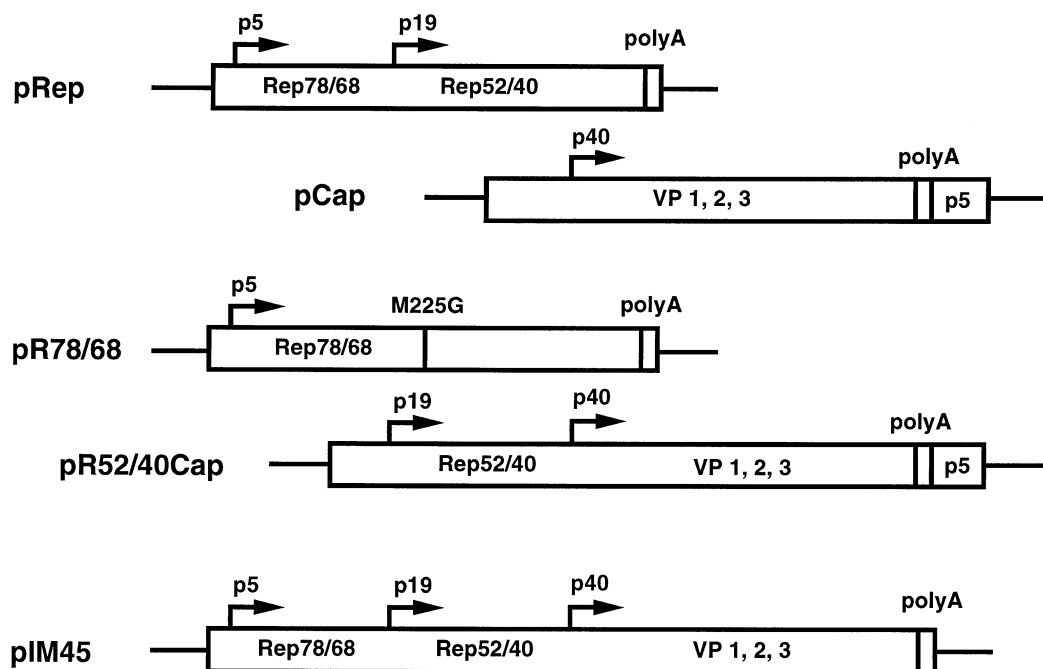


Fig. 1. Schematic representation of the split-type helper plasmids (pRep-pCap type and pR78/68-pR52/40Cap type) and a conventional helper plasmid (pIM45). The plasmids are represented in a linear form with the thick line depicting the backbone plasmid DNA. The rectangular boxes represent the AAV genes with their control regions (see "Materials and Methods" for details).

(Millipore, Tokyo). The membranes were blocked with 4% non-fat milk in Tris-buffered saline with 0.05% Tween 20 for 1 h at room temperature before being incubated with an anti-Rep monoclonal antibody, 294-4 (a kind gift from Dr. Kleinschmidt, Deutsches Krebsforschungszentrum, Heidelberg, Germany), used at a dilution of 1:2000 in TBST or anti-Cap monoclonal antibody, B1 (PROGEN, Heidelberg, Germany), used at a dilution of 1:50 in TBST for 1 h at room temperature.²⁸⁾ Rep and Cap were visualized using the enhanced chemiluminescence detection method (ECL kit; Amersham, Little Chalfant, UK).

AAV-LacZ vector production On the day before transfection, 293 cells were plated onto 10 cm dishes (1×10^6 cells/dish). Cells were transfected with 10 μ g of AAV helper plasmids (pIM45 alone or split-type plasmids), 5 μ g of pAAV-LacZ, and 5 μ g of pladeno by the calcium phosphate procedure as described above. At 72 h after transfection, the cells were collected by gentle pipetting and pelleted by centrifugation (300g, 4°C, 5 min). The pellets were resuspended in 1 ml of Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 8.0], 150 mM NaCl). Following three freeze-thaw cycles and removal of cell debris by centrifugation (10,000g, 4°C, 10 min), the AAV-LacZ vector was obtained and stored at -80°C until use.

Titration of AAV-LacZ by DNA dot blot analysis and X-gal staining The titers of AAV-LacZ were determined by quantitative DNA dot blot hybridization. The AAV-LacZ stock (20 μ l) was first treated with 70 U of DNase I (Takara Shuzo Co., Ltd, Tokyo) in 10 mM Tris-HCl (pH 7.5)/5 mM MgCl₂ at 37°C for 1 h, and then with 500 μ g/ml proteinase K (Boehringer Mannheim Biochemica, Mannheim, Germany) in 10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), and 0.5% SDS at 37°C for 1 h, before being extracted with phenol-chloroform. The isolated DNA and standard DNA (a 1.8-kb *Cla*I-*Pvu*II fragment located within the *lacZ* coding sequence) were then treated with a denaturation solution containing 0.4 M NaOH and 10 mM EDTA (pH 8.0) for 10 min at room temperature. Serial dilutions of the isolated DNA in Tris-EDTA (TE) containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) were applied to a nylon membrane (Hybond N+; Amersham) with a Minifold dot blot apparatus (MINIFOLD; Schleicher & Schuell, Inc., Keene, NH). After UV crosslinking, the filter was prehybridized for 2 h at 42°C and then hybridized with a *lacZ* gene probe (a 1.8-kb *Cla*I-*Pvu*II fragment), radiolabeled with a random primer labelling kit (Amersham), in 50% formamide, 6 \times salt sodium citrate (SSC), 0.5% SDS, 5 \times Denhardt's solution, and 100 μ g/ml denatured salmon sperm

DNA at 42°C overnight. The filter was washed for 15 min at 55°C in 2× SSC-0.1% SDS, 0.5× SSC-0.1% SDS, and 0.1× SSC-0.1% SDS and then exposed to an imaging plate (Fuji Photo Film Co., Ltd., Kanagawa). Blots were directly counted and analyzed on a BAS-2000 imaging system (Fuji Photo Film). The number of AAV vector genomes present was determined by comparison with the standard DNA.

One day before transduction, 293 cells were plated in 12-well plates (2×10^5 cells/well). Subconfluent 293 cells were transfected with various dilutions of the AAV-LacZ. After 24 h, the cells were fixed with phosphate-buffered saline (PBS) containing 0.05% glutaraldehyde for 15 min at room temperature and stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal) in PBS containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, and 1 mM $MgCl_2$ at 37°C for 6 h. The infectious particle titer (functional titer) of AAV-LacZ was determined by scoring the positively stained cells. The results shown are the average of two separate experiments.

RESULTS

Titration of AAV-LacZ vector produced using pRep and pCap AAV-LacZ vectors were produced by using pRep and pCap mixed at various ratios (0:10, 0.1:9.9, 0.3:9.7, 1:9, 3:7, 5:5, 7:3, and 9:1). As a positive control for AAV vector production, a conventional helper plasmid (pIM45) was used. As shown in Fig. 2, maximal vector production was observed when the ratio of pRep to pCap was 1 to 9. The titer was slightly higher than that with pIM45 (pRep:pCap=1:9, $4.9 \pm 2.1 \times 10^{11}$; pIM45, $1.8 \pm 0.16 \times 10^{11}$ vector particles/10 cm dish). When pRep was not transfected (pRep:pCap=0:10), no AAV-LacZ was detected by DNA dot blotting. Infectious particle titers (functional titers) were determined by X-gal staining after 24 h culture in the absence of helper adenoviruses (which enhance the AAV vector-mediated transgene expression) following transduction. In our laboratory, the particle titer to infectious particle titer (X-gal) ratio was roughly 10^4 (pRep:pCap=1:9, 3.7×10^7 ; pIM45, 7.3×10^6 functional titer/10 cm dish). However, if functional titers were determined by X-gal staining after 24 h culture in the presence of helper adenoviruses following transduction, the titers were usually several hundred times higher than those assayed in the absence of adenoviruses.

Rep and Cap expression in 293 cells transfected with pRep and pCap at various ratios The expression of Rep and Cap in 293 cells cotransfected with pRep and pCap at various ratios was analyzed by western blotting (Fig. 3). As negative and positive controls, pUC19 and pIM45 were transfected into 293 cells, respectively.

Rep78 appeared as a doublet as described previously.²⁹⁾ The expression of Rep68 was much weaker than that of

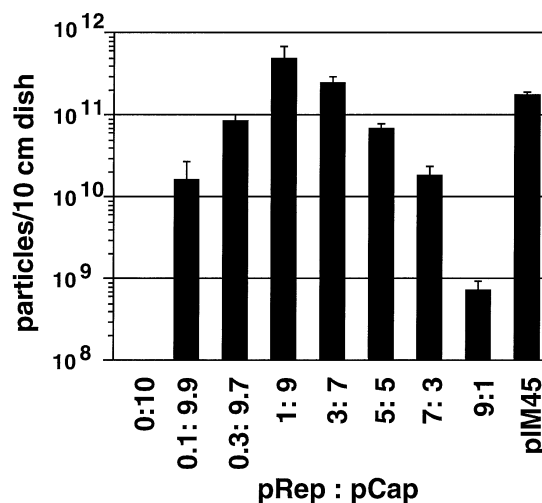


Fig. 2. Titers of AAV-LacZ produced using pRep and pCap at various ratios. The 293 cells (1×10^6 /10 cm dish) were transfected with 5 μ g of pAAV-LacZ, 5 μ g of pladeno, and 10 μ g of helper plasmid (pIM45, or pRep and pCap mixed at various ratios). The AAV-LacZ vectors were recovered from cell lysates at 72 h post-transfection. Titers of AAV-LacZ were determined by quantitative DNA dot-blot hybridization (see “Materials and Methods” for details). Mean values from three measurements are plotted with standard errors.

Rep78. Rep52 was clearly detected as a single band, but for Rep40, two bands of about 40 kDa were detected. All three capsid proteins (VP1, VP2, and VP3) were apparently detected.

When pRep and pCap were mixed at the ratio of 1:9, a smaller amount of Rep and a larger amount of Cap were expressed than that of pIM45. In the case of 0:10 and 0.1:9.9, the amount of Cap expressed was lower than that at the ratio of 1:9, although a larger amount of pCap was transfected.

Titration of AAV-LacZ produced using pR78/68 and pR52/40Cap We produced AAV-LacZ using pR78/68 and pR52/40Cap mixed at various ratios (0:10, 0.1:9.9, 0.3:9.7, 1:9, 3:7, 5:5, 7:3, and 9:1). The titer was highest when the ratio of pR78/68 to pR52/40Cap was 1 to 9, and this was comparable to that with pIM45 (pR78/68:pR52/40Cap=1:9, $2.9 \pm 1.6 \times 10^{11}$ vector particles/10 cm dish, 2.9×10^7 functional titer/10 cm dish) (Fig. 4).

Rep and Cap expression in 293 cells transfected with pR78/68 and pR52/40Cap at various ratios pR78/68 and pR52/40Cap mixed at various ratios were transfected into 293 cells, and the expression of Rep and Cap was analyzed by western blotting (Fig. 5). Rep78, Rep52, and Cap were clearly detected, but Rep68 was observed at a lower level. Two bands of about 40 kDa were detected by the anti-Rep antibody. Since the lower 40 kDa band

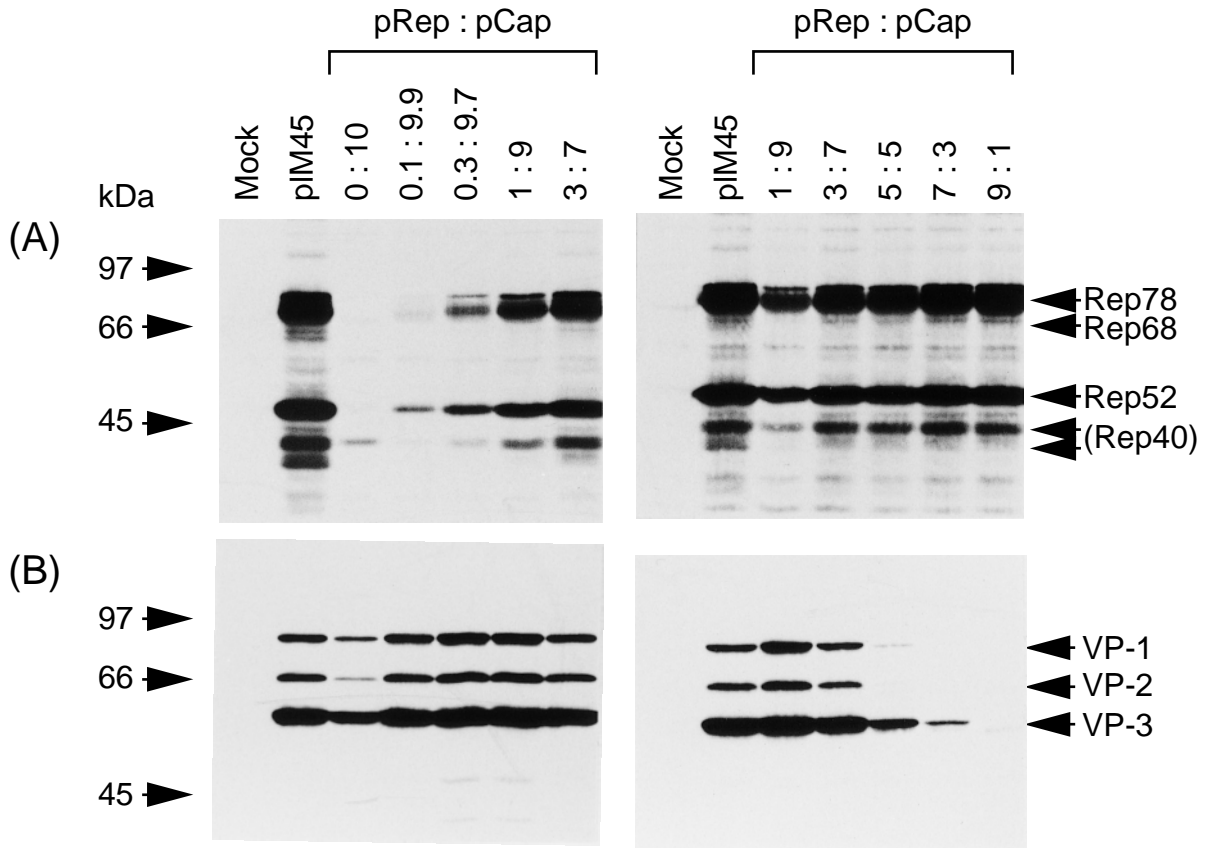


Fig. 3. Expression of Rep and Cap proteins in 293 cells transfected with pRep and pCap at various ratios. 1 ne 293 cells were cotransfected with 5 μ g of pAAV-LacZ, 5 μ g of pladeno, and 10 μ g of helper plasmid (pIM45, or pRep and pCap mixed at various ratios) or with pUC19 in place of helper plasmid as a negative control, and 2 days later cellular extracts were prepared. A 10 μ g aliquot of protein was loaded in each lane (10% polyacrylamide gel) and analyzed by western blotting. (A) Anti-Rep monoclonal antibody (294-4) which recognizes all four Rep proteins. (B) Anti-Cap monoclonal antibody (B1) which interacts with all three Cap proteins (VP1, VP2, and VP3). Molecular size standards (in kilodaltons) are shown on the left, and each of the AAV proteins is indicated on the right.

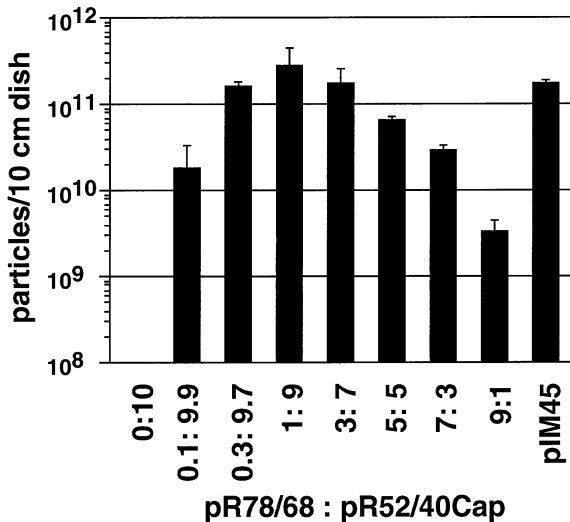


Fig. 4. Titers of AAV-LacZ produced using pR78/68 and pR52/40Cap. The 293 cells (1×10⁶/10 cm dish) were transfected with 5 μ g of pAAV-LacZ, 5 μ g of pladeno, and 10 μ g of helper plasmid (pIM45, or pR78/68 and pR52/40Cap mixed at various ratios). AAV-LacZ vectors were recovered from cell lysates at 72 h post-transfection. Titers of AAV-LacZ were determined by quantitative DNA dot-blot hybridization. Mean values from three measurements are plotted with standard errors.

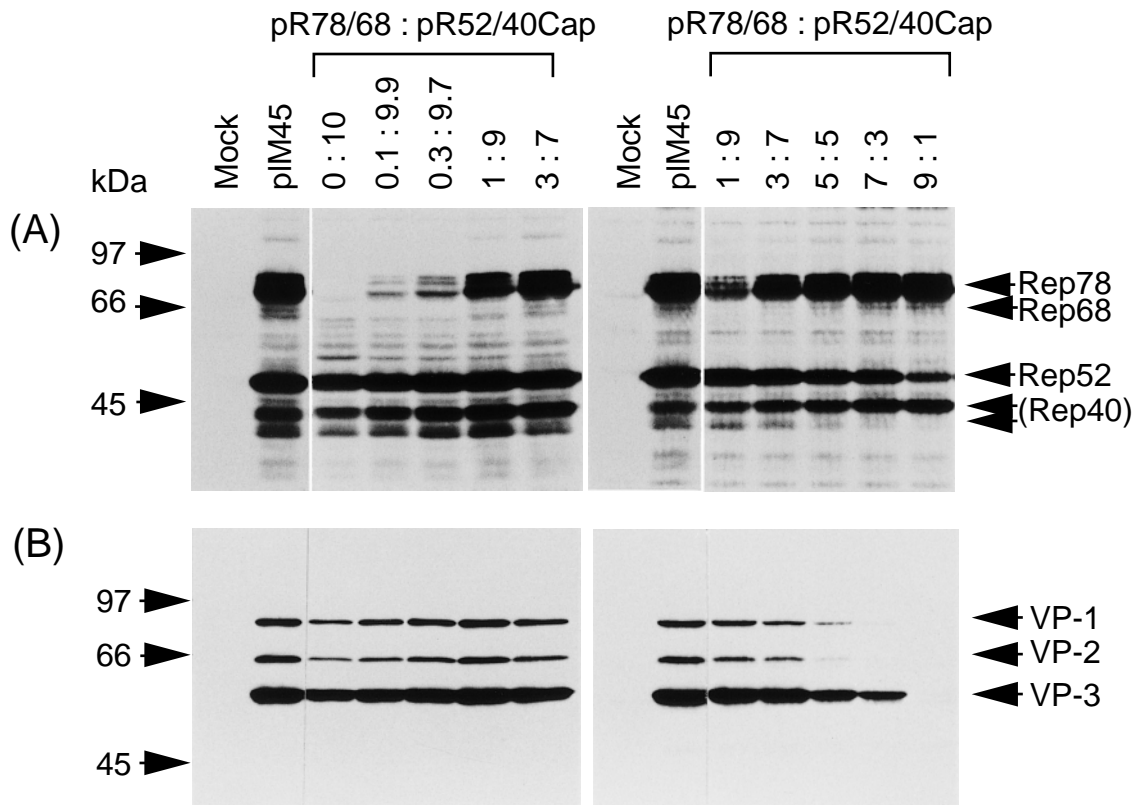


Fig. 5. Expression of Rep and Cap proteins in 293 cells transfected with pR78/68 and pR52/40Cap at various ratios. The 293 cells were transfected with 5 μ g of pAAV-LacZ, 5 μ g of pladeno, and 10 μ g of helper plasmid (pIM45, or pR78/68 and pR52/40Cap mixed at various ratios) or with pUC19 in place of helper plasmid as a negative control, and 2 days later cellular extracts were prepared. A 10 μ g aliquot of protein was loaded in each lane (10% polyacrylamide gel) and analyzed by western blotting. (A) Anti-Rep monoclonal antibody (294-4). (B) Anti-Cap monoclonal antibody (B1). Molecular size standards (in kilodaltons) are shown on the left, and each of the AAV proteins is indicated on the right.

became weaker with a decrease in the ratio of pR52/40Cap to pR78/68, this band may correspond to Rep40. The upper 40 kDa band may be partly derived from the degradation products of the larger Rep proteins, although further experiments will be needed to obtain definitive data on this matter.

When the ratio of pR78/68 to pR52/40Cap was 1 to 9, these helper plasmids produced a smaller amount of Rep78/68 than pIM45 and about the same amounts of Rep52 and Cap. In the case of 0:10, 0.1:9.9, and 0.3:9.7, the amount of Cap was slightly smaller than that at the ratio of 1:9 in spite of a larger amount of pR52/40Cap having been transfected.

DISCUSSION

The present study showed that the AAV helper plasmid can be physically split upstream of the p19 promoter or the p40 promoter without losing the ability to package the

AAV vector genome. A recent study has also demonstrated that the AAV packaging plasmid can be functionally split by arranging the *rep* and *cap* expression cassettes in opposite transcription orientations.³⁰ These findings suggest that it is possible to express each AAV protein in a separate plasmid.

We found that for efficient AAV vector production it is crucial that the AAV proteins are expressed in appropriate amounts. The maximal AAV vector production was obtained when the ratio of split-type helper plasmids was 1:9 with either type (pRep:pCap or pR78/68:pR52/40Cap), and the vector particle titer was comparable to or even higher than that of a conventional helper plasmid (pIM45). Western analysis suggests that the expression of a large amount of Cap and a relatively small amount of Rep is important for efficient vector production.

A previous report showed that the production of capsid proteins is a limiting factor for packaging the AAV vector genome and that overexpression of Rep78/68 leads to a

much reduced AAV vector titer.³¹⁾ On the other hand, down regulation of Rep78/68 expression by using an inefficient translation initiation start signal caused increased capsid protein expression, resulting in much higher AAV vector yields.³²⁾ Thus, our findings are compatible with these earlier reports.

When the ratio of pRep to pCap was 0 to 10 or 0.1 to 9.9, the level of Cap expression was rather lower than that at the ratio of 1 to 9 in spite of the larger amount of pCap transfected. A possible explanation for the decrease in Cap expression is that the amount of Rep78/68 may not be sufficient to enhance the Cap expression, because Rep78/68 is required to transactivate both the p19 and p40 promoters in the presence of adenovirus.²⁵⁾ Since Rep78/68 is also required to replicate the vector DNA, the AAV vector yields may be reduced when the levels of Rep78/68 expression are very low. Thus, for AAV vector production it is important to express appropriate amounts of Rep78/68.

When pRep and pCap were mixed at the ratio of 1 to 9, a smaller amount of Rep52 and a slightly larger amount of Cap were produced than those from pR78/68 and pR52/40Cap mixed at the same ratio, and the titer of AAV-LacZ was a little higher than that with pR78/68-pR52/40Cap type helper plasmids (1:9). These results

suggest that the titer may be mainly affected by differences in Cap expression and that a large amount of Rep52/40 may not be necessary for efficient vector production.

The AAV vector was efficiently produced by modulating the expression pattern of AAV proteins, even when an AAV packaging plasmid was physically split upstream of the p19 promoter or the p40 promoter. These findings will contribute to the development of a more efficient AAV vector production system and provide valuable information for establishing efficient AAV packaging cell lines.

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