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# Development and Characterization of an Antisense-Mediated Prepackaging Cell Line for Adeno-Associated Virus Vector Production

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**One of the limitations of recombinant adeno-associated virus (rAAV) vector systems for gene therapy applications has been the difficulty in producing the vector in sufficient quantity for adequate evaluation. Since the AAV Rep proteins are cytotoxic, it is not easy to establish stable cell lines that express them constitutively. We describe a novel 293-derived prepackaging cell line which constitutively expresses the antisense *rep/cap* driven by a *loxP*-flanked CMV promoter. This cell line was converted into a packaging cell line expressing Rep/Cap for rAAV vector production through adenovirus-mediated introduction of a Cre recombinase gene. Without the introduction of the Cre recombinase gene, the cell line was shown to produce neither Rep nor Cap. rAAV vector was produced ($1 \times 10^9$ genome copies/3.5-cm dish) 4 days after the transduction with Cre-expression adenovirus vector together with transfection of AAV vector plasmid. We further showed that the addition of Cap-expression adenovirus vector caused a 10-fold increase in the yield of rAAV vector. This system is also capable of producing rAAV as a transfection-free system by using a small amount of rAAV instead of vector plasmid.**



***Key Words:*** **adeno-associated virus; adenovirus; vector; antisense; packaging cell; gene therapy.**

Adeno-associated virus (AAV) is a dependent parvovirus with a single-stranded linear genome that contains two open reading frames (*rep* and *cap*) (1). The genome encodes four replication proteins (Rep78, Rep68, Rep52, and Rep40) and three capsid proteins (Cap: VP1, VP2, and VP3). The wild-type virus is capable of infecting a variety of cells and establishing latency via an integrated provirus. Recombinant AAV (rAAV) vectors are promising as a gene delivery vehicle for gene therapy. Their cloning capacity of 4.5 kb can accommodate a variety of cDNAs. Furthermore, no disease has been associated with AAV in either human or animal populations. However, the procedures for growing recombinant virus stocks continue to be awkward. The conventional method for generating vector stocks consists of cotransfection with a plasmid containing the vector genome and a trans-acting helper construct for *rep* and *cap* expression. The difficulty has been in preparing high-quality good manufacturing practice (GMP) grade plasmid stocks for clinical study (2). Recent improvements in vector production include the development of packaging cell lines expressing Rep and Cap (3–5) and ways to transiently express and regulate Rep and Cap (6, 7). However, it is still difficult to establish stable cell lines that express Rep and Cap constitutively long-term because the Rep proteins are toxic to cells (3, 8).

We describe in this article a novel 293-derived prepackaging cell line that can be converted into a packaging cell line expressing *rep* and *cap* through introduction of a Cre recombinase gene. The genetically engineered prepackaging cell line constitutively expresses the antisense *rep* and *cap* driven by a *loxP*-flanked CMV promoter which was designed to be removed when Cre is provided. We further showed that the addition of a Cap-expression adenovirus vector could increase the yield of rAAV.

## MATERIALS AND METHODS

*Plasmid construction.* A plasmid pLCLCR was constructed as follows: A 4.3-kb *Xba*I fragment of pIM45 (identical to pIM29 + 45)

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(9) containing *rep* and *cap* genes with endogenous promoters and polyadenylation signal was ligated to *Xba*I/*Spe*I-digested pBluescript SK+ (Stratagene, La Jolla, CA) to make pSKCR. The cytomegalovirus intermediate-early (CMV-IE) promoter was cloned as a *Mlu*I–*Age*I fragment of pCMV (10) and blunted for insertion into a blunted *Bam*HI site of plox$^2$ (10) so that the CMV-IE promoter was located between the two *loxP* sites (plox$^2$CMV). A *Hin*dIII (blunted)/*Eco*RI fragment of plox$^2$CMV was ligated to the *Sma*I/*Eco*RI-digested pSKCR to allow transcription of the antisense RNA for *rep* and *cap* genes under the control of the CMV promoter. An *Xba*I (blunted)- *Cla*I fragment of the resultant plasmid (pSKLCLCR) was further cloned into the *Pac*I (blunted)/*Nsp*V-digested pAVC2 (11) (pLCLCR). The pLCLCR has the *rep* and *cap* genes with the *loxP*-flanked CMV-IE promoter and SV40 early polyadenylation signal (Fig. 1A).

The AAV vector plasmid, pAAVlacZ (Fig. 1A), harboring a $\beta$-galactosidase expression cassette flanked by ITRs was described before (12). Ad-helper plasmid, pAd, is identical to pVAE2AE4-5 encoding the entire E2A and E4 regions, and VA RNA I and II genes (13).

*Recombinant adenovirus vectors and AAV vectors.* Adex1w1 (Adex; provided from RIKEN gene bank) is a recombinant adenovirus containing a nearly full-length adenovirus type 5 genome with E1 and E3 deletions (14). AxCANCre (AdCre; provided from RIKEN gene bank) is a Cre recombinase expression adenovirus vector (14). AVC2Cap is a recombinant adenovirus expressing Cap driven by the CMV promoter (Fig. 1A). A *Swa*I–*Sph*I fragment of pIM45 containing the *cap* gene sequence was blunted and cloned into the *Sma*I–*Eco*RV site of pBluescript SK+ (pSKCap). The *cap* gene was excised from pSKCap by digestion with *Xba*I and *Cla*I and ligated directly into adenoviral DNA–protein complex AVC2.null digested with *Xba*I and *Nsp*V using the directional *in vitro* ligation technique (11) to obtain AVC2Cap. The ligated DNA–protein complex was introduced into human embryonic kidney 293 cells by calcium phosphate transfection. The viral isolates were screened and amplified by standard techniques (11).

For production of recombinant AAVLacZ, 293 cells were plated onto 10-cm-diameter dishes ($2 \times 10^5$ cells/dish) the day before transfection. Subconfluent 293 cells were cotransfected with AAV helper plasmid (pIM45, 10 $\mu$g; pAd, 10 $\mu$g) and vector plasmid (pAAVLacZ, 10 $\mu$g) using a calcium phosphate-mediated method. Three days after the transfection, AAV vectors were recovered from the cells which were resuspended in 1 ml of Tris-buffered saline [10 mM Tris–HCl (pH 8.0), 150 mM NaCl] by repeated freezing and thawing.

*Isolation of a subline of 293 cells for AAV production.* 293 cells (15) were cultured in Dulbecco's modified Eagle medium (DMEM: Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum (FBS: Life Technologies), 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM glutamine at 37°C with 5% $CO_2$. A plasmid pCMV (10), pIM45, or pLCLCR DNA was linearized and cotransfected with pSV2bsr (Funakoshi Co., Ltd., Tokyo, Japan) containing the blasticidin S-resistance (bsr) gene into 293 cells by the calcium phosphate coprecipitation method (16). One day after the transfection, the cultures were split and stable transformants were selected with bsr (Funakoshi, Tokyo, Japan) at a concentration of 10 $\mu$g/ml for a week. Clones were isolated and parallel cultures of each clone were analyzed by PCR and dot blotting. To compare the ability to produce AAV, each colony was seeded at a density of $2 \times 10^5$ cells per 3.5-cm dish. The cells were transfected with AAV helper plasmid (pIM45, 1 $\mu$g; pAd, 1 $\mu$g) and vector plasmid (pAAVLacZ, 1 $\mu$g) using a calcium phosphate-mediated method. Simultaneously, the cells were transduced with AdCre at an m.o.i. of 1. Three days after the transduction, AAV vectors were recovered from the cells which were resuspended in 1 ml of Tris-buffered saline [10 mM Tris–HCl (pH 8.0), 150 mM NaCl] by repeated freezing and thawing. The clone that produced the most copies of rAAV was isolated and expanded (293CR).

*In vitro recombination assay.* Cre extracts were prepared as described (17). Briefly, $2 \times 10^7$ of 293 cells were infected with $1 \times 10^8$ plaque-forming units (pfu) of AdCre. Twenty-four hours later, cells were harvested and collected by centrifugation at 300*g* for 5 min at 4°C. The cell pellet was washed twice with PBS (−) and resuspended in 5 ml of Cre storage buffer [50% glycerol, 20 mM Tris–HCl (pH 7.5), 300 mM NaCl, and 1 mM EDTA]. The cell suspension was then sonicated for 2 min using an ultrasonic cell disrupter, the Bioruptor (Olympus, Tokyo, Japan), at a power of 200 W and immediately centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was stored at −80°C.

For analysis with a cell-free system, a plasmid pLCLCR DNA (1 $\mu$g) and Cre extract (10 $\mu$l) were incubated at 37°C for 30 min in a 50-$\mu$l volume of buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 100 $\mu$g/ml DNase-free bovine serum albumin, 1 mM PMSF and 5 $\mu$g/ml aprotinin. The recombination efficiency of the substrate was reported to reach a plateau level of 60% when 5 $\mu$l or more of the extract was used (17). The reaction was terminated by phenol/chloroform extraction followed by two cycles of chloroform extraction and ethanol precipitation. The recovered DNA was dissolved and treated with RNase A. A DNA sequence between the vector genome and *cap* sequence was amplified by PCR using the sense primer GTTTACGTGGAGACTCGCCCAGGGCG and antisense primer CAGGCCGGAGCGAGTGACA. The structure of the PCR product, which contains an *Eco*RI site, was confirmed by restriction enzyme digestion. For *in vitro* assay of prepackaging cell line, total chromosomal DNA was prepared by standard techniques from 293CR cells before or two days after the AdCre infection. PCR was performed and the products were analyzed as described above.

Southern blot analysis was performed as follows. The PCR product was transferred to a nylon membrane (Hybond N+; Amersham Life Science, Arlington Heights, IL) by the capillary transfer method. The 2.3-kb Cap probe was isolated from pIM45 by *Pst*I digestion. The probe was radiolabeled with $^{32}$P by using a random primer labeling kit (Amersham Life Science) and hybridized DNA was detected by autoradiography.

*Western blot analysis of AAV proteins.* On the day before infection with AdCre, the 293CR cells were seeded at a density of $2 \times 10^5$ per 3.5-cm dish. The cells were transfected with 1 $\mu$g of pAAVlacZ by the calcium phosphate co-precipitation method. They were infected with Adex or AdCre (m.o.i. of 1) immediately after the transfection, and harvested at 48 h posttransfection. The cells were resuspended in 1 ml of cell-lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 100 IU/ml aprotinin, and 1 mM PMSF). The cell lysates (10 $\mu$g per lane) were loaded onto a 10% polyacrylamide gel, electrophoresed, and transferred to Hybond ECL nitrocellulose membranes (Amersham Life Science). The membranes were blocked and reacted with either anti-Rep monoclonal antibody 303.9 (PROGEN Biotechnik GmbH, Heidelberg, Germany) or anti-Cap monoclonal antibody B1 (PROGEN Biotechnik GmbH). Rep and Cap proteins were visualized by using the enhanced chemiluminescence detection method (ECL kit; Amersham Life Science).

*Dot blot analysis for packaging capacity of the 293CR cell line.* As a standard method to produce rAAV, 293 cells ($2 \times 10^5$ per 3.5-cm plate) were cotransfected with 1 $\mu$g each of pAAVLacZ and pIM45 and then infected with Adex at an m.o.i. of 1. The prepackaging cell line 293CR was transfected with pAAVLacZ and infected with AdCre at various m.o.i.'s, and further used for screening to determine the optimal conditions for the vector production. In the case of the transfection-free system, AAVLacZ was infected at 100 particles per cell instead of pAAVLacZ transfection. When AdCre was introduced for the *loxP*-dependent recombination, the efficiency of the recombination was increased by dosage. However, a high dose of adenovirus would cause cell damage due to the toxicity of the adenovirus itself. Therefore, we estimated the vector production from 293CR after AdCre infection at several m.o.i.'s. The time course of virus production after infection with AdCre was also evaluated. For increased

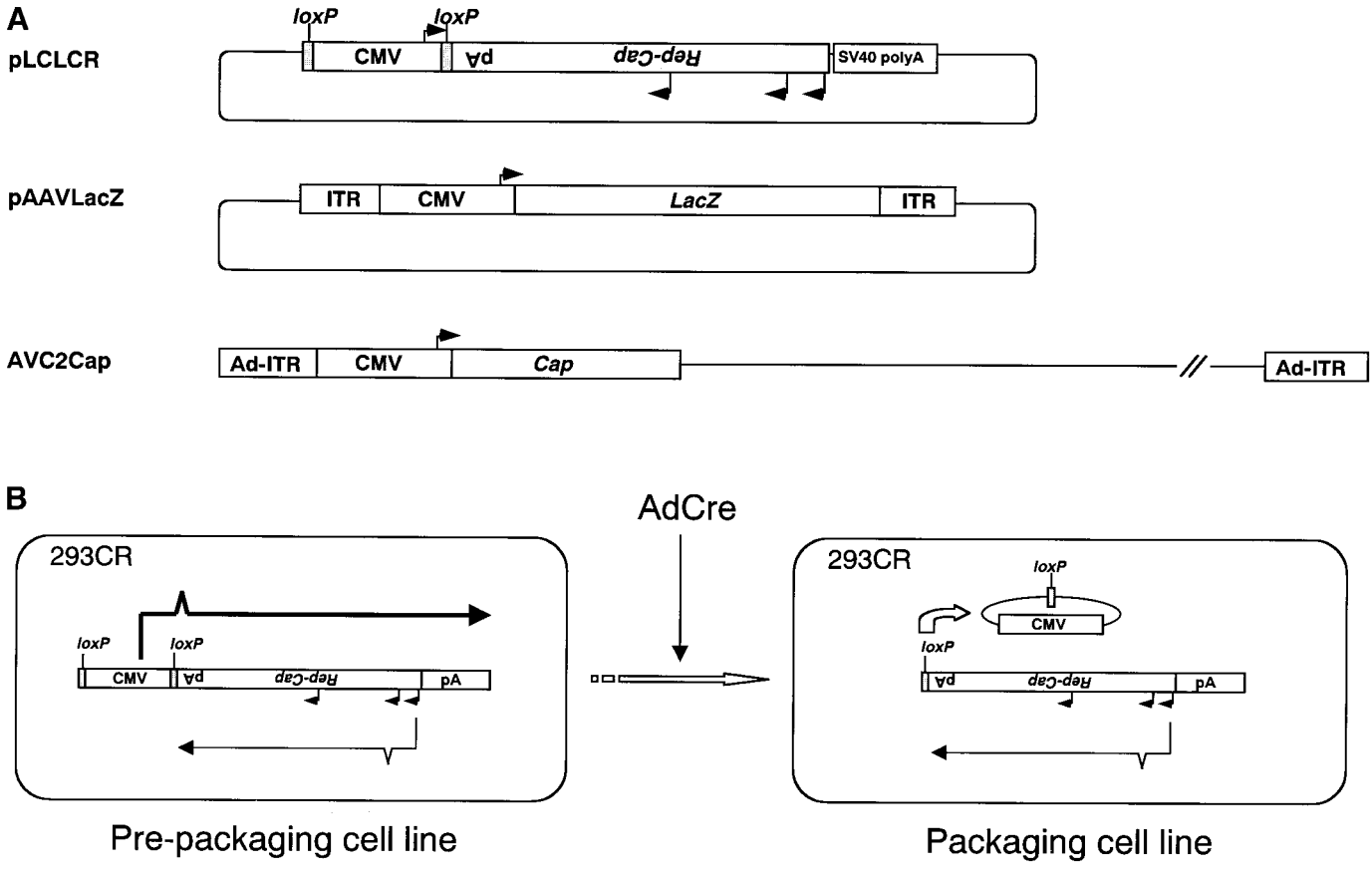


**FIG. 1.** Vector construction for the generation of prepackaging cell lines and a system for Cre-mediated recombinant adeno-associated virus (rAAV) vector production. (A) Structures of pLCLCR, pAAVLacZ, and AVC2Cap. Two *loxP* sequences are tandemly located in pLCLCR. (B) Schematic presentation of the conversion from the prepackaging cell line to the packaging cell line. In the 293CR prepackaging cell line generated by the transfection of pLCLCR into 293 cells, the *rep* and *cap* genes are silent because the antisense RNA is transcribed from a CMV-IE promoter. Cre recombinase expressed by AdexCre in 293CR excises the CMV-IE promoter by site-specific recombination between the two *loxP* sequences and thus converts the prepackaging cell line to the packaging cell line. In the packaging cell line, the *rep* and *cap* genes are now transcribed by the endogenous promoters p5, p19, and p40.

production of the recombinant virus from the 293CR cells, the dose effect of AVC2Cap was also evaluated.

The titers of AAVLacZ were determined by quantitative DNA dot-blotting hybridization. The viral stock of 50 $\mu$l was treated with 70 U DNaseI for 1 h followed by 500 mg/ml proteinase K (WAKO, Osaka, Japan) for 1 h and subjected to phenol–chloroform extraction. The isolated DNA and standard DNA (a 4.4-kb *Not*I fragment of pAAVLacZ) were denatured in 0.4 N NaOH and applied to a nylon membrane (Hybond-N$^+$; Amersham Life Science) with a manifold dot-blot apparatus (Schleicher & Schuell, Keene, NH). The membrane was cross-linked by UV light and hybridized with a *lacZ* gene probe radiolabeled with a random primer labeling kit (Amersham Life Science). The filters were washed and analyzed on a BAS-2000 imaging system (FUJIX, Tokyo, Japan). The number of AAV vector genomes present was determined by comparison with the standard DNA. The data represent the means of triplicate experiments.

## RESULTS AND DISCUSSION

### *Cloning of the Prepackaging Cell Line*

The AAV Rep protein is cytotoxic to most mammalian cells, including 293 derivatives (18). Long and leaky production of the Rep protein would increase the possibility of genetic changes. For the isolation of prepackaging cell lines that express Rep and Cap after the introduction of Cre recombinase, we designed a system in which the CMV-IE promoter is used for antisense transcription in the silencing of the toxic gene (Fig. 1B). The genetic stability of the prepackaging cell lines may be derived from the fact that the *rep* and *cap* genes are under the control of the antisense RNA driven by the CMV-IE promoter. The colony number of pIM45 transfectants was about one third of that of the pLCLCR transfectants, suggesting that the Rep and Cap transcripts from pIM45 are more toxic as expected. Furthermore, the difference between the colony numbers of the control (pCMV + pSV2bsr) and pLALCR transfectants (pLALCR + pSV2bsr) was not significant. These results suggest that the anti-sense expression may have possible use for the regulation of toxic gene expression. These experiments were repeated with similar results. The appearance of clones capable of detection of the PCR products was more frequent in

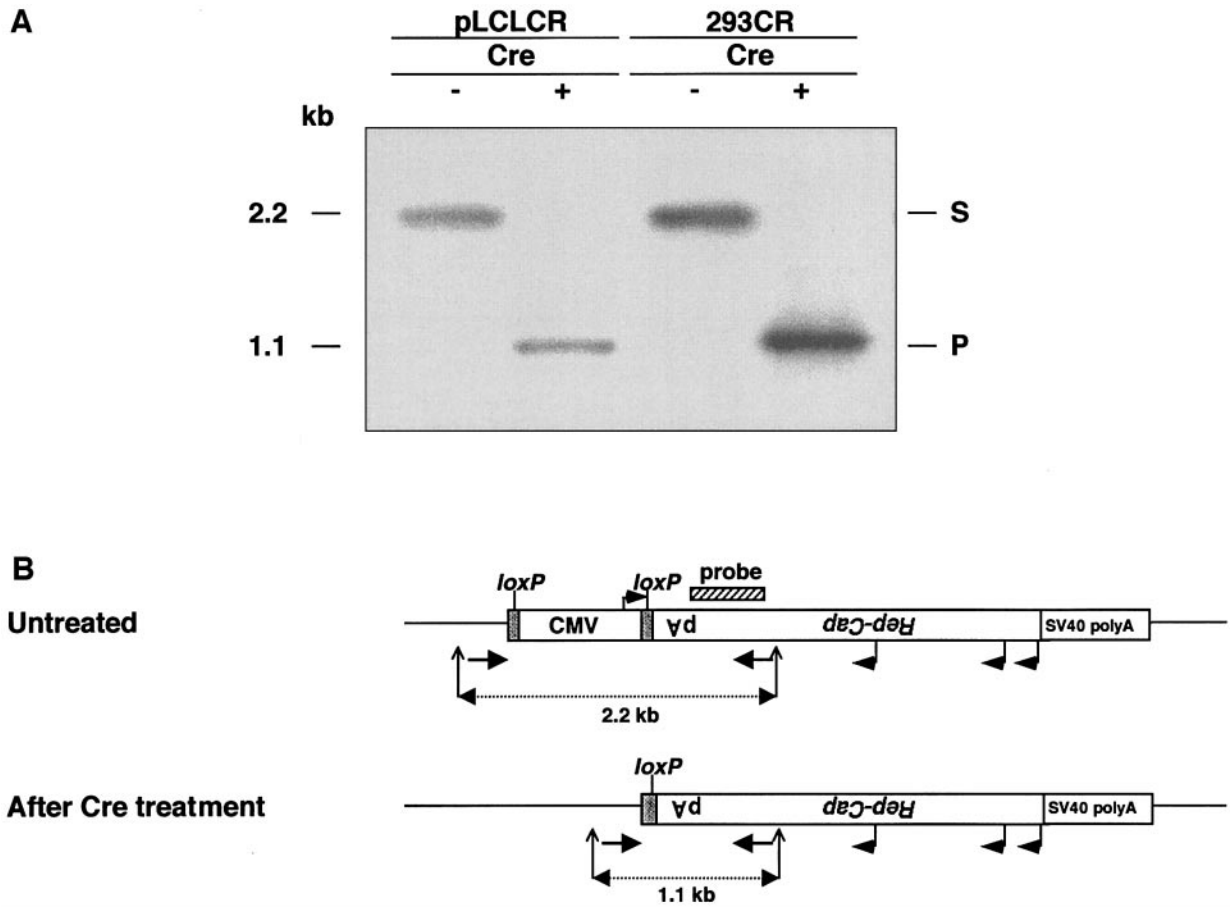


**FIG. 2.** *In vitro* recombination assay. (A) Recombination in pLCLCR in a cell-free system (left) and in the 293CR cell line (right). P and S, product and substrate, respectively. (B) Physical maps and predicted structural changes in pLCLCR after the Cre recombinase expression. Arrows indicate sites of primers used for the PCR analysis. In the case of pLCLCR, the predicted PCR products are 1.1 kb in size after the recombination because of the lack of a CMV-IE promoter.

pLCLCR transfectants (6 of 22 colonies) than in pIM45 transfectants (none of 7). These results indicate that pLCLCR is effective for isolation of clones that harbor *rep* and *cap* genes and support the feasibility of screening the production of rAAV. The positive clones were transfected with pAAVLZ and infected with AxCAN-Cre to estimate the productivity of rAAV by dot-blot analysis. We selected a clone that produced more copies of rAAV than others as a candidate for the prepackaging cell line (293CR).

### *Efficient Recombination in a Cell-Free System and the 293CR Chromosome after the Introduction of Cre Recombinase*

In this study, we established a prepackaging cell line containing an inducible transcriptional unit for the *rep* and *cap* genes with the Cre recombinase–*loxP* system, that is utilized to switch the expression in an all-or-none manner (14). When Cre recombinase is expressed, the prepackaging cell line is efficiently converted into a packaging cell line (Fig. 1B). To examine the recombination activity of Cre in the cell-free system, the Cre extract was mixed with 1 $\mu$g of plasmid pLCLCR containing a pair of *loxP* sequences and incubated at 37°C for 30 min. A single 2.2-kb band was detected as the PCR product from untreated pLCLCR, but it mostly disappeared and a new 1.1-kb PCR product appeared instead after the Cre recombinase transduction (Fig. 2A). This change in fragment size is consistent with the idea that Cre recombinase excised the DNA sequence between the two *loxP* sequences by recombination (Fig. 2B). For recombination analysis of the prepackaging cell line, the 293CR cells were infected with AdCre at an m.o.i. of 1. Chromosome DNA was isolated 2 days after the infection and used for PCR analysis. The same change in the fragment size of the PCR product was observed as seen in the cell-free system (Fig. 2A).

For the rapid and efficient expression of Cre recombinase and Cap, we used the replication-defective adenovirus vectors AdCre and AVC2Cap. The fact that the prepackaging cell line originated from human 293 cells has two advantages: plasmid transfection effi-

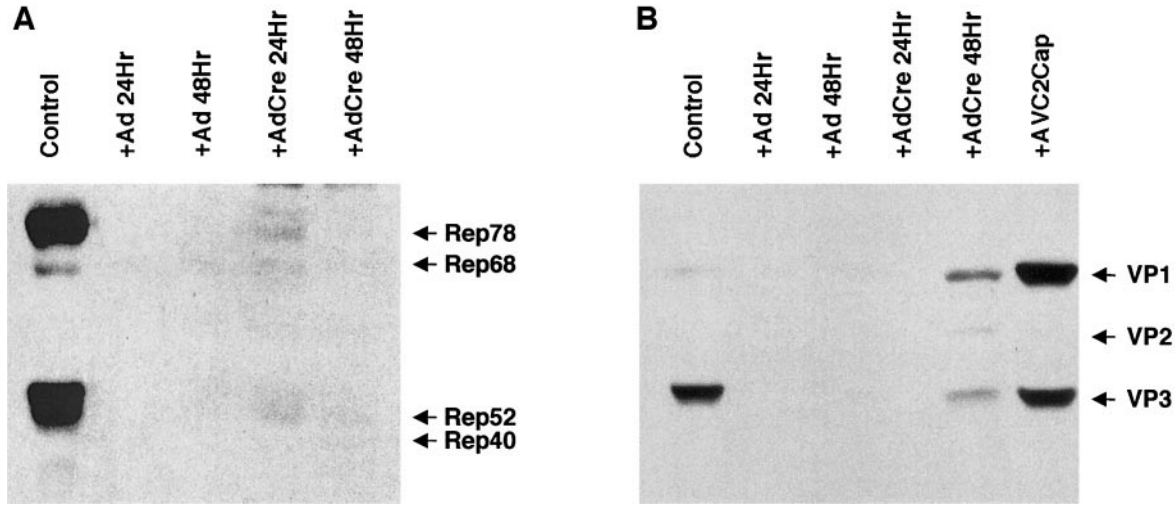


**FIG. 3.** Western blotting of 293CR cell extracts for (A) Rep and (B) Cap expression. Control, positive control generated by transducing 293 cells with pAAVLacZ, pIM45, and Adex. Infection with adenovirus (Ad, Adex; AdCre, AdexCre; or AVC2Cap) is indicated.

ciency is generally higher into 293 cells than other human cell lines (19), and also E1-deleted recombinant adenovirus is capable of amplification in the 293 cells. When the Rep and Cap expression levels were analyzed by Western blotting, the 293CR cells were found to produce detectable levels of Rep and Cap after expression of Cre recombinase (Figs. 3A and 3B), suggesting that Rep and Cap expression was controlled under the anti-sense mediated regulation. Two days after the infection with AdCre, the cell lines produced Cap, while Rep was produced at much lower levels. This also suggested that the limited level of Rep expression seen in our selected clone was favorable for a prepackaging cell. This is consistent with the evidence discussed earlier that the presence of the *rep* gene often resulted in the repression of heterologous gene expression during DNA-mediated transformation or transient expression assays (20). With simultaneous infection with AVC2Cap, the cell lines clearly produced Cap at substantial levels. Interestingly, the ratio of VP1 to VP3 in AdCre- or AVC2Cap-treated cells was higher than that in native AAV. The capsid proteins of wild-type AAV are reported to be composed with a stoichiometry of 1:1:10 for VP1, VP2, and VP3, respec-

**TABLE 1**

AdCre-Mediated rAAV Production in 293CR Cells

| AdCre m.o.i. | Packaging titer (vector particles per 3.5-cm dish)[a] on the day after AdCre infection | | |
|---|---|---|---|
| | 2 | 4 | 6 |
| 0 | ND[b] | ND | ND |
| 0.1 | $2.7 \times 10^6$ | $7.1 \times 10^7$ | $5.2 \times 10^7$ |
| 0.3 | $9.8 \times 10^7$ | $1.6 \times 10^8$ | $2.8 \times 10^8$ |
| 1 | $5.0 \times 10^8$ | $7.6 \times 10^8$ | $5.0 \times 10^8$ |
| 3 | $6.8 \times 10^8$ | $8.2 \times 10^8$ | $3.2 \times 10^8$ |
| 10 | $8.0 \times 10^8$ | $1.0 \times 10^9$ | $1.9 \times 10^8$ |
| 30 | $1.0 \times 10^8$ | $1.2 \times 10^8$ | $6.0 \times 10^7$ |

[a] Titers of the AAVlacZ vectors produced using 293CR after transfection with pAAVlacZ followed by AdCre infection at various m.o.i.'s were examined. The packaging titers were determined by quantitative DNA dot-blot hybridization.

[b] ND, not detectable.

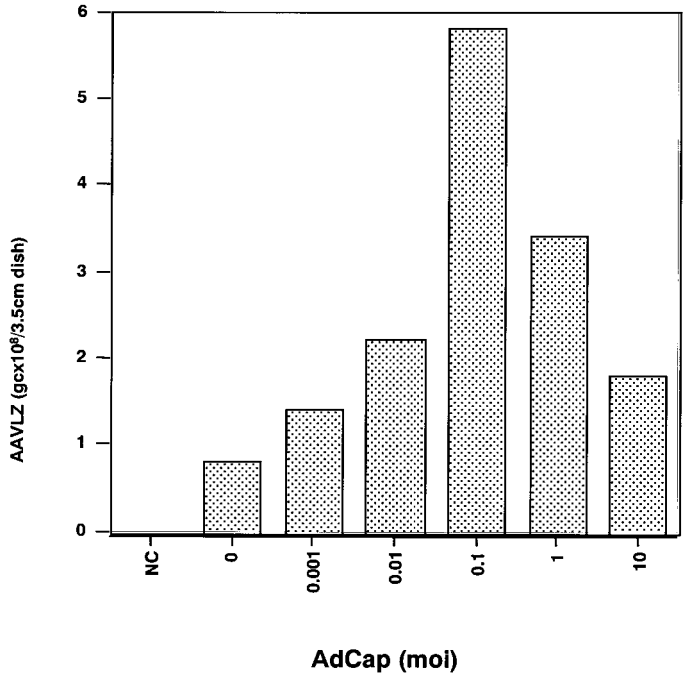


**FIG. 4.** AAV production in the transfection-free system. The 293CR cells were infected with AAVLacZ, AdexCre, and AVC2Cap. The vector production by 293CR after AVC2Cap infection at several m.o.i.'s was estimated. NC, negative control: procedure without Cre introduction.

**TABLE 2**

Effect of AdCre and AVC2Cap on rAAV Virus Production in 293CR Cells

| | Conditions[a] | | | | | Packaging titer[b] (vector particles per 3.5-cm dish) |
|---|---|---|---|---|---|---|
| | Cell line | Vector | AAV helper | Ad helper | Cre | |
| 1 | 293 | pAAVLacZ | pIM45 | Adex | NA[c] | $1.0 \times 10^9$ |
| | 293 | pAAVLacZ | pIM45 | AVC2Cap | NA | $1.1 \times 10^{10}$ |
| 2 | 293CR | pAAVLacZ | — | Adex | — | $<1.0 \times 10^6$ |
| | 293CR | pAAVLacZ | — | Adex | AdCre | $5.0 \times 10^8$ |
| | 293CR | pAAVLacZ | — | AVC2Cap | AdCre | $1.2 \times 10^{10}$ |
| 3 | 293CR | AAVLacZ | — | Adex | — | $<1.0 \times 10^6$ |
| | 293CR | AAVLacZ | — | Adex | AdCre | $7.8 \times 10^7$ |
| | 293CR | AAVLacZ | — | AVC2Cap | AdCre | $5.8 \times 10^8$ |

[a] The cell lines were transfected under various conditions: 1, 293 transfected with a vector plasmid (pAAV-LacZ) and an AAV helper plasmid (pIM45) followed by adenovirus infection of Adex or AVC2Cap; 2, 293CR transfected with a pAAV-LacZ followed by adenovirus infection of Adex with or without AdCre, or AVC2Cap with AdCre; 3, 293CR infected with an AAV-LacZ followed by adenovirus infection of Adex with or without AdCre, or AVC2Cap with AdCre.

[b] Titers of the AAVlacZ vectors produced by using the 293 or 293CR cells were compared.

[c] NA, not applicable.

tively (21). The ratio of virion capsid subunits correlates with the expression levels seen during productive infection (22).

*Conditions for Optimal Virus Production*

We found the optimal range of m.o.i.'s of AdCre for the production of rAAV to be around 1 to 10 (Table 1). Since the growth of 293CR cells was significantly inhibited at an m.o.i. of more than 10, we infected 293CR with the adenovirus vector at an m.o.i. of 1 in subsequent experiments. Transfection-free production of rAAV is also feasible with this system. We measured virus production by 293CR after AdCre infection at an m.o.i. of 1 and AVC2Cap infection at several m.o.i.'s as a transfection-free rAAV production system. We found the optimal range of m.o.i.'s of AVC2Cap for the production of rAAV to be 0.1 (Fig. 4). Cap-expression adenovirus vector produced an approximately 10-fold increase in the yield of AAV vector with either vector plasmid or rAAV (Table 2), consistent with the report that the production of rAAV is dependent on the amount of Cap protein (23). Our previous study has shown that rAAV was amplified effectively when the input virus was used just after transfection of helper plasmids (24). In the study presented here, rAAV was amplified 30-fold compared with the amount of input AAV when AVC2Cap was applied. The yield of the amplified vector did not increase significantly when more than 300 particles/cell of the input virus are used (24). However, production of rAAV without transfection permits the use of crude lysate as a source of vector and eliminates the transfection procedure. The rAAV-producing system presented here is advantageous in that one can control the Rep and Cap expression effectively in 293 cells, and amplify rAAV without a transfection procedure.

We propose that antisense mediated regulation of Rep and Cap expression is feasible approach for the generation of the prepackaging cell line for AAV vectors. However, passage for 2 months resulted in limited growth accompanied with a decrease in Rep and Cap expression (data not shown). Although Rep and Cap expression is suppressed below the level of detection, long-term leaky expression of the *rep* gene product may confer a growth disadvantage on cells. High passage number should be avoided by maintaining aliquots of frozen stock at low passage number for virus propagation. Further improvement of the packaging cell line with more exquisite control system for the *rep* gene expression would ease large-scale production of the recombinant AAV.

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