Highly regulated expression of adeno-associated virus large Rep proteins in stable 293 cell lines using the Cre/loxP switching system

Yoji Ogasawara,1,2† Hiroaki Mizukami,1 Masashi Urabe,1,2 Akihiro Kume,1,2 Yumi Kanegae,4 Izumu Saito,4 John Monahan3 and Keiya Ozawa1,2

1,2Division of Genetic Therapeutics, Jichi Medical School† and CREST, Japan Science and Technology Corporation (JST)2, 3311-1 Yakushiji, Minamikawachi-machi, Tochigi 329-0498, Japan
3Avigen Inc., 1201 Harbor Bay Parkway, #1000, Alameda, CA 94502, USA
4Laboratory of Molecular Genetics, Institute for Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

Since the Rep proteins of adeno-associated virus (AAV) are harmful to cells, it is difficult to obtain stable cell lines that express them constitutively. In this study, stable 293 cell lines were obtained in which large Rep expression was inducible by using the Cre/loxP switching system. To determine the function of the induced Rep proteins, the packaging capacity was examined after supplementation with a plasmid expressing small Rep and Cap proteins. A significant amount of recombinant AAV (5.5 x 108 vector particles per 10 cm dish) was produced by transfection with a vector plasmid and infection with Cre-expressing recombinant adenoaviruses, indicating that the large Rep proteins retained the function required for packaging. These findings indicate that large Rep protein expression can be strictly regulated by the Cre/loxP system and will also serve as a basis for the development of an efficient AAV-packaging cell line.

Adeno-associated virus (AAV) is a dependent human parvovirus with a single-stranded DNA genome of about 4.7 kb. Within the viral genome, two open reading frames for Rep and Cap proteins are located between the two inverted terminal repeats (ITRs) at either end. Rep proteins regulate the replication of the virus; Cap proteins constitute the capsid of the virion structure. AAV vectors have attractive features for gene transfer purposes (Fan et al., 1998; Flotte et al., 1993; Herzog et al., 1999; Kaplitt et al., 1994). The expression of appropriate amounts of Rep and Cap proteins is essential for efficient AAV vector production (Li et al., 1997; Ogasawara et al., 1999). However, these AAV proteins, especially Rep, are known to exert antiproliferative effects and are therefore toxic to cells when overexpressed (Holscher et al., 1994; Yang et al., 1994). Due to this toxicity, constitutive expression of Rep proteins leads to cellular death. Therefore, controlling the expression of Rep proteins is vital to many applications, including AAV-packaging cell lines. An appropriate induction system is required to control protein expression. To date, several systems have been developed and used to control Rep expression, such as tetracycline (Inoue & Russell, 1998), metallothionein promoter (Yang et al., 1994) and mouse mammary tumour virus long terminal repeat (Holscher et al., 1994).

In this report, we investigated the adenovirus-mediated Cre/loxP system (Kanegae et al., 1995) as an inducible genetic switch to overcome the toxicity of the Rep proteins. In theory, cells are transfected with a plasmid in which a ‘stuffer’ sequence, flanked by a pair of loxP recombination target sites, is placed between the CAG promoter and the sequence encoding the AAV proteins. When the cells are infected by a recombinant adenovirus expressing Cre recombinase, the sequence between the two loxP sites should be deleted and the target gene should be activated by the CAG promoter. At the same time, adenoviruses supply helper functions for virus replication. As a result, toxic gene expression can be controlled by Cre-expressing adenovirus vectors.

The structures of the plasmids used in this study are shown in Fig. 1. The SalI–EcoRI fragment of the CAG promoter sequence from pUC-CAGGS, which consists of a variant chicken actin promoter with a CMV-IE enhancer, was blunt-ended with Klenow fragment, ligated with HindIII linkers and then inserted into the HindIII site of plox2 (pCAGLL) (Niwa et al., 1991; Orban et al., 1992). Plasmid plox2 was provided by...
Y. Ogasawara and others

Fig. 1. (a) Plasmid structure of pCAGLBLAAV. A gene for blasticidin S resistance (bsr) was placed between twoloxP sites as a stuffer sequence. AAV rep/cap sequences containing the two native promoters p19 and p40 were placed just downstream of the secondloxP sequence. (b) Plasmid pR52/40Cap contains the rep and cap genes with the p5 promoter sequence placed at the 3' end as an enhancer element.

J. D. Marth (University of California, San Diego, USA) and pUC-CAGGS by Jun-ichi Miyazaki (Tohoku University, Japan). The HindIII fragment of pSV2bsr, containing the blasticidin S-resistance gene (bsr), was subcloned into the HindIII site of pCMV to give pCMVbsr (Urabe et al., 1997).

The XhoI–NotI fragment of pCMVbsr, which carries the bsr gene and SV40 polyadenylation signal, was blunt-ended, ligated with BamHI linkers and inserted into the BamHI site of pCAGLL (pCAGLBL). The p5 promoter-deleted BsaI fragment from pIM45 (nt 285–4458 of the AAV genome), which contains the rep and cap genes (McCarty et al., 1991) and the polyadenylation signal sequence, was isolated, blunt-ended and then inserted into the SmaI site of pCAGLBL to complete pCAGLBLAAV.

Adex1w1 (Adex) is a recombinant adenovirus containing a nearly full-length adenovirus type 5 genome with E1 and E3 deletions. AxCANCre (AdCre) is a Cre recombinase expression adenovirus vector (Kanegae et al., 1995). 293 cells, a human embryonic kidney cell line transformed by adenovirus type 5 (Graham et al., 1977), were maintained as described previously (Ogasawara et al., 1998), and were seeded at a density of 5 × 10⁴ cells per 10 cm dish on the day before transfection. The cells were transfected with 10 µg pCAGLBLAAV by the calcium phosphate procedure (Wigler et al., 1978). The cells were trypsinized, diluted and plated on 10 cm dishes at 24 h post-transfection. The transfected cells were selected in a medium containing 10 µg/ml blasticidin S hydrochloride (Funakoshi), starting at 48 h post-transfection. Fifteen of more than 100 clones were isolated and expanded (CAGLBLAAV).

Western blot analyses of AAV proteins were performed as described previously (Ogasawara et al., 1998). Briefly, on the day before infection with adenovirus, stably transfected 293 cells (CAGLBLAAV) were seeded at a density of 2 × 10⁶ cells per 10 cm dish. The cells were transfected with 20 µg pAAV-LacZ (Maeda et al., 1997) by the calcium phosphate procedure and then infected with Adex or AdCre (m.o.i. of 1) at 6 h post-transfection, and were harvested at 48 h post-transfection. The cells were resuspended in 1 ml 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 µg per lane) were loaded onto a 10% polyacrylamide gel, electrophoresed and transferred to Immobilon membranes (Millipore). The membranes were blocked and then reacted with either anti-Rep monoclonal antibody 294-4 (gift from J. Kleinschmidt, Deutsches Krebsforschungszentrum, Heidelberg, Germany) or anti-Cap monoclonal antibody B1 (Progen). Rep and Cap proteins were visualized by using the enhanced chemiluminescence detection method (ECL kit; Amersham).

Expression profiles for the AAV proteins are shown in Fig. 2. Cell extracts from untreated CAGLBLAAV did not show any recognizable Rep and Cap proteins. However, upon infection with AdCre (m.o.i. of 1), Rep78/68 were strongly induced and Rep52/40 were weakly expressed (Fig. 2a). Induction of Cap proteins was negligible (Fig. 2b). Wild-type adenovirus did not show this effect by itself. Of 15 clones tested, nine showed significant induction of Rep78/68 expression.

To supplement the proteins that were not fully induced by this system, we constructed a Rep52/40 plus Cap expression
plasmid (pR52/40Cap) (Ogasawara et al., 1999). The structure of this plasmid is outlined in Fig. 1. To construct pR52/40Cap, the Sfi–XbaI fragment (nt 537–4488) of pRepCap (Surosky et al., 1997), which contains most of the AAV sequences except the ITRs, was first cloned into pUC18. 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 sequence of the Sfi–XbaI fragment as an enhancer element. A representative CAGBLAAV clone (5 × 10^5 cells per 10 cm dish) was cotransfected with 1 µg pSV2neo and 10 µg pR52/40Cap. After 48 h, the transfected cells were selected in the medium containing 800 µg/ml active-component genetin (Gibco BRL). The genetin-resistant clones were isolated and amplified (CAGBLAAV + pR52/40Cap). Although the cells showed enhanced expression of Rep52/40 and Cap proteins in a constitutive manner, the level of Cap expression was still lower than that resulting from transient transfection with pIM45. No increase in Rep52/40 and Cap expression was observed upon infection of the cells with AdCre (Fig. 2). Rep68 expression was very low in the clone shown in Fig. 2(a), but this seemed to be due to clonal variation.

To determine the function of the large Rep proteins induced by the Cre/loxP system, we measured the packaging capacity of these cell lines. For this purpose, the AAV vector plasmid (pAAV-LacZ) was used, which contains a lacZ gene under the control of the CMV-IE promoter between ITRs. The titres of AAV-LacZ were determined by quantitative DNA dot-blot hybridization. The AAV-LacZ stock (50 µl) was first treated with 70 U DnaseI for 1 h and then with 500 µg/ml proteinase K for 1 h, before being extracted with phenol–chloroform. The isolated DNA and standard DNA (a 188 kb Clal–PvuII fragment located within the lacZ coding sequence) were denatured, applied to a nylon membrane (Hybond-N+; Amersham) with a manifold dot-blot apparatus (Schleicher & Schuell), cross-linked by UV light and hybridized with a lacZ gene probe radiolabelled with a random primer labelling kit (Amersham). The blots were washed and exposed to an imaging plate (Fuji Photo Film). The blots were counted directly and analysed on a BAS-2000 imaging system (Fuji). The number of AAV vector genomes present was determined by comparison with the standard DNA. Transfectants of CAGBLAAV showed a titre of 5 × 10^6 particles per 10 cm dish. Following the addition of the pR52/40Cap plasmid, the packaging efficiency increased 100-fold to 5 × 10^7 particles per 10 cm dish (Table 1). After culturing this cell line for 1 month, the titre had decreased to 4.6 × 10^7 particles per 10 cm dish, accompanied by a decrease in Rep52/40 and Cap expression (data not shown). Two further experiments showed similar results (data not shown). The packaging efficiency of these cell lines was much lower than that of a standard transfection protocol using pIM45 (about 10^11 vector particles per 10 cm dish). Functional titres assayed by X-Gal staining were approximately 10^4-fold less than particle titres in our experiments.

The present study showed that the expression of Rep78/68 was strictly controlled in response to Cre-expressing adenovirus vectors. Functional analysis of the large Rep proteins can be performed with this system. However, the induction of Rep52/40 and Cap proteins was much less significant. Larger amounts of Rep52/40 and Cap proteins were expressed in transient transfection experiments with pCAGBLAAV (data not shown). In the process of establishing clones, the expression of these proteins decreased. The constitutive expression of Rep52/40 and Cap proteins may have caused deleterious effects on 293 cells, even at these low levels, and prevented the establishment of clones with the capacity to express sufficient amounts of these proteins in an inducible manner. Another possibility is that this may be due partly to the lack of p5 promoter regions, which have the ability to enhance both p19 and p40 promoter activities (Pereira et al., 1997). However, even after an additional transfection with pR52/40Cap, containing a p5 element, we could not obtain stable clones expressing Cap proteins in large quantities. The low level of expression of Cap proteins in these clones may be mainly responsible for their inefficient AAV-packaging capacity. In addition, induction of excessive amounts of Rep78/68 may have caused some reduction in AAV vector production. In our recent study, the expression of a relatively small amount of large Rep and a large amount of Cap was shown to be important for the optimal production of AAV vectors (Ogasawara et al., 1999). Since Cap proteins seem to be toxic to the cells, an inducible-expression system for Cap proteins, as well as for Rep proteins, would be essential for the establishment of an efficient AAV-packaging cell line.

One of the problems hampering the clinical application of AAV vectors is the difficulty of large-scale production. To avoid complicated processes in vector production, suitable packaging cell lines are desirable. Several cell lines have been reported utilizing different inducible gene expression systems.

### Table 1. Packaging titres of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Packaging titre (vector particles per 10 cm dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAGBLAAV</td>
<td>5 × 10^6</td>
</tr>
<tr>
<td>CAGBLAAV + pR52/40Cap</td>
<td>5.5 × 10^6</td>
</tr>
<tr>
<td>CAGBLAAV + pR52/40Cap</td>
<td>4.6 × 10^7</td>
</tr>
<tr>
<td>after 1 month</td>
<td></td>
</tr>
</tbody>
</table>

To determine the function of the large Rep proteins induced by the Cre/loxP system, we measured the packaging capacity of these cell lines. For this purpose, the AAV vector plasmid (pAAV-LacZ) was used, which contains a lacZ gene under the control of the CMV-IE promoter between ITRs. The titres of AAV-LacZ were determined by quantitative DNA dot-blot hybridization. The AAV-LacZ stock (50 µl) was first treated with 70 U DnaseI for 1 h and then with 500 µg/ml proteinase K for 1 h, before being extracted with phenol–chloroform. The isolated DNA and standard DNA (a 188 kb Clal–PvuII fragment located within the LacZ coding sequence) were denatured, applied to a nylon membrane (Hybond-N+; Amersham) with a manifold dot-blot apparatus (Schleicher & Schuell), cross-linked by UV light and hybridized with a lacZ gene probe radiolabelled with a random primer labelling kit (Amersham). The filter was washed and exposed to an imaging plate (Fuji Photo Film). The blots were counted directly and analysed on a BAS-2000 imaging system (Fuji). The number of AAV vector genomes present was determined by comparison with the standard DNA. Transfectants of CAGBLAAV showed a titre of 5 × 10^6 particles per 10 cm dish. Following the addition of the pR52/40Cap plasmid, the packaging efficiency increased 100-fold to 5 × 10^7 particles per 10 cm dish (Table 1). After culturing this cell line for 1 month, the titre had decreased to 4.6 × 10^7 particles per 10 cm dish, accompanied by a decrease in Rep52/40 and Cap expression (data not shown). Two further experiments showed similar results (data not shown). The packaging efficiency of these cell lines was much lower than that of a standard transfection protocol using pIM45 (about 10^11 vector particles per 10 cm dish). Functional titres assayed by X-Gal staining were approximately 10^4-fold less than particle titres in our experiments.

The present study showed that the expression of Rep78/68 was strictly controlled in response to Cre-expressing adenovirus vectors. Functional analysis of the large Rep proteins can be performed with this system. However, the induction of Rep52/40 and Cap proteins was much less significant. Larger amounts of Rep52/40 and Cap proteins were expressed in transient transfection experiments with pCAGBLAAV (data not shown). In the process of establishing clones, the expression of these proteins decreased. The constitutive expression of Rep52/40 and Cap proteins may have caused deleterious effects on 293 cells, even at these low levels, and prevented the establishment of clones with the capacity to express sufficient amounts of these proteins in an inducible manner. Another possibility is that this may be due partly to the lack of p5 promoter regions, which have the ability to enhance both p19 and p40 promoter activities (Pereira et al., 1997). However, even after an additional transfection with pR52/40Cap, containing a p5 element, we could not obtain stable clones expressing Cap proteins in large quantities. The low level of expression of Cap proteins in these clones may be mainly responsible for their inefficient AAV-packaging capacity. In addition, induction of excessive amounts of Rep78/68 may have caused some reduction in AAV vector production. In our recent study, the expression of a relatively small amount of large Rep and a large amount of Cap was shown to be important for the optimal production of AAV vectors (Ogasawara et al., 1999). Since Cap proteins seem to be toxic to the cells, an inducible-expression system for Cap proteins, as well as for Rep proteins, would be essential for the establishment of an efficient AAV-packaging cell line.

One of the problems hampering the clinical application of AAV vectors is the difficulty of large-scale production. To avoid complicated processes in vector production, suitable packaging cell lines are desirable. Several cell lines have been reported utilizing different inducible gene expression systems.
(Clark et al., 1996; Hölscher et al., 1994; Inoue & Russell, 1998; Tamayose et al., 1996; Yang et al., 1994). Although recent reports showed great improvements in titres, further improvement is still required. Our system will offer an alternative strategy for the development of the novel AAV-packaging cell lines.

This work was supported in part by grants from the Ministry of Health and Welfare of Japan, Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and a grant from Japanese Foundation for Multidisciplinary Treatment of Cancer.

References


Received 18 March 1999; Accepted 19 May 1999