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A New Strategy for Large-Scale Preparation of High-Titer Recombinant Adeno-Associated Virus Vectors by Using Packaging Cell Lines and Sulfonated Cellulose Column Chromatography

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ABSTRACT

The extensive testing of adeno-associated virus (AAV) as a vector for human gene therapy has been hampered by low efficiency of the current packaging system, which is based on transient transfection with plasmid DNAs and infection with adenovirus in permissive cells. In an effort to resolve this problem, HeLa cell-based packaging cell lines were established. These packaging cells carry multiple copies of the AAV genome lacking the inverted terminal repeat (ITR) sequences. The AAV genes were silent in these cells but inducibly expressed by adenovirus infection. When the AAV vector plasmid containing the neo^R gene flanked by the ITRs was also integrated into these cells, efficient production of the recombinant AAV particles occurred after adenovirus infection. AAV vector particles in cell lysates could be concentrated by sulfonated cellulose column chromatography. Using the packaging cells and the column chromatography technique, it is possible to prepare AAV vectors with the titer of higher than 10^8 cfu/ml or 5×10^{10} particles/ml. This new strategy should be useful for testing AAV vectors *in vivo*.

OVERVIEW SUMMARY

Adeno-associated virus (AAV) is a potential vector for human gene therapy. However, it has been difficult to prepare sufficient amounts of high-titer AAV vectors for *in vivo* application. In this work, we established HeLa cell lines containing multiple copies of the AAV genome and demonstrated that these cells can be used for packaging of recombinant AAV vectors. We also showed that Cellulofine sulfate column chromatography was useful for concentration of infectious AAV particles. Using these two techniques, large-scale preparation of high-titer AAV vectors became possible.

INTRODUCTION

ADENO-ASSOCIATED VIRUS (AAV) is a nonpathogenic, replication-defective parvovirus. For productive infection of AAV, superinfection with helper virus such as adenovirus or herpes simplex virus is required (Berns and Bohenzky, 1987). In the absence of helper virus, AAV stably integrates into a defined region of human chromosome 19 (q13.3-qter)(Samulski *et al.*, 1991; Kotin *et al.*, 1992). The AAV genome is a 4.7-kb single-strand DNA that contains three promoters (p5, p19, p40), a single polyadenylation signal, coding sequences for nonstructural (Rep) and structural (Cap) proteins, and the inverted terminal repeat (ITR) sequences at both ends of the genome. The ITRs are required and sufficient for replication, packaging, and chromosomal integration of the genome.

Recombinant AAV is now considered to be an attractive vector for human gene therapy. The advantages of AAV vectors include lack of pathogenicity, broad host range, efficient and stable integration into cellular genome, ability to infect growth arrested cells (Alexander *et al.*, 1994; Podsakoff *et al.*, 1994), and high physical stability of virus particles. It was demonstrated that AAV vectors are capable of efficient transduction of various hematopoietic cells including $CD34^+$-enriched cells

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from cord blood (Zhou *et al.*, 1994), peripheral blood (Miller *et al.*, 1994), and bone marrow *in vitro* (Muro-Cacho *et al.*, 1992). Successful *in vivo* gene transfer with AAV vectors into post-mitotic cells in the rat brain was also reported (Kaplitt *et al.*, 1994).

A major disadvantage of AAV-mediated gene transfer is the difficulty in preparing a high titer of vector particles. The current method for generation of AAV vectors is based on co-transfection with the recombinant vector and packaging plasmids and infection with adenovirus in permissive cells such as 293 cells and HeLa cells (Hermonat and Muzyczka, 1984; Samulski *et al.*, 1987). The vector plasmid contains the gene of interest and the transcription control elements flanked by the ITRs. The packaging plasmid contains the entire AAV genome sequences except the ITR sequences. In these transfected cells, the recombinant AAV genome flanked by the ITRs is excised, replicated, and encapsidated into viral particles composed of Cap proteins provided *in trans* from the packaging plasmid. The typical yield of AAV vectors from this system is approximately 10^5 colony-forming units (cfu) per 10-cm culture plate (Kaplitt *et al.*, 1994; Miller *et al.*, 1994). Therefore, to obtain the sufficient amounts of AAV vectors for *in vivo* studies, typically more than 100 plates have to be transfected with the plasmids using the time-consuming and complicated calcium phosphate co-precipitation or lipofection method.

The packaging cell lines analogous to those used for retroviral vector production are not available at this moment. Establishment of cell lines that constitutively express the rep gene has not been accomplished. The major cause appears to be the cytotoxicity of the rep proteins. Recently, cell lines inducibly expressing the rep gene have been established using the metallothionein promoter (Yang *et al.*, 1994) or the mouse mammary tumor virus long terminal repeat (LTR) (Hoelscher *et al.*, 1994). However, the utility of such cell lines in generation of the AAV vectors has not been evaluated.

In this study, we established HeLa cell lines bearing high copy numbers of the packaging plasmid. The rep gene is silent in these cells but inducibly expressed by adenovirus infection. We demonstrate that this cell line can be used as an AAV vector packaging cell line for large-scale production of AAV vectors. We also show that sulfonated cellulose column chromatography is efficient for concentration of infectious AAV particles.

MATERIALS AND METHODS

Plasmid construction

The plasmid containing the complete AAV genome (psub201) and the AAV packaging plasmid (pAAV/Ad) have been described previously (Samulski *et al.*, 1987, 1989). The vector plasmid pNAV was constructed by substituting the AAV coding region in psub201 with the 4.3-kb fragment containing the neomycin resistance gene (neo^R) driven by the thymidine kinase promoter from pMC1neo (Thomas and Capecchi, 1987) and the human β-globin second intron, third exon, and polyadenylation signal from pLN (Shimada *et al.*, 1991). pLH contains the hygromycin resistance gene directed by the Moloney murine leukemic virus LTR (Shimada *et al.*, 1991).

Establishment of stable cell lines

HeLa cells grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) were co-transfected with 10 μg of pAAV/Ad and 1 μg of pNAV (for generation of producing cell lines) or 1 μg of pLH (for generation of packaging cell lines) by the calcium phosphate precipitation method on day 1. The cells were trypsinized and replated at various concentrations on day 3, and selection was begun on day 4 in the medium containing 1 mg/ml G418 (active) or 0.4 mg/ml hygromycin B. Drug-resistant colonies were picked up about 20 days after transfection and expanded for analyzing the structure and copy number of integrated genes.

Production and titering of AAV vectors

For generation of AAV vectors by transient plasmid transfection, about 60% confluent 293 or HeLa cells grown in a 10-cm dish were first infected with wild-type adenovirus 5 at a multiplicity of infection (moi) of 10 in 10 ml of DMEM with 2% FCS for 1 hr. The adenovirus-infected cells were co-transfected with 10 μg of pAAV/Ad and 10 μg of pNAV by the calcium phosphate coprecipitation methods, and 6 hr later the medium was replaced with fresh medium. After 72 hr, the cells were harvested in 2 ml of DMEM containing 2% FCS and lysed by four cycles of freezing and thawing. In an improved method, the cells were harvested in 6 ml of the culture medium by scraping, and the cells–medium mixture was frozen and thawed for four times. The cell lysate was centrifuged at 12,000 $\times$ *g* for 10 min to remove cell debris and heated at 56°C for 30 min to inactivate adenovirus.

To prepare AAV vectors from stable cell lines, about 60% confluent producer cells in a 10-cm dish were infected with adenovirus at an moi of 10 in 1 ml DMEM with 2% FCS. After 1 hr, 5 ml of fresh DMEM with 10% FCS were added, and the adenovirus-infected cells were cultured for 3 days. AAV vectors were extracted from the lysed cells as described above.

To determine the biological titer of AAV vectors, mouse 3T3 cells (4×10^4 cells per 35-mm well in 6-well plates) were incubated with AAV vector stock (up to 100 μl) in 3 ml of medium for 2 days. The transduced cells were trypsinized, diluted to 1:10, and replated on 10-cm dishes. After 24 hr, G418 (500 μg/ml active) was added to the culture medium, and the number of G418-resistant colonies were counted 10–14 days after transduction.

For estimation of the total number of vector particles, the vector stock was treated with DNase I, and encapsidated DNA was extracted with phenol-chloroform, precipitated with ethanol, and subjected to slot blot analysis as described (Samulski *et al.*, 1989).

Concentration of AAV vectors by column chromatography

Sulfonated cellulose (Cellulofine sulfate) was obtained from Seikagaku-kougyo Corp. (Tokyo, Japan). The cell lysate containing AAV vectors (up to 200 ml) was applied to the column (17 $\times$ 200 mm), previously equilibrated with phosphate-buffered saline (PBS) by gravity. After washing the column with PBS until the absorbance of the eluate at 280 nm was below 0.01, AAV vectors were eluted with 10 m*M* phosphate

buffer pH 7.2 containing 1.0 *M* NaCl. Fractions of 2.0 ml were collected and titered for the presence of AAV vectors.

Analysis of DNA and RNA

Southern analysis of chromosomal DNA and Northern analysis of total RNA were done as described previously (Fujii and Shimada, 1989). Low-molecular-weight DNA was isolated by the Hirt procedure (Hirt, 1967) at 40 hr after adenovirus infection. The AAV cap probe is a 1.6-kb *Hinc* II fragment from psub201 (nucleotides 2,398–3,983 in the wild-type sequence of AA2CG according to GenBank). The *neo* probe is a 0.9-kb *Pst* fragment from pSV2neo.

RESULTS

Expression of the rep *gene in HeLa cells*

Previous attempts to establish AAV packaging cell lines were hampered by cytotoxic or cytostatic effects of Rep proteins. In the first experiment, we examined the expression of the *rep* gene in transfected cells. RNA analysis showed that both *rep* and cap transcripts were synthesized in 293 cells that express the E1 genes (Fig. 1A). However, no RNA signal was detected in HeLa cells lacking E1 proteins. After adenovirus infection, comparable amounts of transcripts were synthesized in these two cell lines. When the recombinant AAV vectors were generated by the standard transient transfection method, there was no significant difference in the efficiency of vector production between 293 cells and HeLa cells. In both cell lines, the yield of infectious particles was approximately 5×10^5 cfu/ml in 2 ml of cell-lysate from a 10-cm dish. These results indicate that the AAV promoters are silent in HeLa cells. Accordingly, we used HeLa cells for generating AAV packaging cell lines.

Establishment of a recombinant AAV vector producing cell line

HeLa cells were co-transfected with pAAV/Ad and pNAV and selected for resistance to G418. Using PCR screening of genomic DNA, 17 clones containing both the *rep* and neo^R sequences were isolated. These clones were infected with adenovirus, and production of recombinant AAV was monitored by the transduction assay. A clone HAN10 gave the highest titer. After optimization of vector production, it was demonstrated that this clone was capable of producing 3×10^5 cfu/ml in 6 ml of cell-lysate from a 10-cm dish (see below). The growth rate of HAN10 cells was indistinguishable from that of parent HeLa cells.

The copy number and structure of the integrated genes were examined by Southern blot analysis of chromosomal DNA. HAN10 cells contained approximately 20 copies of the packaging sequence and 5 copies of the vector sequence (see Fig. 3, below).

Southern blot analysis of Hirt-extracted low-molecular-weight DNA from HAN10 showed that recombinant AAV genome was excised from integrated pNAV and amplified after adenovirus infection (Fig. 2C). The same assay using the AAV cap probe showed that the wild-type AAV genome did not am-

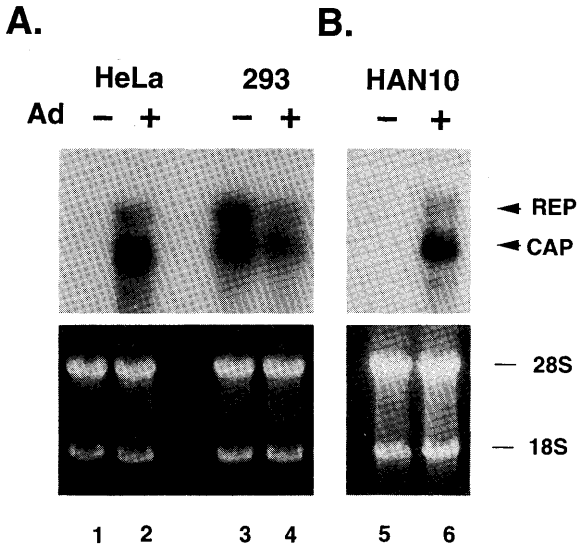

FIG. 1. Inducible expression of the AAV genes. A. Expression of the AAV genes from transiently transfected plasmid DNA. HeLa cells (lanes 1 and 2) and 293 cells (lanes 3 and 4) were transfected with pAAV/Ad. Cells were infected with adenovirus 1 hr before transfection (lanes 2 and 4). RNA was extracted at 40 hr after transfection and subjected to Northern blot analysis using the AAV cap probe. B. Expression of the AAV genes from stably integrated plasmid DNA. RNA was extracted from adenovirus-uninfected (lane 5) or -infected (lane 6) HAN10 cells and analyzed by Northern blot analysis. The upper and lower arrowheads indicate the major *rep* (4.2 kb) and cap (2.3 kb) transcripts, respectively. The lower panels are the ethidium bromide-stained agarose gel.

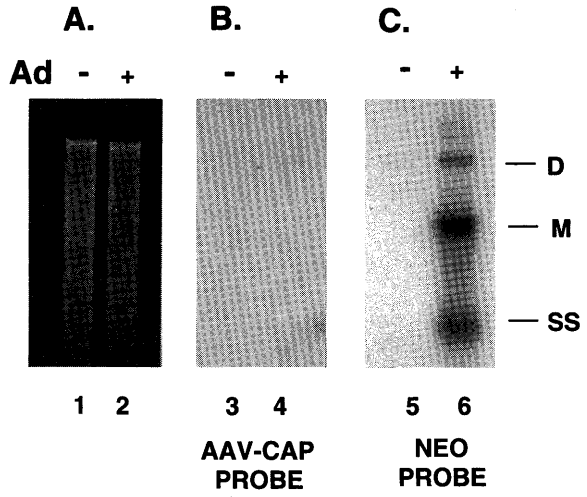

FIG. 2. Excision and replication of the AAV vector genome. Low-molecular-weight DNA was extracted from adenovirus uninfected (lanes 1, 3, and 5) or infected (lanes 2, 4, and 6) HAN10 cells by the Hirt procedure and was blot-hybridized with the AAV cap probe (B) or the *neo* probe (C) after electrophoresis and transfer. A. Ethidium bromide-stained gel. D, Replication-form, double-strand dimer; M, replication-form, double-strand monomer; SS, single-strand monomer.

plified in this system (Fig. 2B). Northern blot analysis of total RNA extracted from HAN10 cells revealed that *rep* expression was efficiently induced by adenovirus infection (Fig. 1B).

Harvesting of AAV vectors

Using an AAV vector-producing cell line, the recombinant AAV vectors could be generated by simple adenovirus infection without plasmid transfection. To improve further the efficiency of preparing AAV vectors, we analyzed the distribution of generated AAV vectors between the cellular fraction and the culture medium. HAN10 cells (3×10^5 cells in a 10-cm dish) were infected with adenovirus at a moi of 10, and after 72 hr the culture medium (10 ml) and the cells were separately collected. The cell lysates in 2 ml of DMEM with 2% FCS were prepared by the standard freezing and thawing procedure. The assay of infectious recombinant AAV showed that 8×10^4 cfu (total) vectors were obtained in the cell-lysate, whereas 2×10^5 cfu (total) were in the culture medium. Approximately 70% of AAV particles were recovered in the culture medium. When we harvested the cells, most of the cells were rounded, probably due to the cytopathic effects of adenovirus but not completely lysed, even though AAV particles appeared to be released from the cells. Therefore, to increase the recovery of AAV vectors, we cultured the cells in 6 ml of DMEM with 10% FCS after adenovirus infection, and the total mixture containing the scraped cells and the original culture medium was subjected to freezing and thawing. Using this strategy, 3×10^5 cfu/ml AAV vectors (2×10^6 cfu/10-cm dish) could be consistently obtained from HAN10 cells.

Establishment of an AAV packaging cell line

We next attempted to establish HeLa cell lines containing the packaging plasmid alone for using as a general packaging cell line. HeLa cells were co-transfected with pAAV/Ad and pLH. A total of nine hygromycin-resistant clones were screened further for the existence of multiple copies of pAAV/Ad by the polymerase chain reaction (PCR) and Southern blot analysis. A clone HA8 carried approximately 20 copies of the AAV/Ad sequence (Fig. 3A).

HA8 cells were transfected with pNAV, and 42 neomycin-resistant clones were isolated. The small-scale biological assay using six-well plates indicated that 11 of 42 clones were capable of producing recombinant NAV vectors after adenovirus infection. The standard biological assay using a 10-cm dish showed that the titers of the 11 producer clones ranged from 5.0×10^2 to 1.8×10^5 cfu/ml (3.0×10^3 to 1.1×10^6 cfu/10-cm dish). These results indicate that the HA8 clone could be used as a general AAV packaging cell line to produce any recombinant AAV vectors.

The recombinant neo^R vector sequence was introduced into HA8 cells by transduction with the recombinant NAV particles at a moi of 1 rather than transfection with pNAV. Seven G418-resistant clones were screened for recombinant virus production. The copy number of the integrated NAV sequence did not exceed three copies/cell, and the maximum titer of these clones was less than 3×10^3 cfu/ml (3×10^4 cfu/10-cm dish).

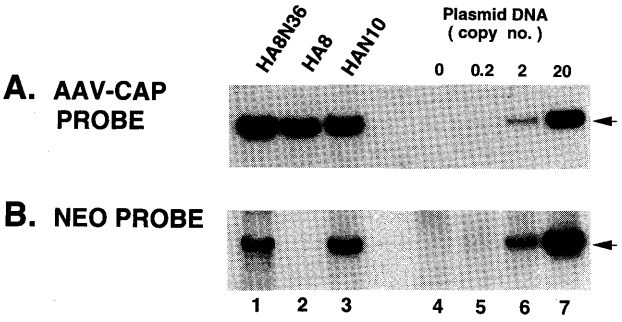

FIG. 3. Southern blot analysis of the integrated plasmid DNAs. Genomic DNAs isolated from HA8N36 (lane 1), HA8 (lane 2), and HAN10 (lane 3) clones were digested with *Xba* I and subjected to Southern blot analysis using the AAV cap probe (A) or the *neo* probe (B). *Xba* I-digested pAAV/Ad (A) or pNAV (B), representing 0.2, 2.0, and 20, copies per cell, was also included in the gel as a copy number standard.

Concentration of AAV vectors by sulfonated cellulose column chromatography

Although the new lysis method described above increased the recovery of AAV vectors from the producing cells, the maximum titer of 3×10^5 cfu/ml is not sufficient for *in vivo* studies. We applied the sulfonated cellulose gel chromatography technique for concentration of AAV particles. A typical elution pattern of this column chromatography is shown in Fig. 4. The cell lysate (165 ml) was loaded on a Cellulofine sulfate gel column and eluted with 1.0 *M* NaCl in 10 m*M* phosphate buffer pH 7.2. The AAV vectors bound efficiently to the gel and eluted with the salt solution. The recovery was nearly 100%, and the concentration factor was more than 50-fold (Fig. 4). Therefore, Cellulofine sulfate column chromatography was proven to be highly efficient for concentration of AAV vectors.

In a separate experiment, AAV vectors prepared using HAN 10 producer cells were characterized by both the biological titering assay and the particle titering assay (Fig. 5). Before column chromatography, the AAV vector stock had the titer of 1×10^6 cfu/ml by the transducing assay, but the particle number was 6×10^8/ml by slot blot analysis. After concentration by column chromatography, the biological titer was estimated to be 1.2×10^8 cfu/ml, whereas the particle number was 6×10^{10}/ml (Fig. 5A,B). The ratio of physical particles to infectious units was calculated to be approximately 500 in both unconcentrated and concentrated preparations. This value is consistent with previous data (Tratschin *et al.*, 1985; Samulski *et al.*, 1989). Using this column chromatography technique, it is possible to prepare the NAV vector with the titer of higher than 5×10^{10} particles/ml. The AAV vector containing the β-galactosidase (β-Gal) gene was also prepared by the transient transfection method and subjected to a sulfonated cellulose column. This vector could be concentrated up to at least 10^9 particles/ml (data not shown).

When the concentrated AAV vector stock was blot hybridized with the AAV cap probe, no signal was detected (Fig. 5C). This confirmed that no wild-type AAV was generated in this AAV packaging system.

DISCUSSION

In this work, we established HeLa cell lines containing multiple copies of the AAV genome lacking the ITRs and demon-

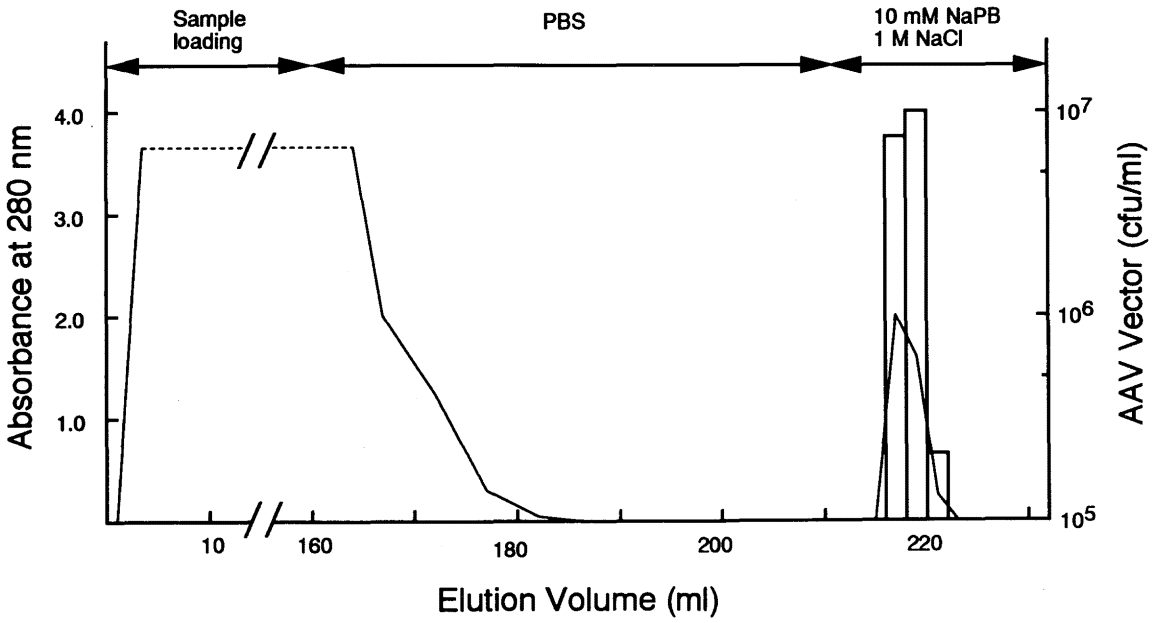

FIG. 4. Cellulofine sulfate column chromatography of the cell lysate containing AAV vectors. The cell lysate (2.2×10^5 cfu/ml of total 165 ml lysate) was obtained from adenovirus-infected HAN10 cells in 20 of 10-cm dishes ($\sim 2 \times 10^6$ cells/10-cm dish). After heat treatment, the lysate was applied to the Cellulofine sulfate column (17×200 mm), washed with PBS, and eluted with 10 m*M* phosphate buffer pH 7.2 containing 1 *M* NaCl. Fractions of 2 ml were collected. The open bars represent the titer of AAV vectors determined by transduction of 3T3 cells.

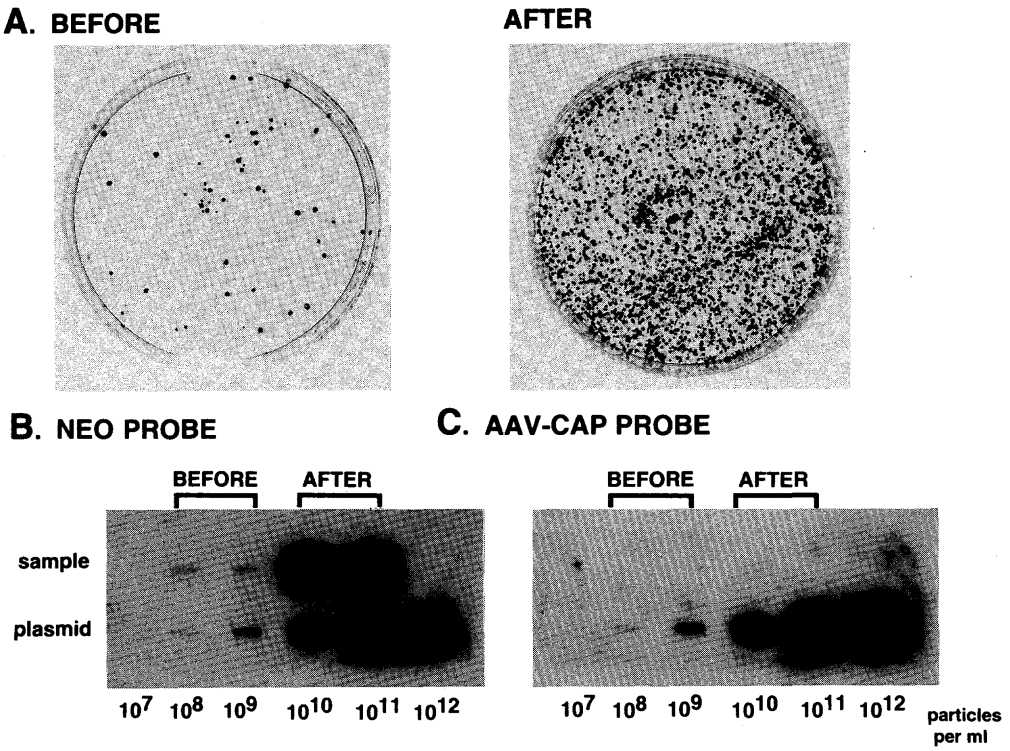

FIG. 5. Titering of AAV vectors prepared using HAN10 producer cells and Cellulofine sulfate column chromatography. A. Biological titering assay. NIH-3T3 cells (4×10^4 cells) were transduced with 0.5 μl of the cell lysate containing AAV vectors before and after column chromatography concentration. The cells were cultured in medium containing G418 as described in Materials and Methods. This concentrated AAV vector stock was shown to have a titer of 1.2×10^8 cfu/ml. B and C. Particle titering assay. AAV vector samples before and after concentration in duplicate were treated with DNase I and extracted DNAs were subjected to slot blot analysis using the *neo* probe or the AAV cap probe. Plasmids, pNAV (B) or pAAV/Ad (C), serially diluted with 10 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA were used for the particle number standard shown in lower slots.

strated that these cells can be used for packaging of recombinant AAV vectors. We also showed that Cellulofine sulfate column chromatography was useful for concentration of infectious AAV particles. Using these two techniques, large-scale preparation of high-titer AAV vectors became possible.

The packaging system for AAV vectors described here is different from that for retroviral vectors in several aspects. Packaging cells for retroviral vectors such as $\psi 2$ and PA317 are constitutively synthesizing all proteins required for assembly of retroviral particles. Therefore, introduction of the vector plasmid into these cells can result in production of recombinant retroviral vectors. However, replication of the AAV genome is dependent upon co-infection with cytopathic adenovirus. Because helper functions of adenovirus have not been completely characterized, the genes necessary for AAV packaging cannot be separated from the full-length adenovirus genome. In addition, the Rep proteins have cytotoxic or cytostatic activities (Mendelson *et al.*, 1988b; Khleif *et al.*, 1991; Yang *et al.*, 1994). Therefore, constitutive expression of the Rep proteins interferes with establishment of stable cell lines. To overcome these problems, we developed a new strategy using inducible packaging cell lines. These cells carry multiple copies of the packaging plasmid in cellular genome, but in the absence of adenovirus, the AAV promoters are silent. When the cells are infected with adenovirus, expression of AAV proteins is induced efficiently. If the vector plasmid is integrated in these cells, replication of the AAV vector genome with the ITRs is also initiated by adenovirus helper functions. Therefore, once such a cell line is established, the recombinant AAV vectors can be generated by adenovirus infection. Because adenovirus infection requires the simple addition of adenovirus stock to the culture cells, large-scale AAV vector production can be easily achieved.

Recently, cell lines inducibly expressing the Rep proteins have been established using heterologous promoters (Yang *et al.*, 1994). In the 293-derived cell lines, the mouse metallothionein promoter was used for inducible expression of the *rep* gene. Addition of heavy metals significantly increased the level of the Rep proteins and inhibited growth rate and plating efficiency as expected. However, even without induction, these cells grew more slowly than nontransfected 293 cells, suggesting that this promoter may have the relatively high level of basal activity in 293 cells. The HeLa-based cell lines containing the AAV genome driven by the mouse mammary tumor virus LTR was also established (Hoelscher *et al.*, 1994). The Rep proteins were synthesized after induction with dexamethasone, but the ratio of each component was different from that observed in cells infected with wild-type AAV. Furthermore, although the AAV-like particles were produced from the adenovirus-infected cells, these recombinant virions were noninfectious. Therefore, it is questionable whether these cell lines can be used for efficient packaging of AAV vectors.

Our study demonstrated that cell lines carrying high copy numbers of the AAV genome can be established without using heterologous inducible promoters. Northern analysis showed that the AAV promoters are silent in HeLa cells and highly inducible by adenovirus infection. These observations appear to be partly inconsistent with previous reports indicating that the AAV promoters are active in various cells. AAV expression without helper virus was demonstrated by using sensitive procedures such as the immunoblotting assay (Mendelson *et al.*, 1988a) or the CAT reporter assay (Tratschin *et al.*, 1986).

Therefore, it is likely that the AAV promoters are weak but function in the absence of adenovirus. Northern analysis may not be sufficiently sensitive to detect the low basal level of AAV expression. However, such low levels of *rep* expression seem not to interfere with the establishment of stable cell lines.

The gel chromatography technique using Cellulofine sulfate was successfully applied for concentration of AAV vectors. Cellulofine sulfate was comprised of spherical beads functionalized with a low concentration of sulfate esters and has been used for concentration of various viruses including rabies, herpes simplex, influenza, and Japanese encephalitis viruses (Amicon Division, 1993). Cellulofine sulfate column chromatography is particularly useful for concentration of AAV particles in a large volume of the cell lysate. However, this technique alone is not sufficient for purification of AAV. A small amount of adenovirus as well as various serum and cellular proteins were always co-eluted with AAV particles from the column. Therefore, the vector preparation must be heat-treated before using transduction experiments. The AAV particles can be purified and concentrated by the cesium chloride ultracentrifugation (Hoggan *et al.*, 1966; Tratschin *et al.*, 1985), but this procedure is applicable only for limited volume of the sample. Therefore, to prepare AAV vectors for *in vivo* experiments, it seems reasonable that AAV particles should be first concentrated by Cellulofine sulfate column chromatography and then further concentrated and purified by cesium chloride centrifugation.

Recombinant AAV is now considered to be an attractive vector for human gene therapy. Although preliminary results suggest that AAV vectors have several advantages compared with the currently used retrovirus- and adenovirus-based vectors, the definitive experiments in animal models have yet to be done. The major reason seems to be the difficulty in preparation of sufficient amounts of high-titer AAV vectors. The new strategy for preparing AAV vectors described here may be useful for development of gene therapy using AAV vectors.

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