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Packaging Cells Based on Inducible Gene Amplification for the Production of Adeno-Associated Virus Vectors

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Although vectors based on adeno-associated virus (AAV) offer several unique advantages, their usage has been hampered by the difficulties encountered in vector production. In this report, we describe a new AAV packaging system based on inducible amplification of integrated helper and vector constructs containing the simian virus 40 (SV40) replication origin. The packaging and producer cell lines developed express SV40 T antigen under the control of the reverse tetracycline transactivator system, which allows inducible amplification of chromosomal loci linked to the SV40 origin. Culturing these cells in the presence of doxycycline followed by adenovirus infection resulted in helper and vector gene amplification as well as higher vector titers. Clonal producer cell lines generated vector titers that were 10 times higher than those obtained by standard methods, with approximately 10^4 vector particles produced per cell. These stocks were free of detectable replication-competent virus. The lack of a transfection step combined with the reproducibility of stable producer lines makes this packaging method ideally suited for the large-scale production of vector stocks for human gene therapy.

Adeno-associated virus (AAV) is a dependent human parvovirus that depends on infection by a helper virus such as adenovirus (Ad) for productive infection but can exist as a latent provirus in the absence of helper virus. The 4.7-kb single-stranded DNA (ssDNA) genome of AAV contains two open reading frames that encode Rep and Cap proteins. The *rep* gene products are essential for viral DNA replication and chromosomal integration, and the *cap* gene encodes the virion capsid proteins. Vectors based on AAV have been developed as gene transfer vehicles able to transduce a wide variety of cell types and integrate into host DNA (reviewed in reference 27). Recent reports of long-term therapeutic levels of transgene products after in vivo delivery of AAV vectors in animal experiments demonstrate the potential of this vector system and suggest that human diseases could be successfully treated by similar approaches (18, 23, 39). These vectors consist of transgene expression cassettes bordered by AAV terminal repeats (TRs) that are packaged by *rep* and *cap* gene products supplied in *trans* from helper constructs.

In spite of the potential advantages of AAV-based vectors, their usage has been limited by the cumbersome and labor-intensive methods required for the production of high-titer vector stocks. In addition, these stocks are often contaminated by replication-competent AAV particles containing viral genes (rcAAV) (2, 4, 33, 35). High stock titers are critical, as expression of AAV vector transgenes requires large numbers of vector particles, with the ratio of vector genome-containing virions to transducing units ranging from 10^6 in stationary human fibroblasts to hundreds in transformed cell lines (17, 32, 37). Stock purification is also important, since contaminating cellular debris can mimic true vector-mediated transduction events and produce artifactual results (1). The conventional method for vector production is cotransfection of a vector plasmid containing the vector genome and a helper plasmid encoding the *rep* and *cap* genes into E1A-transformed human embryonic kidney cells (293 cells) infected with Ad (37). By this method, 100 to 1,000 vector particles per cell, or 10^8 to 10^9 particles per ml of crude stock lysate, can usually be prepared. To obtain the high titers required for experiments, large-scale transfections must be performed and vector virions must be purified from liters of crude lysate. Although several modifications designed to increase DNA transfer efficiency and AAV helper gene expression have been published (4, 10, 12, 25, 42, 43), these protocols still require transfection, electroporation, and/or the preparation of complicated conjugates, so they are not suitable for large-scale vector production.

An ideal approach for AAV vector production would be the development of stable packaging cell lines that can produce vector particles without a transfection step, allowing efficient large-scale stock preparation. The availability of analogous retroviral packaging lines has greatly simplified retroviral vector production and led to their widespread use in basic and clinical research (26). Although packaging lines for AAV vectors have been reported (5, 41), their yields ranged from 300 to 700 vector particles/cell, which was no more than those prepared by the transfection method. This low yield is not due to saturation of virus production in cells, because wild-type AAV (wtAAV) can be produced at a titer of more than 10^5 particles per cell (31). One potential explanation for the different yields of wtAAV and AAV vectors is related to the copy number of viral genes that can be used as templates for expression of the proteins needed for virion production. In cells infected with wtAAV and Ad, replication of the AAV genome produces more than 10^4 copies of the *rep* and *cap* genes per cell (31), while the published packaging cell lines contain 10 to 30 copies that do not amplify further since they lack the AAV TRs required for replication (41). In fact, previous reports suggest that gene expression from the packaging constructs is the rate-limiting step in vector production (8). Unfortunately, AAV vector packaging strategies that rely on overexpression of the viral gene products must first overcome the problems associated with the cellular toxicity of Rep proteins (20, 44), and the requirement that *rep* and *cap* expression levels must be tightly regulated for maximal virion production (25).

We reasoned that an ideal way to increase viral gene expres-

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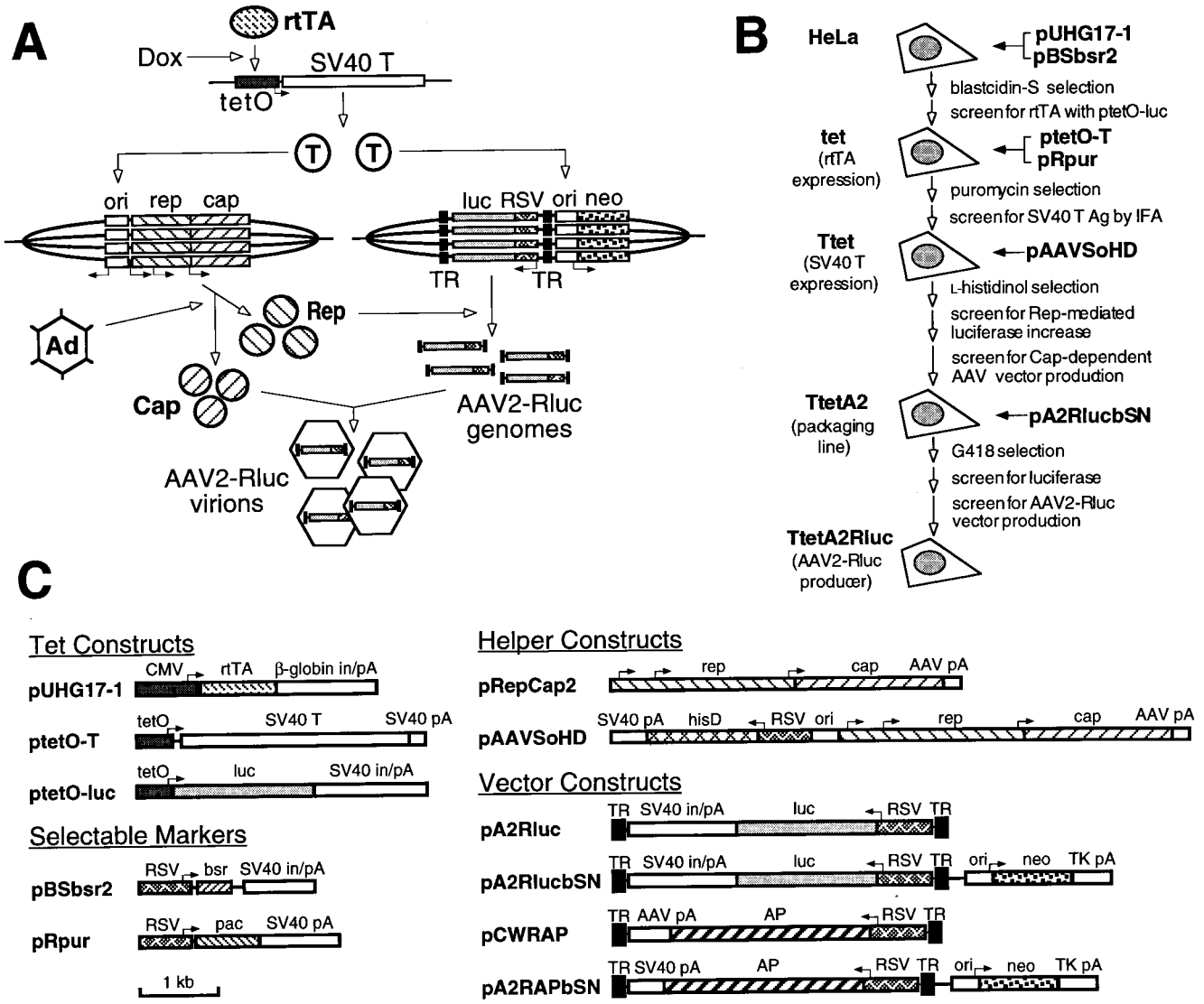

FIG. 1. (A) Scheme for inducible chromosomal gene amplification to produce AAV vectors. Proteins shown include rtTA (15), SV40 T antigen (T), and AAV Rep and Cap proteins. Also shown are AAV2-Rluc excised genomes and packaged vector virions, as well as Ad helper. Amplified helper and vector constructs are shown and labeled as in panel C. AAV2-Rluc virions contain ssDNA. (B) Steps required to generate AAV vector packaging (TtetA2) and producer (TtetA2Rluc) cell lines. (C) Maps of plasmids used in this study (not including bacterial functions). DNA sequences shown include the RSV long terminal repeat promoter (RSV); CMV immediate-early promoter (CMV); SV40 origin and early promoter (ori); CMV minimal promoter with *tet* operators (tetO); SV40 T-antigen coding sequence (SV40 T); rtTA, AAV *rep* and *cap*, and luciferase (luc) genes; AAV TRs; neomycin phosphotransferase (neo), blasticidin-S resistance (bsr), puromycin acetyltransferase (pac), histidinol dehydrogenase (hisD), and human placental AP genes; β-globin, SV40, AAV, and thymidine kinase (TK) intron (in) and/or polyadenylation (pA) sites; and transcription start sites (arrows).

sion during vector production would be to mimic the state occurring during wtAAV infection by amplifying intact fragments of the AAV genome containing the *rep* and *cap* genes at the time of Ad infection. In this study, we describe such a strategy based on inducible amplification of chromosomal loci containing the simian virus 40 (SV40) replication origin in cells that express the SV40 T antigen under the control of the reverse tetracycline-controlled transactivator (rtTA) system (15). When the cell lines that we generated were cultured in the presence of doxycycline (Dox), SV40 T antigen was synthesized, and the integrated AAV helper and vector constructs linked to SV40 origins were amplified. Infection of the producer cell lines with Ad after the addition of Dox resulted in AAV vector production with titers 10 times higher than those obtained by transfection methods and free of replication-competent AAV. The cell lines generated here allow for greatly simplified AAV vector production methods that can easily be scaled up for clinical gene therapy protocols. Our amplification strategy may also prove useful for generating other toxic gene products at high levels.

MATERIALS AND METHODS

Plasmid DNA. Plasmids used in this study are shown in Fig. 1C. pUHG17-1 (15) and pCWRAP (11) were obtained from H. Bujard (University of Heidelberg, Germany) and S. Chatterjee (City of Hope National Medical Center, Duarte, Calif.), respectively. pALAPSN was described previously (32). The other plasmids were constructed by standard techniques (36). pAAVSoHD was assembled in pBluescript II KS(+) (Stratagene, La Jolla, Calif.) from the following pieces: the *rep* and *cap* genes from pAAV/Ad (37), bp 5083 to 5270 of SV40 (Genbank accession no. J02400) containing the origin and early (not late) promoter, the Rous sarcoma virus (RSV) promoter and SV40 polyadenylation

signals from pREP7 (Invitrogen, Carlsbad, Calif.), and the *hisD* gene (16) from pLXSHD (26). pTRbSN was constructed by inserting the SV40 origin-promoter-*neo* cassette of pBK-CMV (Stratagene) between the *Sca*I and *Eco*RI sites of pTR (34). pA2Rluc, pA2RlucbSN, and pA2RAPbSN were constructed by inserting luciferase or alkaline phosphatase (AP) expression cassettes into the *Bgl*II sites of pTR or pTRbSN. pRepCap2 was constructed by inserting the *Xba*I fragment of pAAV/Ad into pBluescript II KS(+). ptetO-luc and ptetO-T are derivatives of pUHD10-3 (15) (a gift of H. Bujard) and contain a firefly luciferase gene and SV40 T-antigen gene, respectively, under the cytomegalovirus (CMV) minimal promoter with *tet* operators. pBSbsr2 is a derivative of pSV2bsr (21) and contains the blasticidin-S resistance gene (*bsr*) and RSV promoter. pRpur was constructed from pPUR (Clontech, Palo Alto, Calif.) encoding the puromycin acetyltransferase gene (*pac*) (6) by replacing the SV40 promoter with the RSV promoter. Further details of construction procedures and the sequences of plasmids are available on request.

Cell lines. 293T (28), HeLa (38), and HT1080 (29) cells were propagated in Dulbecco's modified Eagle's medium supplemented with heat-inactivated 10% fetal bovine serum. Transfection was done by the calcium phosphate method as described previously (36). Concentrations of antibiotics for selection of clones after transfection and for maintenance of resistant cells, respectively, were as follows: blasticidin-S (Calbiochem, San Diego, Calif.), 4.0 and 0.2 μg/ml; puromycin (Clontech), 1.0 and 0.1 μg/ml; L-histidinol (Sigma, St. Louis, Mo.), 20 and 0.5 mM; geneticin (GIBCO BRL, Gaithersburg, Md.), active 1.0 and 0.2 mg/ml. Dox was added to medium at 0.5 μg/ml to induce rtTA-dependent transcription.

Screening assays for cell clones were performed as follows. To screen rtTA-expressing clones, 8×10^4 cells in 15-mm-diameter wells were transfected with 1.6 μg of ptetO-luc for 8 h and cultured in medium in the absence or presence of Dox for 60 to 65 h, and then luciferase expression was assayed. Clones with a >10-fold increase in luciferase expression by Dox treatment were selected. Clones expressing T antigen were detected by an immunofluorescence assay with the monoclonal antibody PAb101 against T antigen as described previously (7) except that fixation was with acetone-methanol (1:1). To screen Rep-expressing clones, 10^4 cells in 10-mm-diameter wells were infected with AAV luciferase vector AAV2-Rluc at a multiplicity of infection (MOI) of 5 to 10 and with Ad at an MOI of 10. Luciferase expression was measured 45 h after infection, and clones with a >20-fold increase in expression compared to HeLa and Ttet cells were chosen. To screen for Cap expression, clones were infected with AAV2-Rluc at an MOI of 5 to 10 and with Ad at an MOI of 10, and vector production in cell lysates was measured by transduction of luciferase activity on HeLa cells. AAV2-Rluc producer clones were screened by the same method after infection with Ad alone.

Preparation of AAV vector stocks. The standard transfection method for vector production was described previously (33). We used 293T cells instead of 293 cells and pRepCap2 in place of pAAV/Ad, as these reagents produced slightly higher vector titers. Stocks were harvested 3 days after Ad infection. Stocks made by transfection of TtetA2 packaging cells were prepared by similar methods, with the following modifications: incubation in Dox for 3 days (unless otherwise indicated) before Ad infection, transfection with only a vector plasmid, and a medium change 16 h after transfection. Stocks were also prepared by infection of TtetA2 cells with vector stocks at an MOI of 10 1 day after Ad infection (Ad was added after culturing in Dox for 3 days). Stocks were prepared from producer cells as follows. Cells grown in the presence of Dox for 3 days were infected with Ad at an MOI of 10, and cell lysates were harvested 3 days later. All crude lysates were prepared by freeze-thawing three times followed by centrifugation to remove debris. For large-scale production, crude lysates were treated with micrococcal nuclease and trypsin as described previously (33), incubated on ice in the presence of 12% polyethylene glycol 3350 and 0.5 M NaCl for 3 h, pelleted by centrifugation (Sorvall HS-4, 6,000 rpm, 30 min), resuspended in phosphate-buffered saline (PBS), and centrifuged through a 40% sucrose cushion (SW28 rotor, 27,000 rpm, 16 h). The pellets were then purified on CsCl gradients (SW41 rotor, 35,000 rpm, 20 to 24 h), and the fractions containing the vectors were dialyzed and stored in Dulbecco's modified Eagle's medium. All stocks were heat inactivated at 56°C for 1 h and stored at −70°C.

rcAAV detection. Vector stocks were tested for contaminating rcAAV by the replication center assay (RCA) described previously (24) and by the sequential amplification assay of Allen et al. (2). Minor modifications of the sequential amplification assay were that cells were harvested at 48 h instead of 72 h postinfection for the second amplification and that DNA from Hirt supernatants (19) was prepared instead of total genomic DNA.

Transduction assays. Transduction of luciferase was performed as follows. HeLa cells were plated at 2×10^4 cells per well in 48-well plates; 22 h later, the cells were infected with Ad at an MOI of 10 and with vector stock dilutions; 44 to 46 h postinfection, the cells were rinsed with PBS and lysed with 100 μl of cell culture lysis reagent (Promega, Madison, Wis.). Then 5 μl of the lysate was mixed with 15 μl of luciferase assay reagent (Promega), and luminescence was measured for 30 s by a luminometer (Lumat LB9507; Berthold System Inc., Aliquippa, Pa.). The luminescence measurement from cells infected with Ad alone was subtracted to calculate luciferase units (LU). One gradient-purified vector stock made from the TtetA2Rluc clone 49 (c.49) producer cell line was diluted serially and used as a standard in luciferase transduction assays to estimate particle numbers of vector stocks based on LU measurements. Transduction of AP was measured by staining cells 2 days after infecting 10^5 HT1080 cells in 35-mm-diameter wells and counting individual stained foci (9).

Quantification of vector genomes. To locate the vector in CsCl gradients, 10 μl of each of approximately 20 fractions obtained from the gradient was mixed with 2 μl of 10% (wt/vol) sodium dodecyl sulfate, boiled for 10 min, and separated on 1.2% alkaline agarose gels (36). DNA was transferred to Hybond-N+ membranes (Amersham, Arlington Heights, Ill.) and hybridized with vector sequences, and the radioactive bands were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) analysis. Fraction densities were determined by refractive indices. Final stock particle numbers were determined by a similar alkaline gel analysis of viral ssDNA prepared from purified virion particles by phenol extraction and ethanol precipitation.

Genomic DNA analysis. Cells were cultured in the absence or presence of Dox and then plated at 4×10^6 cells per 10-cm-diameter dish. On the next day, genomic DNA was prepared from two dishes as described previously (33). The DNA was digested with restriction enzymes, extracted with phenol-chloroform (1:1), precipitated with ethanol, rinsed with 70% ethanol, quantitated by optical density, and analyzed by Southern blotting. Copy numbers were determined by comparison to standards, using PhosphorImager analysis.

RESULTS

Generation of AAV vector packaging cell lines. Our strategy for producing packaging cell lines capable of regulated gene amplification combined rtTA-controlled gene expression and SV40 origin replication as shown in Fig. 1A. In these cells, Dox causes rtTA to initiate transcription of the SV40 T-antigen gene, and then T antigen binds to the SV40 origin linked to an integrated AAV helper construct leading to *rep* and *cap* gene amplification. Infection with Ad results in high level production of Rep and Cap proteins and AAV virion assembly. Because Rep protein is strongly expressed only after Ad infection, this strategy avoids the toxicity of Rep proteins. Producer cells can be generated from these packaging lines by the addition of a vector construct flanked by AAV TRs and linked to the SV40 origin, which will also amplify in the presence of Dox, excise, replicate, and be packaged into virions once Rep and Cap are expressed.

The packaging cells were produced stepwise by cotransfection of expression constructs and selectable marker genes as shown in Fig. 1B and C. First, rtTA-expressing cell lines were obtained by transfection of HeLa cells with plasmids pUHG17-1 encoding rtTA and pBSbsr2 as a blasticidin-S resistance marker. Three of 18 blasticidin-S-resistant clones (tet clones 4, 21, and 22) were identified as having high rtTA expression levels by transient transfection of ptetO-luc and measurement of luciferase expression in the presence of Dox.

In the second step, these rtTA-expressing clones were transfected with ptetO-T encoding the SV40 T-antigen gene under the control of the *tet* operator and pRpur encoding puromycin acetyltransferase. Nine of 79 puromycin-resistant clones expressed SV40 T antigen only in the presence of Dox as determined by immunofluorescence assay with antibody against T antigen. We confirmed that T-antigen expression in these clones led to amplification of plasmid sequences containing an SV40 origin in a transient transfection assay (data not shown). Two of the T-antigen-expressing cell lines (Ttet clones 22-1 and 22-13) showed tight regulation of T-antigen expression even after culturing for 1 month. In these cells, T antigen was present at high levels 2 days after the addition of Dox and persisted for 2 days after the withdrawal of Dox (data not shown).

In the third step, the two T-antigen-expressing cell lines were transfected with pAAVSoHD, which contains the AAV *rep* and *cap* genes, the SV40 origin, and a linked *hisD* selection marker. Fifty L-histidinol-resistant clones were screened for Rep expression by assaying for AAV vector genome replication after infection with Ad and the AAV luciferase vector AAV2-Rluc in the absence of Dox. In 25 of 50 clones, we noted 20- to 100-fold increase in luciferase expression after Ad in-

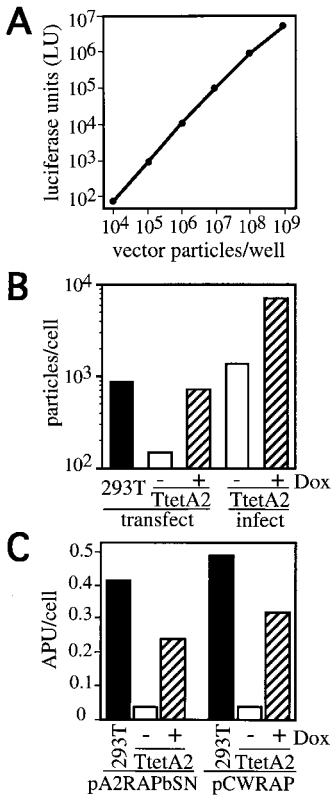

FIG. 2. Vector production by packaging cells. (A) Linearity between LU measurement and AAV2-Rluc infecting particle number. Means of three experiments were plotted. (B) AAV2-Rluc vector was produced by the following procedures. TtetA2 cells were cultured in the absence (−) or presence (+) of Dox for 3 days and then either transfected with pA2Rluc 2 h after Ad infection or infected with AAV2-Rluc stock prepared from a producer cell line (Fig. 4) at an MOI of 10 1 day after Ad infection. Three days after Ad infection, lysates containing vectors were harvested. The number of vector particles produced per packaging cell was calculated by luciferase expression after transduction by these stocks. Results of AAV2-Rluc produced by cotransfection of 293T cells with pA2RlucbSN and pRepCap2 are shown for comparison. (C) AAV2-RAP and AAV2-CWRAP vector stocks were produced by growth of TtetA2 cells in the absence (−) or presence (+) of Dox for 3 days, followed by transfection with pA2RAPbSN or pCWRAP, respectively, 2 h after Ad infection. The number of AP focus-forming units (APU) present in the resulting stocks was determined. Results are shown as APU produced per packaging cell and compared to those obtained by cotransfecting 293T cells with pA2RAPbSN and pRepCap2.

fection, which was presumably due to Rep-mediated vector replication and increased luciferase template. Thirteen of these 25 clones also expressed Cap functions, based on their ability to package AAV2-Rluc vector virions capable of transduction (see Materials and Methods). The clone producing the highest titer of AAV2-Rluc was chosen as a packaging cell line, named TtetA2, and characterized further.

Vector production from the packaging cell line TtetA2. We used the luciferase vector AAV2-Rluc as a convenient measure of vector production from the packaging cell line TtetA2. Because the LU produced after transduction by AAV2-Rluc vector stocks had a linear relationship with the number of infecting vector particles (Fig. 2A), particle numbers were estimated by comparing LU values with those from purified vector standards measured in parallel. AAV2-Rluc vector production in TtetA2 cells transfected with the vector plasmid pA2Rluc or infected with the AAV2-Rluc vector at a low MOI was measured (Fig. 2B). A 3-day treatment with Dox before Ad infection increased vector production by both methods about fivefold. The yield of vector from transfected packaging cells incubated in Dox was similar to that generated by the conventional 293T transfection method, despite the lower transfection efficiency of TtetA2 cells (5 to 10% of TtetA2 cells express a β-galactosidase marker after transient transfection, compared to >50% of 293T cells [data not shown]). There was no difference in titers between stocks made by transfection with pA2Rluc (which lacks the SV40 origin) or pA2RlucbSN (which contains the SV40 origin) when prepared in TtetA2 or 293T cells (data not shown). Presumably T-antigen-dependent replication does not significantly increase vector copy numbers in cells that already contain large amounts of vector plasmid DNA delivered by transfection. When vector stocks were produced by infection of TtetA2 cells with AAV2-Rluc at an MOI of 10, nearly 10^4 vector particles were produced per cell, a yield 10-fold greater than that obtained by the conventional method. This latter method results in a 1,000-fold increase in vector particle numbers without a transfection step.

We also used TtetA2 packaging cells to produce AAV vectors encoding AP. TtetA2 cells were transfected with the AP vector constructs pA2RAPbSN and pCWRAP (11) (Fig. 1C), and the titers of AAV2-RAP or AAV2-CWRAP vector stocks produced were determined directly by counting transduced cells stained for AP expression. Dox treatment increased vector production 5- to 10-fold, resulting in vector titers that were 60 to 65% of those made by the conventional 293T transfection method (Fig. 2C). Production of stocks by infection of TtetA2 cells with AAV2-RAP or AAV2-CWRAP vectors at low MOI resulted in a further increase in vector titer (data not shown). These AP vector results were similar to those obtained with luciferase vectors.

Generation of producer cell lines for AAV2-Rluc. Stable vector producer lines containing integrated copies of the AAV2-Rluc vector genome were made by transfection of TtetA2 cells with plasmid pA2RlucbSN, which consists of the RSV promoter-luciferase expression cassette flanked by AAV TRs, an SV40 origin, and a neomycin phosphotransferase (*neo*) selectable marker (Fig. 1C). The SV40 origin in the vector plasmid is included to allow for Dox-induced gene amplification, which should increase the number of vector templates available for excision and replication in the presence of the AAV Rep proteins. Fifteen of 43 G418-resistant TtetA2 clones transfected with pA2RlucbSN expressed luciferase, and 9 of these produced AAV2-Rluc vector particles after Ad infection. Most of these nine clones produced higher vector titers after Dox treatment, and vector yields from five clones were estimated to be greater than the 1,000 particles/cell obtained by the conventional method, with clone 29 producing 29,000 particles/cell (Table 1). As described below, large-scale, purified stocks were prepared from two of the clones listed in Table 1, and the vector yields based on vector genome measurements were similar to those estimated by luciferase assays. Although vector production from TtetA2Rluc clones 7 and 29 were higher, we chose clones 37 and 49 for further characterization because they grew as well as the parental TtetA2 cell line. There was no relationship between vector production and cell growth rates.

Gene amplification and vector production in vector producer lines. Gene amplification of helper and vector sequences was measured by Southern analysis in the absence of Ad infection to avoid Rep-dependent vector replication (Fig. 3A and B). The copy number of the AAV helper genes in TtetA2 packaging cells was increased fivefold by Dox treatment, reaching 52 copies per cell 4 days after the addition of Dox. A similar amplification of helper sequences was observed in TtetA2Rluc c.37 and c.49 producer cells, with 106 and 78 copies present per cell, respectively, 5 days after addition of Dox (data not

TABLE 1. Comparison of titers among producer clones

Cell line[a]	LU/cell		Increase (+Dox/−Dox)	Estimated particles/cell[c]
	−Dox	+Dox		
TtetA2Rluc				
c.7[b]	28.7	136.5	4.8	14,300
c.14[b]	2.5	3.3	1.3	300
c.29[b]	68.6	275.7	4.0	29,000
c.32	0.0	1.1	27.3	100
c.35	0.3	6.8	23.3	700
c.37	5.5	48.7	8.8	5,100
c.49	4.8	91.3	19.0	9,600
c.54	2.0	17.6	8.8	1,900
c.60	0.1	1.9	26.0	200
Transfected 293T cells	9.2			970[d]

[a] For TtetA2Rluc clones, 4×10^4 cells/35-mm-diameter well were cultured in the presence or absence of Dox for 3 days and then infected with Ad; vector stocks were prepared and titers were determined as described in Materials and Methods.

[b] Cell growth was slow.

[c] Estimated particle numbers produced from the clones treated with Dox were based on the particle number/LU ratio of the stock prepared by transfection of 293T cells with pA2RlucbSN and pRepCap2.

[d] Determined by measuring vector genome numbers.

shown). Vector copy numbers in TtetA2Rluc c.37 and c.49 were increased 10- and 8-fold to 91 and 23 copies per cell, respectively, although the peaks occurred at different times after the addition of Dox. Dox induction also increased vector production when combined with Ad infection, with maximum vector yields more than 10-fold above those from uninduced cells (Fig. 3C). Longer Dox exposures tended to decrease helper and vector copy numbers, as well as vector titers (see Discussion).

Based on these observations, we cultured TtetA2Rluc c.37 and c.49 cells in the presence of Dox for 3 days before Ad infection to prepare large-scale stocks. Particles produced from 5×10^7 cells were fractionated on CsCl gradients, and their genomes were analyzed on alkaline gels (Fig. 4). The particles banded sharply at a density 1.41 g/ml and consisted of ssDNA of the expected size. We calculated that 5,300 (c.49) or 6,400 (c.37) particles per cell were generated from the producer cell lines in these large-scale preparations, based on quantitation of vector genome levels. By luciferase assay, vector yields from c.37 and c.49 were 82.5 and 101.8 LU per cell, respectively. The conventional transfection method produced 970 AAV2-Rluc particles/cell and 8.1 LU/cell, or about a 10-fold-lower yield. The transducing unit-to-particle ratio (LU/particle number) of stocks from the producer cell lines (0.0130 and 0.0205 LU/particle) were slightly higher than those made by the conventional method (0.0084 LU/particle).

Vector stocks made by TtetA2 packaging cells are free of rcAAV. Vector stocks made on the packaging and producer cell lines were examined for the presence of contaminating rcAAV both by RCA (24) and by Southern analysis of DNA prepared after sequential amplification of replicating viral particles (2). To date we have analyzed a total of nine stocks generated by transfection or infection of TtetA2 cells by RCA and never detected rcAAV. These assays included two independent AAV2-Rluc vector stocks prepared by infection of TtetA2 cells (<1 rcAAV particle per 10^7 vector genomes) and an AAV2-RAP stock prepared by infection of TtetA2 cells with a seed stock generated by two prior passages on TtetA2 cells (<1 rcAAV particle per 10^8 vector genomes). Stocks prepared by infecting TtetA2 cells with seed stocks would have amplified any rcAAV particles present in the seed stock, and so this is a very stringent contamination assay. In these cases, the original vector seed stocks were prepared by transfecting TtetA2 cells and were free of rcAAV. The AAV2-Rluc vector stocks prepared from the TtetA2 c.37 and c.49 producer cell lines were also free of rcAAV (<1 rcAAV particle per 10^8 vector genomes). In contrast, contamination of rcAAV was frequently detected in stocks made by the conventional cotransfection method, as also observed by others (2, 35). Examples of these RCA data are shown in Fig. 5A.

We also used a sequential amplification assay to screen for rcAAV (2), which is a more sensitive method for detecting infectious particles (Fig. 5B). With this assay, 10 particles of wild-type AAV2 (as measured by genome numbers) were easily detected. We screened vector stocks made from three dif-

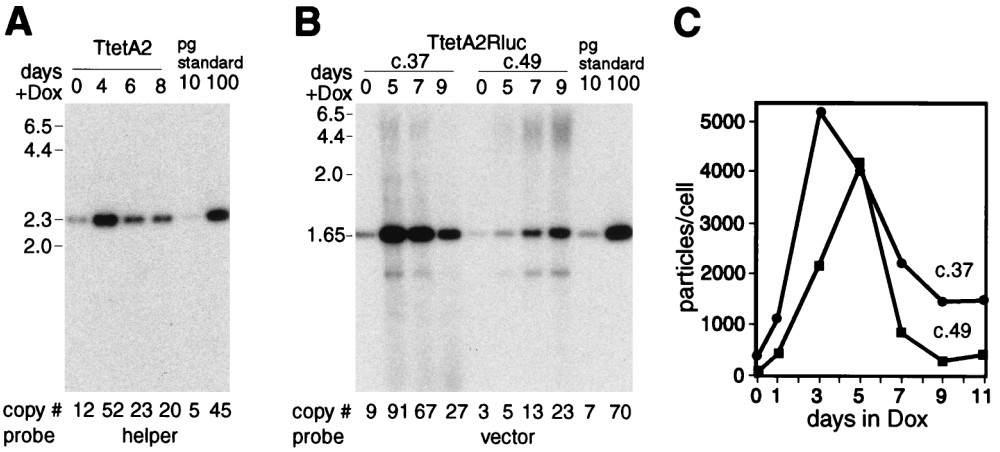

FIG. 3. Gene amplification and vector titer during Dox induction. (A) Southern analysis of genomic DNAs (4 μg/lane) prepared from TtetA2 packaging cells grown in Dox for the indicated number of days, digested with *Pst*I (TtetA2), and probed for *cap* sequences to detect helper construct amplification. *Pst*I-digested pAAVSoHD served as standards. Construct copy numbers per cell are indicated beneath the lanes. Standard fragment sizes (in kilobases) are shown on the left. (B) Southern analysis as for panel A except that genomic DNAs from TtetA2Rluc producer cells c.37 and c.49 were digested with *Xba*I and probed for luciferase sequences to detect vector amplification. *Xba*I-digested pA2RlucbSN served as standards. (C) Effect of Dox incubation time on the production of AAV2-Rluc from TtetA2Rluc c.37 and c.49. Cells (4×10^6/10-cm-diameter dish) grown in Dox for the indicated number of days were infected with Ad at an MOI of 10, and vector stocks were harvested 3 days later. Stock titers were determined by luciferase transduction, and particle numbers were calculated as for Fig. 2A.

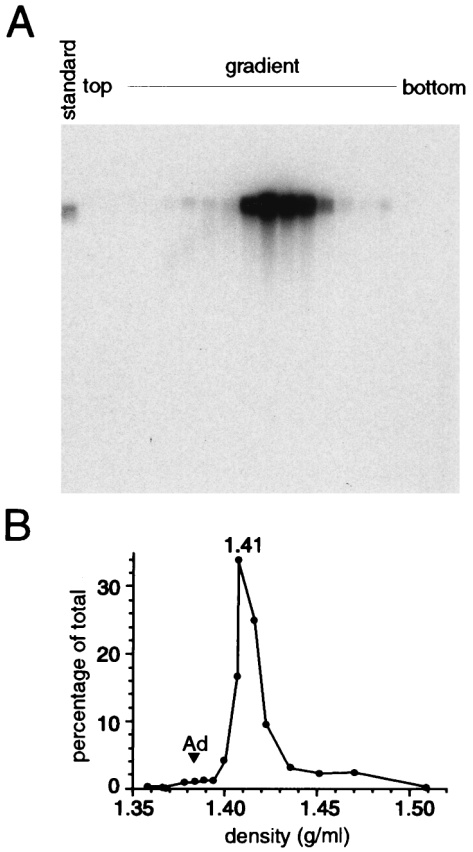

FIG. 4. (A) Southern analysis of an AAV2-Rluc vector stock produced by TtetA2Rluc c.49 cells and fractionated on a CsCl gradient. The AAV2-Rluc ssDNA present in each gradient fraction was released from vector particles, separated on a 1.2% alkaline agarose gel, and probed for luciferase sequences. A 3.85-kb *Bam*HI fragment containing the luciferase expression cassette present in AAV2-Rluc served as a standard. The expected size of the vector DNA is 4.17 kb. TtetA2Rluc c.37 gave very similar results (not shown). (B) The density and percentage of the total amount of AAV2-Rluc genomes present in each gradient fraction were plotted based on quantification of the blot in panel A. The fraction containing Ad is indicated.

ferent vector constructs and produced either by transfected or infected TtetA2 cells or by the producer cell lines TtetA2Rluc c.37 and c.49, and no rcAAV was detected by sequential amplification (10^8 input vector genomes). rcAAV was easily detected in the vector stocks made by the conventional cotransfection method (Fig. 5B). In these experiments we directly compared stocks of the AAV2-LAPSN vector (32) made by both methods, since this was the vector originally shown to contain rcAAV by the sequential amplification method (2). The results from a single round of amplification of wtAAV are also shown to demonstrate that amplification occurred.

DISCUSSION

We have developed an inducible gene amplification system based on rtTA-regulated expression of SV40 T antigen to increase the copy number of chromosomal loci linked to the SV40 origin. We used this system to generate AAV vector packaging and producer cell lines that amplify integrated helper and vector constructs during vector production, resulting in higher vector titers. The producer cell lines that we constructed were capable of generating approximately 10^4 AAV vector particles/cell in a simplified procedure without a transfection step, and the stocks produced were free of detectable rcAAV. These vector yields were 10-fold higher than those obtained by the conventional cotransfection method or by previously published, nonamplifying producer lines (5, 41). The transducing unit/vector particle ratio of the vector stocks obtained from producer lines was equivalent to or better than those made by standard transfection methods, and alkaline gel analyses of the stocks demonstrated that the vector virions contained genomes of the expected size.

The TtetA2 packaging cell line that we constructed could also be used to produce vector stocks without proceeding through a further screening step to generate producer lines. Transient transfection of TtetA2 packaging cells with vector plasmids yielded titers comparable to those obtained by the 293 cell transfection method, and infection of TtetA2 cells with AAV vectors at an MOI of 10 particles/cell led to vector amplification and yields of 10^4 particles per cell. This latter method avoids transfection and generated stocks that were

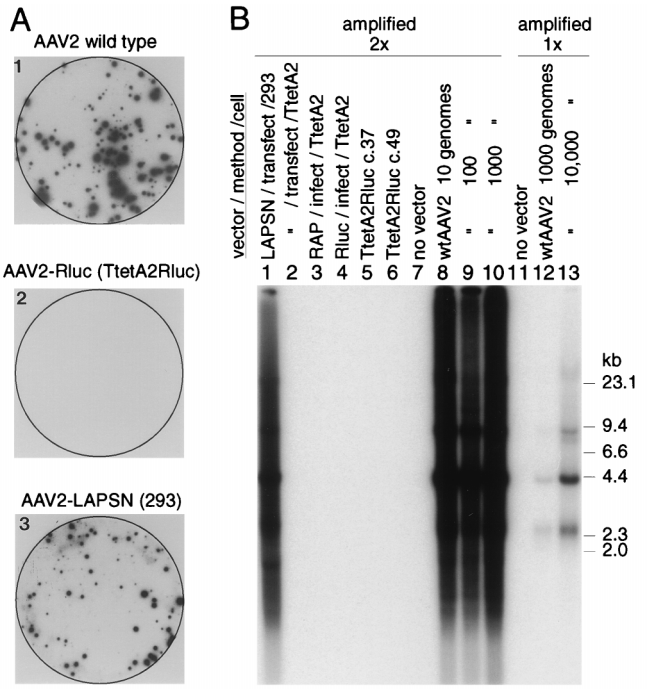

FIG. 5. Assays for rcAAV. (A) RCA. 293T cells were infected with 1.6×10^4 particles of wtAAV2 (filter 1), 9.5×10^8 particles of AAV2-Rluc vector prepared from TtetA2Rluc c.49 (filter 2), or 3×10^5 particles of AAV2-LAPSN vector made by transfecting 293 cells (filter 3) in the presence of Ad (vector and virus particle numbers were determined by Southern analysis of purified virion DNAs on alkaline gels); 28 h later, cells were aspirated onto nylon membrane filters, lysed, and the DNA bound to the filters was hybridized to a fragment containing the entire *rep* and *cap* genes. The circle marks the position of the circular filter exposure on the film. Radioactive spots represent individual foci of rcAAV. (B) Sequential amplification assay. Southern blot of DNA prepared from cells after one round (1×) or two rounds (2×) of rcAAV amplification on 293 cells in the presence of Ad. For the lanes with two rounds of amplification, 10^8 vector particles (lanes 1 to 6), no vector (lane 7), or 10 to 1,000 particles of wtAAV2 (lanes 8 to 10) were used for the first round of amplification, and 0.5-ml aliquots of the resulting lysates were used for the second round of amplification. For the lanes with one round of amplification, uninfected cells (lane 11) or cells infected with 10^3 or 10^4 particles of wtAAV2 (lanes 12 and 13) were analyzed. A DNA fragment containing the *rep* and *cap* genes was used as a probe. AAV2-LAPSN stocks were made by transfection of 293 cells (lane 1) or by transfection of TtetA2 cells (lane 2). The AAV2-RAP stock (lane 3) was made by two sequential rounds of infection of TtetA2 cells after generating the original seed stock by transfection of TtetA2 cells. AAV2-Rluc stocks were made by infection of TtetA2 cells with a seed stock obtained by transfection (lane 4) or were purified from the indicated producer cell lines (lanes 5 and 6).

free of rcAAV, overcoming two of the major limitations of the 293 cell transfection method.

Chiorini et al. (4) also demonstrated that amplification of helper plasmids by the SV40 origin–T-antigen system increased AAV vector production and could generate up to 3,000 vector particles/cell. However, their method was based on transient expression of the helper construct and required electroporation of a helper plasmid containing the SV40 origin into COS cells, so it cannot easily be scaled up. In addition, the stocks produced were contaminated with rcAAV. More recently, Xiao et al. (43) reported a modification of the 293 cell transfection method that incorporated transfection of a deleted Ad helper construct along with the AAV helper and vector plasmids. This approach produced Ad-free vector stocks with yields of over 10^5 particles per cell, which represents a significant improvement over the conventional 293 cell method. The method still suffers from the drawbacks of transfection, and presumably will generate rcAAV just as in other transfection protocols, although rcAAV contamination was not measured in their study.

Our gene amplification method offers certain potential advantages over previously reported strategies. Amplification of genes linked to selectable markers such as dihydrofolate reductase requires a lengthy selection and screening process, and the stability of the amplified array is not ensured (22). The other SV40-based chromosomal gene amplification methods (3, 14, 30) have at least one of the following drawbacks: a complicated manipulation step such as cell fusion, leakiness of T-antigen expression, lack of available human cell lines, or a requirement for temperature shifts. We anticipated that these properties might prevent efficient vector production, as leaky Rep protein expression would be toxic to cells, AAV infection is optimal in human cells susceptible to Ad infection, and the effects of temperature shifts on viral protein production, assembly, and/or infection were unknown. Our method is more versatile, as it allows tight regulation of gene amplification in the entire cell population under normal culture conditions with a simple means of induction. In addition to its use in viral vector production, our regulated gene amplification system could prove useful for the production of large amounts of other gene products, especially when combined with rtTA-controlled expression of a toxic gene product, thereby allowing a coordinated induction of gene amplification and transcription.

In the cell lines established here, copy numbers of the integrated AAV helper and vector constructs were increased 4- to 10-fold by induction with Dox and reached 25 to 100 copies per cell, boosting vector production 5- to 20-fold. Although the yield of 10^4 vector particles/cell was high, there is still room for improvement, as wtAAV yields are at least 10-fold higher, and intracellular wtAAV genome copy numbers are 100-fold higher (31). To further increase the copy number in our cell lines, we must overcome the drop in titer and copy number that occurred after prolonged incubation in the presence of Dox (Fig. 3). One possible explanation for this effect is that smaller origin-containing fragments with truncated helper sequences may amplify and predominate over time, bind and deplete T antigen, and disrupt vector replication or packaging. In some experiments, we observed *rep* gene amplification products of irregular sizes on Southern blots, consistent with this explanation (data not shown). Improvements in helper construct design, such as separation of *rep* and *cap* genes on to smaller cassettes that remain intact during amplification, could avoid this problem. This phenomenon also underscores the need for an inducible amplification system, since continuous T-antigen expression would ultimately decrease vector production.

Elimination of contaminating rcAAV from vector stocks is an important issue, not only for safety reasons (although AAV is not associated with any disease) but also because wtAAV functions can complicate the interpretation of transduction results. Stocks produced by transfection methods are frequently contaminated with rcAAV, even when the vector and helper constructs have no homologous sequences (2, 4, 33, 35). One approach toward reducing rcAAV contamination is to separate and invert the *rep* and *cap* expression cassettes used for transfection (2). In this study, we found both by RCA and a sequential amplification assay that rcAAV was not detectable in stocks prepared from TtetA2 packaging cells or from TtetA2-derived producer cell lines. It is important to note the sensitivity and limitations of rcAAV detection methods. Although the sequential amplification assay (2) can detect rcAAV with more than 100 times the sensitivity of the RCA method (or an analogous single-round amplification assay [Fig. 5]), the method detects only rcAAV containing both functional *rep* and *cap* genes. The RCA method is less sensitive but can detect particles that only contain a functional *rep* gene. Since many of the rcAAV particles being assayed will have been formed by nonhomologous recombination at unpredictable sites in the AAV genome, there could be rep^+ cap^- particles contaminating vector stocks. A more definitive assay for rcAAV would consist of passaging stocks on AAV packaging cells expressing both *rep* and *cap*. This method will amplify any particle containing *cis*-acting AAV TRs, which can then be detected with appropriate probes for viral sequences. After performing this type of analysis with stocks generating by serial passage on TtetA2 cells, we still could not detect rcAAV (Fig. 5).

The producer cell lines reported by others also did not generate rcAAV (<1 rcAAV/10^6 vector genomes) (5). The lack of rcAAV generated from packaging and producer lines suggests that the AAV helper construct is less recombinogenic when integrated than when transfected. In general, the introduction of plasmids into cells by transfection results in a high frequency of recombination compared to plasmids integrated in the chromosome (40), and microinjection experiments have shown that plasmid DNA becomes refractory to recombination 1 h after injection, suggesting that nucleosome assembly may reduce the rate of recombination (13).

The use of AAV vectors in human gene therapy will require large amounts of high-titer stocks and total vector particle numbers far beyond what can realistically be obtained by conventional methods. Systemic production of therapeutic levels of human factor IX and erythropoietin in mouse models has recently been demonstrated by administration of 4×10^{10} to 3×10^{11} AAV vector particles per mouse (18, 23, 39), and the expression was dependent on the dose of vector particles (18, 23). Assuming that the particle numbers required for in vivo vector delivery are proportional to body weight, at least 10^{14} vector particles would be required for therapeutic transduction levels in a single human patient, or the entire vector stock produced from 100 to 1,000 liters of transfected cells by the conventional method. The producer and packaging lines described here should overcome this barrier to AAV-based gene therapy, as they do not require transfection and yield 10-fold-higher titers, and they should be adaptable to suspension culture, allowing convenient, large-scale vector production.

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