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Method to Decrease the Titers of Contaminating Helper Adenovirus during the Production of Recombinant Adeno-Associated Virus

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Recombinant adeno-associated virus (rAAV) vectors are becoming increasingly important in gene transfer applications. However, large-scale production of rAAV to high titer and purity is laborious and time consuming. Traditional production methods lead to contamination with helper adenovirus particles and proteins, which must be eliminated from viral stocks if these are intended for in vivo applications. Recent production protocols have addressed this issue through the design of helper plasmids that contain all adenoviral genes necessary for AAV replication, yielding adenovirus-free stocks of rAAV (7,12,15). However, recent protocols for large-scale production of rAAV rely on producer cell lines that are infected with a helper virus (2,5), and many laboratories continue to use adenoviral helper systems. For this reason, many investigators are exploring new ways of reducing adenoviral contamination, either using replication-defective adenovirus mutants (9) or devising purification systems that eliminate adenovirus particles present in rAAV stocks (3,16).

We explored whether rAAV could be successfully produced using an E1- and E3-deleted helper adenovirus (Ψ 5) in which the packaging signal is flanked by *loxP* sites (4). When this adenovirus infects cells expressing Cre recombinase, recombination between the two *loxP* sites and excision of the packaging signal from the adenoviral genome take place. This leads to the generation of adenoviral genomes that have lost the packaging signal and therefore cannot be packaged into viral particles.

Since conventional methods for the production of rAAV use 293 cells and wild-type helper adenovirus, we hypothesized that replacing these by Cre-expressing 293 cells (Cre8 cells) and Ψ 5 helper virus would lead to rAAV

stocks with much lower levels of contaminating helper adenovirus. To test this hypothesis, we first constructed plasmid pX2luc, which contains the luciferase reporter gene in an expression cassette that includes the myosin light chain 1/3 enhancer upstream of the cytomegalovirus immediate-early promoter. This promoter/enhancer combination has been shown to increase expression levels in C2C12 myotubes in vitro (10). The luciferase expression cassette (3964 bp) was excised with *SalI* and cloned into *XhoI*-digested pAAV.luc.A (8), a derivative of plasmid psub201 (13). The resulting construct, pAAVX2luc, contains the luciferase expression cassette flanked by the AAV inverted terminal repeats. Plasmid pAAV/Ad (14), which contains the *rep* and *cap* genes from AAV2, was used as helper. Either 293 or Cre8 cells were plated on 10-cm dishes to 70%–80% confluence, co-transfected with plasmids pAAVX2luc and pAAV/Ad with the calcium phosphate method for 16 h and infected with Ψ 5 helper adenovirus at an approximate MOI of 2 (Cre8 cells and Ψ 5 adenovirus were a kind gift from Stephen Hardy, University of California San Francisco, San Francisco, CA, USA). Each culture dish was harvested 72 h after infection. After centrifugation, cell pellets were resuspended in 1 mL lysis buffer (50 mM HEPES, 150 mM NaCl, pH 7.6), lysed with three freeze-thaw cycles, centrifuged 20 min at 1000 $\times$ g and the supernatants stored at -80°C.

The total number of viral particles in each preparation was determined by slot-blot hybridization after DNase I treatment, using a probe directed to the transgene, with the ECLTM direct nucleic acid detection system (Amersham Pharmacia Biotech, Barcelona, Spain). The total number of viral genomes (particles) was calculated by quantification of the hybridization signal of the samples and a set of standards prepared from the original plasmid pAAVX2luc. We found the number of viral particles in the lysate from Cre8 cells was approximately twofold higher than in the lysate obtained from 293 cells (Figure 1A). This probably reflects a difference in the total number of cells present at the moment of harvesting, as a result of different survival rates during transfection. Alterna-

Benchmarks

tively, it could be the result of Ψ 5 helper adenovirus replicating at a much lower rate in Cre8 than in 293 cells, leading to a less marked cytopathic effect and a higher number of attached cells at the end of the experiment.

Titration of contaminating helper adenovirus was performed in quadruplicate by the addition of serial dilutions of each viral preparation to 293 cells plated on 96-well plates, and the lowest dilution showing cytopathic effect was scored eight days later. As shown in Figure 1A, the titers of contaminating helper adenovirus were about one order of magnitude lower when Cre8 cells were used, as compared to 293 cells. Taken together, this shows that the number of contaminating infectious adenovirus per each rAAV particle produced is about 22 times lower when Cre8 cells are used.

To confirm that the Ψ 5 adenovirus and Cre8 cell line could successfully

complement all the functions necessary for rAAV production, we also compared the biological activity of the rAAV stocks. Aliquots from each lysate were heat-treated for 5 min at 60°C to substantially inactivate the contaminating adenovirus (6). They were added in duplicate to 293 cells (plated on 6-well plates) that had been previously grown for 2 h either with or without genistein, an inhibitor of tyrosine kinases that has been shown to increase transgenic expression from rAAV (11). Three days later, cells were washed, harvested, lysed with Reporter Lysis Buffer (Promega, Madison, WI, USA) and centrifuged. Luciferase activity was assayed in the supernatants using reagents from Promega in a Lumat LB 9507 luminometer. Figure 1B shows luciferase activity of 293 cells transduced for 72 h with equal amounts of each rAAV stock. Luciferase levels were approximately twofold higher in the

preparations obtained from Cre8 cells, either with or without genistein treatment. The increase in luciferase activity after genistein treatment was also twofold in the viral stock from Cre8 cells. This is in keeping with the difference obtained in total number of rAAV particles from each preparation (Figure 1A) and suggests that the ratio of total to infectious rAAV particles is very similar when either cell line is used. Since nonpurified cell lysates were used in these experiments, it could be argued that a substantial amount of luciferase activity results from pseudo-transduction with luciferase protein present in the cell lysates (1). However, the fact that transgene expression was enhanced by genistein clearly shows that the increase in luciferase activity was the result of true transduction and that rAAV particles produced with Cre8 cells or 293 cells have comparable biological activities.

Figure 1. Efficiency of the modified production method. (A) Total number of rAAV particles, titers of contaminating adenovirus [infectious units (i.u.)/mL] and ratio of adenovirus (i.u.)/rAAV particle produced, using Ψ 5 helper adenovirus with either 293 or Cre8 cells. DNase-resistant rAAV particles were measured by slot-blot; titers of infectious adenovirus were determined in quadruplicate, scoring the lowest dilution that showed cytopathic effect on 293 cells. (B) Transgene expression assay of rAAV prepared using Ψ 5 helper adenovirus with 293 or Cre8 cells. Bars show the average luciferase activity from duplicate experiments (error bars show individual values) in which 293 cells were either not treated (filled bars) or treated with 150 mM genistein for 2 h before the addition of rAAV (hatched bars). Empty bars show the increase resulting from genistein treatment.

In conclusion, our results indicate that rAAV production can be successfully achieved using a helper adenoviral genome in which the packaging signal is flanked by *loxP* sequences, together with a Cre-expressing packaging cell line. Furthermore, this system also leads to reduced contamination by helper virus. We believe that this approach could complement and improve current strategies aimed at increasing the yield and purity of rAAV stocks, particularly those protocols of rAAV production that involve the use of helper adenovirus.

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