A novel method using baculovirus-mediated gene transfer for production of recombinant adeno-associated virus vectors

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Introduction

The baculovirus Autographa californica multiple nucleopolyhedrosis virus causes non-productive infection in mammalian cells. Recombinant baculovirus therefore has the capability to transfer and express heterologous genes in these cells if a mammalian promoter governs the gene of interest. We have investigated the possibility of using baculovirus as a tool to produce recombinant adeno-associated virus (rAAV). AAV has become increasingly popular as a vector for gene therapy and functional genomics efforts, although its use is hampered by the lack of a simple and efficient vector production method. We show here that co-infection of mammalian producer cells with three viruses – a baculovirus containing the reporter gene flanked by AAV ITRs, a baculovirus expressing the AAV rep gene and a helper adenovirus expressing the AAV cap gene – produces infectious rAAV particles. This baculovirus-based chimeric vector method may in future improve large-scale rAAV vector preparations and circumvent present-day problems associated with rAAV production.

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translated to yield four Rep proteins with overlapping amino acid sequences, Rep 78, 68, 52 and 40. Rep proteins have pleiotropic effects on both cellular and viral gene expression and replication. The two larger Rep proteins have regulatory functions and are required for replication, regulation of the AAV promoters, site-specific integration and for rescue of the AAV genome from its integrated state (Berns & Linden, 1995). The function of the two smaller proteins is not known, but they have been implicated in the packaging of the linear single-stranded genome into mature particles (Chejanovsky & Carter, 1989). The three Cap proteins, VP1, VP2 and VP3, also have overlapping amino acid sequences; they are capsid structural proteins ranging in size from 62 to 87 kDa and are expressed from one promoter located at map position 40 (p40). The two ORFs are flanked by 145 nt inverted terminal repeats (ITRs). The ITRs are the only -acting elements necessary for AAV replication, packaging and integration. Recombinant AAV (rAAV) vectors consist of the gene of interest flanked by AAV ITRs. Infections with rAAV in vivo have resulted in efficient, long-term gene transfer in a variety of tissues (Flotte et al., 1993; Kaplitt et al., 1994; Snyder et al., 1997, 1999; Xiao & Samulski, 1996; Xiao et al., 1997). Despite the broad spectrum of possible applications for rAAV-based vectors, their widespread use has so far been hampered by the lack of an efficient production method. In the conventional production method, rAAV is produced by transfecting human cells with two constructs: the rAAV vector plasmid and a Rep/Cap-expressing plasmid. The transfection is usually performed in the presence of replicating human adenovirus to provide the necessary helper functions (Samulski et al., 1989). Virus is then purified from total cell lysates through CsCl gradients, and contaminating adenoviruses are heat inactivated. The titres obtained are considerably lower than the titres obtained when preparing wild-type (wt) AAV, at least partly because of the rate-limiting transfection step. In addition, rAAV preparations are often contaminated by the helper virus, which may trigger an immune response in the transduced host. The future successes of rAAV for gene therapy purposes in humans, as well as for many functional genomics efforts, is therefore directly dependent upon alternative production methods which are easy to perform, give high titres and do not result in contamination with replication-competent helper virus.

As a step towards the development of such an alternative production method, we have investigated the possibility of using baculovirus for transcomplementation of functions necessary to produce rAAV. By co-infecting HEK293 cells with two different baculoviruses harbouring the AAV vector and rep expression cassette respectively, together with a replication-deficient adenovirus expressing the cap gene, infectious rAAV particles were produced. The importance of Rep expression levels and the amount of AAV vector templates for rAAV yields were analysed. The possible use of this system to produce rAAV for gene therapy and functional genomics purposes is discussed.

**Methods**

- **Plasmids.** pVL1392 is an AcMNPV polyhedral baculovirus transfer plasmid used for construction of baculovirus expression vectors (O'Reilly et al., 1994). pBac-CMVRep is a pVL1392-based expression plasmid containing a CMV promoter, the AAV rep gene (position 288–2310 from AAV-2) and a bovine growth hormone polyadenylation (pA) signal. Cloning sites within pVL1392 were BglII and NotI. pBac-p5Rep was constructed by subcloning a 2.2 kb Xbal–HindIII fragment encompassing the endogenous AAV rep gene and promoters from pSub201 (Samulski et al., 1987) into the Xbal/HindIII sites of pBSK2+ (Stratagene). From the subclone, an Apol (blunted)–NotI fragment was transferred to the Xhol (blunt)/NotI sites in pVL1392. pBac-p5mutRep was created by PCR amplification of pBac-p5Rep (12.61 bp) with a phosphorylated primer-pair followed by self-ligation of the linear PCR product. The primers introduce a mutation in the original p5 ATG translation start codon (changing it to ACC) and a C to G mutation 13 bp upstream of the ACG triplet to create a BseHI cleavage site in order to distinguish the PCR product from the template. Forward primer, GCCGCCAGCAGCGG-GTTTTC; rReverse primer, TGCCCGGCTCAACACTCCCGGT. Both pBac-p5Rep and pBac-p5mutRep contain a bovine growth hormone pA signal. pBac-LacZ is a pVL1392-based expression plasmid containing the LTR-RSV promoter, the lacZ reporter gene fused to a nuclear localization signal and the SV40 pA signal. The expression cassette is flanked by AAV-2 ITRs (positions 1–157 and 4489–4680 respectively). The cloning sites within pVL1392 were BglII and a Klenow-treated PvuMI site. pMA41 is a shuttle vector that harbours an adenovirus 5 sequence (position 1–382) followed by a CMV promoter, the AAV-2 cap gene (position 1857–4487) and an additional sequence from adenovirus 5 (position 3446–4419). pMA47 contains a CMV-lacZ expression cassette flanked by AAV ITRs. pXX2, pXX6 and pVL2822 have been described (Xiao et al., 1998; Crouzet et al., 1997).

- **Viruses.** Construction of baculoviruses was done with the BacVector-2000 transfection kit (Novagen). Transfer vectors for construction of vBac-CMVRep, vBac-p5Rep, vBac-p5mutRep and vBac-LacZ were pBac-CMVRep, pBac-p5Rep, pBac-p5mutRep and pBac-LacZ respectively. Correct inserts were verified by PCR and Southern blot analyses. vBac-LacZb (AcNPV-fbgal) and vBac-GFPb (AcNPV-GFP) express their reporter genes from the RSV and CMV promoters respectively, and were kind gifts from Christian Hofmann, HepaVec, Berlin, Germany. Construction of vAd-Cap was done by homologous recombination between pMA41 and pXL2822 in bacteria as described by Crouzet et al. (1997). vAd-COL is a control adenovirus 5 vector lacking the E and E3 regions.

- **Cells and medium.** Sf9 cells (ATCC CRL-1711) were maintained in Grace's insect medium supplemented with 5% foetal calf serum, 2 mM t-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. HEK293 cells (293A from Qbiogene, Middlesex, UK) were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum, 2 mM t-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 mM non-essential amino acids (Gibco).

- **Preparation of high titre baculovirus and adenovirus stocks.** High titre stocks of baculovirus were obtained from single virus plaques as described by O'Reilly et al. (1994). Virus was then concentrated and purified by ultracentrifugation through a 25% sucrose cushion (w/v in 5 mM NaCl, 10 mM EDTA) at 24000 r.p.m. for 75 min at 4 °C (Beckman SW28 rotor). The virus pellet was resuspended in baculo-PBS pH 6.2 (1 mM Na2HPO4, 10.5 mM KH2PO4, 140 mM NaCl, 40 mM KCl) and recentrifuged as above to eliminate all traces of sucrose. The
Results

Baculovirus infection of HEK293 cells

Recombinant AAV is often produced in HEK293 cells, which are highly susceptible to adenovirus infection. These cells express the adenovirus E1 region from an integrated copy, which makes it possible to use an E1 deleted adenovirus as helper. In order to evaluate the efficiency by which baculovirus can infect this cell line, increasing amounts of two baculoviruses containing the reporter genes lacZ (vBac-LacZb) or GFP (vBac-GFPb) were used in separate titration experiments (Fig. 1). Twenty-four hours post-infection cells infected with vBac-LacZb were fixed and stained with X-Gal and the number of blue cells was counted under a light microscope, while cells infected with vBac-GFPb were analysed directly under a fluorescence microscope to visualize reporter gene expression. At an m.o.i. of 25–50, around 70–90% of the cells efficiently expressed the reporter gene and at an m.o.i. of 200 almost all cells were infected. The infected cells did not show any signs of alterations in cell growth or cell morphology due to the virus infection, and they continued to grow well throughout the experiment. A basic characterization of baculovirus infections in HEK293 cells showed that maximal infection occurred when the cell density was near confluence and that infection was not sensitive to serum-containing media in which the complement system had been heat-inactivated (data not shown). In addition, no difference in infection...
efficiency was seen when infection temperatures ranged between 20 and 37 °C (data not shown). Together, these results suggested that it would be feasible to use baculovirus for gene transfer aiming at rAAV production in HEK293 cells.

**Virus characterization**

Several genes are necessary for the production of rAAV; the AAV-specific rep and cap genes, the gene of interest flanked by AAV ITRs (i.e. the vector) and the adenovirus helper genes. To evaluate the feasibility of using baculovirus for trans-complementation in rAAV production, we decided to test the individual components in separate viruses (Fig. 2A). Three recombinant Rep-expressing baculoviruses were constructed. vBac-CMVRep expresses Rep from the strong CMV promoter; vBac-p5Rep and vBac-p5mutRep express Rep from the AAV-specific p5 promoter utilizing the endogenous ATG start codon or an artificial ACG start codon, respectively. A fourth baculovirus was constructed harbouring an RSV-driven lacZ reporter gene flanked by AAV ITRs (vBac-LacZ). The cap gene was cloned behind a CMV promoter within a first generation adenovirus vector (vAd-Cap). The integrity of all five viruses was cloned behind a CMV promoter within a first generation adenovirus vector (vAd-Cap). The integrity of all five viruses was checked by PCR and/or Southern blot analyses (data not shown). Following preparation of high titre virus stocks, expression patterns of the heterologous genes were examined. Western analysis on extracts from HEK293 cells infected with vAd-Cap showed strong expression of all three capsid proteins (Fig. 2B). VP3 was produced in excess compared to VP1 and VP2, which is also seen during a wt AAV infection (Berns & Linden, 1995). The assembly of AAV capsids was analysed by electron microscopy of vAd-Cap-infected HEK293 cells (Fig. 2D). Icosahedral particles corresponding to adenovirus capsids were easily detected in the nucleus. AAV capsids also have an icosahedral structure, but the diameter is approximately 4-fold smaller compared to adenovirus. Based on size comparison, the smaller particles seen in the image might correspond to AAV empty capsids, suggesting that the AAV capsid proteins expressed from vAd-Cap were able to assemble in the cells. Northern analysis was performed to analyse mRNA levels expressed from the different Rep-expressing baculoviruses (Fig. 2C). Specific bands corresponding to the expected sizes of unspliced Rep mRNAs originating from the CMV and p19 promoters was seen when RNA isolated from vBac-CMVRp-infected cells was analysed (lane 2). In contrast, long exposure times were required to detect Rep-specific bands when RNA isolated from cells infected with either vBac-p5Rep or vBac-p5mutRep was analysed (lane 3 and data not shown), showing that Rep mRNA levels expressed from these viruses were significantly lower than from vBac-CMVRp. We could not identify any spliced transcript in this experiment. The integrity of Rep expression cassettes including splice sites has been carefully examined, and DNA rearrangements or mutations cannot explain these results. The reason for the lack of the small, spliced transcripts is therefore unclear, but might reflect a low sensitivity in our experiment. Finally, expression of the reporter gene lacZ in vBac-LacZ was verified by infection of HEK293 cells followed by X-Gal staining (Fig. 3A). A heat inactivation test was performed to ensure that baculovirus was properly heat-inactivated at the temperature used to heat-inactivate the adenovirus helper after the production of rAAV. HEK293 cells were infected with untreated or heat-inactivated vBac-LacZ (Fig. 3). Twenty-four hours post-infection cells were fixed and stained with X-Gal. Examination of the cells under a light microscope revealed that while the majority of cells infected with untreated virus stained blue (Fig. 3A), no blue cells could be seen following infection with the heat-inactivated baculovirus preparation (Fig. 3B). Thus, like the adenovirus helper, baculovirus do not survive the heat-treatment used in the protocol for rAAV production.

**Production of infectious recombinant AAV**

To test if our viruses were able to transcomplement functions required for rAAV production, a small-scale rAAV preparation was performed. HEK293 cells were infected simultaneously with vBac-LacZ, vBac-CMVRp and vAd-Cap. Forty-eight hours post-infection cells and supernatants were collected, freeze–thawed to release virus from cells and heat-inactivated to kill off remaining baculovirus and adenovirus helper. This crude rAAV preparation was then used to infect fresh HEK293 cells in the presence of vAd-CO1, a first generation adenovirus vector which facilitates the production of transcription templates (Fisher et al., 1996). At 24 h post-infection cells were fixed and stained with X-Gal (Fig. 4). Strongly stained cells were easily detected (Fig. 4A), indicating that infectious rAAV particles were indeed produced. This experiment suggested that rep and cap, expressed from vBac-CMVRp and vAd-Cap respectively, were functional. The experiment also implied that the AAV ITR-flanked lacZ transgene within vBac-LacZ could function as a template for replication and that ssDNA rAAV genomes were produced and packaged into functional rAAV particles. Blue cells were only found when using extracts from cells infected with all three viruses simultaneously, and not from cells infected with any two combination of viruses or with vBac-LacZ alone (Fig. 4B and data not shown). The efficiency of rAAV production could not be improved by extending harvest time to more than 48 h post-infection (data not shown).

**Baculovirus-derived rAAV are replication competent**

To investigate whether the rAAV produced were replication competent, a replication assay was performed (Fig. 5). Replication of AAV requires rep and the adenovirus helper genes. Therefore a crude, heat-inactivated rAAV preparation was used to infect HEK293 cells in the presence of vBac-CMVRp and vAd-CO1. vAd-CO1 here serves solely as a source of adenovirus helper genes. Under these conditions, no
Fig. 2. (A) Schematic representation of virus constructs. (B) Western blot analysis on protein extracts prepared 24 h post-infection from either mock infected HEK293 cells (lane 1) or HEK293 cells infected with vAd-Cap at an m.o.i. of 2 (lane 2). The primary antibody used recognizes all three capsid proteins. (C) Northern blot analysis on total RNA prepared 24 h post-infection from either uninfected HEK293 cells (lane 1) or HEK293 new rAAV are being produced due to the lack of capsid proteins. Thirty hours post-infection low molecular mass DNA was prepared by Hirt extraction. DNA was digested with DpnI, an enzyme which digests only methylated and not newly replicated DNA, and DNA was analysed by Southern blot using a lacZ-specific probe. As can be seen in Fig. 5, lane 1, both monomeric and dimeric forms of AAV replication intermediates were formed. No replication intermediates were observed when vBac-CMVRep and vAd-CO1 were infected in the absence of rAAV (lane 2) or when cells were infected with rAAV alone (lane 3). No hybridization signal originating from the single-stranded rAAV genome was observed in cells infected with rAAV alone (lane 3), probably due to the short exposure time of the film (approximately 30 min). These results show that rAAV prepared by the baculovirus-based approach are fully functional and that the rAAV genomes can be mobilized if provided with the necessary helper functions.

**Importance of Rep expression levels and vector amounts for rAAV yields**

To investigate the importance of Rep expression levels and vector amounts for rAAV yields, several small-scale rAAV preparation experiments were performed. First, a titration experiment was carried out in which the amount of vAd-Cap was kept constant at an m.o.i. of 4, while vBac-CMVRep and vBac-LacZ were mixed in equal proportions and used in increasing amounts (Fig. 6A). At 48 h post-infection, cells and supernatants were collected, freeze–thawed and heat-inactivated. The amount of rAAV present in this crude extract was determined by titration on fresh HEK293 cells. At 24 h post-infection cells were fixed and stained with X-Gal and the number of blue cells, which corresponds to the viral titre measured in Transduction Units (TU), was quantified under a light microscope. The maximum number of rAAV particles was found when using baculovirus at an m.o.i. of 50. At this virus dose, $6 \times 10^4$ TU/ml was obtained. When baculovirus concentrations were increased, rAAV titres declined. It has previously been shown that adenovirus gene expression and replication are reduced in the presence of wt AAV coinfection. The inhibition appears to require rep gene expression but the mechanism is not known (Berns, 1996). Decreased replication of vAd-Cap would mean that less template is available for transcription resulting in a reduction in expression of both adenovirus helper genes and AAV capsid proteins. To investigate if vBac-CMVRep infection affected Cap expression in our system, a Western analysis was performed on extracts from HEK293 cells infected with vAd-Cap in the presence of cells infected with either vBac-CMVRep (lane 2) or vBac-p5Rep (lane 3) at an m.o.i. of 9. The Rep-specific probe encompass the whole coding region of rep. (D) Transmission electron micrograph showing deposits of adenovirus and AAV capsids in the cell nucleus of vAd-Cap-infected HEK293 cells. Bar, 500 nm.
increasing amounts of vBac-CMVRep (Fig. 6B). This experiment showed a clear correlation between increasing Rep- and reduced Cap-expression levels, pointing towards at least one explanation for the decrease in rAAV yields. When an experiment was performed in which the amounts of both vAd-Cap and vBac-CMVRep were kept constant at m.o.i.’s of 4 and 25 respectively, and vBac-LacZ was added in increasing amounts, no decline in rAAV titres was seen when using vBac-LacZ above an m.o.i. of 50 (Fig. 6C). Recombinant AAV titres instead increased slightly and reached a plateau at around $1 \times 10^6$ TU/ml. These experiments indicate that the amount of vector is not the limiting factor in this experimental set-up, and that overexpression of Rep is deleterious for rAAV yields. Based on the titration experiment shown in Fig. 1, it appears as if approximately one copy of the CMVRep expression cassette per cell is optimal when using this combination of viruses.

Since the yield of rAAV was clearly dependent on Rep expression levels, we decided to try to produce rAAV with baculoviruses expressing Rep from the less potent AAV p5 promoter. vBac-p5Rep and vBac-p5mutRep are identical except for a mutation in vBac-p5mutRep of the original ATG translation start codon into an inefficient ACG codon, a mutation that was previously shown to increase rAAV yields significantly when producing rAAV by the conventional method (Li et al., 1997). A comparison between the three Rep-expressing baculoviruses clearly showed that both vBac-p5Rep and vBac-p5mutRep produced significantly higher levels of rAAV compared to vBac-CMVRep (Fig. 6D). vBac-p5mutRep reproducibly gave the highest yields. Production of rAAV with vBac-p5mutRep (m.o.i. 25) and vAd-Cap (m.o.i. 3) in the presence of increasing amounts of vBac-LacZ improved the titre slightly to $1 \times 10^6$ TU/ml (Fig. 6E). In summary, our
experiments show that a very low level of Rep expression results in the highest rAAV titres, and that by increasing the amount of vector, a small but significant increase in rAAV yields can be achieved.

**Discussion**

In this paper we demonstrate that baculovirus can be used to transcomplement functions that are necessary to produce rAAV. By co-infection of two baculoviruses, one expressing Rep (vBac-CMVRep, vBac-p5Rep or vBac-p5mutRep) and the other harbouring the lacZ reporter gene flanked by AAV ITRs (vBac-LacZ), together with an adenovirus vector expressing Cap (vAd-Cap), infectious and replication competent rAAV was produced. These results show that an AAV vector constructed within a baculovirus backbone can function as a template for replication and that single-stranded rAAV genomes can be produced and packaged into functional rAAV particles. That the blue cells in Fig. 4(A) really correspond to newly formed rAAV particles, and not to surviving vBac-LacZ, was clear from the fact that baculovirus are heat-sensitive and do not survive the heat treatment used to kill off the adenovirus helper in the crude rAAV preparation (Fig. 3). In addition, the presence of rAAV replication intermediates in Fig. 5 further strengthens the conclusion that rAAV particles were indeed produced. The β-galactosidase protein has previously been reported to withstand heat inactivation and to enter cells by an as yet unknown mechanism resulting in overestimation of rAAV titre values (Alexander et al., 1997). This phenomena, called pseudotransduction, is however not a problem in our system, since no blue cells were seen when the rAAV production protocol was used with vBac-LacZ alone (Fig. 4B).

The absence of pseudotransduction may be the result of the slightly higher temperature used for heat inactivation in our method compared to the conventional protocol (60 °C instead of 56 °C).

In the production protocol described here, the AAV vector and the Rep expression cassette were introduced into the producer cells by different baculoviruses. This enabled us to investigate the requirement for vector and Rep proteins separately. The amount of Rep needed for rAAV production has to some extent been inconclusive. While earlier reports indicated that expression of Rep from strong heterologous promoters was beneficial for rAAV production, later investigations have shown that unregulated overexpression of Rep inhibits rAAV production (Flotte et al., 1995; Li et al., 1997; Ogasawara et al., 1998). Our results clearly demonstrate that attenuated Rep synthesis significantly increased rAAV titres in our baculovirus-based production protocol. Best rAAV yields were achieved when Rep was expressed from the p5 promoter and when the original ATG start codon was mutated into an inefficient ACG codon. However, although this mutation resulted in higher rAAV yields compared to non-mutated rep, the increase was not as dramatic as described when the same mutation was used in the conventional production protocol (Li et al., 1997). In our experimental set-up, the amount of AAV vector did not appear to be limiting, since even though rAAV titres increased slightly with increasing amounts of vector, the increase was not proportional to the increase in vector copies added.

The size of heterologous DNA that can be inserted into a baculovirus backbone is very large. It would therefore be possible to construct a baculovirus harbouring all helper functions necessary to produce rAAV, that is rep, cap and the adenovirus-specific helper genes (vBac-all). The adenovirus genes required for an AAV lytic life-cycle have been determined, even though the mechanisms by which these proteins mediate their helper functions is not fully understood. These are the early region 1 (E1), the E2A transcription unit, E3orf6 and VA (Berns, 1996). Since the cell line we use for rAAV production express E1 from an integrated gene copy, the adenovirus genes that would have to be cloned into baculovirus can be limited to E4orf6, E2A and VA. With such a virus, rAAV would easily be produced by co-infecting vBac-all together with a baculovirus harbouring the gene of interest flanked by AAV ITRs. No adenovirus helper-infection would be required, eliminating the risk of unwanted virus contamination in the final rAAV stock. In the conventional production method the need for adenovirus helper infection has recently been circumvented by the development of adenovirus mini-genomes expressing only the early adenovirus genes needed to supply the essential helper functions.
Fig. 6. Importance of Rep expression levels and vector amounts for rAAV yields. The graphs illustrate the results of one of several typical experiments. (A) HEK293 cells were infected with vAd-Cap at a constant m.o.i. of 4 in the presence of increasing amounts of vBac-LacZ and vBac-CMVRep. Forty-eight hours post-infection virus was harvested and heat-treated at 60°C for 15 min to inactivate adenovirus and baculovirus. This extract was then titrated on fresh HEK293 cells in the presence of vAd-COI at an m.o.i. of 4. Twenty-four hours post-infection cells were stained with X-Gal and the rAAV titre was determined by counting the number of blue cells under a light microscope. Transduction Units per ml (TU/ml) of rAAV were plotted against the amount of baculovirus used. (B) Western blot analysis on protein extracts prepared 24 h post-infection of HEK293 cells. The primary antibody used recognizes all three capsid proteins. vAd-Cap was used at an m.o.i. of 2, while vBac-CMVRep was used at m.o.i.’s of 2 (lane 2), 22 (lane 3) or 122 (lane 4). (C) As in (A) except that vBac-CMVRep was used at a constant m.o.i. of 50. (D, E) Recombinant AAV was produced and analysed as in (A). In (D) vAd-Cap was used at an m.o.i. of 3, vBac-p5mutRep at an m.o.i. of 25 and vBac-LacZ at the amounts indicated.

required for AAV (Grimm et al., 1998; Xiao et al., 1998). A recent report even suggests that rAAV can be produced without the adenovirus E2A and VA if rep and cap are expressed from heterologous promoters (Allen et al., 2000). This shows that functional rAAV can indeed be produced by expression of selected adenovirus genes only. When using the conventional production protocol, every transfected cell harbours perhaps 100 to 1000 copies of the helper plasmids. Although it is not known how many of these plasmids actually reach the nucleus, the conditions used for the conventional production protocol and the baculovirus-based method probably differ considerably. In our protocol, the copy number is
low since the system is non-replicating and every baculovirus that enters the cell will contribute only a single gene copy. Construction of a vBac-all virus will therefore require a careful examination of the proper combination of promoters for all helper-genes in order to obtain the right conditions for optimal rAAV yield.

When directly comparing our baculovirus-based production protocol with the triple transfection protocol in small-scale experiments we find the titres to be comparable. With the baculovirus approach a titre of $1 \times 10^6$ TU/ml was achieved. When using a slightly modified version of the optimized triple transfection protocol described in Drittanti et al. (2001), we reached titres of $4 \times 10^5$ TU/ml (data not shown). Despite repeated attempts, we could not reach the titres (measured as lacZ-forming units) described by Drittanti and co-workers, which underlines the difficulties intrinsic to the transfection procedure. We find the efficiency of the transfection method to be highly dependent on the amount of transfected DNA as well as on the confluence of the cells and the serum concentration. We therefore believe that our baculovirus-based method is less laborious and more reproducible than the triple transfection protocol, and that a further development of this technique may have an impact on future large-scale rAAV vector production.

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