CONFERENCE PAPER

Current issues in adeno-associated viral vector production

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Adeno-associated virus (AAV) is currently one of the most promising systems for human gene therapy. Numerous preclinical studies have documented the excellent safety profile of these vectors along with their impressive performances in their favored target, consisting of highly differentiated postmitotic tissues such as muscle, central nervous system and liver. Clinical trials have been conducted confirming these data, but also emphasizing the requirement of further high-tech developments of the production and

purification procedures that would allow both scaling-up and improvement of vector batch quality, necessary to human application. The scope of this review will be the state of the art in the various production methods of recombinant AAV (rAAV), delimiting their respective perimeter of application and also their main advantages and drawbacks, and thereby shedding light on the main challenges to take in the near future to bring AAV vectors more widely into the clinics.

Gene Therapy (2005) 12, S51–S61. doi:10.1038/sj.gt.3302615

Introduction

Adeno-associated virus (AAV) based vectors are efficient vehicles for gene therapy in humans. An important set of data has accumulated from various preclinical and clinical models (rodents, dog, monkey), providing evidences of the quasi-absence of pathogenicity or toxicity, an excellent safety profile and the ability to confer long-term gene expression in a wide range of tissues.¹

Biology of AAV

AAVs are small nonenveloped single-stranded (ss) DNA viruses with a diameter of 18–25 nm. They belong to the Parvoviridae family and are classified in the Dependovirus genus. In total, 11 strains have been isolated and characterised from humans and primates, and new serotypes are continuously discovered.^{2–6} All serotypes share similar structure, genome size and organization, that is, structure and location of the open reading frames (*orf*), promoters, introns and polyadenylation site. The most divergent serotype is AAV5 with notable differences at the level of the ITR size (167 nucleotides for AAV5 compared to 143–146 for AAV1 to 4 and AAV6) and function.⁷ In addition, at the biological level, they are all dependent on the presence of a helper virus for their replication and gene expression.

AAVs are frequently found in the human populations, 70–80% of individuals having been exposed to an infectious event.^{8,9} No known adverse clinical consequences are associated with AAV infection or latency in humans.

The particle is composed of an icosahedric capsid and one single molecule of the viral genome of either polarity,² and has a density of 1.41 g/cm.³ AAVs are very

resistant to extreme conditions of pH, detergent and temperature, making them easy to manipulate. Finally, wild-type (wt) AAV has the unique property of integration at a specific site into the human genome, which has been extensively described for AAV2.^{10,11} However, because of viral genome manipulation, this propensity is not maintained in vectors.

As the first serotype used to generate vectors, AAV type 2 is so far the best characterised and the majority of gene transfer studies have been based on the use of this serotype.^{12–14} We can and will therefore consider it as a prototype. However, for the last years, rAAV have been actively developed from alternative serotypes. The rationale lies in their very similar structure but different tropism and immunological properties, allowing to design a whole set of vectors. The physical characteristics of the different serotypes are close enough to handle them under similar conditions as AAV2 for production and purification. Only the purification systems based on fine capsid structure (electric property, ligands) should be adapted for each serotype. Many groups are actively evaluating the *in vivo* performances of these serotypes in various animal and disease models (for a recent and extensive review on the serotypes, see Grimm and Kay¹⁵).

The 4.7 kb genome of AAV2 (Figure 1) contains two *orf*, which encode four regulatory proteins, the Rep proteins, and three structural proteins, the Cap proteins.^{16,17} The compacted genome of AAV is framed by two inverted terminal repeats (ITRs), which are base-paired hairpin structures of 145 nucleotides length. The ITRs contain the only necessary regulatory *cis* acting sequences required by the virus to complete its life cycle, namely the origin of replication of the genome, the terminal resolution site and, the packaging and the integration signals. The two major Rep proteins, Rep78 and Rep68, are involved in viral genome excision, rescue, replication and integration¹⁸ and also regulate gene

expression from AAV and heterologous promoters.^{19,20} The minor Rep proteins, Rep52 and Rep40, are involved in replicated ssDNA genome accumulation and packaging.²¹ The cap *orf* is initiated at the p40 promoter and encodes the three structural capsid proteins VP1, VP2 and VP3. Their stoichiometry in the assembled particle is 1:1:10. Finally, all transcripts share the same polyadenylation signal and equal amounts of virions are found containing strands of plus or minus polarity.

Figure 1 AAV-2 genome organization. General organization of the genome and genetic elements of AAV type 2 (a scale of 100 map units is used, 1 map unit being equivalent to approximately 47 nucleotides) are shown. The general organization of the other serotypes is similar, T shaped black boxes indicate the ITRs. The horizontal arrows indicate the three transcriptional promoters. The solid lines indicate the transcripts; the introns are shown by the broken lines. A polyadenylation signal present at position 96 is common to all transcripts. The first *orf* encodes the four regulatory proteins (light grey boxes) arising from the promoters p5 and p19 and the alternative splicing. The second *orf* (promoter p40) encodes the three capsid proteins (dark grey boxes) from two transcripts. VP-1 is initiated from the first cap transcript, and VP-2 and VP-3 are initiated at two different codon sites from the second cap transcript. Note that the initiation site of VP2 is an ACG.

AAV are naturally replication defective viruses, which made them dependent on the presence of an auxiliary virus in order to achieve their productive cycle. This feature gave the name to the Dependovirus gender. Several viruses can ensure the auxiliary functions: adenovirus (Ad), herpes simplex virus and vaccinia virus^{22,23} as well as cytomegalovirus (CMV).²⁴ The infection scheme is fairly simple. Once AAV has entered the cell and been conveyed to the nucleus, its ss genome is converted into a replicative double stranded (ds) form, required for gene expression. In the absence of an auxiliary virus, AAV genome integrates into the host genome and latently persists in a proviral form. In the presence of a helper virus (either by concomitant infection or super infection), the AAV genome can undergo the process of active replication, during which capsid proteins are synthesised and DNA packaged, these steps taking place inside the nucleus.²⁵ AAV does not possess a lytic capability by itself, and in the natural infection process, the liberation of AAV virions usually relies on the lytic effect of the helper virus.

AAV vector design

The design of AAV-based vectors is straightforward, the ITRs being retained, and the exogenous sequences to be transferred cloned in-between. The Rep and Cap functions have therefore to be supplied in *trans*. Similarly, the helper functions from the auxiliary virus have to be provided (Figure 2 depicts the general principle of rAAV design and production). Several systems coexist, with their own advantages and drawbacks. The next chapters of this review deal with the main methods of rAAV production, that is, the means to provide these functions in a cellular-based production system.

Figure 2 rAAV design and production principle. In a transitory production system, a pAAV Helper and a pAAV plasmids will be brought to the packaging cells by transfection. A second step will consist of the infection of the cells (commonly HeLa cells) with a wtAd or with a first generation E1 deleted Ad in 293 cells (see text for details). An improved Ad free protocol is based on the replacement of Ad infection by the transfection of a mini Ad plasmid along with the pAAV Helper and pAAV plasmids. These methods lead to the generation of about 10^3 – 10^4 particles per cell. Prom: promoter, IVS: intervening sequence (eg intron).

Figure 3 Pseudotyped rAAV design and production principle. Pseudotyped vectors have been extensively and preferentially derived since the early 2000's. The general principle for the genetic trans-complementing system is depicted here. The recombinant AAV genome bearing the expression cassette is framed by serotype 2 ITR. Although the Rep protein from all serotypes but 5 can bind and ensure the replication process, it has been found more convenient and efficient to maintain the rep orf from serotype 2 or to create a chimeric rep gene with its 5' part of serotype 2 origin and the 3' of the serotype of interest.⁸² The auxiliary virus functions are provided as for rAAV2 production.

With the discovery and the development of new AAV serotype, the idea has emerged rapidly to develop vectors from these serotypes.^{3,4,26–28} In the vast majority of the studies, a pseudotyping strategy has been adopted for simplicity reason. Basically, all recombinant genomes are based on AAV2 ITRs, and only the capsid is of the serotype of interest (Figure 3). The production strategies for pseudotyped particles are exactly the same as for the classical AAV2 vectors.

In laboratory scale, most of the current methods of production of viral particles are still based on transient transfection of human embryonic kidney (HEK) 293 cells with minor variations. To step toward clinical application, various strategies allowing scaling-up of the amount of virus produced are being actively investigated. Here we shall review and discuss the former and the latter of these approaches.

Although out of the scope of this review, the protocols for purification of rAAV have evolved over the last decade. The most classical and easy to acquire for a non-specialised laboratory remain the ultracentrifugation on a CsCl²⁹ or iodixanol gradient.³⁰ These methods of purification are limited by the capacity of cell lysate volume that can be processed and by the low purity achieved. However, these methods are still widely used and most of the time sufficient for fundamental studies and early *in vivo* preclinical evaluation of the vectors. More complex techniques based on chromatography are now well developed for the generation of high purity grade and up-scaled production suitable for human clinical application.^{31–33}

Traditional methods for the production of AAV vectors

The classical production methods for rAAV are based on transient transfection protocols of target/producer cells.

This is a trans-complementation and transitory transfection based approach, which requires the following genetic elements: (i) the sequence of the rAAV genome, usually cloned into a plasmid, called pAAV. It consists of the ITRs framing the expression cassette, which cannot exceed 4.5 kb; (ii) the sequence encoding the two AAV orf rep and cap, usually cloned into a plasmid, called pHelper or pRepCap and complementing the missing rAAV functions and (iii) the required helper functions encoded by the natural auxiliary virus, usually Ad (Figure 2).

Production based on transient transfection and infection

The first production system of the current AAV vectors, that is, totally deleted for rep and cap orf, was based on a two-plasmids system, pAAV and pHelper, in which overlapping AAV sequences had been totally eliminated, leading to the considerable reduction of wtAAV generation in the recombinant virus batch.³⁴ In addition, the exact set of Ad genes providing the auxiliary functions were not clearly identified. This led to the very first method based on double transfection of target cells (HeLa cells) with the pAAV and pHelper, followed by an infection step few hours later with wt Ad at low multiplicity³⁴ (Figure 2). After 2–3 days, rAAV accumulation within the cells has reached a maximum and because of the cytolytic effect of Ad, cells are harvested for further purification. It is important in this protocol to harvest the cells before the Ad-induced lysis has occurred, to allow for the purification of rAAV from the cell lysate only. Processing the cellular medium for AAV purification would be extremely laborious due to the limited capacity of ultracentrifuge rotors. This method, in addition of being extremely laborious and time consuming requires extensive purification and heat inactivation, in order to eliminate the contaminating auxiliary wt Ad, a highly undesirable contaminant. Nevertheless, wt Ad contamination of rAAV stocks, associated with the possibility of its propagation, was a persistent problem in the initial studies of gene transfer with rAAV, raising serious questions about the safety of such produced vectors.³⁵

Replacement of the wt Ad by a replication defective Ad has been a partial solution to this safety problem, at least limiting the contamination to only nonreplicative Ad. A first generation adenoviral backbone vector deleted for E1 genes (Δ E1) or thermo-sensitive mutants^{36,37} were used instead of the wt. Because E1 was recognized as being necessary for AAV replication, HEK 293 cells were chosen as the transitory producer cell for AAV vectors. These cells are an Ad5-transformed cell line expressing E1 (E1A and E1B) and are the conventional cells used for recombinant Ad production. In this protocol, 293 cells are transfected with the vector plasmid and the helper plasmid (Figure 2), followed by infection with Ad Δ E1, in similar conditions as those described above based on wt Ad. This method led to reasonably high titers of 10^{11} – 10^{12} physical particles (also called viral genome, vg) per ml (10^2 – 10^3 vg/cell) and also to substantial levels of Ad Δ E1 contamination, which is still undesirable because of the toxicity and immunity associated with Ad administration. In addition, heat inactivation does not allow complete Ad elimination and further results in losses in rAAV titers.

Production based on transient transfection

An important step was achieved in the late 1990s by replacing the Δ E1 adenoviral infection step by a mini-Ad plasmid encoding the complementary adenoviral helper functions E2, E4 or f6 and VAI.^{37–39} This led to the establishment of a tri-transfection method, which allowed for the total elimination of adenoviral particles in the process of production and therefore as a contaminant of AAV preparation (Figure 2). Following a single-step transfection of 293 cells (expressing E1) with the three plasmids at an equimolar ratio, the cells are incubated for 48–72 h and harvested. However, with such a protocol, a single cell has to be hit three times in order to produce rAAV virions, thereby affecting the efficiency of production on a per-cell basis.

Generating a two-plasmid system made a further improvement of this transfection-only procedure. A two-component packaging plasmid containing the AAV *rep* and *cap* genes and the Ad auxiliary functions was generated.^{40,41} This system, requiring only a double-hit cell transfection with the hybrid pAd/Helper and pAAV plasmids, resulted in up to 10-fold higher titers than those achieved using conventional protocols, production ranging between 10^2 and 10^4 vg/cell.

Modification of the regulation of *rep* and *cap* in the pHelper plasmid

At the level of the Rep and Cap coding sequences, studies have highlighted the requirement for a tight regulation between *rep* and *cap* genes leading to few modifications. It was shown that for optimum virion production per cell, the *rep* gene must be weakly expressed, whereas the *cap* gene must be overexpressed, capsid protein being a limiting factor for virion accumulation in the cell.^{42–44} However, it was also shown that capsid protein expression is rate limiting until a threshold of assembled capsids is reached in the cell, and that beyond this limit, efficient encapsidation is more likely to be rate limiting.⁴⁰

With all these transitory methods of production, several important limitations persist. First, they are laborious, and titers within the range of $10^{12–13}$ vg per ml are attainable, which translate to about $10^{9–12}$ infectious particles per ml. Such titers, although suitable for most of fundamental biology and preclinical studies in rodent and mid-size animal models, are hardly sufficient for early clinical evaluation in humans, and efficient transfection procedure turned out to be difficult to scale up. Second, 'pseudo-wt AAV' or replication competent (rc) AAV particles arise from nonhomologous recombination between vector and helper AAV plasmids,^{37,45} which is a matter of concern from a safety point of view.

Advanced methods for the production of AAV vectors

Although the traditional approaches for the production of rAAV are widely used for the production of non-GMP and also GMP grade vectors, they are still affected to their low-scale dimension. The adaptation of transient methods to a reactor environment and the further scale-up to an industrial scale are difficult or almost impossible, although large-scale transient production of recombinant proteins using 293 cells cultivated under

serum-free conditions is feasible for developmental purposes.^{46,47} In addition, under GMP-conditions for clinical applications, the transient transfection method requires the certification of several components (plasmid DNA, transfection reagents, and cell lines). Thus, transfection-independent methods have been developed and evaluated for the production of rAAV. These methods are based either on the use of packaging/producer cell lines, which produce rAAV after induction or on the use of the baculovirus/insect cell system. Finally, another expectation of such methods is to allow for the reduction of rcAAV.

Mammalian packaging and producing systems for rAAV

Ideally, an AAV packaging system should, in a first instance, circumvent the problem of the transfection process and provide a production technology based on the use of one or several infection steps of the packaging cells containing one or several helper functions. The main approaches for stable mammalian production systems are the packaging cells and producer cells strategies (Figure 4a). The packaging cells approach is an incomplete system, which only harbour a part of all the necessary AAV genetic elements, usually the AAV helper sequences alone (indicated rep–cap in Figure 4a – packaging cell). The more evolved producer cell lines are complete trans-complementing systems, which bring all necessary AAV derived components, that is, AAV helper sequences (rep–cap) together with the vector sequence (indicated AAV vector in Figure 4a – producer cell). Induction of rAAV production from a packaging cell line is carried out by either transfection of plasmid containing the rAAV sequence (pAAV) followed by an infection with Ad. As this approach does not alleviate the problem of the scalability associated with transfection, it will not be further developed here. The alternative is to coinfect the packaging cells with a recombinant hybrid Ad Δ E1 bringing in the rAAV sequence, and wt Ad bringing the early function E1A. When using the producer cells, the production of rAAV only relies on the infection of the cells with a helper virus. If wt Ad or herpesvirus (HSV-1) is to be used to induce the production of rAAV in either of these approaches, the cells concomitantly produce the helper virus.

The choice of the mother cell line to establish the production system depends on several criteria among which its capability to efficiently assemble large quantities of rAAV. In a first glimpse, the 293 seems to be the cell line of choice. However, constitutive expression of Ad E1A gene that activates AAV p5 promoter and expression of the large Rep proteins was shown to be toxic for the cells.⁴⁸ This led to the evaluation of other cell lines, such as HeLa in many studies. In addition, it was suggested that integrated copies of rAAV vectors were more easily rescued from HeLa cells than from 293 cells¹⁴ and seem to assemble AAV particles more efficiently than 293 cells in a transfection assay.⁴⁹ Other cells such as A549 have also been used to derive packaging and producer cells for rAAV.^{50,51,63}

Packaging cell associated with a rAAV/Ad-hybrid virus. In this approach, stable packaging cells containing the AAV *rep* and *cap* genes integrated in their genome

Figure 4 The principle of packaging and producer cell lines. (a) Approaches for HeLa-based packaging and producer cells. The stable transfection of HeLa cells with a plasmid encoding the AAV *rep* and *cap* genes leads to a packaging cell line. The transfected HeLa cells can then be used for the production of rAAV by either transfecting them with an pAAVvector (not shown here) followed by the infection with an helper virus (here the Ad) or can be infected twice, first with wt Ad, and later with a rAAV/Ad-hybrid virus. These so infected cells produce in any case wt Ad and rAAV. The stable transfection of the packaging cells with a plasmid containing the rAAV vector or their infection with an rAAV vector leads to a producer cell line. The infection of the producer cell with a helper virus leads to the production of the helper virus, the rescue of the integrated rAAV, and its production. (b) Structure of a rAAV/Ad-hybrid vector. A first generation E1 deleted Ad contains the complete AAV vector sequence inserted into the E1 region. AAV ITR are indicated by the black boxes and frame the expression cassette.

are inducibly expressed upon Ad infection.⁵² The yield of rAAV is dependent upon the *rep* and *cap* copy number per cell.^{53,54} In these cell lines, the copy number of integrated *rep* and *cap* genes, under the control of their native promoters and devoid of ITRs, varies from 5 to 20 per cell.^{55–57} In addition, the *rep* and *cap* genes were shown to be rapidly amplified upon infection with wt Ad, up to about 100 copies per cell.^{58,59}

In HeLa-based packaging cells, rAAV production is achieved by using a rAAV/Ad-hybrid viral system, in which the rAAV genome is embedded in the replication-deficient Δ E1 Ad (Figure 4b). However, this system requires a prior infection with wt Ad to supply the Ad E1 function and activate the native AAV promoters.

The optimal production of rAAV vectors requires the careful optimization of several production parameters, the most important being the MOI for each wt Ad and rAAV/Ad hybrid viruses, the time of infection (TOI) for both viruses, the time interval between both viral infections, and the optimal time to harvest the virus from cell lysate and/or supernatant.

Several studies indicated that a MOI of 10 to 100 for the wt Ad led to optimal rAAV production.^{55,57} Similar range of MOI of the rAAV/Ad hybrid virus was shown to be required. Exceeding these MOI led to the reduction of rAAV production, although in various extent, or to the absence of yield improvement, depending on the studies.^{20,57} The differences observed between these studies may be explained by the fact that the packaging cell systems described, although based on HeLa cells, differed in several parameters (different *rep* and *cap* constructs inserted).

No specific study has addressed the question of the optimal TOI of the packaging cells. In most protocols, adherent cells were infected 24 h after plating.^{55,57} However, the rational guide for the choice of the TOI should be the physiological state of the cells, which have to be in their exponential growth phase. One should also pay attention to the time interval between the two viral infections. The cells must be infected first with the wt Ad and 15–24 h later with the rAAV/Ad hybrid virus^{55,57} to allow a temporally regulated programme of gene expression, with E1A protein peaking at 20–24 h leading to the induction of high levels of *rep* and *cap* expression. Other infection schedules failed to produce high amount of rAAV.⁵⁷

The optimal timing for harvesting rAAV has not been the subject of specific studies in the context of this production system. Generally, rAAV is harvested 72 h after the initial infection with wt Ad,⁵⁵ then cells are lysed and the cell lysate further processed for downstream purification of rAAV.

The most relevant parameter to give an overall estimate of the system is the final rAAV yield. Under optimised conditions, similar or higher rAAV yields can be obtained with the packaging/rAAV/Ad hybrid system (Table 1) in comparison to the classical transient production methods based on 293 cotransfection or cotransfection/Ad infection.^{55,57}

However, the packaging approach offers the main disadvantage of wt helper virus production in parallel to the rAAV. Not only to massively use the cellular resources, thereby no more available for rAAV growth, it leads to the contamination of rAAV stocks, which

Table 1 Production yields of rAAV using the transient, packaging and producer cells, HSV-1 and baculovirus based systems

Production method	Yield (vg/cell)	Scale-up	rcAAV	References
<i>Plasmid transfection</i>				
293, transfection+wt Ad5 or AdΔE1	10 ³ –10 ⁴			34,36,37 55
293, transfection only	10–10 ³		<1 rcAAV/10 ⁶ vg	37–41 37
<i>Packaging cell+rAAV/Ad hybrid</i>				
HeLa B50+wt Ad5	6 × 10 ³ –2 × 10 ⁴		<1 rcAAV/10 ⁹ vg	52–55,57–59 55
HeLa B50+Ad sub100r	3 × 10 ³ –6 × 10 ⁵			55
HeLa B50+wtAd5		100 1		66
A549 based+wt Ad5				50
<i>Cell line+Ad helper virus</i>				
HeLa based+wt Ad5	8 × 10 ³ –5 × 10 ⁴		<1 rcAAV/2 × 10 ⁹ vg	58,59,62,63,67,68 62
HeLa based+wt Ad5	10 ³ –2 × 10 ⁴		<1 rcAAV/10 ¹¹ vg	59
HeLa based+wt Ad5	10 ⁴ –10 ⁶			49
HeLa based+wt HSV-1 or rHSV	10 ⁴ –10 ⁶			64
A549 based +Ad ts	>10 ⁴	15 1		50,63
<i>HSV-1 based system</i>				
BHK cells+rHSV	10 ⁵			69,70 71
<i>Baculovirus based system</i>				
Sf9 cells+rBac	5 × 10 ⁴ 10 ⁴ –10 ⁵	20 1		80,81

Comparison of different systems and studies is often difficult due to the different methods used to measure the viral particles and the different signification given to units. Infectious units (or particles, i.u. or i.p.) may refer in some studies to transgene expressing cells (such as GFP or LacZ), or to the RCA (recombination centre assay) infectious particles, which measure the capability for a viral particle to enter the cell, traffic up the nucleus, synthesized the complementary strand and being amplified. Infectious particles finally can refer to particles capable of self-production in a cellular system (provided the helper virus is present). Transducing units can be found in many papers, but this measurement is highly dependent on the assay and its sensitivity, as well as the target cells. These methods of measurement are therefore only relevant within identical studies comparing different production systems. As a consequence, viral genome (vg) counting appears to be the most useful approach to compare different studies, as it is based on a physical measure of viral genome against a DNA standard. Here are given the main references for the diverse approaches and when available specific vector production in vg per cell.

therefore requires a highly efficient purification process. To minimise the extent of wt Ad contamination, E1-expressing replication deficient Ad were tested with varying results.⁶⁰ Among various temperature-sensitive mutants of E2b gene tested by Gao *et al.*,⁵⁵ some did not affect rAAV yields, or even led to an increased rAAV production (mutant sub100r) in comparison to a wt Ad infection.

Because HeLa is a potentially dangerous cell line due to the presence of the HPV 18 sequence,⁶¹ other cell lines have been considered to establish packaging cell for rAAV. The human alveolar carcinoma cell line A549-based K209, while containing less copies of integrated *rep* and *cap* genes than the HeLa-based B50 packaging cell line, also amplified these sequences to similar levels,⁵⁰ depicting an amplification capacity more than 20 times higher than the B50 cells. AAV vector production by staggered infection with a wt Ad and rAAV/Ad-hybrid virus led to comparable productivity. Moreover, the K209 cell line requires optimal wt Ad MOI of 1, significantly lower than for the B50 line, which is an additional advantage in terms of cost and purification requirements.

Tessier *et al.*⁵¹ tested two other *rep-cap* stable cell lines derived from other cell backgrounds (293, TE671). In contrast to HeLa and A549 based packaging cells for which a more than 100- and 2000-fold amplification of *rep* and *cap* sequences, respectively, after Ad infection was observed, 293 cells poorly amplified the *rep* and *cap* genes (two- to three-fold), whereas no amplification was

observed for the TE671 based cells, emphasizing the importance of the cellular background.

As mentioned, the use of the rAAV/Ad hybrid virus system in conjunction with packaging cells expressing *rep* and *cap* genes offers several benefits over classical transient production systems. In addition to comparable levels in cell-specific production,⁵⁷ it provides a real possibility for a scaling-up to reactor sizes. Another advantage of this production system is its versatility. A single master cell needs to be established and characterised to produce all different AAV vectors, and each desired AAV vector can be easily generated from Ad ΔE1, whose generation is a straightforward process. In addition, the formation of rcAAV appears to be reduced with such a system.^{55,57} However, some problems persist, among which the stability of the rAAV/Ad hybrid virus, and the question of the acceptability of human-based transformed cell lines (HeLa cells contain papilloma sequences)⁶¹ for the production of human medicinal products.

rAAV producer cell lines. These approaches are usually based on the above-described packaging cells into which the rAAV sequence has been integrated (Figure 4a). Production of AAV vector solely relies on helper virus infection. Like packaging cells, from which they often derived, producer cells contain the same 1–20 copies of *rep* and *cap* gene.^{50,58,62,63} Interestingly, no correlation between rAAV sequence copy number and

the level of rAAV particles produced was found, with both Ad or HSV-1 as helper.⁶⁴

Establishment of producer cells can be made either by transfection of the packaging cells with a pAAV sequence and a resistance gene (either on the same or a separate plasmid) allowing selection^{58,59} or by infection of the packaging cells with the AAV vector.^{62,63} This latter method requires a rather cumbersome manual selection process of the best clones usually by PCR screening and AAV vector production capacity induced by Ad infection. The overall cloning efficiencies appeared greatly variable between studies and generally low.^{49,62,63}

Optimization of producer cells is based on the same parameters as for the packaging cell systems. Wt Ad or replication defective Ad or HSV can be used for the induction of rAAV production. As for the packaging cells, Ad presents the advantage of being moderately pathogenic but the production peaks at about 2–3 days postinfection. Replication-deficient Ads may be used⁶³ but were found sometimes instable.⁴⁹ Protease deficient Ad (AdΔPS)⁶⁵ offered an alternative but led to reduced levels of rAAV production (our unpublished results). When HSV-1 was used as the helper, it was shown to rapidly induce rAAV production (which peaks within 24 h), leading to similar or higher yields than those obtained with Ad5.⁶⁴ In addition, the action of the HSV-1 is independent of the physiological conditions of the cells (Jenny *et al*, unpublished results). Finally, screening for various replication defective HSV-1 showed that some of them could induce rAAV assembly at a level similar to that obtained with wt HSV-1 or Ad5.⁶⁴

Optimal TOI of the producer cells with Ad in serum-containing media is optimum 24–48 h after the cell plating, the time for the cells to reach the early exponential growth phase. We observed that serum-free conditions rendered the production yields independent of the culture phase and cellular physiology at the TOI when wt Ad or HSV was used (our unpublished results).

As discussed for the packaging cells, Ad infection leads to amplification of *rep* and *cap* gene amplification,⁵⁸ as well as the integrated vector genome (more than 10⁶-fold) at Ad MOI of 10–100.⁵⁹ Low MOI (1–5) were sufficient to induce *rep* and *cap* gene amplification and rAAV assembly with wt HSV-1 or replication-defective HSV-1 strains.⁶⁴

In general, the rAAV production levels reached with these cell lines are within a range of 10³ to 10⁵ vg per cell upon wt Ad induction^{56,59,62} (for comparison, see Table 1). Similar levels were reached when wt HSV-1 (MOI = 5) was used, with one exceptionally high producer cell line being beyond 10⁶ particles per cell.⁶⁴

The single infection step allowed by the producer cell system allows for scaling up to 130 l bioreactors with the HeLa-based B50 cell line⁶⁶ and large-scale production to 15 l in serum-free medium in suspension with A549-based producer cells.⁶³

Other approaches have been taken to establish producer cell lines and should be mentioned here. An inducible HeLa-based cell line containing the AAV *rep* and *cap* genes, the AAV vector DNA and the whole Ad5 set of helper functions (E1A–E1B E2, E4, and VA RNA) under the tet-off system was established. However, although rAAV production was achieved, the cell lines were not stable and vector yields were low.⁶⁷

The same authors developed a very elaborated helper-virus-free inducible AAV 293-based producer cell line using conditional disruption of all four *rep* sequences using the Cre/Lox system.⁶⁸ Upon induction of AAV *rep* gene expression by an Ad deleted for E1A, E1B and E3 and carrying the *cre* gene, these producer cells amplified the *rep* and *cap* gene five- to ten-fold during vector production. Yields were higher than with the HeLa-based cell lines and classical transient transfection methods. In addition, the ratio of vg/transducing unit was improved compared to other methods.⁶⁸

Producer cell system based on recombinant HSV-1.

The minimal set of HSV-1 genes required to provide the auxiliary function to AAV production has been identified as the early genes UL5, UL8, UL52 and UL29.²³ These genes encode components of the HSV-1 core replication machinery – the helicase, primase and primase accessory proteins and the single-stranded DNA binding protein.

Conway *et al*⁶⁹ developed a rAAV-2 production system based on the use of a replication defective rHSV-1 (ICP27 deleted) containing AAV *rep* and *cap* genes under control of their native promoters (rHSV-1 d27.1-rc). By infecting 293 cells, which had been transiently or stably transfected with a pAAV sequence, high level of all four Rep proteins and rescue of the rAAV from proviral rAAV genome was obtained. High MOI (over 10) were less efficient, likely because of cytotoxicity of immediate early genes. The main advantage of this system is the absence of replication of the rHSV-1 in 293 cells. In addition, this system holds the possibility of a scaleable and flexible production system, conveniently allowing for the production of rAAV with various transgenes (by modifying the producer cell line) or different rAAV serotypes (by modifying the rHSV-1).

A similar approach was developed for the production of rAAV5 vectors, by using a negative rHSV-1-ΔICP27 virus harbouring the AAV5 *rep* and *cap* gene.⁷⁰ In contrast to the results obtained for rAAV2 production,⁶⁹ rAAV5 production was significantly lower than by the classic transient transfection method.⁷⁰

Booth *et al*⁷¹ recently reported an HSV-1-based rAAV production system in which all functions are supplied by a single virus (bearing the *rep–cap* genes and the rAAV sequence) or two helper viruses (one with the *rep–cap* genes and another bearing the rAAV sequence). Non-replicating (IPC27 deleted) rHSV-1 viruses were compared with replication competent viruses. The most efficient rAAV production process was that based on the use of nonreplicating HSV/AAV hybrid vectors (Table 1) but required higher MOI. Although these systems offer the advantages of simplicity and scalability, in comparison to the production methods currently used, the presence of HSV-1 derived contaminants will require special attention in the purification process.

The main advantages of producer cell lines lie in the sole need of a single infection step with a helper virus to induce the production of rAAV. Other advantages are that, for industrial GMP production, limited biological components have to be characterised (the cell line and the helper virus), adaptation to serum-free media is possible, large-scale production in bioreactors is achievable, and global yields are at least as good as with the

classical methods. However, some drawbacks remain. One is the necessity to establish a new producer cell line for each desired AAV vector. In addition, helper virus particles and components contamination of the rAAV stocks is a persistent problem, requiring highly efficient downstream purification methods. Finally, although scaling-up was achieved to some extent for these systems, mammalian cell-based systems may not be the most suitable to reach large-scale production for human application. The baculovirus-insect cell system may thus offer a good alternative.

Production based on the baculovirus expression vector system (BEVS)

The *Baculoviridae* are a family of large, enveloped DNA viruses.⁷² Baculoviruses infect preferentially arthropod with the vast majorities of permissive species falling within the order of Lepidoptera. Several continuous cell lines such as Sf9, Sf21 or High Five allowing *in vitro* baculovirus propagation are commercially available.⁷³

Recombinant baculoviruses derived from *Autographa californica* multinuclear polyhedrosis virus (AcMNPV) are the most commonly used in biotechnology,⁷⁴ in particular for the production of recombinant proteins⁷⁵ or of virus like particles (VLP ie shells devoid of viral nucleic acids).⁷⁶

The main advantages of the BEVS can be summed up as following: (i) the presence of very strong promoters (polyhedrine or p10) enable the production of high quantity of heterologous proteins without gene size limitation; (ii) the insect cells possess the ability to perform the main post-translational modifications, thereby allowing the production of biologically active proteins; and (iii) the baculovirus technology can be easily implemented, scale-up is readily achievable, cells are grown in suspension, and various serum-free media are commercially available.

Assembling viral particles is a more complex process than expressing a single protein. However, it was shown that VLP based on HBV, B19 parvovirus, rotavirus, human papillomavirus could be successfully produced⁷⁷ with the BEVS. It was further demonstrated that during VLP production, the viral structural proteins expressed by baculovirus infected cells could assemble into 3D multimeric structures.⁷⁶

Ruffing *et al*⁷⁸ described first the possibility to produce AAV-like particles. To study the specific requirements to empty capsid synthesis, the AAV2 capsid proteins were expressed separately on three different recombinant baculovirus, by mutating the internal translational initiation sites of VP2 and/or VP3. As observed for the autonomous parvovirus B19 and canine parvovirus, AAV-based VLP production was achievable with this system, but further pointed to a strict requirement in the minor capsid component VP2 for efficient formation of AAV-like particles in Sf9 cells. In addition, when VLPs were generated from three separate recombinant baculoviruses, the correct VP1:VP2:VP3 stoichiometry of 1:1:10 was not achieved.

The potential of making bioactive recombinant AAV2 using baculovirus as a gene delivery agent has been shown in human 293 cells by Sollerbrant *et al*.⁷⁶ In this approach, two recombinant baculoviruses harbouring the *rep* gene and the AAV vector were produced, and the

cap gene was inserted into a first generation Ad Δ E1. Infection of 293 cells with these two baculoviruses and Δ E1 Ad led to the production of infectious rAAV.⁷⁹ These results were the first demonstration that an rAAV sequence cloned into a baculovirus backbone could provide an efficient template for recombinant viral DNA replication and that this replicated rAAV genome could be efficiently packaged, leading to functional rAAV particles generation. The global yield obtained with this approach was equivalent to that obtained by transient transfection but adherent cells were still used limiting scale up feasibility.

More recently, BEVS has been successfully used for rAAV2 production by Kotin's group.⁸⁰ This strategy consisted of splitting the two trans-complementing functions Rep and Cap and the AAV vector in three different recombinant baculoviruses: rBac-Rep; rBac-Cap and rBac-ITR-GFP-ITR as the rBac-rAAV in this study (Figure 5a). Similar constraints as those mentioned earlier for the classical production system and packaging cell lines stand for the baculovirus system: tight regulation of the Rep proteins is required, by reducing the large Rep78/68 and overexpressing the small Rep52/40.^{42,44} The baculovirus constructions were therefore modified with respect to these constraints.

To establish Rep encoding baculovirus, the large and small *rep* *orf* were split and placed under the transcriptional control of two baculovirus promoters of different strength: the polyhedrine promoter from the AcMNPV driving the small Rep52 and the truncated promoter of the immediate-early 1 gene (Δ E1) of the *Orgyia pseudotsugata* nuclear polyhedrosis virus driving the large Rep78. Under these conditions, rep52 was highly expressed whereas the expression of Rep78 was reduced, thereby limiting the cytotoxicity of the latter. In addition, the replacement of natural *rep* promoters by insect promoters allows for the elimination of the helper virus in the production process, thereby improving the overall safety of the system.⁸⁰

The capsid proteins VP1, VP2 and VP3 in the wild-type context are under p40 promoter control and are synthesised from two differently spliced mRNA (Figure 1). In the baculovirus backbone, a native VP expression cassette containing the *cap* intron failed to produce VP proteins in insect cells.⁸⁰ To circumvent the problem, the p40 promoter was replaced by the AcMNPV polyhedrine promoter. In addition, to maintain the correct VP stoichiometry in Sf9 cells, the ATG codon of VP1 was mutated to ACG and the nine-upstream nucleotide element of the ACG initiation codon of VP2 was introduced at a similar position relative to VP1. These modifications allowed for the expression of all three VP polypeptides from a single transcript without RNA splice and in the correct ratio.⁸⁰

There is no specific modification in the AAV vector sequence cloned into the baculovirus bearing AAV vector: the AAV ITR frame the gene of interest along with the chosen regulatory sequences as in other production systems.

In this system, rAAV2 production is based on a triple infection of the Sf9 cells at their mid-exponential growth phase ($\sim 10^6$ cells/ml) with each recombinant baculovirus stocks in a 1/1/1 stoichiometry with an overall MOI of 5 (Figure 5b). At 3 days postinfection, Sf9 cells and supernatant were harvested and AAV particles were

Figure 5 The baculovirus/insect cell production system for rAAV. (a) Genetic constructions of the recombinant baculoviruses. The two-split rep orf are driven by two insect promoters, the Polyhedrin promoter (pPol) of AcMNPV and a truncated form of the immediate-early 1 gene promoter (pAIE1) of *Orgyia pseudotsugata* nuclear polyhedrosis virus. The difference of the promoter strength allows high expression of the small Rep and a reduced expression of the toxic large Rep. rBac-Cap express capsid proteins. The three proteins are directly translated from one transcript. rBac-GFP carries a rAAV-GFP genome. The presence of a CMV and p10 promoter allows GFP expression in both mammalian and insect cell. (b) AAV production is carried out by a triple infection of Sf9 cells. The three recombinant baculoviruses are used at a ratio 1/1/1 with an overall MOI of 5. At 3 days postinfection rAAV are harvested from cells and supernatant and purified.

purified. Comparison of specific rAAV yield between BEVS and transient transfection showed a nine-fold increase of rAAV production in insect cells (45×10^3 versus 5×10^3 vg per Sf9 and 293 cells, respectively) (Table 1). Moreover, the Sf9-produced rAAV-GFP transduced mouse retinal cells *in vivo* as efficiently as did 293-produced rAAV-GFP.⁸⁰

We evaluated this system for a pseudotyped rAAV2/1 production. The rBac-Cap encoding AAV1 VP proteins was not initially optimised for the ATG of VP1. AAV2/1 vector was produced as described for rAAV2 (identical cell density, baculoviruses ratio and overall MOI). In this system, the specific production of a rAAV2/1-GFP in Sf9 cells was 10^4 vg/cell (Geny *et al*, unpublished data). *In vivo* evaluation of a rAAV2/1-mSEAP (encoding a secreted form of the murine alkaline phosphatase) produced with the BEVS showed a slight decrease of the mSEAP level (about three-fold) compared to the same vector produced by transient tri-transfection in 293 cells. However, capsid composition analysis with the B1 antibody showed a reduced incorporation of VP1 in AAV capsid. Optimized rBac-Cap at the ATG of VP1 was sufficient to restore a correct ratio of VP proteins and *in vivo* performances equivalent to those of an rAAV produced by tri-transfection in 293 cells.

The possibility to produce biologically active rAAV with BEVS is the first attractive alternative to conventional methods using transient transfection of adherent

mammalian cells. Among the advantages described above, the major points to emphasise are the capacity to produce AAV without scale up limitation,⁸¹ and the complete absence of the auxiliary virus, thereby considerably improving the safety of the production system. However, this process still presents some drawbacks, among which the genetic instability of the baculovirus stocks, but more importantly the production of baculovirus concomitantly with rAAV. The development of rAAV purification processes will have to be done in parallel to face the increasing production scale and to allow the complete elimination of the recombinant baculoviruses. Finally, common to all production processes, the level of rcAAV that arise with this technology will have to be carefully evaluated.

Conclusions

Recombinant AAV production has considerably evolved over the last two decades. Although the most promising techniques for a wider application in clinics are in a reachable hand, several issues remain to be solved.

Scaling-up is now an attainable goal, both with producer lines and the baculovirus-based production systems, and similar or higher vector production levels (vg/cell) than with the transfection-based system can be obtained with these advanced production approaches (Table 1). However, the producer cell approaches still hold the question of the stability of the integrated components over the time and the real feasibility of scaling-up in a serum-free, suspension culture system. In addition, these producer cell lines still require an infectious event with Ad or HSV-1, even if replication defective. This implies the development of highly efficient, large-scale purification methods, which has been largely initiated over the last years, but also to the development of elaborated assays to ensure the absence of auxiliary virus and the establishment of stringent specifications in order to be acceptable by regulatory agencies.

The baculovirus production approach is also very promising, as scaling-up in the absence of serum has been established for the production of other biologicals. However, there are challenges to face: the baculovirus has also to be carefully eliminated in the process of purification and specifications as well as the potential toxicity associated with virus or viral protein contamination has to be carefully evaluated. An additional problem lies in the genetic instability of baculovirus, and it may be necessary to reduce the amount of baculovirus used for the production. For instance, a single baculovirus bearing all genetic elements required for rAAV production may be designed.

Finally, the general problem of rAAV stocks is their contamination with variable amounts of rcAAV, which cannot be eliminated by any purification method. It was shown that these particles arose from nonhomologous recombination between the rAAV and AAV helper (*rep* and *cap*) sequences during the process of production. However, these rcAAV could be significantly reduced using a modified helper construct expressing *rep* and *cap* genes in opposite direction.⁴⁵ Their presence will have to be carefully measured with these various approaches, and methods allowing their reduction to an acceptable level in term of safety and clinical acceptability will need to be further developed.

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