Since recombinant adeno-associated virus (rAAV) was first described as a potential mammalian cell transducing system, frequent reports purportedly solving the problems of scalable production have appeared. Yet few of these processes have enabled the development of robust and economical rAAV production. Two production platforms have emerged that have gained broad support for producing both research and clinical grade vectors. These processes differ fundamentally in several aspects. One approach is based on adherent mammalian cells and uses optimized chemical transient transfection for introducing the essential genetic components into the cells. The other approach utilizes suspension cultures of invertebrate cells. Baculovirus expression vectors are used for introducing the AAV genes into the cells. In addition, the baculovirus provides the helper functions necessary for efficient AAV DNA replication. The use of suspension cell culture provides an intrinsically more scalable platform system than using adherent cells. The upstream processes for suspension cultures are amenable for automation and are easily monitored and regulated to maintain optimum conditions that produce consistent yields of rAAV. Issues relating to developing new and improving existing rAAV production methods are discussed.

INTRODUCTION

Of the many successful tissue culture and small animal studies using recombinant adeno-associated virus (rAAV) vectors, few have advanced to clinical trials. Preclinical efficacy testing, especially in large animal models and toxicology studies, requires vector quantities not easily produced in laboratory and most research-grade vector core facilities. [Albeit, many reasons contribute to this lack of progress, reliable and economical access to vector certainly is a major factor.] Even though murine models of human genetic diseases serve a critically important role for research, predicting successful clinical outcomes from successful mouse studies remains problematic. Whether using alternative animal models may be better predictors of clinical outcomes remains to be seen. However, due in part to limited experience with human trials, many basic elements of human gene therapy are unresolved, such as determining whether the causes of immunogenesis are due to transgene expression (2) or AAV structural proteins (3,4). More clinical experience with different transgenes, AAV serotypes, routes of administration, etc. would be beneficial for improving the human applications. With so many potential disease targets, vector production concerns should not constrain the research. To address the limited availability of rAAV, several groups have established reliable methods for producing research grade and clinical grade vectors. Some of the issues involved in producing large quantities of vector are discussed in the sections that follow.

RECOMBINANT AAV PRODUCTION

Reagents for rAAV production have evolved since the first reported use of AAV as a transducing vector (1), however, the basic requirements remain unchanged. All current rAAV production methods require a common set of factors acting in trans with respect to the vector genome (vg). These components consist of the AAV non-structural proteins, the AAV structural proteins, a set of helper-virus gene products and cellular factors necessary for macromolecular syntheses, including DNA polymerase components for vg replication. Both the AAV genome and vg are linear, single-stranded DNA with interrupted terminal palindromes, usually referred to as inverted terminal repeats or ITRs. The ITRs are the
only cis element required for rAAV DNA rescue from a duplex form and subsequent replication (5–9), packaging and possibly for stabilizing the vg in the transduced cell (10). The linear, single-stranded DNA genome is unique to the Parvoviridae and, therefore, the replication pathway is unique to the Parvoviridae as well. The replication strategy is reminiscent of rolling-circle replication (RCR) systems (11), and ‘rolling hairpin’ replication was a term used to describe parvovirus DNA replication (12). Even the phylogenetically divergent RCR proteins from prokaryotes, plant viruses and DNA viruses retain structural similarities and conserved catalytic domain elements (13,14). The AAV genome has two extensive open-reading frames that code for the non-structural proteins (located on the ‘left’ half of the AAV genome) and structural or virion proteins (VPs) (located on the ‘right’ half of the AAV genome). The non-structural proteins are expressed from the rep genes consisting of one open-reading frame regulated by a 5′-terminus promoter (p5) and an internal promoter (p19). Both the p5 and p19 transcripts utilize a common splice donor and acceptor resulting in two mRNAs from each promoter. These mRNAs are translated into two p5 derived and two p19 proteins: Rep 78 and Rep 68, and Rep 52 and Rep 40. In a wild-type AAV infection, the Rep protein levels are temporally regulated transcriptionally, i.e. by promoter activity and ‘strength’, and post-transcriptionally, i.e. by splicing efficiency (15). All four Rep proteins are not necessary for rAAV production; one p5 protein and one p19 protein are sufficient. The three AAV VPs, VP1, VP2 and VP3, are encoded by the cap genes, and are expressed as a single primary transcript, two splice acceptors are differentially processed producing one transcript as a monomer, and converted into higher complexity replication intermediate forms, vector DNA replication is relatively low compared with the level attained with wild-type AAV infection (unpublished data). Whether this inefficient rescue and replication results from incomplete helper functions or gene dosage is unresolved, but rAAV yields improve with greater transfection efficiency.

METHODS FOR LARGE-SCALE PRODUCTION OF RAAV

Reported yields of recombinant AAV produced per cell ranged from ≤1 to ~1 000 000 vg containing particles depending on the production method (summarized in 32). Interestingly, the reported yields of rAAV per cell seem to converge to a 104 to 105 rAAV particles produced per cell constitue a consensus value for production and processing, apparently independent of production platform. Several factors, both trivial and meaningful, contribute to the particle per cell value. These include: (i) quantitative analysis; (ii) cis-acting transgene effecting either replication or packaging; (iii) trans-acting effects due to expression of the vg in producer cells; (iv) up-stream processing effects, e.g. transfection efficiency; (v) down-stream processing, viz rAAV recovery from the biomass.

TRANSFECTION

Scaling up rAAV production basically requires increasing the cell number in a manner compatible with the upstream production process. For chemical transfection-based processes, adherent cells are typically grown as monolayers in plastic cell culture plates or roller bottles. The available surface area determines the maximum number of cells and, therefore, the amount of rAAV produced. CellSTACK® (Corning Corp.) and Nunc Cell Factories (Thermo Scientific) represent products for large-scale adherent cell cultivation. The 100 layered CellCube® unit (85 000 cm2) has the potential
for growing \( \geq 10^{10} \) cells that have the potential of yielding \( 10^{14} \text{–} 10^{15} \) rAAV particles.

Alternatively, suspension cultures allow cell expansion based on volume rather than area. The conversion factor for adherent cell number to suspension cell number is \( \approx 10 \text{–} 50 \text{ cm}^2 = 1 \text{ cm}^3 \), depending on cell density for either format. Transfection methods for producing rAAV using either inorganic compounds, e.g. calcium phosphate, or organic compounds, e.g. polyethyleneimine (PEI), or non-chemical, e.g. electroporation, have been extensively described using adherent cells (33–35). However, for suspension cells, transfection using either calcium phosphate (36,37) or PEI (37–40) are most represented. The use of PEI appears more advanced, presumably due to the reproducibility and reliability of using a commercially available, single reagent rather than the more complicated (and idiosyncratic) calcium phosphate transfection procedure (39). PEI-mediated transfection of suspension culture-adapted 293 cells in 1 l produced rAAV equivalent to transfecting fifty 50 cm diameter plates of adherent cells. Assuming that the cell densities were \( 1 \times 10^5 \) per cm\(^2\) for adherent cells and \( 2 \times 10^5 \) per cm\(^3\) for suspension cells (which probably increased during the production time), then the specific yield was about five times greater for the suspension culture cells than for the adherent cells. A similar increased specific yield of rAAV production compared with adherent cells was reported using calcium phosphate transfection of 293 cells. However, the suspension cultures were smaller and issues concerning scalability were not addressed (36,37).

RECOMBINANT AAV PRODUCTION IN SF9 CELLS

To an extent, baculovirus expression vectors for producing rAAV in Spodoptera frugiperda (SF9) cells recapitulate the wild-type AAV infection. First, the recombinant baculovirus initiates a productive infection and subsequently the progeny baculovirus can secondarily infect additional cells in the culture. With \( \approx 100 \) infectious baculovirus particles released per cell, the entire cell culture population becomes infected within one or two infection cycles depending on the initial multiplicity of infection. Secondly, analysis of the low molecular weight DNA indicates that the lepidopteran SF9 host cells replicate the vector DNA much more efficiently than HEK 293 cells (41). Thirdly, the baculovirus provides the helper functions necessary and sufficient for rAAV genome replication (unpublished data).

The physical, biochemical and biological characteristics of SF9 cell-produced rAAV indicate that rAAV produced in SF9 cells is equivalent to rAAV produced in HEK 293 cells (41–43). Analysis of vector-derived genomes demonstrated that up to 4.7 kb of linear, single-stranded DNA is efficiently packaged (42).

Volumetric scalability is a key benefit of using the baculovirus–insect cell system: the process is linearly scalable from 0.02 to \( \approx 200 \) l (submitted for publication). The limitations of the baculovirus expression vectors–insect cell system for rAAV production results from baculovirus genetic and physical instability. The genetic instability limited the expansion to less than six passages and the physically unstable particles require regular re-derivation from an early passage master virus bank (44,45). Reconfigured baculovirus expression vectors resulted in increased genetic stability for at least six passages (46). In addition, the physical instability has been resolved by adapting the titerless-infected-cells preservation and scale-up (TIPS) (47,48). Cryopreserved in liquid nitrogen, the baculovirus-infected insect cells (BIIC) retain viability without diminished infectivity for long periods of time. The BIIC are then aliquoted for single use and provide a solution for the physical stability concerns.

DOWN-STREAM PROCESSING

Reduced to the simplest terms, recovery of rAAV from the biomass involves liberating rAAV from cells, separating rAAV from other cell and media components and concentrating the product. Fortunately, the AAV capsids are very resilient and physically robust; therefore, downstream processes may exploit conditions that are often avoided, such as prolonged exposure to elevated temperatures, repeated freezing and thawing cycles, acidic conditions and exposure to organic solvents.

The initial steps in downstream processing are determined by the cell culture format. Adherent cells may be lysed in situ or detached from the growth substrates and lysed in a small, disposable vessel by freeze-thaw lysis, mechanical homogenization or chemically via the use of surfactants. Large volume suspension cultures may be treated with surfactants, e.g. Triton X-100, or homogenized with a mechanical device. Nuclease treatment may be incorporated following lysis to reduce viscosity and facilitate subsequent filtration and chromatography steps.

Following cell lysis, the insoluble cell components are removed either by filtration (convenient for large volumes) or centrifugation. For small-scale production, polyethylene glycol may be used to efficiently precipitate rAAV from the clarified cell lysate. Following low-speed centrifugation, the pellet containing the rAAV may be resuspended in buffer and further concentrated and purified by density gradient centrifugation, e.g. cesium chloride isopycnic gradients or iodixanol step gradients. For large volume cultures, a series of filters with decreasing pore sizes are used to prevent clogging and improve rAAV recovery. Tangential flow filtration (TFF) is a convenient technique for concentrating rAAV, and also enables buffer exchange.

Many strategies have been published describing chromatographic purification of rAAV. The capsids are recoverable from the clarified cell lysate by ion exchange chromatography; both cation and anion exchange media have been described (49–56). A recently developed immunoaffinity chromatography medium produced with a recombinant single-chain antibody binds several AAV capsid serotypes, including AAV1, AAV2, AAV6 and AAV8 (46). The antibody appears to bind capsids with high specificity and affinity. ‘Polishing’ via size-exclusion chromatography produces a near-homogenous final product. Following TFF concentration and sterile filtration, rAAV produced by these methods can be used in pre-clinical studies and, with cGMP compliant practices, in clinical studies.
DISCUSSION

Baculovirus expression vectors in combination with Sf9 cells system provide an alternative approach to using mammalian cells for rAAV production. Sf9 cells are easily grown in serum-free or animal-derived component-free media and are amenable to upstream and downstream processing at any scale. The AAV rep and cap genes, vg and helper virus functions are introduced efficiently into Sf9 cells by baculovirus infection. Originally developed using three baculoviruses (41), more recent improvements resulted in consolidating the rep and cap genes into one baculovirus (57). Thus, substantially increasing the probability that a single Sf9 cell is co-infected with two of the required baculoviruses.

In contrast to chemical transfections, the baculoviruses contribute an essential biological component to rAAV production both as an efficient method for delivering the genetic elements required for rAAV production to nearly all of the cells used in the bioreactor and as an AAV helper virus for vector DNA replication.

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Conflict of Interest statement. Portions of the technology described in this report are covered by United States and European patents assigned to the Secretary of the Department of Health and Human Services. A fraction of the licensing fees and royalty payments made to the NIH are distributed to the inventors in accordance with US Government and NIH policy.

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