Advantages of homologous recombination- over transposition-based systems to generate recombinant baculovirus for AAV vector production

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Abstract

Viral vectors have a great potential for gene delivery, but manufacturing at pharmaceutical scale is a big challenge for the industry. The baculovirus-insect cells system is one of the most scalable platforms to produce clinical grade recombinant Adeno-Associated Virus (rAAV) vectors, however, the standard procedure to generate recombinant baculovirus based on Tn7 transposition is time consuming and still suffers technical constraints. Indeed, we recently shown that baculoviral sequences adjacent to the AAV ITRs are preferentially encapsidated into the rAAV vector particles. This observation raised concern about safety for clinical applications due to the presence of bacterial and antibiotic resistance coding sequences with Tn7-mediated system for the construction of recombinant baculoviruses. Here, we investigated a faster and safer method to generate baculovirus reagents based on homologous recombination (HR) for its use in rAAV manufacturing compared to the Tn7-based system. First, we confirmed the functionality of inserted cassette and the absence of undesirable genes into HR-derived baculoviral genomes. Strikingly, we found that the exogenous cassette shown an increased stability over passages when using HR system. Finally, we tested these materials to produce rAAV vectors. The baculoviruses originated from either system lead to high rAAV vector genome yields, with the advantage for the HR method being that the rAAV lots are exempted of undesirable gentamycin and kanamycin genes derived sequences which provides an additional level of safety for the manufacturing of rAAV vectors. Overall, this study highlights the importance of the upstream process and starting biologic materials to generate safer rAAV biotherapeutic products.

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ABSTRACT

Viral vectors have a great potential for gene delivery, but manufacturing at pharmaceutical scale is a big challenge for the industry. The baculovirus-insect cells system is one of the most scalable platforms to produce clinical grade recombinant Adeno-Associated Virus (rAAV) vectors, however, the standard procedure to generate recombinant baculovirus based on Tn7 transposition is time consuming and still suffers technical constraints. Indeed, we recently shown that baculoviral sequences adjacent to the AAV ITRs are preferentially encapsidated into the rAAV vector particles. This observation raised concern about safety for clinical applications due to the presence of bacterial and antibiotic resistance coding sequences with Tn7-mediated system for the construction of recombinant baculoviruses. Here, we investigated a faster and safer method to generate baculovirus reagents based on homologous recombination (HR) for its use in rAAV manufacturing compared to the Tn7-based system. First, we confirmed the functionality of inserted cassette and the absence of undesirable genes into HR-derived baculoviral genomes. Strikingly, we found that the exogenous cassette shown an increased stability over passages when using HR system. Finally, we tested these materials to produce rAAV vectors. The baculoviruses originated from either system lead to high rAAV vector genome yields, with the advantage for the HR method being that the rAAV lots are exempted of undesirable gentamycin and kanamycin genes derived sequences which provides an additional level of safety for the manufacturing of rAAV vectors. Overall, this study highlights the importance of the upstream process and starting biologic materials to generate safer rAAV biotherapeutic products.

keywords: Baculovirus Expression Vector, Tn7 Transposition, Homologous recombination, rAAV vectors, Manufacturing, Gene Therapy.

INTRODUCTION

Gene therapy gives rise to hopes for a large spectrum of genetic diseases, that are mainly untreatable using conventional pharmacology. Over the past decade, recombinant Adeno-Associated viruses (rAAV) have been the most studied viral vectors and were successfully used in clinical trials for *in-vivo* gene transfer. In the case of Duchenne Muscular Dystrophy (DMD) which is the most common lethal muscle genetic disease, animal-based studies suggest that a whole-body treatment to achieve efficient systemic gene delivery would require infusion of high doses of rAAV vector within the range of 10^{14} vector genomes/kg (Guiner et al., 2013; Le Guiner et al., 2017). Although several strategies have been developed to optimize vector transduction in targeted muscle cells, the large-scale production of high-quality rAAV vectors is still a limiting step to move forward for clinical trials into large patient population.

Most commonly, rAAVs are produced in HEK293 adherent mammalian cells by co-transfection of two or three plasmids containing (i) the AAV genes, (ii) the essential adenoviral helper genes that are supplemented in*trans* , and (iii) the viral genome with a maximal size of 4.7kb which is framed by inverted terminal repeats (ITRs) required for genome replication and encapsidation (Galibert and Merten, 2011). This method has been used worldwide, including the manufacturing of AAV2 and AAV8 reference-standard materials (RSM) to be exploited by the scientific community and regulatory agencies (Lock et al., 2010; Ayuso et al., 2014) in order to provide common quantification methods for the particle and genome. However, the expansion and transfection of adherent cells may limit manufacturing of rAAV biotherapeutic products. To scale-up the procedure, suspension systems have been developed and optimized during the last decade, using suspension mammalian cells and serum-free medium (Grieger et al., 2016) or the insect cells/baculovirus system (Cecchini et al., 2011; Kotin and Snyder, 2017).

The baculovirus expression vector (BEV) platform has become an established manufacturing platform for the

production of viral vaccines and gene therapy vectors and it offers many advantages over plasmid transfection of mammalian adherent cells such as manufacturing speed, cost efficient and scalability. Of note, the insect cell-based system has shown to be adapted and efficient for industrial application with examples in the flu vaccine industry (Cox and Hashimoto, 2011; Milian and Kamen, 2015) using bioreactors of 20,000 liters (Flublock, Protein Sciences).

The standard method to generate a recombinant baculovirus is based on site-specific Tn7 transposition of an exogenous DNA cassette carrying the gene of interest (GOI) from a plasmid donor to the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) baculovirus DNA called "bacmid" (Kubo and Craig, 1990; Luckow et al., 1993). Recombinant baculoviruses are produced in Sf9 insect cell line (*Spodoptera frugiperda*) which has been adopted as workhorse in many laboratories for baculoviral vectors amplification because of its ability to combine both a high production yield of infectious particles, and growth in suspension at 27degC-31degC without CO_2 in serum-free media, an advantage for large-scale cGMP manufacturing. Originally, BEV system has been adapted for rAAV production by co-infecting insect cells with three recombinant baculoviruses (*rep, cap* and GOI) where AAV promoters were replaced by baculovirus ones to allow expression of AAV genes into insect cells (Urabe et al., 2002). Later, R. Kotin and collaborators developed a simplified dual baculoviruses system by combining the *rep* and*cap* helper functions into one unique construct (Smith et al., 2009).

The baculovirus/Sf9 platform has proven its efficiency for the manufacturing of rAAV viral vectors in particular through the market authorization of Glybera, a gene therapy product indicated for the treatment of Lipoprotein Lipase Deficiency. However, the regulatory agencies requested to the company uniQure for residual baculovirus DNA assessment due to a potential side effects (Bryant et al., 2013). For this purpose, we have developed a protocol based on high-throughput sequencing to identify and quantify residual DNAs, called the*single-stranded virus sequencing* (SSV-Seq), and we demonstrated that baculoviral DNA is the major source of DNA contamination in the final rAAV product with up to 2.1% of total NGS reads (Penaud-Budloo et al., 2017). Worryingly, the gentamycin resistance gene coding sequence, that has been used to select recombinant baculovirus after Tn7 transposition, was detected by qPCR in research-grade rAAV lots. Finally, it has also been reported in the literature that BEV genome derived from the Tn7-bacmid system is subjected to instability and spontaneously deletion, leading to the generation of defective interfering viruses (DIs) which accumulate at the expense of the intact ones and limiting scale-up of batch production (Pijlman et al., 2004, 2003). Altogether, these technical concerns prompted us to reconsider other options to overcome these barriers and to define new standards to produce baculoviral reagents for rAAV vector manufacturing.

MATERIALS AND METHODS

Plasmid constructions

The donor plasmid pFastBac1 (pFB) was supplied in a commercial kit with the Tn7-bacmid DNA (Bac-to-Bac(r) system) (ThermoFisher Scientific, MA). It contains a selective gene coding for ampicillin (AmpR) and the two Tn7 sites left (L) and right (R) that are surrounding a polylinker to clone the gene of interest and another selective gene coding for gentamicin (GmR). We have generated a first construct containing the cytomegalovirus (CMV) promoter, the enhanced green fluorescent protein (eGFP) reporter gene and a human β-globin (HBB) polyadenylation signal, all flanked by two flop-oriented ITRs of AAV serotype 2 derived from the pSub-201 plasmid (Samulski, 1987) (**Supplementary Fig. 1**). The ITR upstream of the CMV promoter lacks 15 bp in the external A region, and the ITR downstream of the polyA is truncated by 17 bp with respect of the wild-type ITR2. The second construct was kindly provided by R.M. Kotin (NIH, Bethesda, USA) and carries the coding genes "Rep2" and "Cap8" that follows the design described by Smith and colleagues allowing production of rAAV vectors using a dual baculovirus system (Smith et al., 2009). We additionally generated Anc80 construct changing the ATG start codon of Anc80 capsid orf in ACG and the CAG start codon of the putative assembly-activating protein (AAP) in CTG as described in the patent number WO2019067982A3. The optimized Anc80 orf was cloned between the p10 baculovirus promoter and the herpes simplex virus (HSV) thymidine kinase (tk) polyadenylation signal sequence in the donor plasmid allowing the expression of AAV2 Rep78 and Rep52 proteins under the control of the polyhedrin promoter

and followed by the simian virus SV40 polyadenylation signal sequence. The related baculovirus constructs were also generated with the donor plasmid pBac-1 supplied in a commercial kit with the HR-bacmid DNA (BacMagic-2?, Merck or flashBAC GOLD?, Oxford Expression Technologies), containing a selective gene coding for ampicillin (AmpR) and a polylinker to clone the gene of interest which is surrounded by the two wild-type homologous baculoviral sequences *lef2/orf603* and *orf1629* . The BacMagic-2 and flashBAC GOLD kits contain *chi-a* and *v-cath* deleted parental bacmid. The pBac-1 is linearized by enzymatic digestion with the "PmeI/AclI" or "PmeI/DrdI" restriction endonucleases before the transfection in Sf9 cells. All these plasmids were validated by Sanger sequencing and subsequently used for the generation of the recombinant baculoviruses.

SF9 insect cells culture

Spodoptera frugiperda Sf9 insect cells (ThermoFisher Scientific) were grown at 27°C in Sf-900 III SFM in 100 to 250 ml glass spinner flask (ThermoFisher Scientific).

Generation of recombinant baculovirus

The recombinant baculoviruses BEV rep2/cap8, BEV rep2/capAnc80 and BEV ITR-GFP were generated using either the kit Bac-to-Bac® (ThermoFisher Scientific, MA) for the Tn7 system or the BacMagic-2? (Merck Millipore) or flashBAC GOLD? (Oxford Expression Technologies, Oxford, England) kit for the HR-based system.

Tn7 site-specific transposition of the cassette from donor plasmid pFastBac1 (pFB) to the bacmid backbone derived from bMON14272 was performed by transformation of 10 ng of the donor plasmid in *Escherichia coli* DH10Bac competent bacteria in accordance with the instructions of the supplier (Bac-to-Bac[(r)] user guide, ThermoFisher Scientific, MA). The recombinant bacmid was validated for the presence of the cassette by PCR using the set of primers M13-pUC-F 5'- CCAGTCACGACGTTGTAAAACG and M13-pUC-R 5'- AGCGGATAACAATTTCACACAGG from either side of the insert and the set of primers M13-pUC-F and BAC-G 5'- AGCCACCTACTCCCAACATC targeting the selective gene sequence of the cassette, and then confirmed by Sanger sequencing. One microgram of Tn7-bacmid DNA was then transfected in 1ml of Sf9 insect cells at a density of $1x10^6$ cells/ml cultivated in 6-wells plate, using 9μl of Cellfectin-II reagent (Thermo Fisher Scientific, MA). The supernatant (P0) was harvested at 96 hours post-transfection. Baculoviral clones are isolated from the P0 stock by plaque assay and five of them are amplified in T25 flask (P1) before to perform up to ten serial passages in order to validate the genetic stability of the cassette. When meeting specifications, i.e. sequence identity and genetic stability, a unique clone is selected, and a larger stock is then generated by amplification in Sf9 insect cells seeded in spinner-flask (P2).

Homologous recombination occurred by transfecting 500ng of linearized donor plasmid derived from the pBac-1 and 100ng of the HR-bacmid in 1ml of Sf9 insect cells at a density of $1x10^6$ cells/ml cultivated in 6-wells plate and using 5μl of Cellfectin-II reagent in accordance with the instructions of the supplier (BacMagic?-2 user guide, Merck Millipore, Billerica, MA). As previously described, the supernatant (P0) was recovered 96 hours post-transfection and five clones were then amplified and characterized equally up to P2 stock.

Recombinant baculovirus isolation by plaque assay

Isolation of recombinant baculovirus by viral plaque assay was performed by seeding 6-wells plate with 1ml of cell suspension at concentration equal to $1x10^6$ viable Sf9 cells per ml. After 30 min at 27°C, attached cells were infected with serial dilutions of baculovirus. An initial dilution was realized from 50μl of the baculovirus stock in 500μl of Sf-900 III serum-free medium and sequential 1:3 dilutions were done in 48-wells plate adding 250μl of the initial dilution in 500μl of medium. Typically, to be able to quantify the lysis plaque for a baculovirus stock of $>1x10^7$ plaque-forming units (pfu) per ml, 200μl of the dilutions $7.2x10^{-4}$, $2.2x10^{-5}$ and $6.5x10^{-5}$ were added directly into each well, in duplicate. The plate was incubated for 3h at 27°C in a humidified chamber. To prepare plaquing overlay, 4ml of 4% agarose gel (ThermoFisher Scientific, MA) that was preheated at 70°C was mixed with 12ml de SF900 medium 1.3X (ThermoFisher Scientific, MA), the

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inoculum was removed from each well from high to low dilution and replace with 2ml of the diluted agarose. After gel solidification, the plate was carefully placed in a humidified chamber and incubated between 9 to 11 days at 27°C. Isolated clones are picked-up from the solid medium and individually amplified and finally characterized (P1).

Recombinant baculovirus titration by Cell Size Assay (CSA)

In this work, we exploited an easy and fast method for baculovirus titration based on cell size Assay (CSA) which was reported in literature (Janakiraman et al., 2006). This method is based on the measurement of infected viable cells diameter. Briefly, dilutions were prepared in a 48-well plate as following: 48µl of the virus stock was added to 252µl of Sf-900 III SFM and seven additional 1:2 serial dilutions were realized adding 150µl of the previous dilution to 150µl of medium. A volume of 875µl of Sf9 cell suspension at $1.15x10^6$ cells/ml were dispensed into each well of a 24-wells low-attachment plate and completed with 125µl of each dilution. An uninfected control and a concentrated control were also prepared by adding 125µl of medium or 125µl of the undiluted virus stock, respectively. The plate was immediately placed at 27°C under shaking at 175 rpm for 17 to 20h. After incubation, the cell diameter was measured for each dilution using a Vi-CELL counter (Beckman Coulter, CA).

Characterization of recombinant baculovirus

The identity of the baculoviral genomes was verified by Sanger sequencing from PCR products of DNA extracts. The copy numbers of the AAV genome and RepCap cassette were determined after DNA extraction using a High Pure viral nucleic acid kit (Roche Life Science) followed by a qPCR using the sets of primers and probes described previously (Penaud-Budloo et al., 2017).

For the stability study of the baculovirus clones over passages, 2ml of Sf9 cells were seeded per well at a density of $1x10^6$ cells per ml in a 6-wells plate. For each clone, cells were infected with the baculovirus supernatant of either of the passages P1 to P10 at multiplicity of infection (MOI) of 1 infectious unit as determined by CSA assay and incubated for 72 hours at 27°C in a humidified chamber. After low speed centrifugation 5 min at 500 x g, the cells pellet was recovered at each passage in order to verify AAV Rep and Cap proteins expression by SDS-PAGE and western blotting. Total proteins were extracted in RIPA buffer supplemented with 1X protease inhibitor cocktail (Sigma-Aldrich, MO) and protein concentration was determined using the kit DC protein assay (Bio-Rad, CA). Five micrograms of total proteins were loaded on a 10% Tris-Glycine mini-gel (ThermoFisher Scientific, MA) and run at 1ml/cell, 100V for 2.5 hours. Proteins were transferred onto a PVDF membrane (ThermoFisher Scientific, MA) for 7 min at 25V, 1.3 A constant, using a Trans-Blot Turbo Transfer System (Bio-Rad, CA). Membranes were incubated overnight at 4°C in blocking buffer (1X PBS, 0.1% Tween 20, 5% milk powder) and 1h at room temperature in the primary antibody solution (1X PBS, 0.1% Tween 20, 0.5% milk powder, primary antibody). The Rep303.9 antibody or the anti-AAV VP1/V2/VP3 B1 antibody (Progen, Heidelberg, Germany) are used at a dilution 1:20 or 1:2,000 for the detection of Rep and Cap proteins, respectively. Membranes were washed three times for 10 min in the washing buffer (1X PBS, 0.1% Tween 20) and incubated for 1h at room temperature with the secondary antibody HRP-linked goat anti-mouse diluted at 1:2,000 (Agilent Dako, CA). After three additional washes for 10 min, the chemiluminescent signal was revealed using the Western Pierce ECL substrate (ThermoFisher Scientific, MA) and exposure with Amersham Hyperfilm ECL (Cytiva GE Life Sciences, MA).

rAAV production and purification

Sf9 cells were infected at a density of 10^6 cells/ml with the combination of BEV rep2/cap8 or BEV rep2/capAnc80 and BEV ITR-GFP at MOI 1 IU per baculovirus. Four days after infection, cells were lysed by the addition of 0.5% Triton X-100 (Sigma-Aldrich, MO) for 2.5 hours at 27°C with agitation. The crude bulk was clarified by centrifugation for 15 min at 500·g and 20°C, the supernatant is then filtrated through a polyethersulfone (PES) membrane with 0.2 µm (Merck Millipore, Billerica, MA). rAAV vectors were purified by immune affinity chromatography with a single POROS CaptureSelect AAV8 column (ThermoFisher Scientific, MA) and formulated in Dulbecco's phosphate buffered saline (Lonza, Verviers, Belgium)

containing 0.001% Poloxamer 188 (Merck KGaA, Darmstadt, Germany).

rAAV characterization

For real-time PCR analyses, 3µl of each purified rAAV stock was pretreated or not with 20U of DNase I (Roche, Basel, Switzerland) before DNA extraction in a total volume of 200µl of DNase reaction buffer (13 mM Tris pH 7.5, 0.12 mM $CaCl_2$, 5 mM $MgCl_2$) for 45 min at 37°C. The vector genome (vg) copy number was determined after DNA extraction, using a High Pure viral nucleic acid kit (Roche Life Science). Baculoviral DNA contamination was quantified by qPCR assays targeting resistance genes kanamycin ("kana") using the set of primers Kana-F 5'- GGGCGCCCGGTTCTTTTTGTC, Kana-R 5'- GCCAGTCCCTTC-CCGCTTCAGTG and Kana-Pr 5'- CCGACCTGTCCGGTGCCCTG and the gentamycin ("genta") using the set of primers Genta-F 5'- AGCCCGCATGGATTTGAC, Genta-R 5'- GGGCATCATTCGCACATGTA and Genta-Pr 5'- TGGTCAGGGCCGAGC. The copy numbers of the rep-cap sequences and the Sf9 genome were determined by quantification of the rep2 gene and Sf ribosomal protein L37A gene amplicons, respectively, as previously reported (Penaud-Budloo et al., 2017).

For rAAV vector characterization, SDS-PAGE was performed from 2.5 to $5x10^9$ vector genomes and western blotting was realized as described above for the detection of AAV Cap proteins. Vector purity was also evaluated by SDS-PAGE followed by silver staining (PlusOne silver stain kit; GE Healthcare Life Sciences) of 2.10^{10} vector genomes of each rAAV stock.

Quantifications and statistical analyses

Statistical analysis was applied in presence of at least n = 3 biologic replicates, and only descriptive statistics are reported. Data were expressed as mean ± SD, where one-tailed nonparametric Mann-Whitney test was performed to compare two independent groups. In all the analyses, the significance threshold was set at 0.05. Differences were considered statistically significant at *$p<0.05$. Analyses were performed using GraphPad Prism v8.4.1 (GraphPad Software, San Diego, CA).

RESULTS

HR- and Tn7-mediated systems operate with an artificial bacterial chromosome (BAC) integrated in baculoviral DNA

The generation of BEVs by Tn7 transposition in bacteria is a well described method in the literature and is commercialized as Bac-to-Bac[?] system (Bac-to-Bac, 2015, ThermoFisher Scientific). The circular baculoviral DNA derived from the *Autographa californica* nucleopolyhedrovirus (AcMNPV) has been engineered to insert a bacterial artificial chromosome (BAC) into the *polyhedrin* locus (Smith et al., 1983) that contains the kanamycin selective gene (KnR), the mini-F bacterial replicon and a mini-attTn7 site inserted into the LacZα region (Luckow et al., 1993) (**Fig. 1a**). This shuttle vector named "bacmid" can easily be modified and amplified using conventional *E. coli* bacterial transformation in which a helper plasmid (pMON7124) provides the functions required *in trans* for Tn7 transposition. The bacmid with DNA insert is selected under gentamycin (GmR) antibiotic pressure (**Fig. 1b**). More recently, an alternative system to Tn7 transposition has emerged (Possee et al., 2008; Zhao, 2003) based on homologous recombination (HR) mechanism and inspired from a previous study (Kitts and Possee, 1993). The bacmid used for the generation of BEV by HR has been commercialized as BacMagic[?] (Merck Millipore, Novagen) or flashBAC[?] systems (Oxford Expression Technologies). In these systems, HR system does not require the use of bacterial step to perform gene cloning into baculovirus backbone, which is laborious and time-consuming. Nonetheless, the parental bacmid contains a chloramphenicol resistance (CmR) gene to allow its amplification and selection in bacteria. By directly co-transfecting insect cell line with the defective parental bacmid DNA (*orf1629⁻*) and a donor plasmid containing the gene of interest (GOI) flanked by homologous sequences *lef2* and non-deleted *orf1629* , HR events induce bacmid knock-out (KO) of the bacterial replicon from the targeted *polyhedrin* locus and knock-in (KI) of the GOI that simultaneously restores the *orf1629* entire sequence allowing BEV genome replication and the generation of viable recombinant baculoviruses progeny named P0 seed stock (**Fig. 1b**).

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Whatever the system used to generate the P0, Tn7 or HR, one round of viral plaque isolation is required to eliminate DIs, then to amplify and characterize few P1 clones. In this work, five P1 clones have been selected per construct. Finally, one clone that meets the required specifications, i.e. sequence identity and genetic stability, will be selected and used to generate the master viral seed P2 and P3 (**Fig. 1b**). Importantly, commercial sources for the HR system also offer bacmid versions that are deleted in some "accessory" genes. In this study, HR-derived BEV devoid of chitinase (AcORF-126, *chiA*) and cathepsin (AcORF-127, *v-cath*) genes were used. The two encoded proteases favor postmortem liquefaction of the larvae and release of occlusion derived viruses in the environment (Hawtin et al., 1997) but are non-essential for baculovirus replication *in vitro* (**Fig. 1a**). Several AAV capsids, including 1, 3, 6, 7, 8 and rh10, have been shown to be susceptible to the baculovirus cathepsin (Galibert et al., 2018). In particular, the subsequent VP1/VP2 cleavage of serotype 8 capsid has been documented to impair *in-vivo* infectivity. The sequences of both capsid variants, AAV8 and AAVanc80, used in this study contain the major predicted cathepsin cleavage site.

HR system is an easy, fast and safe system to generate recombinant baculovirus

In order to evaluate the HR system for the generation of recombinant baculoviruses intended for rAAV vector production, we first transfected Sf9 insect cells with the donor plasmid pBac. The plasmid pBac-rep/cap carries the AAV2 *rep* and AAV8 *cap* ORFs that were optimized for the expression in Sf9 cells. This plasmid was digested (linear) or not (circular) with restriction enzymes on both sides of the GOI. Indeed, we hypothesized that a linearized donor plasmid would be more prone to provide efficient homologous recombination as suggested in previous genome editing report (Song and Stieger, 2017). We collected and amplified five individual clones per condition to be further characterized by assessing the number of infectious particles. The circular pBac leads to heterogenous clones with low infectivity (<5.0 x 10^8 IU/ml) (**Fig. 2a**). Inversely the linear donor plasmid produced clones with high infectivity with a mean of 9.5 ± 1.1 x 10^8 infectious unit (IU)/ml. Interestingly, chloramphenicol coding sequence was still detectable by PCR amplification in the cleared supernatant for the circular plasmid meaning that the parental bacmid contaminates the P1 baculovirus stock, contrary to the linear plasmid condition (data not shown). Next, the genetic stability of the recombinant baculovirus was analyzed for each clone by two qPCR assays targeting the AAV Rep sequence (insert) and the baculovirus DNA polymerase gene (baculovirus backbone). The results are represented as a ratio "bac/rep" (**Fig. 2b**). The ratio insert/backbone was close to 1 in all clones produced with the linear plasmid, confirming that all five baculoviral clones carried the exogenous cassette "rep2/cap8". We also evaluated the functionality of the cassette "rep2/cap8" to express AAV proteins in Sf9 cells by Western blot analysis for each P1 clone. The expression of Rep78, Rep52, and VP1, VP2, VP3 proteins was detected only for baculoviruses derived from the linear pBac plasmid (**Fig. 2c**) and was stable over 10 serial passages. Indeed, no signal was detected for clones derived from circular pBac plasmid confirming the absence of the expression casette in the baculovirus population. Altogether, these observations suggested that is preferable to use linear donor template to favor HR efficiency, and drastically reduce the proportion of DI baculoviruses.

HR system allows better genetic stability of GOI over serial passages

Next, we decided to investigate the stability of recombinant baculovirus generated by HR and Tn7 systems. A baculovirus vector carrying a GFP reporter gene flanked by the AAV2 ITRs was produced either using the HR- or the Tn7-based method. Two types of baculovirus "rep/cap" were generated, one allowing the expression of AAV8 capsid and the other one the expression of AAVanc80 capsid variant. Of note, AAVanc80 has been recently identified as an *in silico* reconstructed ancestral AAV capsid with high transduction efficiency *in-vivo* (Zinn et al., 2015). We selected five clones per construct, and we performed side by side comparison of the baculovirus stability over 10 serials passages in Sf9 cells. To that purpose, the copy number of AAV ITR (itr) or Rep sequence (rep), and the copy number of the baculovirus DNA polymerase gene (bac) was determined by qPCR at passages P1, P4, P7 and P10. HR system showed extended genomic stability of the insert with a consistent ratio bac/itr or bac/rep up to ten passages (**Fig. 3a − left**). Whatever the insert, the baculoviruses generated with Tn7 system are more heterogeneous between clones and has the tendency to loose the insert after 7 passages (**Fig. 3a - right**). Finally, the expression of the Rep and Cap proteins required for the rAAV vector production was assessed by Western blotting. Positive signals

for VP1, VP2, VP3 proteins were observed up to passage P10 for each HR-derived clone (**Fig. 3b - top**), while a drastic drop in capsid protein expression was observed from passage P8 for the Tn7-derived clones (**Fig. 3b - bottom**). Altogether, these observations suggest that HR system robustly prevents the loss of inserted cassettes upon passages, which is a major advantage for large-scale manufacturing of recombinant baculovirus, not only for rAAV production but also for vaccine or recombinant protein production.

Baculoviruses derived from HR or Tn7 systems allow the generation of recombinant AAV viral vectors at similar production yields

We performed rAAV production using the co-infection strategy described by Smith and collaborators with the recombinant baculoviruses derived either from HR or Tn7 system (Smith et al., 2009). To this end, Sf9 cells were infected with a BEV ITR-GFP and a BEV rep/cap at a multiplicity of infection (MOI) of 1 infectious unit each, as determined by CSA assay. Insect cells were harvested 96 hours post-infection to maximize production of rAAV using low MOI (Cecchini et al., 2011). We first evaluated rAAV production yields by qPCR both at harvest in the cleared lysate. Interestingly, baculovirus vectors derived from HR and Tn7 systems yielded similar levels of rAAV particles whatever the capsid variant (**Fig. 4a**). The HR-derived BEV seems to lead to slightly more empty particles, even if statistically not significant (**Fig. 4b**). This may be related to the absence of *chiA* and *v-cath* proteases, as suggested previously (Chen, 2017). After purification, the infectivity of rAAV stocks was analyzed calculating the ratio vector genomes/infectious particles. No difference in term of infectivity was observed between vectors by using the infection center assay (ICA) method (**Fig. 4c**), which evaluate the capability of rAAV particles to enter HeLaC32 cells and replicate in the nucleus in the presence of Rep and Adenovirus (François et al., 2018). Altogether, these observations suggested that the HR system is comparable to Tn7 to produce rAAV in suspension Sf9 insect cells.

Advantages of HR system over Tn7 system in term of product safety

We designed qPCR assays to amplify specifically product-related DNA impurities, i.e. gentamycin and kanamycin gene sequences, in the newly produced rAAV batches. In the Tn7-derived rAAV batches, we detected $>10^9$ copies of gentamycin out of 10^{12} viral genomes while this value is remarkably lower $<5,0$ x 10^7 copies in HR-derived batches (**Fig. 5a**). The kanamycin resistance gene sequence was not detectable whatever the system used to produce recombinant baculoviruses (data not shown). LOQ = 7.0 x 10^6 copies/ml.

Finally, the identity and purity of rAAV vectors derived from HR and Tn7 systems were determined by western blot analysis and SDS-PAGE silver staining (**Fig. 5b**). The profile of the proteins detected by silver stained SDS-PAGE was comparable in all cases, but the capsid signal was more intense in rAAV stocks produced with the HR system when loading the same amount of vg per well, and the same was true for western blot analysis. It correlates with previously observed increased of total capsids in HR condition as measured by ELISA (**Fig. 4b**). Altogether, these observations suggested that HR system definitively reduces the risk of bacterial sequences encapsidation into the final rAAV biotherapeutic and, thus, it increases the safety profile of the product as recommended by regulatory agencies, taking into account that the delta chitinase-cathespin version may lead to more empty rAAV particles at harvest.

DISCUSSION

Our study compares two systems to generate recombinant baculovirus vectors in the context of rAAV production. Indeed, baculovirus backbone can be engineered by exploiting both homologous recombination (HR) or Tn7 transposition, the latter being the most widely used system. First, we show that the recombinant baculoviruses derived from the Tn7 system tends to loose the exogenous expression cassette after few rounds of viral amplification while HR system preserves baculoviral genome stability over multiple passages. This is an advantage for manufacturing at industrial scale, because multiple rounds of baculovirus amplifications could be needed to infect large volumes of culture. Secondly, we show that BEVs generated with the HR system preserve rAAV yields and infectivity comparable to the standard Tn7 system. Importantly, HR system is devoid of any bacterial DNA sequences in rAAV biotherapeutic final product which is an additional safety advantage to meet requirements from the regulatory agencies.

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The genetic instability, and thus, substantial loss of the expression cassette, observed during recombinant baculovirus amplification after Tn7 transposition was specific to this system. The mechanism underlying this genetic instability is not fully understood, although the presence of residual bacterial elements could be detrimental for baculovirus genome replication. Our data show that HR system resulted in increased genetic stability of the inserted transgene, due to knock-out the BAC sequence from parental bacmid, when homologous recombination occurred. Nonetheless, some limitations of HR system have also been identified, i) the low frequency of natural HR events that can occur in Sf9 insect cell could lead to the generation of fewer number of clones of recombinant baculoviruses; ii) the risk to generate baculovirus defective interfering particles (DIs) due to cross-complementation of ORF1629 essential protein from viable recombinant baculovirus. Here we show that the use of circular donor plasmid induced very low HR frequency and lead to accumulation of DIs that are not carrying the cassette of interest. However, we solved this issue by using a linearized donor plasmid that maximizes the on-target cassette integration and finally leads to similar yield of baculoviral clones than Tn7 system. This observation suggests again the need for HR system to still proceed with one round of viral plaque isolation to definitively eliminate DIs and generate a pure baculoviral reagent before amplification for generating master viral seed (MVS). Whereas the standard Tn7 system seemed to be limited to a few numbers of passages, in this study we show that HR system may allow extended number of rounds (up to 10 passages) without cassette instability. This is a major advantage for industrial applications if large batches of biotherapeutic products require the use of high doses of baculovirus reagents.

Our study also shows that HR and Tn7-derived BEVs generated similar amounts of rAAV vectors, despite the fact that total capsid titer seemed to be slightly higher with HR. This observation was confirmed by SDS-PAGE analyses when running AAV proteins normalized by the vector genome copy number. Further investigations are necessary to determine if the higher proportion of empty AAV capsids in the HR system is due to the absence of chitinase and cathepsin in the BacMagic-2 system, as suggested by another group (Chen, 2017), or a direct consequence of the higher genetic stability of Cap expression along the rAAV production.

Importantly, we show that final rAAV products generated with HR-derived BEV are exempted of encapsidated bacterial sequences including antibiotic resistance genes, such as gentamycin and kanamycin. In contrary to the Tn7 system, the HR system knock-out the BAC sequence from parental bacmid. Gentamycin sequence is detectable in rAAV generated with Tn7-derived BEVs, likely due to the proximal location with the AAV ITRs. Nonetheless, none of the two methods can avoid this reverse packaging phenomenon of baculoviral sequences from the AAV ITRs (Penaud-Budloo et al., 2017).

In summary, we conducted a side-by-side comparison of different methods to generate recombinant baculovirus vectors and concluded that all methods are equally efficient to generate high yields of rAAV vectors. HR-derived BEVs showed higher genetic stability over passages and lower amounts of nucleic acid impurities (i.e. bacterial sequences) which are significant advantages for large scale manufacturing of rAAV biotherapeutics.

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AUTHOR CONTRIBUTIONS

A.J. designed the study, performed research, experiments, interpreted data and wrote the manuscript; L.B. and P.J. performed research, experiments, and interpreted data; L.M. performed baculovirus and AAV vectors production and characterization. M.B. and F.B. provided technical help with qPCR design and AAV vectors purification and characterization were supervised by V.B. and C.R. L.H.V. provided Anc80 material and reviewed the manuscript. O.A. provide funding and supervised research. M.P-B and E.A. designed the study,

coordinated the work, supervised research, interpreted data, provide funding and wrote the manuscript.

COMPETING INTERESTS

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Figure 1: Gene transfer systems to generate recombinant baculoviruses.

a) Tn7 (Bac-to-Bac®) and HR (BacMagic-2? or flashBAC GOLD?) bacmid systems are derived from wild-type circular baculoviral genome by deletion of structural *polyhedrin* gene (Ac8) and insertion of bacterial artificial chromosome (BAC) as described by Luckow et al., 1993 and Possee et al., 2008. Commercial HR systems are optionally deleted for accessory genes, i.e. *chitinase* (Ac126) and/or *v-cathepsin* (Ac127) which are of interest for our study. **b)** Tn7 system is handled in bacteria where both "pFastBac" donor plasmid and Tn7-bacmid DNA are maintained and amplified thanks to the selective genes coding for ampicillin (AmpR) and gentamycin (GmR). Tn7 transposition is induced with the help of pMON7124 helper plasmid that expresses transposition proteins (*TnsABCD*) in *trans* leading to gene of interest (GOI) transfer from the donor plasmid pFastBac to the Tn7-bacmid DNA by attTn7 site recognition, including the selective gene coding for GmR. The presence of the expression cassette into the Tn7-bacmid DNA is characterized by PCR, before to be purified from bacteria and then transfected into Sf9 insect cell line, where the first BEV progeny is generated (P0). Viral clones are isolated by plaque assay (P1) and characterized (i.e. titer, sequence identity and genetic stability). One stable clone meeting specification is then amplified to generate a master viral seed (P2 or P3). HR system, "pBac" donor plasmid and the HR-bacmid DNA are directly co-transfected in Sf9 insect cell line, without the need of a bacterial step. Homologous recombination can

occur by homology arms recognition of *lef2* and non-deleted*orf1629* sequences from the donor plasmid up-to the HR-bacmid DNA. The cassette is transferred into the bacmid in place of the existing BAC sequence carrying the Mini-F$^?$, and the selective gene chloramphenicol (CmR), that are finally deleted from the final construct. Moreover, the *orf1629* sequence is fully reconstituted leading to viable BEV progeny generation (P0). Similarly, to Tn7 system, one clone (P1) is then characterized and amplified up to MVS build-up (P2 or P3).

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Figure 2: Generation of baculoviral reagents with HR system.

Characterization of five BEV rep2cap8 clones (P1) generated with HR system and derived from circular or digested (= linearized) "pBac" donor plasmid. **a)** Infectious titers for each clone using the cell size assay method (n = 1). **b)** Viral genome qPCR quantification using specific "bac" and "rep" amplicons. A ratio (bac/rep) is calculated and plotted to compare clones altogether (n = 2). Mean +- SD. **c)** Western blot analysis of "Rep" proteins (Rep78 and Rep52) and "Cap" proteins (VP1, VP2 and VP3) after serial steps of clone amplification (P1, P5 and P10).

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Figure 3: Side by side characterization of baculoviral reagents derived from HR or Tn7 systems. **a)** Characterization of BEV clones where genome stability is monitored by qPCR quantification using specific amplicons along serial passages of amplification. BEVs are derived from the HR- or Tn7-based system with a total of 5 clones per system. The ratio bac/amplicon is calculated and plotted to compare clones (n = 1 or 2). Mean +- SD. **b)** Western blot analysis of "Cap" proteins (VP1, VP2 and VP3) after serial steps of BEV rep2/cap8 clonal amplification derived from HR and Tn7 systems.

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Figure 4: Recombinant AAV production using both HR and Tn7 derived baculoviral reagents.

a) Total vector genomes (vg) quantification by qPCR at harvest time or after purification for AAV2/8-GFP (n = 3) or AAV2/anc80-GFP (n = 2) preparations. Titers were normalized for a standard production volume equal to 125ml. **b)** Quantification of total AAV2/8-GFP viral particles (vp) by ELISA assay or total viral genomes (vg) by qPCR in clarified crude bulk (n = 3). **c)** Left: quantification of infectious units (iu) of AAV particles by ICA assay in purified AAV8-GFP (n = 3) and AAVanc80-GFP (n = 2) batches; Right: representation of infectivity expressed as a ratio "vg/IU" for the indicated constructs. Mean +- SD.

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Figure 5: rAAV characterization derived from HR and Tn7 reagents.

a) Total gentamycin copies quantified by qPCR in purified AAV8-GFP (n = 3) or AAVanc80-GFP (n = 2) batches and normalized to 10^{12} total viral genome (vg). Mean +- SD. **b)**Top: western blot analysis of "Cap" proteins (VP1, VP2 and VP3) for different replicates of purified AAV preparations carrying indicated constructs; Bottom: silver staining analysis of total proteins and purity for different replicates of purified AAV preparations carrying indicated constructs.

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Supplementary Figure 1: Design of constructions. Linear representation of different expression cassettes that are used to produced recombinant AAVs.