Development of an insect cell-based adeno-associated virus packaging cell line employing advanced Rep gene expression control system

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INTRODUCTION

Adeno-associated viruses (AAVs) are one of the leading gene delivery platforms. The development of novel AAV vector-based therapies is rapidly expanding, with more than 260 clinical trials currently initiated. Therefore, the need for a robust and scalable production system for AAV is also in high demand. Conventionally, AAV vector production relies on the transient transfection of mammalian cells. However, this system cannot easily be scaled up to produce the amount of AAVs required for late-stage trials or routine clinical use.

The baculovirus expression vector (BEV) system is a leading platform for scalable production of AAVs. The previously described One-Bac system consists of an insect packaging cell line harboring the AAV Rep and Cap genes and a BEV carrying the transgene and AAV inverted terminal repeats. Here we describe a new system where we successfully translated the molecular design of a double AAV Rep expression cassette to inducible plasmid vectors. These optimized plasmid vectors employ non-canonical late promoters and alternative start codons that alleviate promoter-promoter competition. Because too much Rep expression can be toxic to the host cells, tighter regulation of AAV Rep expression is warranted. This has been achieved by adopting alternate baculovirus homologous region enhancers.

Inoculation of the resultant stable insect Rep packaging cell line by a recombinant BEV produced high-titer recombinant AAV (rAAV) preparations (1 × 10^11 genome copies/mL). Sequential batch reactor experiments indicate that this system is amenable to large-scale AAV production. We generated an insect packaging cell line that employs an optimized Rep gene control system, ensuring stable and appropriate Rep expression. This platform produces potent and high-yield AAV particles and demonstrates potential for scale up.

The insect cell-based baculovirus expression vector (BEV) system is a leading platform for scalable production of AAVs. The previously described One-Bac system consists of an insect packaging cell line harboring the AAV Rep and Cap genes and a BEV carrying the transgene and AAV inverted terminal repeats. Here we describe a new system where we successfully translated the molecular design of a double AAV Rep expression cassette to inducible plasmid vectors. These optimized plasmid vectors employ non-canonical late promoters and alternative start codons that alleviate promoter-promoter competition. Because too much Rep expression can be toxic to the host cells, tighter regulation of AAV Rep expression is warranted. This has been achieved by adopting alternate baculovirus homologous region enhancers. Inoculation of the resultant stable insect Rep packaging cell line by a recombinant BEV produced high-titer recombinant AAV (rAAV) preparations (1 × 10^11 genome copies/mL). Sequential batch reactor experiments indicate that this system is amenable to large-scale AAV production. We generated an insect packaging cell line that employs an optimized Rep gene control system, ensuring stable and appropriate Rep expression. This platform produces potent and high-yield AAV particles and demonstrates potential for scale up.

The baculovirus expression vector (BEV) system is a well-established alternative to mammalian cell culture that was used to produce the first AAV gene therapy drug (Glybera) to gain regulatory approval.

Urabe et al. first described the generation of functional recombinant AAV vectors using Spodoptera frugiperda (Sf9) insect cells. This system was called “Triple Bac” because it required co-infection of Sf9 cells with three recombinant baculoviruses (BEVs), each delivering a separate gene (Rep, Cap, and the inverted terminal repeat [ITR]-flanked transgene of interest) essential for recombinant AAV (rAAV) production. The Triple Bac system has several advantages over the transient transfection method. However, it was not widely adopted because of several limitations, including passage-dependent loss of Rep expression. Bakker and Hermens and Hermens et al. designed a construct (Rep 183) that optimized Rep expression, resulting in a scalable system that has been used to produce AAV-based gene therapy vectors for several clinical trials. However, the issues of low co-infection ratio and cost and the process complexity imposed by using three rBEVs persisted. Novel next-generation BEV systems such as OneBac 1.0 were developed to address these concerns. The OneBac 1.0 platform employs an Sf9-based packaging cell line that has been genetically modified to incorporate inducible copies of the Rep and Cap genes. This system is capable of producing rAAVs at 10^9 genome copies (GCs)/cell upon a single inoculation of the BEV harboring the AAV ITRs and the transgene of interest. However, a considerable proportion of the rAAV particles produced contained packaged Rep and Cap sequences; therefore, the system could not support development for clinical applications. Several improvements have been made to the OneBac 1.0 system. Removal of the Rep binding element (RBE) reduced collateral packaging of non-vector DNA over 1,000-fold, and introduction of a strong VP1 codon and a synthetic intron rescued viral proteins (VP) stoichiometry. Wu et al. fused the Cap gene together with the ITR-transgene-ITR (Cap-Trans) in the baculovirus vector genome while maintaining the inducible Rep gene integrated in the packaging Rep Sf9 cells. The system produced more than 10^9 vector genomes (VGs)/cell of several AAV serotypes (AAV2, AAV8, and AAV9), and yield was stable over 4 passages.

Here we designed an inducible plasmid vector (expressing replicase) that is optimized for Rep expression in at least two ways. First, it employs alternative baculovirus promoters to regulate AAV gene expression. This platform produces potent and high-yield AAV particles and demonstrates potential for scale up.

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expression. To date, the polyhedron promoter (polH) has been widely adopted in BEV systems used for rAAV production. Alternative late promoters (such as p10) share a host factor with polH, and others exhibit different temporal profiles and induction strengths. Promoter use for rAAV production in insect cells (outside of polH) has not been fully explored. Second, tighter regulation of the expression of AAV Rep, which can be toxic for host cells when present too early and/or too abundantly, was achieved by using alternative baculovirus enhancer sequences. Use of the baculovirus homologous region (hr) 2 or hr2.09 enhancer sequence in combination with polH is the default molecular design for the inducible OneBac platform. By examining use of alternative baculovirus promoters in combination with other baculovirus hrs, we optimized this production system. This platform employs an improved Rep gene control system, ensuring stable and appropriate Rep expression, and has proven production of potent and high-yield AAV particles with demonstrated potential for scale up.

RESULTS

Promoter-promoter interaction is present during recombinant BEV-dependent trans-activation, which influences AAV Rep protein expression from single Rep cassette expression plasmids

The single Rep cassette is frequently used to express AAV2 Rep proteins in a BEV system, especially via trans-activation. Expression of Rep78 and Rep52 relies on polH (a single upstream baculovirus-derived late promoter). The weak non-canonical ACG start codon drives expression of Rep proteins at the correct ratio, increasing AAV particle production. How the regulatory elements in the single Rep design exert control and behave in the presence of similar cis-regulatory elements in the genome of the trans-activating agent is not fully understood. Therefore, a BEV inducible and polH-dependent Rep expression plasmid was generated to examine the interaction between the Rep promoters within the BEV genome and the plasmid. This plasmid construct (pCLD 002) contains an upstream hr2.09 enhancer to support polH-dependent trans-activation in combination with a full-length AAV2 Rep cassette with an attenuated ACG start codon. pCLD 002 was transiently transfected into ExpresSF+ cells and trans-activated via inoculation with the indicated recombinant baculovirus (Figure 1A). Upon Bac Trans inoculation, the pCLD 002 construct expressed Rep78 and Rep52, mirroring the Rep expression from the Bac Rep183-positive control (Figure 1B; pCLD 002). Expression of AAV2 Rep52 and Rep78 was only observed when trans-activation was performed using Bac Trans. The other baculoviruses (Bac polH Cap and Bac polH Cap Trans) only induced AAV2 Rep52 expression. This suggests (1) promoter-promoter interaction in the recombinant baculovirus trans-activation system and (2) that Rep78 and Rep52 expression are differentially regulated in the single AAV Rep cassette (possibly by the endogenous AAV p19 promoter in the full-length Rep gene cassette). Other studies have shown that native AAV promoters are constitutively active in BEV systems during wild-type AAV production. To confirm this, partial Rep-nano-luciferase reporter constructs were designed with the same upstream hr2.09 enhancer, combined with a late conservative promoter (polH or p10), and a similar experiment was performed. A partial 12-amino-acid Rep was incorporated to mimic the influence of the downstream nucleotides during translation initiation. The nano-luciferase reporter is "split" from the non-functional Rep peptide using a 2A self-cleaving peptide (P2A) that is most efficient in insect cells. The reporter induction profile was determined after trans-activation by inoculation with (Bac polH Cap Trans) or without (Bac Trans) recombinant conservative late promoters. The weak trans-activation associated with an "empty" promoter and an hr2.09 enhancer is expected because other enhancers have previously demonstrated weak promoter activity. To confirm that there was no difference in infectivity between recombinant baculoviruses, native AAV2 promoters (p5 and p19) were also tested. In the presence of an hr enhancer, the p5 and p19 promoters were constitutively active in insect cells (Figure 1C, circle), confirming previous observations. However, their expression was enhanced upon baculovirus trans-activation, with no marked difference in induction profiles when Bac Trans or Bac polH Cap Trans were used (Figure 1C, squares versus triangles), demonstrating that the recombinant baculoviruses have similar infectivity and trans-activation capacities. Conversely, strong induction of reporter expression was observed after Bac Trans trans-activation when nano-luciferase (Nano-Luc) is regulated by polH and p10, as early as 24 h after infection. At the same time point, trans-activation by Bac polH Cap Trans resulted in lower reporter gene expression compared with Bac Trans (Figure 1C). The p10 promoter reporter showed a tendency toward increased luciferase signal upregulation compared with polH after Bac polH Cap Trans trans-activation (Figure 1C, 24 h post infection [h.p.i.]). The reporter study demonstrates distinct baculovirus promoter-promoter interaction, which is influenced by cis-regulatory elements in the trans-activation system.

Use of short hr enhancers, late promoter p10, and a strong ATG start codon generates an optimal single Rep cassette design inducible by baculoviruses harboring a recombinant polH promoter

A variety of baculovirus hr sequences possess transcription enhancer activity. The number of IE-1 DNA binding site sequences within the hr may correlate with its activity. To determine the influence of enhancers (which are also cis-regulatory elements) on single Rep cassette trans-activation, the activity of hr2/hr2.09 (which has 7 IE-1 DNA binding sites) and others (i.e., hr1, hr3, hr4b, and hr5) combined with polH, as reference promoter, was profiled using the Nano-Luc reporter constructs upon trans-activation with recombinant baculoviruses (see Figure 1A approach). Comparison of these hr enhancers demonstrated significant nucleotide differences (Figure 2A) despite the intermittent presence of the IE-1 DNA binding sites. All hr sequences (irrespective of their directionality) could enhance the promoter activity upon transactivation with baculoviruses, but with different degrees of activity (Figure 2B). The synthetic hr, engineered to have 8 IE-1 DNA binding sites, and hr3 failed to enhance promoter expression beyond hr2.09, which has 7 sites. Overall, hr2.09 was the most efficient, followed by hr4b, which is one of the shortest hr sequences with the fewest IE-1 binding elements.
Figure 1. The use of an alternative late baculovirus p10 promoter in inducible plasmid vectors ameliorates promoter-promoter competition caused by incorporation of a polH promoter in the recombinant baculovirus.

(A) Schematic of transient transfection and baculovirus trans-activation study involving the reporter or pCLD expression construct. Luciferase activity was measured for the partial Rep nano-Luc reporter study, and western blotting was performed to determine Rep expression from the pCLD construct. GSG-P2A is a self-cleaving peptide.21 (B) Expression profiles of AAV Rep proteins from the pCLD harboring the indicated regulation element under the influence of different baculovirus trans-activations 48 h post infection (h.p.i.). T, Bac Trans; CT, Bac polH Cap Trans; Bac Rep 183, baculovirus expressing AAV2 Rep.8 GFP-expressing pDNA was included as a transfection control. (C) The kinetics and intensity of reporter gene expression (Nano-Luc) are regulated by the indicated promoter upon the indicated baculovirus trans-activation. Relative luciferase units (RLUs) were measured in a 30-μL sample volume. Mock (orange circles): inoculated using an equal volume of fresh medium. Bac Trans (red squares): recombinant baculovirus harboring only AAV ITR-transgene-ITR. Bac polH Cap Trans (blue triangles): recombinant baculovirus harboring the polH regulated AAV2 Cap gene and ITR-transgene-ITR. Bac polH Cap: recombinant baculovirus harboring the polH regulated AAV2 Cap gene only. Empty and GFP-expressing pDNAs were included as experimental controls. Each data point represents an independent experimental replicate performed by different operators at different times.
(Figures 2A and 2C, pCLD 012). However, relatively high basal expression was observed from the mock-treated hr2.09 sample compared with the pGFP mock (Figure 1C) and other hr sequences (Figure 2B). However, these differences were not statistically significant. To compare the effect of hr enhancers on AAV Rep expression, several plasmid vectors (pCLDs) were made and tested for regulation of expression upon baculovirus trans-activation (Figure 2C). Western blotting (Figure 2C) demonstrated that all hr enhancers improved polH-mediated AAV2 Rep upregulation. Distinct expression strengths were observed (with hr2.09 > hr4b > hr5) on AAV2 Rep78 (supporting the earlier observations shown in Figure 2B) but not on Rep52 expression, confirming that, in the single AAV Rep expression cassette, endogenous p19 regulates expression of the smaller downstream Rep52, whereas expression of the upstream Rep78 is differentially regulated (by the polH promoter). A single Rep cassette was designed using comparatively weak hr enhancers (e.g., hr4b and hr5), to create an inducible platform with relatively low basal expression. The p10 promoter had a tendency toward stronger induction upon transactivation with Bac polH Cap Trans; hence, the promoter was combined with an hr4b enhancer to create a single cassette AAV2 Rep with a strong wild-type ATG start codon (Figure 2D). To confirm the reduction of promoter-promoter
The utility of short hr enhancers in combination with alternative baculovirus promoters to inducibly regulate a double Rep cassette design

The single Rep cassette is the most frequently used configuration for trans-activated Rep. However, the double Rep cassette design (delivered by Bac Rep 183) is currently employed (in a BEV and insect cell system) to produce AAV vectors that have been used in clinical trials. Because optimization results in high Rep78 levels, which can impede AAV yield, our design replicated the AAV Rep expression profile in a previously published mammalian production system. In the double Rep cassette, Rep52 and Rep78 expression are, respectively, regulated by the polH promoter and a truncated IE-1 (ΔIE-1) baculovirus promoter, which limits expression of Rep78. However, ΔIE-1 is a constitutive promoter (Figure 3A), which is ineffective in a trans-activation system. To overcome this, the delayed early 39k promoter was used as an alternate regulator of Rep78 expression. Early expression (3–6 h after baculovirus trans-activation) was mediated by the 39k promoter, making it a potential ΔIE-1 temporal mimic (Figure 3A). Luciferase expression driven by the 39k promoter was less impeded by Bac polH Cap Trans but higher compared with ΔIE-1, especially at later time points (Figures 3A, 24–72 h time points). A luciferase reporter assay (construct as per Figure 3B) was used to screen for non-canonical start codon alternatives that could reduce 39k-driven protein expression. Most of the tested non-canonical start codons mediate functional protein translation in insect cells, with AGC, TTG, and ATT causing lower protein expression compared with wild-type ATG, CTG, and GTG. Replacing the ATG start codon with the suboptimal AGC/TTG/ATT codon reduced 39k promoter strength at later time points to approximately that of the ΔIE-1 profile (Figure 3B). Based on the reporter study, pCLDs (019 and 020) incorporating the weak AGC start codon were assessed in combination with a 39k promoter to determine their effect on the full-length AAV2 Rep expression profile in the presence or absence of an hr enhancer sequence (Figure 3C). Upon baculovirus trans-activation, pCLD 020 (in which the hr2.09 is still present) was the only construct that resulted in AAV2 Rep78 expression, even when transactivated by Bac polH Cap Trans (Figure 3C). This confirms that transactivation of the 39k promoter is also hr enhancer dependent, and alternative baculovirus promoters can be used to initiate promoter-promoter interaction upon trans-activation. Expression of Rep78 remained high under the 39k promoter, and the Rep78:Rep52 ratio did not reflect the Bac Rep 183 Rep expression profile (Figures 1B and 2D). To circumvent this, expression of Rep78 was reduced by employing a weaker enhancer sequence (hr4b), and Rep52 was concomitantly enhanced by use of an additional strong late promoter in an artificial intron, as described for a previously published molecular design (Figure 3D). Several late promoters (with the least promoter-promoter competition with polH) were also tested. Despite the presence of a strong hr2.09 enhancer, using polH (even as the intronic promoter) failed to trigger Rep52 expression when transactivated by Bac polH Cap Trans (Figure 3D, pCLD 043). Rep52 expression was partially restored by replacing the intronic promoter with p10 or p6.9 (Figure 3D, pCLD 105 and 106). This provides more evidence of promoter-promoter competition among some baculoviral promoters. Despite adoption of an intronic promoter, which reduced promoter competition, the level of Rep52 expression was different from controls (Rep 183; Figures 1B and 2D). Therefore, several double Rep cassette constructs were designed, utilizing short hr enhancers to reduce Rep78 expression, and an additional copy of codon-shuffled Rep52 under regulation of a baculovirus late promoter in reverse complement orientation (Figure 3E, pCLD 050–054). Because enhancer activity is known to be...
bidirectional (Figure 2B, hr4b(−)), we tested the capacity of the single hr4b (pCLD 050 and 051) and compared it with the hr4b-hr5 combination (pCLD 052, 053, and 054). The expression kinetics and strength of several non-conservative baculovirus late promoters, such as p6.9 and pSel120, were profiled using the luciferase reporter assay to assess their ability to regulate the extra copy of Rep52 and their propensity to cause promoter-promoter competition. All early baculovirus promoters (ΔIE-1 and 39k) were trans-activated by Bac Trans and Bac polH Cap Trans with similar strength (Figure 3A). The maximum difference was observed with the Bac polH Cap Trans trans-activation of the p6.9 promoter at lower strength after 48 h.p.i, which is similar to the effect of the p10 promoter (Figure 1C). The trans-activation of pSel120 with Bac polH Cap Trans was more marked at 72 h.p.i. To evaluate the effect of these constructs on Rep protein expression, the (pCLD 050–054) plasmids were transfected, and Rep expression upon trans-activation was determined by western blotting (Figure 3E). As expected, use of the 39k promoter resulted in inducible Rep78 expression regardless of use of hr enhancer or recombinant baculovirus (Figure 3E). Although Rep52 expression by these constructs was trans-activated by any baculovirus, distinct expression intensity profiles were observed when Bac Trans and polH Cap Trans were used, especially for the p10 regulated construct (Figure 3E, pCLD 052). The use of alternative late p6.9 and pSel120 promoters partially ameliorated the promoter-promoter competition after Rep52 trans-activation by Bac polH Cap Trans. Here we showed the potential utility of alternative baculovirus promoters in inducible AAV double Rep designs to drive expression of AAV genes at the correct time and intensity. This approach also provides a solution for the promoter-promoter competition observed when using recombinant baculoviruses harboring the same promoters as the expression plasmids.

An optimized inducible double Rep cassette design produces rAAV particles after a single inoculation by a baculovirus harboring the recombinant polH promoter

To determine whether plasmid vectors, pCLD 046 and pCLD 050–054, could be used to produce intact rAAV particles, small transient rAAV production experiments were performed in ExpresSf+ cells (Figure 4A). Different Bac polH Cap Trans viruses encoding different AAV Cap serotypes and transgenes were used as trans-activating agents. Transient rAAV production using the inducible single (pCLD 046) and double Rep cassette (pCLD 050–054) plasmid vectors consistently produced DNase-resistant rAAV particles from several production batches with an average GC titer of $\pm 1 \times 10^{10}$ GC/mL in crude lysate buffer (CLB) (Figure 4B). The expression profile of AAV Rep, notably the temporal expression of Rep78, differed between the single and the double Rep cassette constructs (Figure 4C). The capsid ratio (VP1:2:3 ratio) exhibited by the purified rAAV particles produced via transient transfection of the pCLDs was similar (Figure 4D). rAAV particles derived from the inducible double Rep cassette plasmid vectors, notably from the pCLD 052 and 053 constructs, exhibited higher potency, particularly relative to the single Rep cassette (pCLD 046)-derived particles (Figure 4E).

The combination of non-canonical start codons, short baculovirus hr enhancers, and alternative baculovirus promoters generated optimized inducible Rep cassette plasmid vectors with flexible expression profiles that can be trans-activated by baculoviruses. These modifications also ameliorated promoter-promoter competition. The pCLD 046, 052, and 053 vectors were selected to generate stable packaging insect cell lines with an inducible Rep (iRep) expression profile (iRep) (Figure 5A). All iRep cell lines (iRep 046, iRep 052, and iRep 053) produced rAAV particle titers of at least $1 \times 10^{11}$ GC/mL titer and $1 \times 10^{5}$ GC productivity per cell (Figure 5B). rAAV particle function and capsid ratios (VP1:2:3 ratio) exhibited by the AAV2/5 materials were comparable with each other (Figure 5C). Use of the iRep 052 and 053 cell lines produced AAV particles with potency similar to triple Bac-derived material, which was also more potent than those produced by single Rep cassette iRep 046 cell lines (Figure 5D), confirming the previous transient transfection results (Figure 4E). Integrated Rep gene expression stability was examined using representative iRep 052 stable cells in a sequential batch reactor (SBR) study without selective antibiotic pressure. Stable Rep78 and Rep52 expression was noted at all time points studied up to passage 9 (20 generations). The expression ratio of Rep78 and Rep52 (low Rep78 and high Rep52) (Figure 5E) was comparable with expression from the benchmark control Bac Rep 183 (Figures 1B and 2D). The average DNase-resistant AAV particle yield in filtered crude lysed bulk (FCLB) samples was more than $1 \times 10^{11}$ GC/mL, which remained relatively stable but with a slight decline in AAV yield (Figure 5F). These overall observations confirm the presence of the integrated Rep genes cassette in the heterogeneous iRep 052 cell line and the need to perform a single-cell isolation step before master cell banking and using them for clinical-grade and large-scale rAAV production.

Figure 4. An optimized inducible double Rep cassette design produces high titer and potent rAAV particles after inoculation by a baculovirus harboring the recombinant polH promoter

(A) Schematic of transient AAV production. (B) GC titer (GC/mL) of nuclelease-resistant AAV particles in crude lysate buffer (CLB) harvested 3 days after transient AAV production using the indicated pCLD transfection and baculovirus–trans-activation. Bac Cap5 FIX (orange circles): recombinant baculovirus harboring the polH regulated AAV Cap5 gene and ITR-FIX-ITR. Bac Cap2/5 sNano-Luc (blue triangles): recombinant baculovirus harboring the polH regulated AAV Cap2/5 gene and ITR-secreted-Nano-Luc-ITR. (C) AAV Rep kinetics and expression profiles from the transient AAV production experiment in comparison with the triple (3-ple) Bac platform. (D) Capsid VP1:2:3 profile of AVB purified AAV2/5 sNano-Luc and AAV5 FIX, loaded in equal volume. (E) Top panel: schematic of the AAV potency assay in Huh7 cells. The AVB purified AAV2/5 particles produced from the transient AAV production were normalized and used to inoculate Huh7 cells at a dose of $10^{6}$ GC/cell. At 3 d.p.i., the potency of AAV2/5 particle transduction was determined by quantification of Nano-Luc (RLU) secreted into the supernatant. Bottom panel: potency comparison of purified AAV produced from transient AAV production using the indicated inducible Rep plasmid vectors (pCLD), 3-ple Bac material: AAV material harboring the same transgene produced from the lab-scale 3-ple baculovirus production platform as described previously. Each data point represents an independent replicate of an AAV batch production performed by different operators at different times.
DISCUSSION

Despite rapid advancements in development of gene therapy drugs, difficulties in scaling up AAV production may hinder clinical trials and routine application of AAV-based gene therapy. Therefore, advancements in production methods will have a real-world impact. OneBac 1.0 systems consist of an insect Sf9 packaging cell line that supplies the AAV Cap and Rep helper genes, and the AAV genome (ITR) and gene of interest are contained in a single BEV.10 This simplified and cost-effective system can produce high-yield AAVs upon a single baculovirus infection. Several modifications have been made to the OneBac 1.0 system, resulting in appropriate VP stoichiometry, enhanced BEV stability, and reduced encapsidation of non-vector DNA.12,13

These inducible rAAV insect packaging cells contained AAV single Rep cassette and Cap expression plasmids under control of the polH promoter.10 Discovery of the late-acting promoter polH was fundamental for development of BEV systems that could produce heterologous proteins in insect cells.23 Therefore, use of the polH promoter was optimized and widely adopted for rAAV production by BEV systems. Although extensive studies of polH have been conducted to date, the utility of alternate baculovirus promoters exhibiting different kinetics or induction strengths remains unknown.16-18 We demonstrated competition between the polH promoter in the expression plasmid vectors and the recombinant baculovirus during trans-activation. Remarkably, adoption of an alternative late promoter (p10) in the expression plasmid vectors partially ameliorated this. The modified OneBac system described by Wu et al.13 used a p10 promoter to regulate Cap gene expression in the baculovirus trans-activator, which unexpectedly resulted in lower promoter-promoter interaction.13 These findings show that using different promoters...
can optimize a system. A wide range of promoters are available that can be used in numerous combinations to potentially enhance future system development.

A variety of baculovirus hr enhancer sequences have previously demonstrated the ability to stimulate transcriptional activity. These sequences have been adopted and used in conjunction with baculovirus promoters to create recombinant expression plasmids. The OneBac system adopted similar strategies to generate baculovirus trans-activatable AAV gene expression plasmid vectors. The OneBac hr2.09 sequence markedly enhanced AAV Rep and Cap expression by plasmid vectors upon trans-activation with a recombinant baculovirus. Comparatively high basal expression was observed when using the strongest enhancer (hr2.09). However, alternative enhancers (hr4b or hr5) can be used to reduce basal gene expression. We showed that all hr sequences, regardless of their directionality, enhanced baculovirus promoter activity upon trans-activation with baculoviruses with different degrees of efficiency. The IE-1 DNA binding site sequence acts as a transcriptional regulator, and its presence is thought to be integral for the enhancer function of hr. It has been supposed previously that the amount of binding sites correlated with enhancer strength. However, we showed that the synthetic hr, engineered to have 8 IE-1 DNA binding sites, failed to enhance promoter expression beyond an enhancer (hr2.09) that only contained 7 binding sites, implying that additional mechanisms regulate hr enhancer strength. Different hr effect strengths (hr2.09 > hr4b > hr5) were only observed on AAV2 Rep78 expression (but not on Rep52). This implies the presence of a distinct regulation system in the single Rep cassette, where the native endogenous AAV p19 promoter is functional in the presence of any hr and inducible irrespective of baculovirus addition. Further evaluation of these features may provide additional methods to optimize induction of AAV Rep and the BEV system overall.

We also designed an optimized insect cell-based packaging system where alternative baculovirus promoters were adopted in an inducible AAV double Rep design. The double Rep cassette design is favored for AAV production using the BEV platform because of the high-quality rAAV produced. The advantages of the double Rep cassette are strong and temporal control of Rep expression, which can be modified to replicate the dynamics of wild-type AAV Rep protein expression. In a mammalian host, wild-type AAV requires the largest Rep proteins (Rep78 and 68) for viral DNA replication. The smaller Rep proteins (Rep52 and 40) are largely responsible for accumulation and packaging of single-stranded genomes. Therefore, we hypothesize that large Rep proteins are required earlier to propagate and provide sufficient AAV genomic substrates that are later loaded by smaller Rep proteins into assembled capsids. Use of DAE-1, an immediate-early baculovirus promoter, to regulate the large Rep78, and polH, a late baculovirus promoter, to regulate the majority of small Rep52 in a double Rep cassette BEV system, such as Bac Rep 183, may replicate this. The temporal synchronicity of Rep protein expression driven by the selected promoters may explain the improved full: empty ratio of AAV particles generated by the triple baculovirus system, which incorporates a double Rep design.

Prior attempts to adapt double Rep cassette design to plasmid vectors amenable to baculovirus trans-activation were unsuccessful. This may have been due to the constitutive ΔIE-1 promoter and cis-trans competition of the polH promoter adopted. We successfully translated the double cassette AAV Rep design to inducible plasmid vectors by utilizing an alternative 39k promoter. This delayed early promoter seems to reduce promoter-promoter interaction/competition with the polH promoter, which is a cis-acting element in Bac PolH Cap Trans. The distinct temporal activity of the baculovirus promoters evaluated (one is a delayed early promoter and the others are late promoters) may explain why similar activities were obtained with the immediate-early ΔIE-1 promoter. The baculovirus early promoters are regulated by IE0 and IE1 transcription factors however, IE0 is not required for late promoter polH function. Therefore, the requirement for temporally distinct transcription may explain the promoter-promoter interaction. Adopting this principle allowed us to generate inducible AAV double Rep cassettes designed to mediate the correct temporal expression of AAV genes without deleterious promoter-promoter interactions.

Stable iRep insect cell line generation was performed, using the most promising (pCLD 046, 052, and 053) inducible AAV-Rep plasmids. The stability and appropriate expression of Rep78 and Rep52 by the optimized iRep (052) stable pool cells was determined by expansion in 2-L SBRs (with a 2,000-mL working volume) without antibiotic selective pressure, which demonstrated the stability of the integrated Rep genes over multiple cell passages. At passage 9, the ratio of Rep78 and Rep52 was comparable with Rep expression by the triple baculovirus expression platform (Bac Rep 183). Although a stable cell line was used in this study, the cells were a heterogeneous population because sorting or single clone isolation had not been performed. Cellular heterogeneity may have contributed to the variability in protein expression profile. Removal of selection pressure and the ability to maintain AAV yield up to passage 9 expansion, replicating a more than 1,000-L single-batch production process, implies that the integrated iRep is still present in this heterogeneous cell population. The decline in Rep expression (despite an AAV yield above 1 × 1011 GC/mL) underscores the importance of including a single cell isolation step to obtain monoclonal cells with the best genetic stability.

Conclusion

We evaluated use of alternate, non-conservative baculovirus promoters (p10, 39k, p6.9, and pSel120) with distinct expression intensities and temporal profiles to create an inducible expression construct regulating wild-type single or double cassette AAV Rep. The resultant plasmid vector construct is less prone to promoter-promoter competition upon recombinant baculovirus trans-activation. Adoption of baculovirus hr enhancers with lower basal activity enabled tight regulation of the inducible plasmid vector construct. This new design limits expression of toxic AAV genes, such as Rep, which could otherwise be detrimental to cell viability. Use of this optimized production platform resulted in AAV particles that demonstrated improved in vitro potency. Yields from this system
exceeded $1 \times 10^{11}$ GC/mL. SBR experiments indicated that his insect cell platform is amenable to large-scale AAV production, and Rep stability was demonstrated (up to passage 9). We described the development and characterization of an insect packaging cell line that employs an optimized Rep gene control system, ensuring stable and appropriate Rep expression. Use of this optimized platform produced potent and high-yield AAV particles and demonstrated the potential for scale-up.

MATERIALS AND METHODS

Cell culture

Huh7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Waltham, MA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA) at 37°C, 5% CO₂. ExpressSf+ and S9 cells were maintained in SF-900 II (Thermo Fisher Scientific, Waltham, MA) medium alone or supplemented with 10% FBS (v/v) (Thermo Fisher Scientific, Waltham, MA), respectively, in shaker flasks at 28°C, 135 rpm.

Inducible expression plasmids and recombinant baculovirus construction

Inducible expression plasmids (pCLDs) and Nano-Luc reporter constructs were created using GeneArt services (Thermo Fisher Scientific, Waltham, MA). To generate a recombinant baculovirus containing only the ITR-transgene-ITR (Bac Trans), the AAV Cap expression cassette alone (Bac polH Cap2/5), or the AAV Cap expression cassette and ITR-transgene-ITR (Bac polH Cap5 [human Factor IX (FIX)] or Bac polH Cap2/5 [secreted Nano-Luc (sNano-Luc)]), S9 cells were transfected with pVD-ITR-transgene-ITR (SEAP transgene) or pVD-polH-Cap (polH Cap2) or pVD-polH-Cap-ITR-transgene-ITR (polH Cap Trans) (Cap5 FIX, Cap2/5 sNano-Luc) and linearized baculovirus genome using Cellfectin II reagent (Thermo Fisher Scientific, Waltham, MA). The in-house-developed linear baculovirus genome contains the lacZ gene, which is replaced after successful homologous recombination with the plasmid DNA. Positive cell plaques were identified using blue-white screening and transferred onto adherent S9 cells. 72 h after transfection, the supernatant from S9 cells was passaged and amplified in ExpressSf+ cells until passage 4 and stored in liquid nitrogen. The baculovirus expressing AAV2 Rep (Bac Rep183) was generated as described previously. Bac Rep183 is also called the double cassette AAV Rep.

AAV vector production

AAV variants were generated by infecting transiently transfected ExpressSf+ insect cells with freshly amplified recombinant baculovirus stocks at passage 4 or 5, as described previously. After 72-h incubation at 28°C, cells were lysed with 1% Triton X-100 (Merck, Darmstadt, Germany) for 1 h. Genomic DNA was digested using benzonase at a final concentration of 9 U/mL (Merck, Darmstadt, Germany) for 1 h at 37°C. Cell debris was removed by centrifugation for 15 min at 1,900 × g.

The clarified lysate was stored at 4°C until purification and pre-treated again with DNase at a final concentration of 200 µg/mL (Merck, Darmstadt, Germany). DNase-resistant AAV particle titers were determined using quantitative polymerase chain reaction (qPCR) with primers and probes directed against the promoter region of the CMV promoter or the FIX transgene. The clarified lysate was purified using AVB Sepharose (GE Healthcare, Chicago, IL), and AAV titers were determined by qPCR.

Transient transfection and expression analysis

ExpressSf+ cells were adherently seeded and transfected using Cellfectin II (Thermo Fisher Scientific, Waltham, MA) with 1 pg of plasmid DNA encoding the inducible Nano-Luc reporter or the Rep gene. This method typically resulted in approximately 50% transfection efficiency. Cells were inoculated 1 day after transfection with 1% (v/v) concentration of passage 5 baculovirus. Up to 72 h after infection, secreted Nano-Luc reporter gene expression was measured using a luciferase assay kit (Promega, Madison, WI) and a Glomax luminometer (Promega, Madison, WI).

SDS-PAGE electrophoresis and western blot

Transfected cells (48 h after transfection) were lysed with Radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO) and protease inhibitors (Roche, Basel, Switzerland). The cell lysates were prepared with loading buffer containing the reducing agents β-mercaptoethanol and then separated on a 4%–12% Bis-Tris gel (BioRad, Hercules, CA). Electrophoresed proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using the Turbo-blot Turbo system (BioRad, Hercules, CA). The membrane was blocked using SuperBlock T20 blocking buffer (Thermo Fisher Scientific, Waltham, MA) before probing with α-AAV2-Rep, 303.9 (Progen, Heidelberg, Germany) and horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO). Bound antibodies were detected with the enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, Waltham, MA) and imaged using a Chemidoc imager (BioRad, Hercules, CA). AAV VP protein composition was determined by equal-volume loading of denatured AVB purified AAV particles, which were electrophoresed using Minit-protein Stain-free 4%–12% Bis–Tris polyacrylamide gels (BioRad, Hercules, CA). Gels were imaged using the Chemidoc system and analyzed using Image Lab software (BioRad, Hercules, CA).

In vitro potency assay

Huh7 cells were infected with AAV variants expressing secreted Nano-Luc at different multiplicities of infection (MOIs) (in GC/cell). Co-infection with a wild-type adenovirus (MOI 30) was performed to stimulate second-strand synthesis. Forty-eight hours after the start of infection, secreted Nano-Luc expression was measured using a luciferase assay kit (Promega, Madison, WI) and a Glomax luminometer (Promega, Madison, WI).

Generation of ExpressSf+ stable cells with inducible expression of AAV2 Rep/iRep Sf+ cells

The parental ExpressSf+ cells were transfected with a mix of DNA (1 pg DNA/cell): Cellfectin II liposome (Thermo Fisher Scientific, Waltham, MA). Cells were pressure selected and maintained in
blasticidin (25 μg/mL) antibiotic (InvivoGen, Toulouse, France) from day 3 after transduction for ~1.5 months, when the cell pool was banked.

**iRep stable cell pool pre-culture for SBR study**

To evaluate the stability and expression of the integrated Rep genes in the stable cell pool, gene expression was examined over different cell passages in shake flasks and expansion in an SBR. Pre-cultures of iRep 052 (iRep) stable pool cells were produced in 1-L shake flasks, and different passages were harvested for AAV production in SBRs. The SBR studies were performed without selective antibiotic pressure. iRep 052 cells (0.5 × 10^6 VC/mL in 2 L of SF-900 II medium [Thermo Fisher Scientific, Waltham, MA]) were added to a 2-L SBR with a 2,000-mL working volume (UniVessel SU bioreactor [Sartorius, Göttingen, Germany]). The SBR was operated under defined conditions that ensured reproducibility. After 48–72 h of cultivation, the viable cell density (VCD) of passage 4 cells was assessed. A calculated volume of cells was removed from the bioreactor and replaced with fresh SF-900 II medium (Thermo Fisher Scientific, Waltham, MA), resulting in a final VCD of 0.5–1.0 × 10^6 viable cells/mL (depending on culture conditions) in a working volume of 2 L. This cycle was repeated up to cell culture passage 9. AAVs were generated from different cell passages (up to passage 9) by inoculating iRep 052 cells with freshly amplified recombinant baculovirus stocks at passage 4 or 5, as described previously. After 72-h incubation at 28°C, cells were lysed. DNase digestion, clarification, and AAV titer estimation were performed as described above.

Material and reagents commercially procured will be shared upon request. Reagents such as AAVs generated by the authors will not be shared unless a memorandum of understanding (MOU) is signed between the research-sponsoring institution and the requester. Methods are available to disclose.

**Statistical methods**

Prism software v.9 (GraphPad Software, San Diego, CA, USA) was used for data analysis, using a one-way analysis of variance (ANOVA) multiple-comparisons test. *p = 0.05 and **p = 0.01 were considered statistically significant.

**Data availability**

All data associated with this study are present within the manuscript.

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**AUTHOR CONTRIBUTIONS**

F.M., F.L., H.R., and A.K. were involved in design of the research, interpretation of the data, and/or development of the paper. All authors provided critical feedback on the manuscript and approved the final version of the paper prior to submission.

**DECLARATION OF INTERESTS**

All authors are paid employees of uniQure biopharma B.V. uniQure has filed an international patent application, WO2021/198,510, related to the subject matter of this paper. Dr. Anggakusuma is a named inventor.

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