# Evaluation of an Early, Late, Very Late Expressed Rep in a Recombinant Baculovirus to Produce a More Potent AAV-based Gene Therapeutic in Insect Cells.

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## SUMMARY

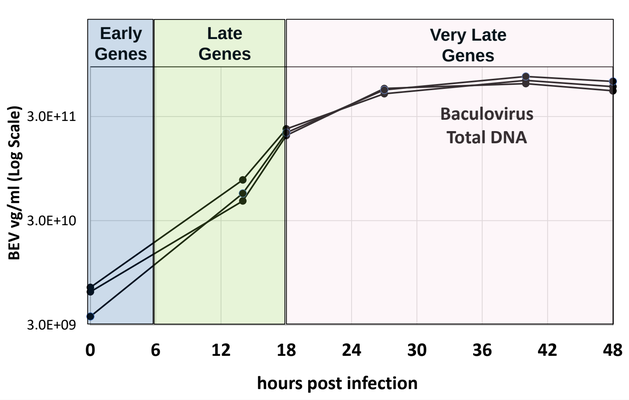
- Earlier expression of Rep in the BEV system correlated with higher amounts of ITR transgene replication and packaging in recombinant AAV9 (rAAV9) capsids
- ITR-SEAP transgene containing rAAV9 capsids made when Rep was expressed earlier had higher transduction potency on HEK-293 cells
- Despite having the same p10 promoter and translational context for the AAV9 capsid ORF, the earlier expression of Rep coincided with higher abundance of VP1 in assembled AAV capsids and this may have improved potency
- Earlier expression of Rep did not affect ITR transgene percent full in rAAV9 capsids
- Q-PCR data indicate some half length dsDNA genome packaging in capsids instead of full-length ssDNA genomes
- In CsCl density gradient AAV capsids fractions, half length genomes were less prevalent when Rep was expressed earlier

## ABSTRACT

The baculovirus expression vector (BEV) insect cell platform is an economical way to produce AAV-based human therapeutics at scale for large patient populations. The common method to produce AAV is to co-infect insect cells with a Rep/Cap and ITR-transgene BEVs. In this regime Rep and Cap genes are synchronously expressed from very late baculovirus polh and p10 promoters respectively. We have investigated asynchronously expressing Rep earlier than Cap to initiate ITR-transgene replication prior to the production of capsid proteins. This was achieved by expressing Rep from a synthetic early, late, very late (ELVL) baculovirus promoter based combining the OpMNPV gp64 promoter with the AcMNPV polh promoter. When this ELVL-Rep design was compared it to the common Polh-Rep design in BEV infected insect cells we observed comparable AAV titers and productivity. However, the AAV capsids generated by ELVL-Rep were more potent than the AAV capsids generated by the common Polh-Rep design.

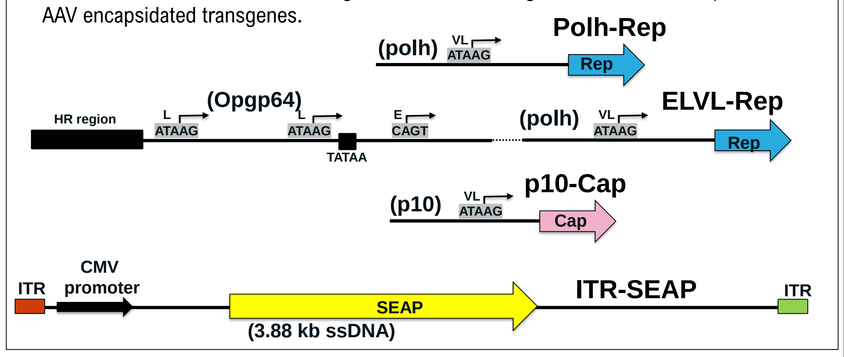
## Introduction

- The baculovirus replication cycle is a triphasic temporal cascade of gene expression that occurs over 48 hours post initial infection of insect cells. The three temporal classes of baculovirus genes are; Early genes, Late genes and Very Late genes. Most rAAV BEV expression designs use Very Late promoters for Rep and Cap genes. Baculovirus DNA replication initiates during late gene expression when the baculovirus DNA polymerase is present. rAAV transgene replication in recombinant BEVs requires the baculovirus DNA polymerase and the AAV Rep protein. We wanted to determine if expressing the rAAV Rep during the baculovirus DNA replication phase improved the replication of ITR-Transgenes and impacted the quality of recombinant AAV.



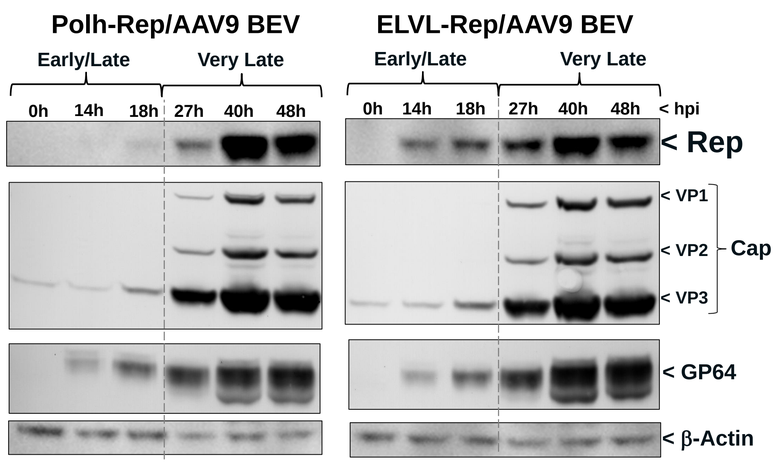

## Method

- The AAV Rep gene was engineered into the BEV with a synthetic baculovirus promoter that combined a baculovirus HR enhancer element, the Early/Late promoter of the *Op*MNPV baculovirus gp64 gene and the Very Late *Ac*MNPV polh gene promoter. This "ELVL-Rep" design was cloned into a recombinant BEV which also had a p10 Very Late promoter-AAV9 capsid (Cap) gene. We then compared the ELVL-Rep Rep/Cap BEV with a Rep/Cap BEV that had a Polh-Rep and p10-AAV9 Cap. The ELVL-Rep and Polh-Rep BEVs were co-infected with a recombinant BEV containing an ITR-SEAP transgene to evaluate for production of AAV encapsidated transgenes.




## Figure 1. Time Course Western Blot Analysis

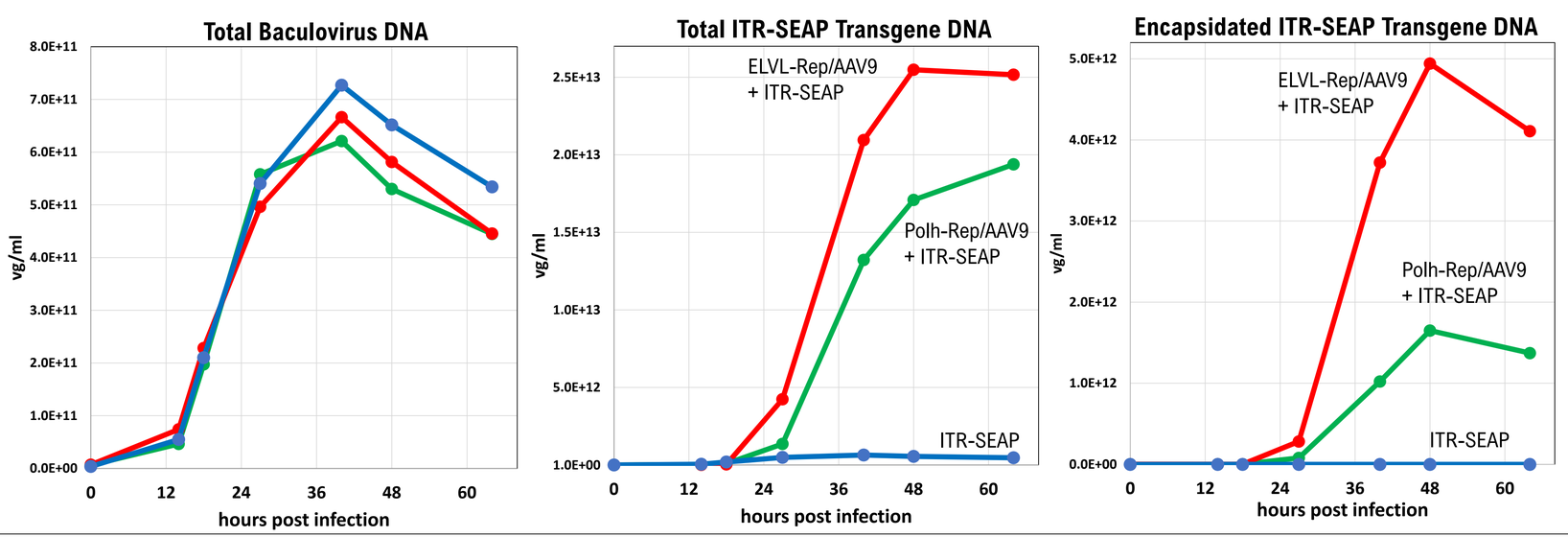
- Sf9 insect cells were synchronously infected with either the Polh-Rep/AAV9 BEV or the ELVL-Rep/AAV9 BEV. Infected cells were harvested at different hours post (hpi) infection and proteins were fractionated by denaturing SDS-PAGE followed by Western blot transfer to nitrocellulose membrane. Western blots were probed with protein specific antibodies and secondary HRP conjugated antibodies to enable visual detection by ECL.



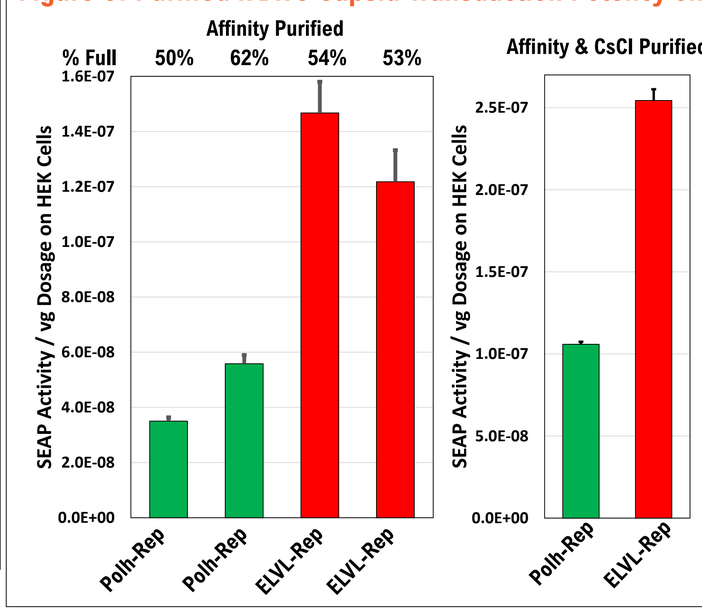

- The ELVL-Rep BEV expressed Rep earlier than the Polh-Rep BEV as designed

## Figure 2. Time Course of Total AAV and BEV Genome Replication & Encapsidated AAV Genomes

- Sf9 insect cells were synchronously infected with Polh-Rep/AAV9 BEV & ITR-SEAP BEV (—●—), ELVL-Rep/AAV9 BEV & ITR-SEAP BEV (—●—), or ITR-SEAP BEV (—●—). Infected cells were harvested at different times post infection and quantified by Q-PCR with primer probes specific the baculovirus genome or the ITR-SEAP transgene ORF. Total DNA in cells and DNaseI resistant encapsidated DNA were measured.



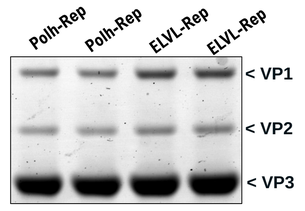

## Figure 3. Purified rAAV9 Capsid Transduction Potency on HEK-293T Cells

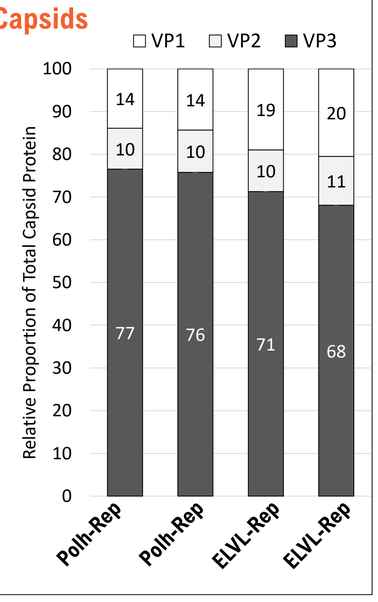



- Sf9 cells were infected with either Polh-Rep BEV & ITR-SEAP BEV or with ELVL-Rep BEV & ITR-SEAP BEV.
- rAAV9 capsids were purified by immunoaffintity chromatography and then purified by CsCl density gradient ultracentrifugation.
- HEK-293T cells were transduced with AAV capsids.
- HEK cell supernatants were assayed for alkaline phosphatase activity.

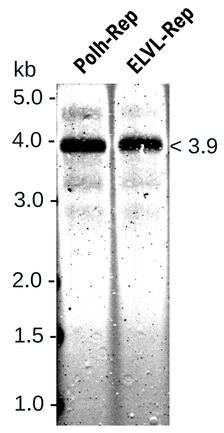
## Figure 4. Relative VP1, VP2, VP3 Abundances in Affinity Purified rAAV9 Capsids

- Affinity purified rAAV9 capsids were fractionated in a reducing SDS-PAGE gel and Coomassie stained.
- The Coomassie stained gel was imaged, and proteins were quantified using ImageJ software



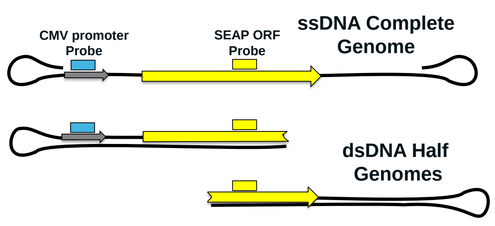




## Figure 5. Denaturing Electrophoresis of Packaged Genomes from rAAV9 Capsids




- Sf9 cells were infected with either Polh-Rep BEV & ITR-SEAP BEV or with ELVL-Rep BEV & ITR-SEAP BEV.
- Affinity purified rAAV9 capsids were mixed with SDS/NaOH denaturing dye and liberated 3.88 kb ITR-SEAP transgene genomes were fractionated by agarose gel electrophoresis.
- Polh-Rep and ELVL-Rep rAAV9 samples appeared similar

## Figure 6. Dual Probe Q-PCR Analysis of Transgene in rAAV CsCl Density Gradient Fractions




- Sf9 cells were co-infected with either Polh-Rep BEV & ITR-SEAP BEV or with ELVL-Rep BEV & ITR-SEAP BEV.
- Affinity-purified AAV9 capsids from Sf9 lysates were fractionated by CsCl density gradient ultracentrifugation.
- CsCl fractions were probed with either CMV promoter or SEAP ORF specific Q-PCR probes.
- CMV and SEAP Q-PCR titers are the same when complete genomes are packaged
- SEAP Q-PCR titers will be higher when partial dsDNA genomes are packaged
- Fractions 8-11 showed ELVL-Rep generated capsids that had more complete ITR-SEAP transgenes packaged