Journal of Biotechnology 255 (2017) 37–46

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Improved pFastBac™ donor plasmid vectors for higher protein production using the Bac-to-Bac® baculovirus expression vector system

Hui Shang[a,b,1], Tyler A. Garretson[b,1], C.M. Senthil Kumar[b,2], Robert F. Dieter[b], Xiao-Wen Cheng[a,b,*]

[a] Graduate Program in Cell, Molecular, and Structural Biology, Miami University, Oxford, OH, 45056 USA
[b] Department of Microbiology, Miami University, Oxford, OH, 45056 USA

ARTICLE INFO

Keywords:
Cis element
Expression vector
Polyadenylation
Protein expression
Untranslated region

ABSTRACT

The Autographa californica multiple nucleopolyhedrovirus (AcMNPV)-based Bac-to-Bac® expression system consists of a bacmid and five pFastBac™ donor transfer vectors. It has been widely used for eukaryotic gene expression in insect cells to elucidate gene function in biotechnology laboratories. The pFastBac™ vectors contain a 50 bp AcMNPV polyhedrin (*polh*) promoter and a 127 bp SV40 polyadenylation (pA) signal for cloning a gene of interest into the bacmid, resulting in unsolved lower gene expression levels than the wild type (wt) AcMNPV in insect cells. Therefore, the purpose of this research is to understand why the Bac-to-Bac system produces lower gene expression levels. Here, we determined that bacmids transposed with pFastBac™ vectors produced 3–4 fold lower levels of certain proteins than the wt AcMNPV. We found that an 80 bp *cis* element 147 bp upstream of the 50 bp *polh* promoter and a 134 bp *polh* pA signal are required in pFastBac™ to achieve bacmid protein expression levels equivalent to wt AcMNPV in High Five insect cells. Therefore, researchers currently using pFastBac™ vectors for protein expression can transfer their genes of interest into the improved vectors in this report to elevate protein expression yields in insect cells to reduce protein production costs.

1. Introduction

Insect-specific baculoviruses in the family *Baculoviridae* have circular, double-stranded, DNA genomes in the range of 88–180 kb (Herniou et al., 2012). Baculovirus research focuses on molecular and genetic studies, protein display as well as eukaryotic gene expression (Grabherr and Ernst, 2010; Passarelli and Miller, 1993; Rodems and Friesen, 1993; Smith et al., 1983). Of all the baculoviruses, Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the most studied, and it is the foundation of the baculovirus expression vector system (BEVS) (Hopkins et al., 2010). AcMNPV is preferred because it has the propensity to replicate efficiently in IPLB-Sf21-AE (Sf21), Sf9 (cloned from Sf21) and BTI-Tn-5B1-4 (High Five™) insect cells and can produce a high concentration or titer of budded virus (BV) (Cheng et al., 2013; Granados et al., 1994; Summers and Smith, 1987).

AcMNPV cell infection is accompanied by high levels of expression of a virus-encoded protein called polyhedrin, which forms large paracrystalline particles of 0.5–15 μm in diameter in the nuclei during late phase infection (Tanada and Haya, 1993). Production of these particles, formally known as polyhedra, requires large amounts of polyhedrin protein. This high level of protein is generated from a huge pool of mRNA produced under a very strong polyhedrin (*polh*) promoter. High-level *polh* promoter-mediated transcription requires 19 late expression factors (*lef*), one very late expression factor-1 (VLF-1), and a multi-functional protein (FP25K) (Cheng et al., 2013; Lu and Miller, 1995). Due to the high protein expression level mediated by the *polh* promoter in insect cells, AcMNPV has been used commercially to produce prophylactic vaccines, such as Cervarix® to fight against cervical cancer caused by human papillomavirus (HPV) and FluBlok® to reduce influenza virus infection in humans (Cox and Hashimoto, 2011; Harper, 2009).

The most widely used AcMNPV *polh* promoter-based BEVS in the biotech industry and research laboratories is the Bac-to-Bac system®, constructed in the late 1990's and marketed by Invitrogen (Carlsbad, CA) (Luckow et al., 1993). The Bac-to-Bac system® involves site-specific transposition between a clonal copy of the AcMNPV genome (bacmid)

Abbreviations: polh, polyhedrin; pA, polyadenylation; AcMNPV, Autographa californica multiple nucleopolyhedrovirus; HPV, human papillomavirus; UTR, untranslated region

* Corresponding author at: Department of Microbiology, 32 Pearson Hall, Miami University, Oxford, OH, 45056, USA.
E-mail address: Chengx@miamioh.edu (X.-W. Cheng).

[1] Both authors contributed equally to this manuscript.
[2] Current address: Indian Institute of Spices Research, Marikunnu, P. O., Kozhikode, Kerala, 673012, India.

http://dx.doi.org/10.1016/j.jbiotec.2017.06.397
Received 18 January 2017; Received in revised form 14 April 2017; Accepted 15 June 2017
Available online 20 June 2017
0168-1656/ © 2017 Elsevier B.V. All rights reserved.

and a pFastBac™ donor plasmid to produce recombinant bacmid DNA in DH10Bac™ *Escherichia coli* cells with the aid of a helper plasmid. The helper plasmid expresses a transposase to transfer the gene of interest from the pFastBac™ donor plasmid to a specific site within the bacmid *in vivo*. The Bac-to-Bac® system eliminates the lengthy (up to 6 months) plaque-purification step required by the conventional homologous recombination method to produce the recombinant virus (Kitts et al., 1990; Smith et al., 1983). Due to the ease with which foreign genes can be cloned into the AcMNPV bacmid, the Bac-to-Bac™ system along with its five pFastBac vectors (pFastBac1, pFastBac Dual, and pFastBacHT-a, −b, −c) have become a powerhouse second only to the *E. coli* expression system for eukaryotic protein structure studies, as shown in the worldwide Protein Data Bank (Gabanyi and Berman, 2015).

It was reported that the Bac-to-Bac® system expresses lower protein yields than the conventional BEVS (Gomez-Sebastian et al., 2014). However, the elements at the *polh* locus that regulate protein expression yields are unknown to date. Therefore, the aim of this project is to identify these elements to improve protein production yields in insect cells.

In this report, we show that certain protein expression levels using the Bac-to-Bac® system are not as high as the wild type (wt) AcMNPV in certain insect cell lines. The donor plasmid vectors such as pFastBac™1 and pFastBac™ Dual lack an 80 bp *cis* DNA element and contain a 127 bp SV40 polyadenylation (pA) signal. When the 80 bp *cis* DNA element was inserted upstream of the 50 bp *polh* promoter and the SV40 pA was replaced with an AcMNPV *polh* pA signal in pFastBac™1 and pFastBac™Dual, certain protein expression levels equaled that of the wt AcMNPV in High Five cells using the Bac-to-Bac® system.

2. Materials and methods

2.1. Cell lines, viruses and plasmids

Insect cell lines used in this project included High Five, Sf21 and Sf9 cells, all obtained from Invitrogen. The wild type (wt) virus used in this study was AcP3, a plaque-purified AcMNPV E2 strain originally received from Dr. Max Summers of Texas A & M University (Cheng et al., 2013). Plasmids and bacterial strains used were pFastBac™1, pFastBac™Dual, and the host bacterial strain DH10Bac, obtained from the Bac-to-Bac® system kit (Invitrogen).

2.2. Modification of pFastBac™1 to produce improved donor transfer vector pFastBac-M1

Although the exact reason for the poorer protein expression yield of the Bac-to-Bac® system compared to the wt AcMNPV was unknown, we first amplified a 1.5 kb DNA fragment by PCR using a forward primer AcPolh-F-XbaI and reverse primer AcPolh-R-XhoI and cloned it into the pGEM-T Easy vector (Promega, Madison WI) to produce pGEM-PolhE (Table 1). This 1.5 kb fragment contained the *polh* ORF with 319 bp of DNA sequence upstream of the *polh* ORF start codon ATG. The 319 DNA sequence included the 50 bp *polh* promoter and additional upstream sequences. The 1.5 kb fragment also included the *polh* downstream untranslated region (UTR) containing a 472 bp *polh* polyadenylation signal (pA) between nucleotides (ntd) 739–1211 (Figs. 1 A1 and 2).

To evaluate the effect of this 1.5 kb fragment on polyhedrin protein expression using the Bac-to-Bac® system, the 1.5 kb fragment from pGEM-PolhE was retrieved with restriction endonucleases (REN) XbaI and XhoI (NEB, Ipswich, MA) and inserted into these sites in pFastBac™1 to generate a clone (pAcBac-PolhE) (Fig. 1B; Fig. 2). Competent DH10Bac cells were transformed with pAcBac-PolhE and recombinant bacmid clones were screened and selected using X-gal and IPTG on antibiotic plates, following conditions recommended by Invitrogen. One confirmed recombinant bacmid with the 1.5 kb *polh* fragment was used to transfect High Five cells to generate AcBac-PolhE budded virus (BV).

Table 1
A list of primers used in this study.

Primer names	Primer sequences (restriction enzyme sites underlined)
AcPolh-F-XbaI	5′-tctagagcatagtacgcagcttcttc-3′
AcPolh-R-XhoI	5′-ctcgagtaacacgcccgatgttaaa–3′
AcPolh-F-EcoRI	5′-gaattcatgccggattattcatacc-3′
Hind-F	5′-ataaagctaggacatatttaacatcgggcgtgttag-3′
Hind-R	5′-atgtcctagctttatttaacgtgtttacgtcgagtc-3′
Polh-F1-HindIII	5′ −cccaagcttcttgtagcagcaatctag-3′
Polh-R-BamH1	5′-cggatccaatatttataggtttttttattacaaaactg-3′
Promoter-R1	5′- gttaatccggtgctgc-3′
Promoter-R2	5′-aaaagggaggtgaactg-3′
Promoter-R3	5′- gtctcattacaatggctg-3′
Promoter-R4	5′-ctatatattgatagacatttccag-5′
promoter-F	5′-gatatcatggagataattaaaatg −3′
CisF1	5′-gtagcatagtacgcagcttctt-3′
Polh-R-BamH1	5′-cccggatccaatatttataggtttttttattacaaaactg-3′
Ac-Polh-F-EcoRI	5′-gaattcatgccggattattcatacc-3′
Ac-Polh-R-XbaI	5′ −tctagattaatacgccggaccag-3
HPV16 L1-F1-XbaI	5′-tctagaatggaggtgacttttatttacatc-3′
HPV16 L1-R1-HindIII	5′-aagcttttacagcttacgtttttgcg-3′
AcpolhF	5′-cccagatctatgccggattattcatac-3′
AcpolhR1	5′-ggggtcgacgataacggcacctaaat-3′

The AcBac-PolhE construct had two *polh* promoters; one from the parental pFastBac1 vector and one from the upstream sequences of the 1.5 kb DNA fragment (Fig. 2). Also, AcBac-PolhE had two pAs; the SV40 pA from the pFastBac1 vector and the *polh* pA from the 1.5 kb DNA fragment (Fig. 2). To delineate the functionality of the 1.5 kb insert in AcBac-PolhE, the vector *polh* promoter and SV40 pA of pAcBac-PolhE were deleted. The vector *polh* promoter was deleted by digestion of pAcBac-PolhE with BstZ17I and XbaI, followed by Klenow enzyme treatment and self-ligation with T4 DNA ligase (NEB) to generate the plasmid pAcBac-PolhED. The SV40 pA was deleted by digestion of pAcBac-PolhED with XhoI and AvrII, followed by Klenow enzyme treatment and self-ligation with T4 DNA ligase to generate plasmid pAcBac-PolhED-XX. To use the unique HindIII site of the donor vector for cloning genes, the HindIII site in the UTR of *polh* was mutated from AAGCTT to AAGCTA by site-directed mutagenesis using the primer pair Hind-F and Hind-R (Table 1) and the QuikChange II Site–Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). This resulted in the generation of the plasmid pAcBac-PolhED-XXH (Fig. 2), which was necessary for the subsequent steps of engineering pFastBac1-M1.

Inverse PCR was used to produce pFastBac-M1. A pair of primers (Polh-F1-HindIII and Polh-R-BamH1) using pAcBac-PolhED-XXH DNA as a template and the high fidelity *pfu* enzyme (Agilent Technologies) produced a linear DNA fragment that was digested with HindIII and BamHI (Table 1). The digested linear DNA fragment was then ligated with T4 DNA ligase into the multiple cloning site (MCS) fragment retrieved from pFastBac™1 digested with HindIII and BamH1, thus producing pFastBac-M1 (Fig. 1B).

To determine if all the upstream sequences of the *polh* promoter were required for the improved protein expression yield of pFastBac-M1, four reverse primers (Promoter-R1, −R2, −R3 and −R4, Table 1) were designed to map the 240 bp region upstream of the promoter (Fig. 1A2; Table 1). Each of the four reverse primers was paired with primer promoter-F in inverse PCR, in order to delete a defined length of DNA sequence in the 240 bp region immediately upstream of the *polh* promoter, using pAcBac-PolhED-XXH DNA as a template with the *pfu* DNA polymerase. The promoter-F and promoter-R3 reaction ultimately produced the clone pAcBac-MR3-Polh, which was missing 144 bp (ntd −240 to −96, Fig. 1A3) of the 240 bp upstream region but maintained the rest of the plasmid sequences, including an 80 bp DNA sequence upstream of the 50 bp *polh* promoter and the *polh* pA (Fig. 2). Competent DH10Bac™ cells were transformed with pAcBac-MR3-Polh DNA to generate AcBac-MR3-Polh. This bacmid DNA was transfected into High Five cells to produce BV for infection of High Five cells, which were

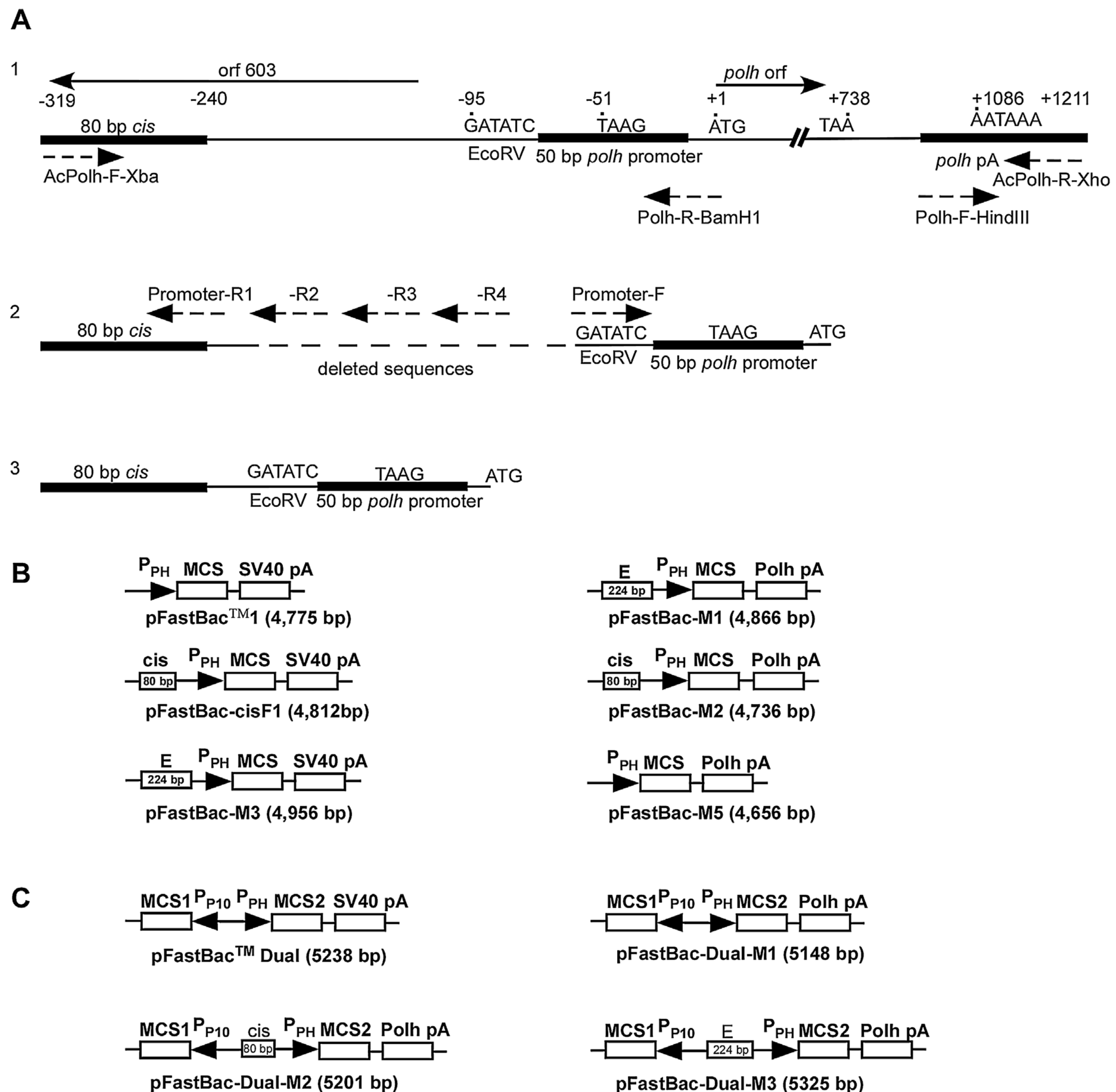

Fig. 1. Manipulation of the polyhedrin (polh) locus of AcMNPV for improved donor plasmid vector construction (not drawn to scale). A, The *polh* locus and flanking regions. 1, Schematic of the polh gene and the flanking regions containing the 80 bp cis element, the 50 bp AcMNPV polh promoter and the *polh* polyadenylation signal (polh pA). 2, Deletion of the upstream sequences of the 50 bp AcMNPV polh promoter in inverse PCR. 3, Fusion of the 80 bp cis element with the 50 bp AcMNPV polh promoter. B, A list of donor plasmid vectors derived from pFastBac1. pFastBac1 is the commercial vector that served as the source for construction of the different donor plasmid vectors developed in this study. E, extended sequences. Cis, cis element. C. Comparison of commercial dual vector pFastBac-Dual with improved dual expression vectors. E, extended sequences. Cis, cis element.

used to compare polyhedra production with AcP3.

2.3. Generation of additional donor plasmid vectors

To generate pFastBac-cisF1, a pair of primers (CisF1 and Polh-R-BamH1) and pAcBac-MR3-Polh DNA as the template were used to amplify a 136 bp (ntd +2 to −319, Fig. 1A3) fragment that contained the 80 bp *cis* element and the *polh* promoter, with the ATG sequence of the *polh* ORF mutated to ATT (in Polh-R-BamH1) (Table 1). This PCR product was agarose gel purified and cloned into the SnaBI and BamHI sites of pFastBac™1 to generate the pFastBac-cisF1 donor plasmid vector (Fig. 1B). Ultimately, this vector had an 80 bp *cis* element upstream of the *polh* promoter and SV40 pA.

To generate pFastBac-M2, a fragment containing the 80 bp *cis* element and the *polh* promoter was retrieved by digestion of pFastBac-cisF1 with SnaBI and BamHI. This fragment was then inserted into the SnaBI and BamHI sites of pFastBac-M1, thus producing pFastBac-M2 (Fig. 1B). pFastBac-M2 contained the 80 bp *cis* element upstream of the *polh* promoter and *polh* pA (Fig. 1B).

To generate pFastBac-M3, the extended *polh* upstream fragment (224 bp plus *polh* promoter) from pFastBac-M1 was retrieved by digestion with SnaBI and BamHI, and inserted between the SnaBI and BamHI sites of pFastBac™1, thus producing pFastBac-M3 (Fig. 1B). pFastBac-M3 had an 224 bp extended sequence upstream of the *polh* promoter and SV40 pA (Fig. 1B).

To generate pFastBac-M5, pFastBac™1 and pFastBac-M1 were separately cleaved by a double-digestion with BamHI and EcoRV, and the digested DNA fragments were separated by agarose gel electrophoresis. The fragment containing the 50 bp *polh* promoter and Tn7R from pFastBac™1 and the fragment containing the *polh* pA and Tn7L were both gel-extracted and ligated by T4 DNA ligase for transformation to produce pFastBac-M5 (Fig. 1B).

2.4. Modification of a dual expression vector, pFastBac Dual

To improve this dual vector, the AcMNPV *polh* pA sequence was first

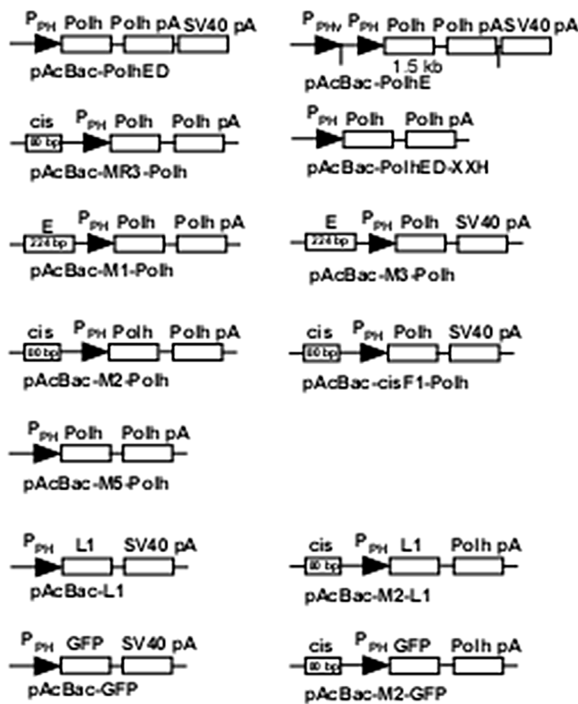

Fig. 2. A list of transfer donor vecctors that were used for the production of the viruses. P_{PH}, polyhedrin gene promoter. $P_{PHv,}$ polyhedrin gene promoter of the vector. Polh, polyhedrin gene. Polh pA, polyhedrin gene polyadenylation signal sequence. SV40 pA, SV40 polyadenylation signal sequence. E, extended sequences. Cis, cis element. L1, HPV16 L1 major capsid protein gene. GFP, green fluorescent protein gene.

cloned into pFastBac Dual. This was achieved by a digestion of pFastBac-M2 and pFastBac Dual separately with HindIII and EcoRV. The DNA fragments were separated by agarose gel electrophoresis. The 3374 bp HindIII/EcoRV fragment that contained the *polh* pA sequence and the 1774 bp HindIII/EcoRV fragment that contained the MCS and the *p10* promoter were gel purified and ligated for the production of pFastBac-Dual-M1 (Fig. 1C).

To insert the 80 bp *cis* element into pFastBac-Dual-M1, the plasmid pFastBac-M2 was digested with SnaBI and BamHI, and pFastBac-Dual-M1 was digested with BstZ17I and BamHI, followed by agarose gel electrophoresis. The 201 bp fragment containing the 80 bp *cis* element and the 50 bp *polh* promoter from the pFastBac-M2 digestion, plus the larger fragment from the pFastBac-Dual-M1 digestion, were gel purified and ligated for the production of pFastBac-Dual-M2 (Fig. 1C). To further confirm the significance of the 80 bp *cis* element in enhancing the 50 bp *polh* promoter activity, the 224 bp *polh* upstream fragment in pFastBac-M1 was retrieved by a digestion of SnaBI and BamHI and ligated into the BstZ17I and BamHI sites of pFastBac-Dual-M1 by T4 DNA ligase (NEB) to produce pFastBac-Dual-M3 (Fig. 1C).

2.5. Cytoplasmic particle purification and identification

High Five cells at 5×10^5 cells per 35 mm tissue culture dish were infected with AcP3, AcBac-PolhE, AcBac-PolhED or AcSDP33-35 at an MOI of 1. At day 3 post-infection (P.I.) or when cytoplasmic particles appeared, the medium was removed and 1% SDS was added to lyse the cells. The lysates were filtered with a Whatman Nuclepore Track-Etch membrane (pore size 8 μm). Particles retained on the membrane were washed with TE in 1.5 ml microcentrifuge tubes. An aliquot from each infection was examined under a microscope for the purity of cytoplasmic particles. To determine the nature of these cytoplasmic particles, the purified particles were first solubilized by treatment with 0.1 M Na_2CO_3 (pH 10.5) (Cheng et al., 1998). The concentrations of the solubilized proteins were determined by a Bradford protein assay kit (Bio-Rad) following recommended procedures. About 50 μg of solubilized particle proteins from each virus infection was analyzed on 12% SDS-PAGE. A duplicate gel was used to transfer proteins to a Protran nitrocellulose membrane (Scheicher & Schuell, Keene, N.H.) for antibody detection. An anti- polyhedrin polyclonal antibody of *Choristoneura fumiferana* MNPV was provided by Dr. Basil Arif of the Great Lake Forestry Center, Canada and was used at 1:10000 to bind to proteins on the blot. Following the primary polyhedrin antibody binding, the blot was incubated with horseradish peroxidase (HRP)-linked anti-rabbit IgG at 1:1000 (Cell Signaling, Danvers, MA). Antibody binding was visualized using HRP color development Reagent (Bio-Rad, Hercules, CA) in a western blot analysis.

2.6. Protein expression assay

For polyhedrin protein expression comparisons between different viral constructs, the ORF of AcMNPV *polh* was amplified using a pair of oligo primers (Ac-Polh-F-EcoRI and Ac-Pol-R-XbaI, Table 1**)** with *Taq* and cloned into pGEM-T Easy (Promega). After sequencing confirmation, the *polh* ORF was digested with EcoRI and XbaI to clone into commercial pFastBac™1 and the improved donor vectors in order to construct pAcBac1-Polh, pAcBac-M1-Polh, pAcBac-cisF1-Polh, pAcBac-M2-Polh, pAcBac-M3-Polh, and pAcBac-M5-Polh (Fig. 2). To generate viruses for *polh* expression comparison, competent DH10Bac cells were transformed with plasmid DNA from these constructed vectors to produce recombinant bacmids, following the procedures recommended by Invitrogen. High Five cells were transfected with recombinant bacmids by the polyethylenimine (PEI) method to produce BVs of AcBac-Polh, AcBac-M1-Polh, AcBac-cisF1-Polh, AcBac-M2-Polh, AcBac-M3-Polh and AcBac-M5-Polh (Ogay et al., 2006). High Five cells were infected with AcP3, AcBac-PolhE, AcBac-PolhED at an MOI of 1 in triplicate. At day 4 P.I., infected cells were photographed and the media were removed and replaced with 1 ml of 1% SDS to lyse the cells and release polyhedra by rocking for 30 min at room temperature (23 °C). Polyhedra yields were enumerated by taking images of polyhedra and counted using the OpenCFU program (Geissmann, 2013). Due to the size differences of polyhedra from the various viral infections, the purified polyhedra from each infection were solubilized in 0.1 M Na_2CO_3 (pH 10.5) (Cheng et al., 1998). Bovine serum albumen (BSA) of known concentration (NEB) was serially diluted with 0.1 M Na_2CO_3 (pH 10.5). A Bio-Rad protein assay dye reagent concentrate system was used to construct the standard curve and estimate the protein yield of solubilized polyhedra for statistical comparison.

To support *polh* expression differences between AcBac-Polh and AcBac-M2-Polh in High Five cells, the green fluorescent protein (GFP) gene was used for the comparison. The GFP gene was retrieved from pBlueGFP by double digestion of BamHI/XhoI and cloned between the BamHI and XhoI sites of pFastBac-M2 to generate pAcBac-M2-GFP (Fig. 2) (Cheng et al., 2001). Ultimately, AcBac-M2-GFP virus was generated in High Five cells using the Bac-To-Bac system following procedures recommended by Invitrogen. To compare GFP expression yields between the two vectors, AcBacGFP from Cheng et al. and AcBac-M2-GFP were used to infect High Five cells in triplicate as described above (Fig. 2) (Cheng et al., 2013). At day 4 P.I., GFP expression yields from High Five cells infected with the two viruses were estimated using a FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). GFP expression differences were analyzed using Excel (Microsoft).

In addition to the use of polyhedrin and GFP for protein expression comparison between different donor plasmid vectors, an HPV16 major capsid protein L1 was used to quantify protein expression levels of the donor vectors developed in this project. The L1 gene was amplified using a pair of primers (HPV16 L1-F1-XbaI and HPV16 L1-R1-HindIII; Table 1) with plasmid pML2D, which contains a copy of HPV16 L1 (Durst et al., 1983), as the template in PCR. The PCR product was cloned into the pGEM-T Easy vector (Promega) and confirmed by sequencing. The L1 gene was retrieved by digestion with XbaI and HindIII

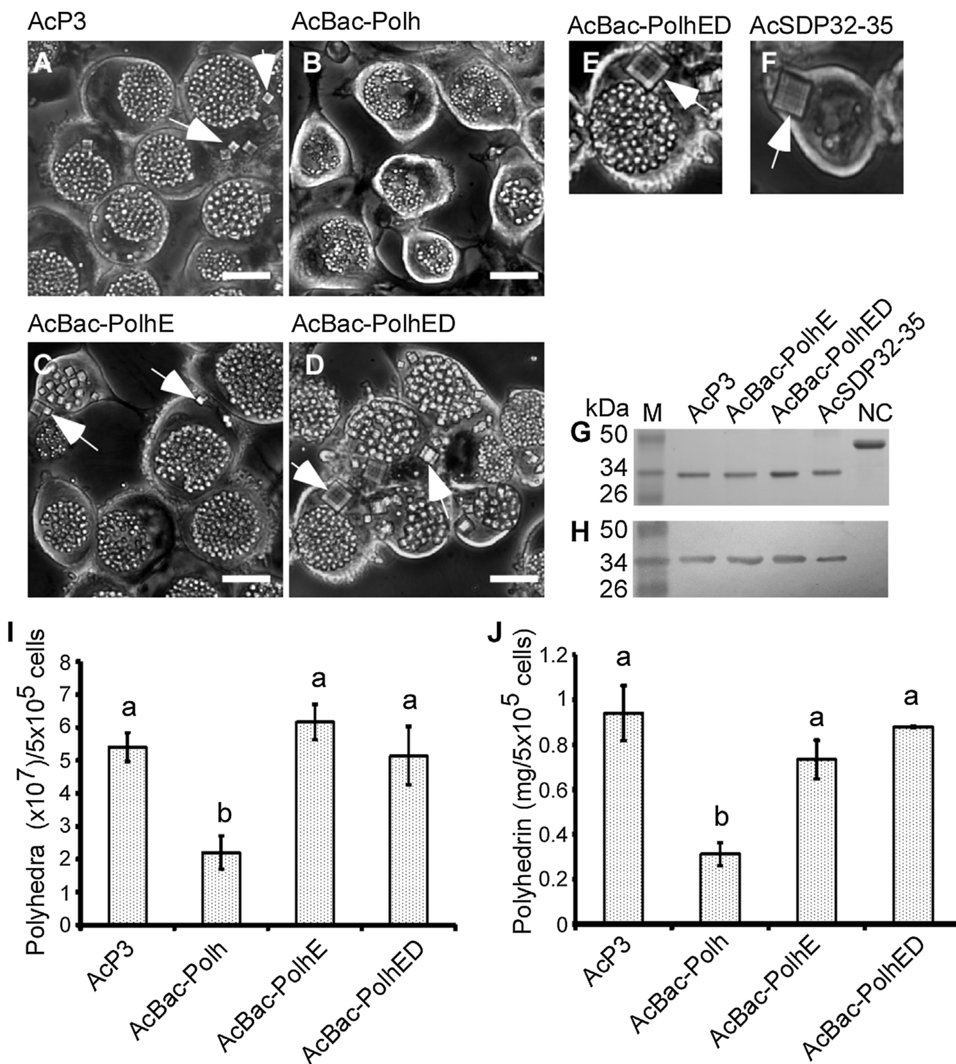

Fig. 3. Comparison of polyhedrin protein expression in High Five insect cells infected with the wild type AcMNPV and pFastBac1-based bacmid. A, B, C and D, Phase contrast microscopy of polyhedra in High Five cells infected with wt AcMNPV (AcP3), AcBac-Polh derived from pFastBac1, AcBac-PolhE containing an extend *polh* gene fragment in AcMNPV-based bacmid and AcBac-PolhED with vector polh promoter detected, respectively. Arrows point to cytoplasmic crystal formation. E and F, comparison of cytoplasmic crystals (an enlargement of a cell in D) in High Five cells infected with AcBac-PolhED and AcSDP32-35 having a mutated nuclear localization signal in the *polh* gene. G and H, identification of cytoplasmic crystals by SDS-PAGE and western blot with an anti-polyhedrin antibody, respectively. NC, negative control. I and J, Quantitative comparison of the production of polyhedra (I) and polyhedrin protein (J) in High Five cells infected with different viruses that contain polh expressed as means ± standard error of the mean. Means were calculated from three independent cell infections.The means with the same letter had no significant difference at p = 0.05.

(NEB) and ligated into the XbaI/HindIII sites of pFastBac™1 and pFastBac-M2 to produce pAcBac1-L1 and pAcBac-M2-L1, respectively, for transformation of DH10Bac cells (Fig. 2). The resulting recombinant bacmid AcBac-L1 and AcBac-M2-L1 DNAs were transfected into High Five cells as described above and BV was harvested for subsequent infection. High Five cells in 6-well plates were infected in triplicate with various viruses constructed for L1 expression at an MOI of 1 (O'Reilly et al., 1992). High Five cells infected with AcBacGFP lacking L1 were used as a negative control (Cheng et al., 2013). At 72 h P.I., cells were harvested and lysed in a radioimmunoprecipitation assay buffer (RIPA; 25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and sonicated for SDS-PAGE. Equal amounts of proteins in the lysates (100 μg) were loaded on two identical acrylamide gels for protein separation. Proteins on the gel were then transferred to nitrocellulose membranes. One blot was probed with a mouse anti-HPV16 L1 monoclonal antibody (BD Pharmingen, San Jose, CA) for L1 expression yield comparison and the other blot was probed with a *Naegleria gruberi* alpha-tubulin monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa) for protein loading normalization. A goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (Bio-Rad) was used to bind to the primary antibodies (L1 and tubulin) for color development. The blots were photographed, the L1 and tubulin signals were quantified by densitometry using ImageJ, and results were statistically tested using the T-test of Excel (Microsoft) (Schneider et al., 2012).

2.7. Transcriptional analysis

To understand whether protein expression differences of these vectors are correlated with gene transcription, *polh* mRNA levels between AcBac-Polh and AcBac-M2-Polh were compared. High Five cells were infected separately with AcBac-Polh and AcBac-M2-Polh at an MOI of 5 in triplicate (Fig. 2). At day 4 P.I., infected cells were harvested and total RNA extracted using Tri Reagent (Molecular Research Center, Cincinnati, OH) following the protocol recommended by the reagent provider. Total RNA (1 μg) from each isolation was digested with RQ1 RNase-Free DNase (Promega) to remove DNA contamination following the protocol recommended by the enzyme provider. The DNA-free RNA was used for cDNA synthesis using primers oligo dT and 28S-R with a DyNAmo cDNA synthesis kit (NEB) (Xue et al., 2010). cDNA was used as the template in *polh* mRNA level comparison between AcBac-Polh and AcBac-M2-Polh normalized to the housekeeping 28S gene. The 28S-F and 28S-R primer pair was used for 28S and AcpolhF and AcpolhR1 primer pair for *polh* in separate reactions in the same run for real-time qPCR analysis using a Bio-Rad iCycler iQ system according to Xue and Cheng with the modification only at the annealing temperature that was changed to 64.5 °C (Xue and Cheng, 2010). The inverse of the threshold cycle (Ct) between *polh* mRNA levels of AcBac-Polh and AcBac-M2-Polh relative to 28S Ct was statistically analyzed by the T-test of Excel (Microsoft).

3. Results

3.1. Cloning of a polh fragment into pFastBac™1 yielded polyhedrin protein expression levels similar to wt AcMNPV levels

To understand why protein expression levels of the Bac-to-Bac® system are lower than in wt AcMNPV (AcP3) (Cheng et al., 2013), a 1.5 kb *polh* fragment was cloned into pFastBac™1 (Invitrogen) to produce pAcBac-PolhE for bacmid virus, AcBac-PolhE generation (Fig. 2). This 1.5 kb *polh* fragment included the *polh* open reading frame (ORF) in addition to 319 bp (ntd +1 to −319) upstream and 473 bp (ntd +738 to +1211) downstream sequences (Fig. 1A1). At the same time, a DNA fragment containing only the *polh* ORF was cloned into pFastBac™1 to produce pAcBac-Polh for the generation f a bacmid virus, AcBac-Polh (Figs. Fig. 1A1, 2 and 2). Infection of High Five cells with AcP3, AcBac-PolhE, and AcBac-Polh resulted in no apparent difference between the production of polyhedra in AcP3 and AcBac-PolhE infected samples, while AcBac-Polh infection had clearly reduced levels of polyhedra (Fig. 3A–C).

In addition, some High Five cells infected with AcP3 and AcBac-PolhE generated cube-shaped cytoplasmic particles in the size range of 2–12 μm in diameter (Fig. 3A, C). These particles appeared indistinguishable from the cytoplasmic polyhedra formed by polyhedrin lacking the nuclear localization signal (NLS), which are produced by AcSDP32-35 infection in High Five cells (Fig. 3A, C, E, F) (Jarvis et al., 1991). Unlike what was observed in the AcBac-PolhE cell infection, High Five cells infected with AcBac-Polh did not produce these cytoplasmic particles (Fig. 3B). When the particles from AcP3, AcBacPolhE, and AcSDP32-35 infected High Five cells were purified by filtration and analyzed by SDS-PAGE, they all showed similar mobility, suggesting they may be composed of the polyhedrin protein (Fig. 3G). These large cytoplasmic particles were specifically recognized by an anti-polyhedrin antibody in a western blot analysis, and thus confirmed to be composed of polyhedrin (Fig. 3H). Sf21 and Sf9 cells infected with either AcP3 or AcBac-PolhE did not produce these cytoplasmic polyhedra (data not shown), suggesting that High Five cells could support higher polyhedrin expression than either Sf21 or Sf9 cells. Phenotypic variation among the polyhedra produced during High Five infection with the different viral constructs was informative but not quantifiable.

To provide quantitative insight, the levels of polyhedra produced during infection with the different virus constructs were determined. When High Five cells were infected with AcP3, AcBac-PolhE, and AcBac-Polh, no differences in the number of polyhedra produced were detected between AcP3 and AcBac-PolhE; however, the number of AcBac-Polh polyhedra recovered was about 3-fold less than in the other infections (Fig. 3I). Since polyhedra from the three virus infections were different in sizes (Fig. 3A–C), the polyhedra from each viral infection were solubilized (Cheng et al., 1998) and the polyhedrin protein yields were estimated by the Bradford method. As with the number of polyhedra, the polyhedrin protein yields were similar between AcP3 and AcBac-PolhE, and both had about 3-fold more than AcBac-Polh (Fig. 3I, J). Collectively, these data suggest that DNA sequences present in pFastBac-PolhE but missing from pFastBac™1 and from the *polh* ORF can provide higher polyhedrin production using the Bac-to-Bac® system in High Five cells.

3.2. PFastBac-PolhE exhibited polyhedrin protein expression levels similar to AcP3 even after deletion of the vector polh promoter

In the donor plasmid pFastBac-PolhE there are two copies of the *polh* promoter, one from pFastBac™1 (vector *polh* promoter) and the other from the upstream region of the *polh* ORF in the DNA fragment that was inserted into the multiple cloning site (MCS) (Figs. 1 A1, 2 and 2). One hypothesis for similar polyhedrin yields between AcP3 and AcBac-PolhE could be that more *polh* mRNA was transcribed from the two *polh* promoters in AcBac-PolhE, thus leading to more polyhedrin protein production. To test this hypothesis, the vector *polh* promoter was deleted from pAcBac-PolhE to generate another donor plasmid, pAcBac-PolhED, and subsequently the bacmid virus construct AcBac-PolhED (Fig. 2). Infection of High Five cells with either AcBac-PolhED or AcBac-PolhE yielded similar polyhedrin protein levels with many polyhedra per cell (MP phenotype), as seen in Fig. 3C, D. The production of polyhedra by AcBac-PolhED was also comparable to that of AcP3; both AcBac-PolhED and AcP3 showed cytoplasmic polyhedra during infection (Fig. 3A, C–E). Taken together, these data confirm that additional sequences upstream of the 50 bp *polh* promoter of pFastBac™1 are needed to achieve higher polyhedrin expression levels using the Bac-to-Bac® system in High Five cells.

3.3. Improved donor vector pFastBac-M1 showed enhanced protein expression levels

Since AcBac-PolhED, which has the extended *polh* promoter upstream sequences and *polh* pA, produced similar polyhedrin protein levels to AcP3 (Fig. 3I, J), we constructed our first improved donor plasmid vector by using inverse PCR to generate pFastBac-M1 (Fig. 1B). This donor vector has a DNA fragment with the extended upstream sequences (E), the 50 bp *polh* promoter, an MCS, and a *polh* pA fragment (Fig. 1B). We then cloned the *polh* ORF into pFastBac-M1 and generated the AcBac-M1-Polh virus (Fig. 2). Infection of High Five cells with AcBac-M1-Polh resulted in the same MP phenotype seen with AcP3, along with the production of cytoplasmic polyhedra (Fig. 4A). Comparison of polyhedra yields between High Five cells infected with either AcBac-M1-Polh or AcP3 showed no difference, and both generated about 3-fold more polyhedra than AcBac-Polh (Fig. 4B). However, the DNA elements in pFastBac-M1 responsible for elevated polyhedra production in High Five cells remained unknown.

3.4. An 80 bp cis element upstream of the polh promoter elevated protein expression yields

To understand why AcBac-PolhED and AcBac-M1-Polh were able to produce more polyhedrin than AcBac-Polh, inverse PCR was used in an attempt to map the 227 bp region upstream of the EcoRV site of the *polh* promoter in pFastBac-PolhED (Fig. 1A2, Table 1). Inverse PCR with the promoter-F forward primer paired with promoter-R1, −R2 or −R4 reverse primers did not yield any useful clones. However, inverse PCR using the promoter-F/promoter-R3 primer pair produced an unexpected 144 bp deletion in the middle of the 227 bp *polh* upstream region, thus producing donor plasmid vector pAcBac-MR3-Polh (Fig. 2), This vector, containing an 80 bp sequence upstream of the 50 bp *polh* promoter, the *polh* ORF, and *polh* pA sequences, was used to generate the bacmid virus AcBac-MR3-Polh via the Bac-to-Bac® system. Infection of High Five cells with AcBac-MR3-Polh showed MP and cytoplasmic polyhedra similar to those of AcP3, AcBac-PolhED and AcBac-M1-Polh (Figs. 3 D and 4 A).

3.5. SV40 pA in pFastBac™1 contributed to lower protein expression levels

Since the 80 bp *cis* element of AcBac-MR3-Polh was able to improve the *polh* promoter-mediated polyhedrin protein expression levels to match AcP3, AcBac-PolhED and AcBac-M1-Polh (Fig. 3; Fig. 4A, B), we wanted to investigate whether insertion of this 80 bp *cis* element upstream of the *polh* promoter of pFastBac™1 could enhance *polh* expression. To test this hypothesis, the 50 bp *polh* promoter of pFastBac™1 was replaced with the DNA fragment containing the 80 bp *cis* element and 50 bp *polh* promoter from pFastBac-MR3-Polh, but the SV40 pA fragment was retained, to generate donor plasmid vector pFastBac-cisF1 (Fig. 1A2). The *polh* ORF was cloned into pFastBac-cisF1 to generate pAcBac-cisF1-Polh for the production of the bacmid virus AcBac-cisF1-Polh. Infection of High Five cells with AcBac-cisF1-Polh showed lower polyhedra production compared to that of AcBac-MR3-Polh

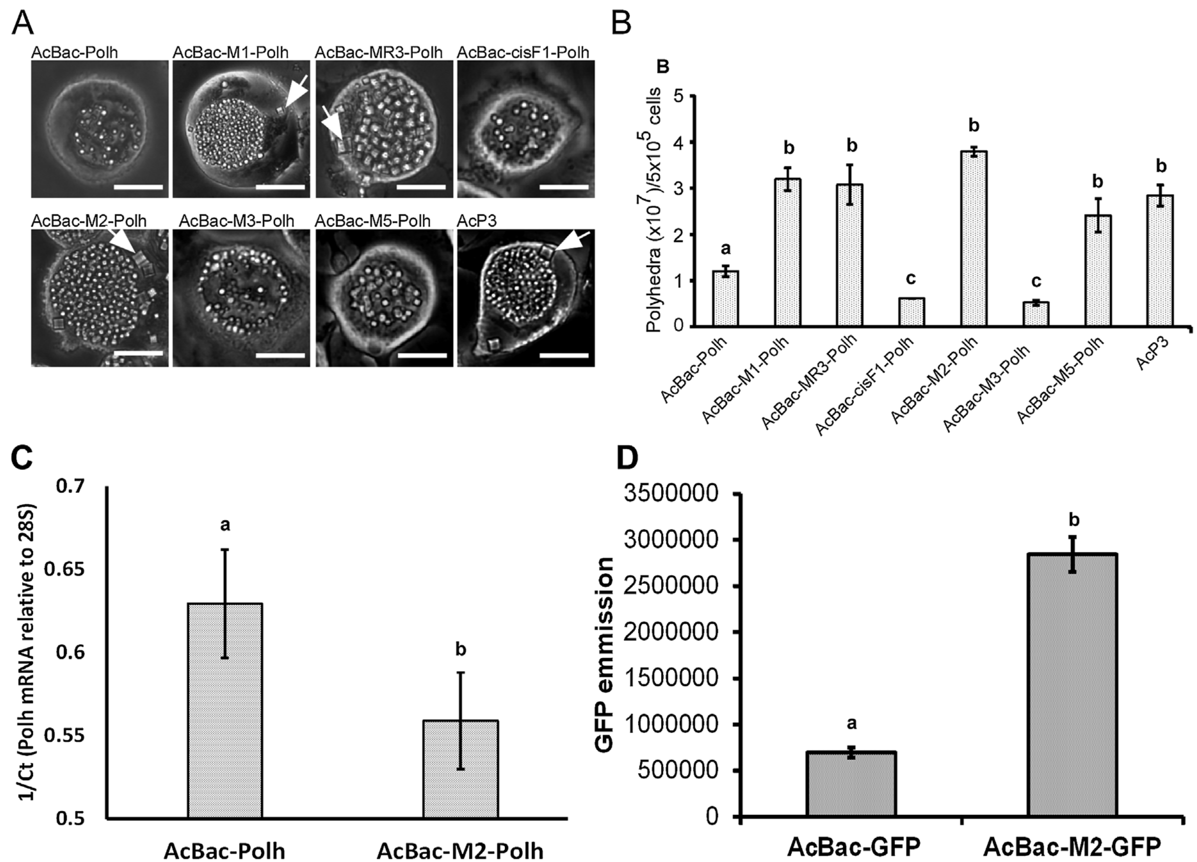

Fig. 4. Comparison of protein production in High Five cells infected with bacmids transposed with different donor plasmid vectors. A, Phase contrast microscopy of High Five cells infected with different viruses derived from vectors presented in Fig. 1B, AcBac-MR3-Polh is an intermediate vector for AcBac-cisF1-Polh production (not shown in Fig. 1B). Both AcBac-MR3-Polh and AcBac-cisF1-Polh have the 80 bp *cis* element, but AcBac-MR3-Polh has polh pA whereas AcBac-cisF1-Polh has SV40 pA. Arrows point to cytoplasmic polyhedrin crystals. Scale bar = 10 μm. B, Quantitative comparison of the production of polyhedra in High Five cells infected with different viruses. C. Comparion of *polh* mRNA levels between AcBac-Polh and AcBac-M2-Polh in High Five cells. High Five cells were infected with AcBac-Polh and AcBac-M2-Polh. Total RNA were isolated from infected cells for transcription level comparion of polh mRNA by real-time qPCR. Ct, threshold cycle. **D.** Comparion of GFP expression between AcBacGFP and AcBac-M2-GFP in High Five cells. All experiments were conducted in triplicates. Error bar, the standard error of the mean. Means with the same letter had no significant difference at p = 0.05.

(Fig. 4A, B). These data suggest that the SV40 pA signal reduced the production of polyhedra.

To confirm this observation, the *polh* ORF was cloned into pFastBac-M3, which contained the 227 bp sequences upstream of the *polh* promoter but had the SV40 pA signal, and ultimately the bacmid virus AcBac-M3-Polh was generated (Fig. 2). Infection of High Five cells with AcBac-M3-Polh resulted in polyhedra production lower than AcBac-M1-Polh (Fig. 4A, B). Thus, both donor plasmid vectors confirmed that the SV40 pA signal reduces polyhedra production. These data also suggest that in order to improve expression levels, this SV40 pA signal should be replaced with an AcMNPV viral pA, or more specifically the *polh* pA, since AcBac-M1-Polh contained the *polh* pA signal.

3.6. Polyhedrin pA was required in donor plasmid vectors for higher protein expression

To test if *polh* pA could help donor plasmid vectors such as pFastBac™1, pFastBac-cisF1 and pFastBac-M3 produce more polyhedrin protein, the SV40 pA sequence in pFastBac™1 and pFastBac-cisF1 was replaced by *polh* pA to generate pFastBac-M5 and pFastBac-M2, respectively (Fig. 1B). Subsequently, the *polh* ORF was cloned into pFastBac-M2 and pFastBac-M5 to generate pAcBac-M2-Polh and pAcBac-M5-Polh for the production of the bacmids AcBac-M2-Polh and AcBac-M5-Polh (Fig. 2). Infection of High Five cells with either AcBac-M2-Polh or AcBac-M5-Polh yielded levels of polyhedra similar to AcBac-M1-Polh and AcP3, which were all higher than AcBac-Polh, AcBac-cisF1-Polh and AcBac-M3-Polh (Fig. 4A, B). However, AcBac-M2-Polh resulted in the highest level of polyhedra production among the viruses containing the *polh* pA signal (Fig. 4A, B).

To support the *polh* expression data that indicated pFastBac-M2 is the best donor vector developed in this study for higher protein expression, the HPV16 L1 genes was cloned into the commercial pFastBac™1 and improved pFastBac-M2 vectors to generate pAcBac-L1 and pAcBac-M2-L1 for the production of AcBac-L1 and AcBac-M2-L1 bacmid viruses, respectively (Fig. 2). Similar to the higher expression level of AcBac-M2-Polh relative to AcBac-Polh, High Five cells infected with AcBac-M2-L1 also showed about 4-fold more L1 expression than AcBac-L1 (Figs. 4 A, B and 5 A, B). Furthermore, the GFP expression level of AcBac-M2-GFP showed 3-fold higher than AcBacGFP in High Five cells (Fig. 4D).

3.7. AcBac-Polh produced more polh mRNA than acBac-M2-Polh in high five cells

Since all the pFastBac-M2-derived viruses (AcBac-M2-Polh, AcBac-M2-L1 and AcBac-M2-GFP) showed 3–4 fold more protein production than the viruses derived from the standard commercial vector pFactBac1 (AcBac-Polh, AcBac-L1 and AcBacGFP) in High Five cells (Fig. 4A, B, D, Fig. 5A, B), we wondered if this observation was correlated with the mRNA levels. When *polh* mRNA levels of AcBac-Polh and AcBac-M2-Polh were compared by real-time qPCR, *polh* mRNA from AcBac-Polh showered about 1-fold higher than that from AcBac-M2-Polh in High Five cells (Fig. 4C).

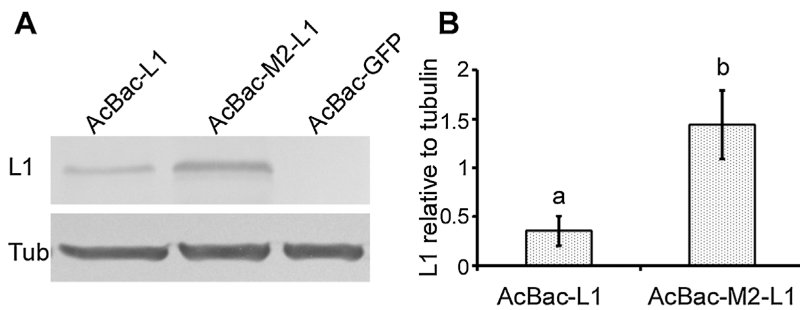

Fig. 5. Comparison of HPV16 L1 protein expression levels in High Five cells infected with AcBac-L1 and AcBac-M2-L1. High Five cells were infected separately with AcBac-L1 and AcBac-M2-L1 in triplicate. At day 3 P.I. infected cells were harvested for L1 expression analysis. Equal amounts of protein (100 μg) were loaded to each lane in SDS-PAGE. A, Western blotting analysis of L1 protein expression levels in High Five cells infected with AcBac-L1 derived from pFastBac1 or AcBac-M2-L1 derived from pFastBac-M2. AcBacGFP was used as a negative control. B, Quantitative comparison of L1 expression. Western blotting signals for A in triplicate were quantified by densitometry. Error bars, the standard error of the mean. Means with the same letter had no significant difference at p = 0.05.

3.8. PFastBac™-Dual vector required the 80 bp cis element and polh pA for higher polyhedrin expression

Improved pFastBac™1-based donor plasmid vectors such as pFastBac-M1 and pFastBac-M2 all have the 80 bp *cis* element and *polh* pA in addition to the 50 bp *polh* promoter (Fig. 1B). Whether these elements were also required in the pFastBac™ Dual vector for improved protein expression in High Five cells remained unknown. To test the effect of these elements, the *polh* ORF was inserted into the commercial pFastBac™ Dual and the newly generated pFastBac-Dual-M1, pFastBac-Dual-M2 and pFastBac-Dual-M3 vectors for bacmid production of AcBac-Dual-Polh, AcBac-Dual-M1-Polh, AcBac-Dual-M2-Polh and AcBac-Dual-M3-Polh, respectively (Fig. 1C). The levels of polyhedra produced from AcBac-Dual-Polh and AcBacBac-Dual-M1-Polh infections were lower than those from AcBacBac-Dual-M2-Polh and AcBacBac-Dual-M3-Polh (Fig. 6A, B). Moreover, AcBac-Dual-M1-Polh produced 2-fold more polyhedra than AcBac-Dual-Polh and AcBacBac-Dual-M2-Polh, and AcBacBac-Dual-M3-Polh yielded 3-fold more than AcBac-Dual-Polh (Fig. 6A, B). Since pFastBac-Dual-M2 and pFastBac-M2 are similar in that they both contain the 80 bp *cis* element and *polh* pA, the improved *polh* expression using pFastBac-Dual-M2 supports the findings that these elements are required for high protein expression in pFastBac-M2. Similar *polh* expression levels between AcBac-Dual-M3-Polh and AcBac-Dual-M2-Polh further support the finding that the 80 bp *cis* element and *polh* pA are the only additions to the donor plasmid vectors required to elevate protein yields in the Bac-to-Bac® expression system (Fig. 6A, B).

4. Discussion

In this report, we identified an 80 bp *cis* element 147 bp upstream of the 50 bp *polh* promoter of AcMNPV and the *polh* pA that are required for the commercial pFastBac™ vectors to transpose certain genes into the bacmid to achieve expression levels to that of the wt AcMNPV in High Five insect cells. This sequence was discovered when the first baculovirus genome was sequenced, but not yet characterized (Ayres et al., 1994). Therefore, this 80 bp *cis* element and the *polh* pA can be used to modify many baculovirus expression vectors to improve protein expression levels in High Five insect cells.

Among all the BEVSs developed since the 1980's, the emergence of the Bac-to-Bac® system represented a key milestone for biotechnology and eukaryotic protein expression because this system has overcome a major drawback of the conventional BEVSs, the requirement for several rounds of plaque assay to obtain a purified recombinant virus (Jarvis, 2009; Luckow et al., 1993). Instead, the Bac-to-Bac® system uses *E. coli* for recombination, isolation of pure colonies, and extraction of bacmid DNA for cell transfection, to produce pure recombinant virus for protein expression in 7–10 days (Luckow et al., 1993). However, donor plasmid vectors with the *polh* promoter are needed to recombine the gene of interest into the bacmid in *E. coli* cells.

The *polh* promoter used in the donor plasmid vectors is one of the strongest baculovirus promoters during insect cell infection (Adang and Miller, 1982). The 50 bp AcMNPV *polh* promoter was mapped by linker-scan mutations in the *polh* promoter region of the AcMNPV genome (Ooi et al., 1989). Following this discovery, the 50 bp *polh* promoter has been inserted into multiple donor plasmid vectors, such as the popular pFastBac™1 plasmid vector studied in this project, to recombine the gene of interest into the bacmid. It is apparent why previous *polh* promoter mapping did not find this 80 bp *cis* element, since the mapping was directed downstream toward the *polh* mRNA transcription start site TAAG (Ooi et al., 1989), whereas the 80 bp *cis* element is 147 bp upstream of the *polh* promoter (Fig. 1A1, 2).

Initially we deemed this 80 bp DNA element to be an enhancer, but later decided it is instead a *cis* element because there is another copy of the sequence in the non-essential ORF 603, which is separated from the *polh* promoter by a 3.2 kb DNA plasmid sequence in the recombinant bacmid (Gearing and Possee, 1990; Luckow et al., 1993). A previous study discovered a 2555 bp AcMNPV sequence upstream of *polh* that includes ORF 603, lef2, ORF5 and ORF4, and that enhances the promoter activity of cytomegalovirus (CMV), heat shock 70 from *Drosophila*, and *p35* of baculovirus (Lo et al., 2002). In addition, another study reported that over expression of IE1 and IE0 as well as a

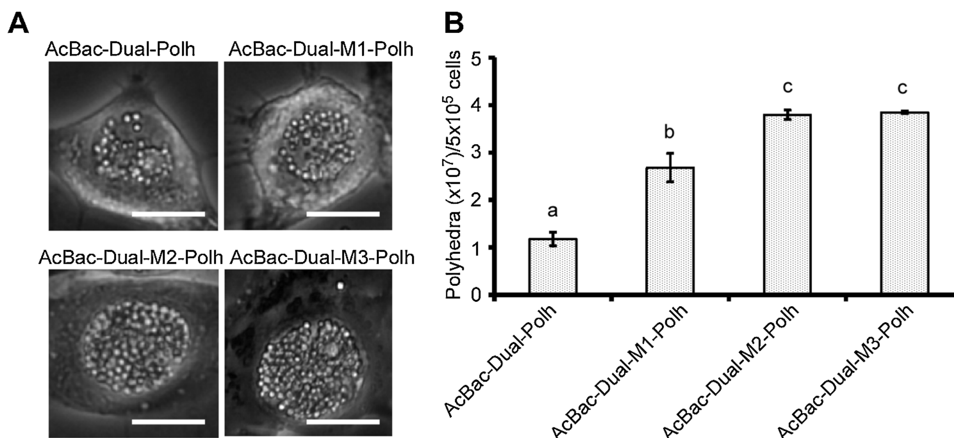

Fig. 6. Improved pFastBac-Dual vectors and comparison of the production of polyhedra in High Five cells infected with bacmids derived from the various dual vectors. High Five cells were infected separately with different viruses in triplicate. At day 3 P.I. data were collected from infected cells. A, Phase contrast microscopy of High Five cells infected with different viruses derived from the dual vectors expressing AcMNPV *polh*. Scale bar = 10 μm. C, Quantitative comparison of the production of polyhedra in High Five cells infected with different viruses. Polyhedra were extracted from infected cells for enumeration. Means with the same letter had no significant difference at p = 0.05.

homologous repeated transcription enhancer sequence of AcMNPV enhances *polh* promoter activity (Gomez-Sebastian et al., 2014). However, these elements are either much larger than the 80 bp *cis* DNA sequence or the factors are different from what we discovered in this report. Furthermore, this 80 bp *cis* element is different from another 293 bp enhancer-like element located 1 kb upstream of the ATG site of *polh* that was reportedly able to enhance the *polh* promoter activity of *Bombyx mori* NPV (Acharya and Gopinathan, 2001).

Although the 80 bp *cis* element enhanced the *polh* promoter of pFastBac™1 to allow the bacmid to produce higher levels of protein, it is uncertain whether the entire 80 bp sequence is needed for enhanced protein expression. In addition, adding this *cis* element alone did not allow the bacmid to produce more polyhedrin protein; instead, the optimized enhancement was observed only in combination with the *polh* pA signal (Figs. 4–6). This suggests that the downstream SV40 pA plays a negative role by reducing protein expression levels.

The rationale for inserting SV40 pA into the early donor plasmid vectors was to facilitate transcription termination and mRNA polyadenylation, thereby improving mRNA stability for anticipated higher protein expression (Westwood et al., 1993). It was observed that a gene expression cassette with SV40 pA expressed less reporter protein than one with the *p10* 3′UTR (van Oers et al., 1999). Furthermore, it has been argued that additional pA signal sequences should not be added to baculovirus expression vectors (O'Reilly et al., 1992). Our data in this report showed that AcBac-Polh that has SV40 pA displayed more *polh* mRNA levels than AcBac-M2-Polh that has *polh* pA (Fig. 4C). This result is supported by our early study that SV40 pA increases mRNA levels but reduces protein expression levels (Salem et al., 2015). It is unclear why the levels of *polh* expression regulated by the SV40 pA sequence and *polh* promoter were lower than with the *polh* pA (Fig. 4, 6). One may speculate that since SV40 pA is foreign to AcMNPV and High Five cells, there might be small RNA, micro RNA or protein(s) from the virus or the host cells that interact with SV40 pA to negatively regulate protein synthesis.

Previously, High Five cells showed higher protein expression yields than Sf9 cells infected with recombinant AcMNPV (Wilde et al., 2014). It is unknown why cytoplasmic polyhedra were produced in High Five cells infected with AcP3, AcBac-PolhE, AcBac-PolhED, AcBac-M1-Polh and AcBac-M2-Polh but not in Sf21 and Sf9 cells, supporting the finding of higher protein expression in High Five cells than in Sf9 cells (Fig. 3 and 4) (Wilde et al., 2014). It is possible that the larger cell size of High Five that is about twice the sizes of Sf9 and Sf21 enables High Five cells to synthesize more proteins (Cheng et al., 2013). It is also possible that polyhedrin crystallization in the cytoplasm reflects the higher level of cytoplasmic polyhedrin (much higher than that needed for crystallization), allowing it to crystalize prior to transport to the nucleus.

5. Conclusions

Since the pFastBac-M2 and pFastBac-Dual-M2 vectors have the same MCS as commercial pFastBac™1 and pFastBac™ Dual, researchers who are using these vectors for protein expression can simply transfer their genes of interest into the improved pFastBac-M2 and pFastBac-Dual-M2 vectors to achieve higher protein expression yields and reduce protein production costs. All other baculovirus vectors using the 50 bp *polh* promoter and SV40 pA can also be modified to include the 80 bp *cis* element and to replace SV40 pA with the *polh* pA fragment for improved protein expression yields in High Five cells.

Conflicts of interest

The authors declare no financial or commercial conflict of interest.

Author contributions

XWC, TAG and HS planned experiments; TAG, HS, CMSK and RFD performed experiments; XWC, TAG and HS analyzed data; XWC and TAG wrote the paper

Acknowledgments

The authors would like to thank Drs. Natasha Finley and Racheal Morgan-Kiss for providing the bacterial protein and filter, respectively, used in identifying the cytoplasmic crystals. Dr. Don Jarvis is credited with providing the virus for identification of the cytoplasmic polyhedrin crystals and suggestions in the writing of this manuscript. We also thank Dr. Susanne Wells for providing the HPV16 plasmid and Dr. Susan Hoffman for proofreading this manuscript. This work was supported by the US Department of Agriculture (US-Egypt Science and Technology Joint Fund project no. 58-3148-7-164) and the Miami University Interdisciplinary Research Round Table Fund Project.

References

Acharya, A., Gopinathan, K.P., 2001. Identification of an enhancer-like element in the polyhedrin gene upstream region of Bombyx mori nucleopolyhedrovirus. J. Gen. Virol. 82, 2811–2819.
Adang, M.J., Miller, L.K., 1982. Molecular cloning of DNA complementary to mRNA of the baculovirus Autographa californica nuclear polyhedrosis virus: location and gene products of RNA transcripts found late in infection. J. Virol. 44, 782–793.
Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M., Possee, R.D., 1994. The complete DNA sequence of Autographa californica nuclear polyhedrosis virus. Virology 202, 586–605.
Cheng, X.W., Carner, G.R., Fescemyer, H.W., 1998. Polyhedrin sequence determines the tetrahedral shape of occlusion bodies in Thysanoplusia orichalcea single-nucleocapsid nucleopolyhedrovirus. J. Gen. Virol. 79, 2549–2556.
Cheng, X., Krell, P., Arif, B., 2001. P34.8 (GP37) is not essential for baculovirus replication. J. Gen. Virol. 82, 299–305.
Cheng, X.H., Hillman, C.C., Zhang, C.X., Cheng, X.W., 2013. Reduction of polyhedrin mRNA and protein expression levels in Sf9 and Hi5 cell lines, but not in Sf21 cells, infected with Autographa californica multiple nucleopolyhedrovirus fp25k mutants. J. Gen. Virol. 94, 166–176.
Cox, M.M., Hashimoto, Y., 2011. A fast track influenza virus vaccine produced in insect cells. J. Invertebr. Pathol. 107 (Suppl), S31–S41.
Durst, M., Gissmann, L., Ikenberg, H., zur Hausen, H., 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. U. S. A. 80, 3812–3815.
Gabanyi, M.J., Berman, H.M., 2015. Protein structure annotation resources. Methods Mol. Biol. 1261, 3–20.
Gearing, K.L., Possee, R.D., 1990. Functional analysis of a 603 nucleotide open reading frame upstream of the polyhedrin gene of Autographa californica nuclear polyhedrosis virus. J. Gen. Virol. 71, 251–262.
Geissmann, Q., 2013. OpenCFU, a new free and open-source software to count cell colonies and other circular objects. PLoS One 8, e54072.
Gomez-Sebastian, S., Lopez-Vidal, J., Escribano, J.M., 2014. Significant productivity improvement of the baculovirus expression vector system by engineering a novel expression cassette. PLoS One 9, e96562.
Grabherr, R., Ernst, W., 2010. Baculovirus for eukaryotic protein display. Curr. Gene Ther. 10, 195–200.
Granados, R.R., Guoxun, L., Derksen, A.C.G., McKenna, K.A., 1994. A new insect cell line from Trichoplusia ni (BTI-Tn-5B1-4) susceptible to Trichoplusia ni single enveloped nuclear polyhedrosis virus. J. Invertebr. Pathol. 64, 260–266.
Harper, D.M., 2009. Currently approved prophylactic HPV vaccines. Expert Rev. Vaccines 8, 1663–1679.
Herniou, E.A., Arif, B.M., Becnel, J.J., Blissard, G.W., Bonning, B.C., Harrison, R.L., Jehle, J.A., Theilmann, D.A., Vlak, J.M., 2012. Family baculoviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus Taxonomy Classification and Nomenclature of Viruses, Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Inc., San Diego, pp. 163–173.
Hopkins, R., Esposito, D., Gillette, W., 2010. Widening the bottleneck: increasing success in protein expression and purification. J. Struct. Biol. 172, 14–20.
Jarvis, D.L., Bohlmeyer, D.A., Garcia Jr., A., 1991. Requirements for nuclear localization and supramolecular assembly of a baculovirus polyhedrin protein. Virology 185, 795–810.
Jarvis, D.L., 2009. Baculovirus-insect cell expression systems. In: Burgess, R.R., Deutscher, M.P. (Eds.), Methods in Enzymology. Elsevier, pp. 191–222.
Kitts, P.A., Ayres, M.D., Possee, R.D., 1990. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. Nucleic Acids Res. 18, 5667–5672.
Lo, H.R., Chou, C.C., Wu, T.Y., Yuen, J.P., Chao, Y.C., 2002. Novel baculovirus DNA elements strongly stimulate activities of exogenous and endogenous promoters. J. Biol. Chem. 277, 5256–5264.
Lu, A., Miller, L.K., 1995. Differential requirements for baculovirus late expression factor genes in two cell lines. J. Virol. 69, 6265–6272.
Luckow, V.A., Lee, S.C., Barry, G.F., Olins, P.O., 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign

genes into a baculovirus genome propagated in *Escherichia coli*. J. Virol. 67, 4566–4579.

O'Reilly, D.R., Miller, L.K., Luckow, V.A., 1992. Baculovirus Expression Vectors, A Laboratory Manual. W. H. Freemon and Company, New YorK.

Ogay, I.D., Lihoradova, O.A., Azimova Sh, S., Abdukarimov, A.A., Slack, J.M., Lynn, D.E., 2006. Transfection of insect cell lines using polyethylenimine. Cytotechnology 51, 89–98.

Ooi, B.G., Rankin, C., Miller, L.K., 1989. Downstream sequences augment transcription from the essential initiation site of a baculovirus polyhedrin gene. J. Mol. Biol. 210, 721–736.

Passarelli, A.L., Miller, L.K., 1993. Identification and characterization of lef-1, a baculovirus gene involved in late and very late gene expression. J. Virol. 67, 3481–3488.

Rodems, S.M., Friesen, P.D., 1993. The hr5 transcriptional enhancer stimulates early expression from the Autographa californica nuclear polyhedrosis virus genome but is not required for virus replication. J. Virol. 67, 5776–5785.

Salem, T.Z., Seaborn, C.P., Turney, C.M., Xue, J., Shang, H., Cheng, X.W., 2015. The influence of SV40 polyA on gene expression of baculovirus expression vector systems. PLoS One 10, e0145019.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675.

Smith, G.E., Summers, M.D., Fraser, M.J., 1983. Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol. Cell. Biol. 3, 2156–2165.

Summers, M.D., Smith, G.E., 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agric. Exp. Station Bull. 16, 1–57.

Tanada, Y., Haya, H.K., 1993. Insect Pathology. Academic Press Inc.

Westwood, J.A., Jones, I.M., Bishop, D.H., 1993. Analyses of alternative poly(A) signals for use in baculovirus expression vectors. Virology 195, 90–99.

Wilde, M., Klausberger, M., Palmberger, D., Ernst, W., Grabherr, R., 2014. Tnao38, high five and Sf9?evaluation of host-virus interactions in three different insect cell lines: baculovirus production and recombinant protein expression. Biotechnol. Lett. 36, 743–749.

Xue, J.L., Cheng, X.W., 2010. Using host 28S ribosomal RNA as a housekeeping gene for quantitative real-time reverse transcription-PCR (qRT-PCR) in virus-infected animal cells Curr Protoc Microbiol Chapter 1, Unit1D 2.

Xue, J.L., Salem, T.Z., Turney, C.M., Cheng, X.W., 2010. Strategy of the use of 28S rRNA as a housekeeping gene in real-time quantitative PCR analysis of gene transcription in insect cells infected by viruses. J. Virol. Methods 163, 210–215.

van Oers, M.M., Vlak, J.M., Voorma, H.O., Thomas, A.A., 1999. Role of the 3' untranslated region of baculovirus p10 mRNA in high-level expression of foreign genes. J. Gen. Virol. 80, 2253–2262.