Figure

Figure

Figure

Figure

Figure

For: Plasmid

Insect Cell Transformation Vectors that Supports High Level Expression
and Promoter Assessment in Insect Cell Culture

Paul D. Shirk^{1,*} and Richard B. Furlong¹

¹Center for Medical, Agricultural and Veterinary Entomology, Agricultural Research Service,
US Department of Agriculture, 1700 SW 23rd Drive, Gainesville, Florida 32608 USA

*To whom correspondence and proofs should be sent: Paul D. Shirk, USDA ARS CMAVE,
1700 SW 23rd Drive, Gainesville, FL 32608. E-mail: paul.shirk@ars.usda.gov, Telephone: 352-
374-5720, Fax: 352-374-5863

[Keywords] genetic transformation, densovirus integration, transfection, expression, *Spodoptera*
frugiperda Sf9 cells, *Bombyx mori* Bm5 cells

Abstract

A somatic transformation vector, pDP9, was constructed that provides a simplified means of producing permanently transformed cultured insect cells that support high levels of protein expression of foreign genes. The pDP9 plasmid vector incorporates DNA sequences from the *Junonia coenia* densovirus that are involved in integration of the densovirus in insect cell chromosomes and a promoter/enhancer system that results in high levels of expression. The plasmid also contains two markers that permit selection of transformed insect cells by antibiotic resistance or by cell-sorting for fluorescent protein expression. Transformation of *Bombyx mori* Bm5 or *Spodoptera frugiperda* Sf9 cultured cells with the pDP9 vectors results in the integration of the pDP9 plasmid into genomic DNA of Bm5 and Sf9 cells. pDP9 contains a multiple cloning site (MCS) 3' of the densoviral P9 promoter and insertion of a protein coding sequence within the MCS results in high level expression by pDP9 transformed cells. P9 driven transcription in the pDP9 transformed Sf9 cells produced foreign gene transcript levels that were 30 fold higher than actin 3 driven transgenes and equivalent to hr5IE1 driven transgenes. The pDP9 vector transformation results in the efficient selection of clones for assessment of promoter activity.

1. Introduction

Stable somatic transformation of *Spodoptera frugiperda* Sf9 cell lines has been achieved with plasmids containing a modified genome of the insect parvovirus from the subfamily Densovirinae isolated from the Buckeye butterfly, *Junonia coenia* (Lepidoptera: Nymphalidae) (*JcDNV*) (Bossin et al., 2003). Transfection of *S. frugiperda* Sf9 cells with a *JcDNV* plasmid vector that included an EGFP reporter, pJGFPH, resulted in the selection of clonal cell lines that maintained strong, stable, long-term GFP expression due to chromosomal integration of the pJGFPH plasmid (Bossin et al., 2003). Sequencing of plasmid integration sites from host cell chromosomes showed that almost all plasmid recombination events were localized within the inverted terminal repeat associated with the densoviral P9 promoter (P9ITR) (Bossin et al., 2003). Moreover, the insertions involved the near entirety of the plasmid into the host cell chromosome. In addition to transformation of insect cell lines, the *JcDNV* somatic transformation vectors have been used effectively to somatically transform various dipteran and lepidopteran species by microinjecting syncytial embryos (Royer et al., 2001; Bossin et al., 2007). Microinjection of *JcDNV* vectors led to the highly efficient recovery of somatic transformants where promoter specific expression was maintained from the larval through the adult stage (Royer et al., 2001; Bossin et al., 2007; Shirk et al., 2007).

In all of these investigations, the identification of somatically transformed cell lines and insects has relied primarily on the expression of a chimeric protein constructed between the viral capsid proteins (VP) and a reporter protein. The DNA strand containing the P9 promoter produces a 2.6 kb transcript that codes for four VPs (Dumas et al., 1992). Translation from the 2.6 kb transcript utilizes four different AUG start codons to produce the four VPs which all share a common C-terminal amino acid sequence that includes a nuclear localization signal. A reporter

gene coding sequence inserted in frame with the VP gene and 3' to the VP4 ATG translation initiation codon results in P9 regulated expression of the VP-reporter chimeric protein including the nuclear localization signal. Expression of the P9 regulated VP-reporter has been detected in the cells of the midgut, hind gut, Malpighian tubules, fat body and cardiac cells (Royer et al., 2001; Bossin et al., 2007; Shirk et al., 2007). The transcriptional activity of the P9 promoter is positively influenced by a 557 bp sequence located within the overlapping 3' sequences of the VP gene and the opposite strand non-structural protein genes (NS) (Shirk et al., 2007). Deletion of the 557 bp sequence reduced the P9 driven expression below detectable levels while inclusion of the sequence in either orientation maintained expression.

Somatic transformation by the *JcDNV* vectors does not depend on the presence of the opposite strand ITR, the associated P93 promoter (P93ITR) or the non-structural genes (Giraud et al., 1992; Bossin et al., 2007). Deletion of all densoviral sequences 3' of the SV40 polyadenylation site of the VP-DsRed chimera reporter in pJDR did not affect the efficiency of the somatic transformation (Shirk et al., 2007). However, this deletion, which included the 557 bp enhancer sequence, did reduce VP-DsRed expression to below detectable levels and detection of somatic transformation was established by the product of a secondary expression cassette included in the construct. Insertion of the 557 bp enhancer fragment in either orientation restored the P9 driven expression.

On the other hand, deletion of part or all of the NS-genes does affect the number of chromosomal integration sites and the copy number of plasmid concatemers at each site of integration (Bossin et al., 2003). Based on both Southern blots and primed *in situ* synthesis (PRINS) analyses, integrations of the *JcDNV* vectors expressing the full complement of NS-genes were found to integrate at single chromosomal sites with a single copy of the plasmid

sequence present. When the NS-3 gene was deleted, multiple chromosomal integration sites were observed and as with the intact viral sequence the integrations involved single copies of the plasmid. Deletion of all three NS genes again resulted in multiple chromosomal integration sites but the integration resulted in head to tail concatemers of the plasmid sequence with approximately 60 plasmid copies per integration site.

We report here that sequences from the *J. coenia* densovirus genome which result in integration within the host cell chromosome and enhanced P9 promoter activity (Shirk et al., 2007) were inserted into a plasmid that contained two selection markers to produce new vectors. The new vectors capitalize on selected properties of the *JcDNV* somatic transformation vectors which result in stable chromosomal integration of high copy numbers of the plasmid sequence that promotes strong transcriptional activity. Based on the findings reported here, this somatic transformation vector provides many of the features necessary for high-efficiency expression coupled with the convenience of cell transformation and selection using a plasmid that is not dependent upon a viral infection.

2. Materials and Methods

2.1 Insect Strains

The *Drosophila melanogaster* w[m] strain has a *white* mutation with an M cytotype was obtained from the Bloomington Stock Center (Indiana University, Bloomington IN USA).

2.2 Plasmids

The two inverted terminal repeats of the *JcDNV* were individually cloned into Litmus 28i (Invitrogen/ThermoFisher Scientific, Waltham MA). A 692 bp *BmgBI/HindIII* fragment that contains the P9ITR from pJDR (Bossin et al., 2007) was ligated into *StuI/HindIII* digested Litmus 28i (Lit-P9ITR). Similarly, an 886 bp *BsrBI/BsrGI* fragment that contains the P93ITR from pJDR was ligated into *Acc65I/ZraI* digested Litmus 28i (Lit-P93ITR). A 2138 bp *BstZ17I/BglII* fragment from pSL-hr5IE1DsRed (Bossin et al., 2007) that contains hr5IE1DsRed was ligated into *BfrBI/BglII* digestions of both Lit-P9ITR and Lit-P93ITR to produce Lit-P9ITR/hr5IE1DsRed and Lit-P93ITR/hr5IE1DsRed.

The pDP9 plasmid vector (Fig. 1A) was constructed in the following manner. The *BsaI/DraIII* fragment of pEGFP-N1 (Clontech, Mountain View CA) containing a kanamycin/neomycin (Kan/Neo) resistance cassette was isolated and the over-hanging ends were filled with T4 DNA polymerase (New England BioLabs Inc., Ipswich MA). The Kan/Neo cassette was ligated into *StuI* digested pSLfa1180fa (Horn and Wimmer, 2000) to produce pSL-Kan/Neo. The *BstZ17I/BglII* fragment containing the *Bombyx mori* actin A3EGFP reporter cassette (BmA3G) from pPIGA3EGFP (Tamura et al., 2000) was cloned into *BstZ17I/BglII* digested pSLfa1180fa to produce pSL-BmA3G. Subsequently, the BmA3G expression cassette was removed from pSL-BmA3G by digestion with *SmaI/AflII* and ligated into pSL-Kan/Neo

digested with *NruI/AflIII* to produce pSL-Kan-BmA3G. The *NciI* fragment containing LITR-P9 promoter was isolated from pJDR (Shirk et al., 2007) and ligated into *PmlI* digested pSL-Kan-BmA3G to produce pSL-ITRP9-Kan-BmA3G. The *J. coenia* densovirus enhancer region (*JcES*) (Shirk et al., 2007) was isolated by digestion with *MluI/SacII* from pJDR and ligated into pSL-ITRP9-Kan-BmA3G digested with *MluI/SacII* to produce pSL-ITRP9-ESs-Kan-BmA3G. Various superfluous restriction sites were removed by digestion, followed by filling overhanging ends with T4 DNA polymerase (New England BioLabs, Inc.) and subsequently re-ligating to produce the pDP9 transformation vector (pSL-ITRP9-MCS-Es-Kan-BmA3G) (Fig. 1A).

The components of pDP9a-DsRed (Fig. 1B) were constructed to test the activities of two additional expression cassettes regulated by the P9 promoter or the hr5IE1 promoter. The ITRP9 sequence from pJDR was subcloned into pSL-Kan using a *HindIII* site to produce pSL-ITRP9-Kan using a common *HindIII* site. The ESs enhancer was cloned from Lit-JcESsdBS-ND using and *AgeI/EcoRV* fragment into pSL-ITRP9-Kan at the *AgeI/SnaBI* site to produce pSL-ITRP9-ESs-Kan. The DsRed coding sequence *NcoI/SspI* was cloned from pDsRed-N1 (Clontech) into pSL-fa1180fa. The DsRed coding sequence was then removed from pSL-DsRed by digestion with *NcoI*. The *NcoI* ends were filled by T4 DNA polymerase (New England BioLabs Inc.) and the fragment was ligated into pSL-ITRP9-ESs-Kan digested with *NruI/NsiI* placing the DsRed coding sequence 3' of the *JcDNV* P9 promoter producing pSL-ITRP9-DsRed-ESs-Kan. The forward orientation of the DsRed coding sequence was selected. The hr5ie1EGFP cassette in phr5IE1EGFP was removed with *ApaI/HpaI* and cloned into pSL-ITRP9-DsRed-ESs-Kan to produce pDP9a-DsRed (pSL-ITRP9-DsRed-ESs-Kan-hr5IE1EGFP).

2.3 Embryo injections, somatic transformation and marker detection

The microinjection of the DNA solutions were as described previously (Bossin et al., 2007). Essentially, the embryos were collected within 7 min after deposition to achieve optimal exposure of the embryonic nuclei to the plasmid DNA solutions and the highest probability of a chromosomal integration occurring in somatic nuclei. The syncytial embryos were mounted on cellophane (Bio-Rad, Hercules CA) strips without being dechorionated and were microinjected with 1 µg plasmid DNA/µl injection buffer (5 mM KCl; 0.1 mM sodium phosphate pH 6.8) within 15 min after deposition. This procedure resulted in low embryo hatch rates (5-30%) but assured that embryonic nuclei were exposed to the transforming DNA at a developmental time when only a limited number of nuclei were present. Following injection, the strips with *D. melanogaster* embryos were hydrated, placed on *Drosophila* diet and then kept at 20° C until hatching.

The embryos, larvae, pupae and adults were scored for somatic transformation by detection of fluorescent protein expression. The DsRed fluorescent proteins were detected with a MZ FLIII fluorescent stereozoom microscope (Leica Microsystems, Buffalo Grove IL) using an EGFP/DsRed filter set (Excitation 480/40 nm and 560/40 nm; Emitter 525/50 nm and 630/60 nm) (Chroma Technology Corp., Bellow Falls VT). Digital photographs were made using a DC 500 CCD camera operated with Leica IM50 software (Leica Microsystems). To detect fluorescence, the insects were removed from diet and viewed individually.

2.4 Cell Transformation and Selection

Bombyx mori Bm5 and *Spodoptera frugiperda* Sf9 cells were maintained in Grace's insect medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 10 µg/ml gentamycin (Sigma-Aldrich, St. Louis MO) at 24° C. The Bm5 and Sf9 cells were

grown in 25 cm² polystyrene culture flasks (Corning Inc., Corning NY) and media was exchanged weekly. Doubling time for the Bm5 cells under these conditions was approximately 72 h and for the Sf9 cells was approximately 24 h. The Bm5 and Sf9 cells were transfected with a mixture of Lipofectin (Invitrogen) plus 2 µg/ml of plasmid DNA according to the manufacturer's protocol. Following the transfection period, the each cell type was cultured in growth medium with FBS and 10 µg/ml gentamycin with or without 50 µg/ml G418 (A. G. Scientific, San Diego CA) as specified in the experiment. The cultured cells were observed using a DM IL fluorescent microscope equipped with Leica IMC (Leica Microsystems). GFP fluorescence was observed using a GFP filter (excitation 470 nm/dichromatic 500 nm/suppression 525 nm). DsRed fluorescence was observed using an Y3 filter (excitation 535 nm/dichromatic 565 nm/suppression 610 nm). The microscope was also equipped with a DC500 digital camera (Leica Microsystems), and the images of the cells were captured using the LM50 software (Leica Microsystems). The digital images were processed using Adobe CS5 12.0 (Adobe Systems Inc., San Jose CA). The Bm5 and Sf9 cell populations were profiled for GFP and DsRed expression at various times following transfection by microscopy determining both the frequency of cells expressing the reporter and the intensity level of expression (arbitrarily scaled from 0-3). The Bm5 cell populations were profiled for GFP expression 50 days after transformation by flow cytometry using a FACScan (BD Biosciences, San Jose CA) at 488 nm excitation and 553 nm emission or by sorting using a FACSsort (BD Biosciences) at 488 nm excitation and 553 nm emission. Cell distributions were calculated with CellQuest software (BD Biosciences).

2.3 Quantitative-PCR (qPCR), Gene Copy Number Estimation and Quantitative Reverse
Transcription PCR (RT-qPCR)

Genomic DNA was isolated from Bm5 or Sf9 cultured cells that were collected and combined from 4 bottles in 1 ml of serum-free Grace's medium using the Wizard Genomic DNA Isolation System (Promega, Madison WI) according to the manufacturer's protocol for *Drosophila* S2 cultured cells. Plasmid DNA was isolated from Bm5 or Sf9 cultured cells that were collected and combined from 4 bottles in 1 ml of serum-free Grace's medium using the Zyppy Plasmid Miniprep Kit (Zymo Research, Irvine CA) according to the manufacturer's protocol for bacteria.

Direct PCR of genomic DNA from pDP9 transformed cells was conducted with the *Jc*ITR primers (Table 1) which produced a 1.8 kb fragment from the pDP9 plasmid that extends from within the pSLfa1180fa backbone to the 3' of the *J. coenia* enhancer sequence. The PCR reactions were run using 100 ng genomic DNA with *Taq* DNA polymerase (NEB) in a MJ Research PTC-100 Programmable Thermal Controller (MJ Research|Bio-Rad) programmed for 30 cycles of 97° C for 30 sec, 58° C for 30 sec, and 72° C for 90 sec with a final extension reaction at 72° C for 120 sec. The PCR products were resolved by electrophoresis in 1% agarose and stained with ethidium bromide along with a 1 kb DNA Ladder as a standard reference.

Quantitative PCR of the genomic DNA was conducted using the IQ SYBER Green mix (Bio-Rad) with various amounts of genomic DNA template plus a specific primer and beacon set (Integrated DNA Technologies, Coralville IA) (Table 1). All samples were run in triplicate. The reactions were run in a MiniOpticon (Bio-Rad) programmed for 35 cycles of 97° C for 30 sec, 56° C for 30 sec, and 72° C for 30 sec. Quantitation of gene copy number was based on comparison with a standard dilution series of a quantified plasmid containing the corresponding

208 DNA sequence. For all plasmids used as standards, the linear regressions had r^2 greater than
209 0.95.

210 Total RNA was extracted from cultured cells using the Ribopure kit
211 (Ambion|ThermoFisher, Carlsbad CA) according to the manufacturer's instructions. cDNA was
212 produced from 1 μ g total RNA extract for each treatment using AffinityScript QPCR cDNA
213 synthesis kit (Stratagene|ThermoFisher, La Jolla CA) as directed by the manufacturer. RT-qPCR
214 was conducted on cDNAs using IQ SYBER green mix (Bio-Rad) with a designated primer and
215 beacon set (Integrated DNA Technologies) (Table 1). All biological samples were run in
216 triplicate with triplicate RT-qPCR reactions conducted from each biological sample. RT-qPCR
217 reactions were run in a MiniOpticon (Bio-Rad) programmed for 35 cycles of 97° C, 30 sec; 56°
218 C, 30 sec; 72° C, 30 sec. Quantitation of copy number was conducted as described above. To
219 assure that there was no interference from DNA carried over from the RNA extract, reactions
220 were made using the RNA extract and none contained more than 0.1% of the cDNA measured.
221 The relative levels of a specific transcript were standardized as a ratio to the 28S transcript level
222 (Barbu and Dautry, 1989; Goidin et al., 2001; Denz and Dube, 2005) using the 2- $\Delta\Delta$ Ct method
223 (Livak and Schmittgen, 2001) to analyze the data.

3. Results

3.1 Somatic Transformation with *JcDNV* ITRs

To determine if either of the ITR sequences of *JcDNV* without any accompanying viral sequences was sufficient to promote somatic transformation, the P9ITR and P93ITR were cloned separately into plasmids containing the hr5IE1DsRed reporter cassette to produce Lit-P9ITR-hr5IE1DsRed and Lit-P93ITR-hr5IE1DsRed. Microinjection of either of the plasmids into *D. melanogaster* embryos resulted in persistent DsRed fluorescence in larvae, pupae and adults (data not shown) from both the plasmid containing the P9ITR sequence and the plasmid containing the P93ITR sequence. The frequency of larvae expressing DsRed when treated with either plasmid (Table 2) was similar to that of pJDR reported previously (Bossin et al., 2007; Shirk et al., 2007).

3.2 pDP9 Chromosomal Integration

Because the *JcDNV* ITRs apparently led to somatic transformation independent of all other viral sequences, new somatic transformation vectors, pDP9 and pDP9a-DsRed were constructed that included at least two selectable markers (visible and antibiotic) and a configuration that provided the possibility of expression from the densovirus P9 promoter. Transfection of Sf9 cells with pDP9a-DsRed resulted in observable levels of the fluorescent marker proteins within 24 h (Fig. 2). The level of expression for each of the two expression cassettes within the vector was variable at this time. Some cells were producing significant amounts of both DsRed and GFP (Fig. 2 A, B – arrows) while in other cells the expression was higher for one or the other fluorescent protein.

Transfection of pDP9 into Bm5 cells resulted in persistent GFP fluorescence over 50 days while expression from phr5IE1EGFP had fallen to background by 30 d (Fig. 3A). Bm5 cells transfected with pDP9 or pDP9a-DsRed still retained detectable GFP fluorescence in more than 46% of the cells at 108 d (Table 3; Fig. 4A, B). The continued observable GFP fluorescence is in contrast to pJDR and pJDR-3xP3G which had less than 11% showing DsRed fluorescence or less than 0.05% GFP fluorescence from the pJDR-3xP3G transfected cells after 50 d.

At 48 h after transfection, both Bm5 and Sf9 cells treated with pDP9 were either placed in media containing G418 for selection or were subjected to cell sorting. The pDP9 transfected Sf9 cells that were grown with G418 selection were kept in the original flasks with only media changes until growth was confluent across the surface of the flask. Expression of the fluorescent protein marker was confirmed by visual inspection at each change of the media. Sixty percent of the surviving G418 selected Bm5-pDP9 cells contained detectable GFP fluorescence at 120 d after transfection when viewed by FACScan (Fig. 4B). Isolation of both genomic and plasmid DNA from pDP9 transfected Sf9 cells after 22 d showed that the pDP9 plasmid was associated with genomic DNA. Direct PCR of the genomic DNA with primers that flanked the *JcDNV* ITR-P9 region of the pDP9 vector demonstrated the persistence of the vector in the Sf9 chromosomal DNA (Fig. 5). The production of a 1.8 kb fragment which corresponds to an intact *JcDNV* ITR flanking region of pDP9 was also consistent with a concatemeric insertion of the vector in the chromosomes. Isolation of plasmidic and genomic DNA fractions from the transfected Sf9 cells were subjected to qPCR with primers for EGFP and showed that there were 5.25×10^5 (SD $\pm 1.15 \times 10^4$) copies per cell of the pDP9 sequence in the plasmidic fraction still persisting in the cells while there were 1.89×10^3 (SD $\pm 1.53 \times 10^3$) copies in the genomic fraction.

To determine the level of the vector in relationship to chromosomal genes, genomic DNA was isolated from transfected Sf9 cells at 70 days after transfection with pDP9a-DsRed. qPCR of the Sf9 genomic DNA using primers for EGFP and *S. frugiperda* hsp90 was conducted. The hsp90 gene was previously determined to be present at 60 copies in the *S. frugiperda* genome (Landais et al., 2001). In the genomic DNA isolated from the Sf9 cells transfected with pDP9a, the ratio of pDP9a copies to hsp90 copies was 0.9 demonstrating that the pDP9a vector was present in the genome as multiple copies in numbers that indicated concatemerization which was similar to that reported previously for *JcDNV* vectors (Bossin et al., 2003).

3.3 P9 Driven Expression by pDP9 Vectors: Transcripts

Total RNA was isolated from pDP9-DsRed transfected Sf9 cells and used to determine the levels of transcripts produced from integrated vector sequences. RT-qPCR was conducted on total RNA for DsRed transcript which was regulated by the P9 promoter and EGFP which was regulated by the BmA3 promoter. As a standard, the transcript levels for 28S rDNA (Barbu and Dautry, 1989; Goidin et al., 2001; Denz and Dube, 2005) were determined. The transcript level for DsRed which was regulated by the P9 *JcDNV* promoter/enhancer was 0.13 times the level of 28S transcript (Table 4). On the other hand, the transcript level for EGFP which was regulated by the actin 3 promoter was only 4.75×10^{-3} times the levels of the 28S transcript or 29 fold less than the P9 driven transcripts.

When pDP9a-DsRed was transfected into Bm5 cells, both reporter cassettes were active at similar levels. The transcript levels for 28S rDNA were used for normalizing the other transcript measured. The transcript level for DsRed, regulated by the P9 *JcDNV* promoter/enhancer, was 0.16 times the levels of the 28S transcript (Table 5). The transcript level

291 for EGFP, regulated by the hr5IE1 promoter/enhancer was 0.19 times the level of 28S transcript.
292 The transcript levels for both DsRed and EGFP were both higher than those for the basal
293 expression of the hsp90 gene which was 0.1 times the 28S transcript. When determined in non-
294 transfected cells, the ratio between 28S and hsp90 transcripts was the same.

4. Discussion

Inclusion of either of the *J. coenia* densovirus ITR sequences within a plasmid resulted in the integration of the plasmid into the genomic DNA of the host cell producing somatic transformation efficiencies similar to the observations for the transcriptionally interrupted *J. coenia* densovirus (Bossin et al., 2007; Shirk et al., 2007). The only sequence necessary for somatic transformation was the 527 bp that included the *J. coenia* densovirus ITR plus the P9 or P93 promoter. Inclusion of a single ITR in a plasmid that otherwise showed background levels of persistent fluorescence resulted in a pattern of fluorescence from the hr5IE1DsRed expression cassette in *D. melanogaster* larvae, pupae and adults which was similar to that from somatic transformations achieved with the complete *JcDNV* vectors that included both of the ITRs (Bossin et al., 2007; Shirk et al., 2007).

When transfected into cultured cells, expression of the fluorescent marker proteins under the control of the P9 and hr5IE1 promoters, was detected within 24 h after transfection. Although the vector contained both expression cassettes, not all of the cells were uniformly expressing the fluorescent proteins. At this early time after transfection, the expression observed was most likely transient (Bossin et al., 2003) and therefore not consistent from cell to cell. The expression from the pDP9 vectors persisted for up to 120 d and appeared to be permanent.

Although plasmid vector was still present in the cells, integrations of the pDP9 vectors into genomic DNA had occurred within 22 d after transfection. The integrations were primarily as concatemers of approximately 60 copies each. The organization of the concatemeric integrations described here were consistent with those previously reported for *JcDNV* vectors that lack all of the NS genes but retained both inverted terminal repeats (Bossin et al., 2003). Integration of the human parvovirus adeno-associated virus (AAV) within a 100 bp specific site

on chromosome 19 often results in monomeric or tandem head-to-tail concatemers as well (Yang et al., 1997). AAV has its highest efficiencies of integration when both ITRs are present and is only 20% as efficient when one ITR is included in a transforming construct (Nakai et al., 2003). The mechanism that results in the integration of the AAV genome begins with the formation of circular intermediates (Duan et al., 1998; Duan et al., 1999). The *in vivo* annealing of complementary plus and minus single strand genomes with random intermolecular end joining between the various orientations of monomeric double stranded DNAs gives rise to the single and concatemeric integration vectors (Nakai et al., 2000) that insert into breaks in the chromosomal DNA (Miller et al., 2004). The formation of hairpin DNA structures within the TR sequences is critical for the recombinational integration of AAV and for the formation of persistent circular episomal concatemers (Choi et al., 2005). The site specific integration is mediated by DNAPKcs and ligase IV members of the nonhomologous end joining pathway (Daya et al., 2009). While ligase IV is required for the site specific integration, the presence of DNAPKcs increases the frequency of the integrations.

The mechanism of integration for the pDP9 vectors appears to be different than that for the AAV. Even though *JcDENV* ITRs produce hairpin loop structures necessary for replication similar to those described for AAV replication and integration (Ding et al., 2002), the lack of a requirement for the inclusion of both ITRs for efficient integration demonstrates fundamental differences between the mechanisms involved in the integration the two modified parvovirus sequences.

Expression resulting from the activity of the *JcDENV* P9 promoter/enhancer was comparable with that from the hr5IE1 enhancer promoter. When both reporter constructs were included in the same vector pDP9a-DsRed, the levels of transcripts resulting from each of the

expression cassettes were similar and at 30 fold greater levels than the transcript produced under the control of the *B. mori* actin 3 promoter from similar vector integrations. The hr5IE1 is one of the more active promoters (Jarvis et al., 1996; Douris et al., 2006) and provides strong expression of the GFP or DsRed reporter to facilitate selection of pDP9 transformants using cell sorting. Although the P9 promoter/enhancer shows similar activity, the inclusion of other promoters such as the synergistic double enhanced *BmNPV* ie-1 gene with the HR3 element and the *B. mori* A3 cytoplasmic actin promoter (Lu et al., 1996) may provide much greater expression activity. Insertion of alternate promoter-structural gene constructs in pDP9 can be easily achieved because of the inclusion of a multiple cloning site situated 3' of the *JcDNV* P9 promoter.

The pDP9 somatic transformation/expression vectors were found to be highly efficient vehicles for high level expression in cultured insect cells. The concatemeric integration of the vectors increases the potential for even higher levels of expression beyond those previously reported for other expression systems (Douris et al., 2006). They provide a useful alternative to the baculovirus based expression systems and offer versatility in being applicable to cultured cells from at least two orders, Lepidoptera and Diptera. With this flexibility, high level expression will not be limited to a few cultured cell lines but will potentially be applicable to most insect cell lines. The ability to produce enhanced quantities of targeted proteins within insect cells as a result of the sequence concatemerization will also provide a cell based system for assaying proteins with potential antimicrobial activities, as an enhanced coupled intracellular reporter system or for assessment of metabolic pathways where multiple genes could be expressed from within the same chromosomal insertion. The potential of these expression activities are being explored for selecting active agents for inclusion in transgenic systems.

ACKNOWLEDGEMENTS

The authors wish to thank Neal Benson of the Flow Cytometry Core Laboratory, ICBR, University of Florida for assistance with the cell sorting analysis.

The use of trade name, commercial product, or corporation in this publication is for the information and convenience of the reader and does not imply an official recommendation, endorsement or approval by the U. S. Department of Agriculture or the Agricultural Research Service for any product or service to the exclusion of others that may be suitable.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

375 REFERENCES

- 376 Barbu, V. and Dautry, F. (1989) Northern blot normalization with a 28S rRNA oligonucleotide
377 probe. *Nucleic Acids Research* 17, 7115.
- 378 Bossin, H., Fournier, P., Royer, C., Barry, P., Cerutti, P., Gimenez, S., Couble, P. and Bergoin,
379 M. (2003) *Junonia coenia* densovirus-based vectors for stable transgene expression in Sf9
380 cells: influence of the densovirus sequences on genomic integration. *Journal of Virology*
381 77, 11060-71.
- 382 Bossin, H., Furlong, R.B., Gillett, J.L., Bergoin, M. and Shirk, P.D. (2007) Somatic
383 transformation efficiencies and expression patterns using the *JcDNV* and *piggyBac*
384 transposon gene vectors in insects. *Insect Molecular Biology* 16, 37-47.
- 385 Choi, V.W., Samulski, R.J. and McCarty, D.M. (2005) Effects of adeno-associated virus DNA
386 hairpin structure on recombination. *Journal of Virology* 79, 6801-7.
- 387 Daya, S., Cortez, N. and Berns, K.I. (2009) Adeno-associated virus site-specific integration is
388 mediated by proteins of the nonhomologous end-joining pathway. *Journal of Virology*
389 83, 11655-64.
- 390 Denz, C.R. and Dube, D.K. (2005) The benefits of 28S rRNA for standardization of reverse
391 transcription-polymerase chain reaction for studying gene expression. *Analytical*
392 *Biochemistry* 341, 382-4.
- 393 Ding, C., Urabe, M., Bergoin, M. and Kotin, R.M. (2002) Biochemical characterization of
394 *Junonia coenia* densovirus nonstructural protein NS-1. *Journal of Virology* 76, 338-345.
- 395 Douris, V., Swevers, L., Labropoulou, V., Andronopoulou, E., Georgoussi, Z. and Iatrou, K.
396 (2006) Stably transformed insect cell lines: tools for expression of secreted and
397 membrane-anchored proteins and high-throughput screening platforms for drug and
398 insecticide discovery. *Advances in Virus Research* 68, 113-56.
- 399 Duan, D., Sharma, P., Yang, J., Yue, Y., Dudus, L., Zhang, Y., Fisher, K.J. and Engelhardt, J.F.
400 (1998) Circular intermediates of recombinant adeno-associated virus have defined
401 structural characteristics responsible for long-term episomal persistence in muscle tissue.
402 *Journal of Virology* 72, 8568-77.
- 403 Duan, D., Yan, Z., Yue, Y. and Engelhardt, J.F. (1999) Structural analysis of adeno-associated
404 virus transduction circular intermediates. *Virology* 261, 8-14.
- 405 Dumas, B., Jourdan, M., Pascaud, A.M. and Bergoin, M. (1992) Complete nucleotide-sequence
406 of the cloned infectious genome of *Junonia coenia* Densovirus reveals an organization
407 unique among parvoviruses. *Virology* 191, 202-222.
- 408 Giraud, C., Devauchelle, G. and Bergoin, M. (1992) The densovirus of *Junonia coenia* (*JcDnv*)
409 as an insect cell expression vector. *Virology* 186, 207-218.
- 410 Goidin, D., Mamessier, A., Staquet, M.J., Schmitt, D. and Berthier-Vergnes, O. (2001)
411 Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-
412 actin genes as internal standard for quantitative comparison of mRNA levels in invasive
413 and noninvasive human melanoma cell subpopulations. *Analytical Biochemistry* 295, 17-
414 21.
- 415 Horn, C. and Wimmer, E.A. (2000) A versatile vector set for animal transgenesis. *Development*
416 *Genes and Evolution* 210, 630-7.
- 417 Jarvis, D.L., Weinkauff, C. and Guarino, L.A. (1996) Immediate-early baculovirus vectors for
418 foreign gene expression in transformed or infected insect cells. *Protein Expression And*
419 *Purification* 8, 191-203.

- Landais, I., Pommet, J., Mita, K., Nohata, J., Gimenez, S., Fournier, P., Devauchelle, G., Duonor-Cerutti, M. and Ogliastro, M. (2001) Characterization of the cDNA encoding the 90 kDa heat-shock protein in the Lepidoptera *Bombyx mori* and *Spodoptera frugiperda*. *Gene* 271, 223-31.
- Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 25, 402-408.
- Lu, M., Johnson, R.R. and Iatrou, K. (1996) Trans-activation of a cell housekeeping gene promoter by the IE1 gene product of baculoviruses. *Virology* 218, 103-13.
- Miller, D.G., Petek, L.M. and Russell, D.W. (2004) Adeno-associated virus vectors integrate at chromosome breakage sites. *Nature Genetics* 36, 767-73.
- Nakai, H., Montini, E., Fuess, S., Storm, T.A., Meuse, L., Finegold, M., Grompe, M. and Kay, M.A. (2003) Helper-independent and AAV-ITR-independent chromosomal integration of double-stranded linear DNA vectors in mice. *Molecular Therapy* 7, 101-11.
- Nakai, H., Storm, T.A. and Kay, M.A. (2000) Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver *in vivo*. *Journal of Virology* 74, 9451-63.
- Royer, C., Bossin, H., Romane, C., Bergoin, M. and Couble, P. (2001) High amplification of a densovirus-derived vector in larval and adult tissues of *Drosophila*. *Insect Molecular Biology* 10, 275-80.
- Shirk, P.D., Bossin, H., Furlong, R.B. and Gillett, J.L. (2007) Regulation of *Junonia coenia* densovirus P9 promoter expression. *Insect Molecular Biology* 16, 623-33.
- Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E., Kamba, M., Komoto, N., Thomas, J.L., Mauchamp, B., Chavancy, G., Shirk, P., Fraser, M., Prudhomme, J.C. and Couble, P. (2000) Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. *Nature Biotechnology* 18, 559-559.
- Yang, C.C., Xiao, X., Zhu, X., Ansardi, D.C., Epstein, N.D., Frey, M.R., Matera, A.G. and Samulski, R.J. (1997) Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration *in vivo* and *in vitro*. *Journal of Virology* 71, 9231-47.

Tables

Table 1. Gene specific primers and beacons for gene quantitation and RT-qPCR.

Target Gene		Primer Sequence (5' → 3')	Annealing Temperature
EGFP	Forward	AGAAGAACGGCATCAAGG	54° C
	Reverse	GTGCTCAGGTAGTGGTTG	
	Beacon	CGCGATCTCGAGGACGGCAGCGTGCAGGAATCGCG	
DsRed	Forward	CCGTAATGCAGAAGAAGACC	54° C
	Reverse	ACTTGAACCTCCACCAGGTAG	
	Beacon	CGCGATCCTCCACCGAGCGCCTGTACGATCGCG	
28S Ribosomal RNA	Forward	GGGGAGGAAAAGAACTAAC	56° C
	Reverse	CAACTTTCCTTACGGTACT	
Sf-Hsp90	Forward	CACGCCTCTCGCATTTAC	56° C
	Reverse	TTAATCCACTTCCTCCATACG	
pDP9 JcITR	Forward	CGGCATCAGAGCAGATTG	58° C
	Reverse	CGTGTATTATTATGGAATGAACC	

Table 2. The frequency of insects with persistent DsRed fluorescence in *D. melanogaster* larvae following microinjection of embryos.

Vector	Number of Eggs Injected	Number of Fluorescent Eggs	Larvae Observed	Number of Fluorescent Larvae
Lit-JcLITR/hr5IE1DsRed	395	254 (64%)	57 (15%)	20 (57%)
Lit-JcRITR/hr5IE1DsRed	503	30 (6%)	114 (23%)	29 (25%)
pJDR ^a	970		112 (12%)	55 (49%)
phr5IE1DsRed ^a	1712	595 (35%)	205 (12%)	1 (0.5%)

^aData from Bossin et al. (2007).

Table 3. Distribution of GFP and DsRed expressing Bm5 cells transfected with *JcDNV* and pDP9 vectors. The pDP9 and pDP9a transfected cells were maintained for 108 d prior to scan while the pJDR and pJDR-3xP3G were maintained for 50 d.

Vector	GFP Expression (% Cells)	DsRed Expression (% Cells)
pDP9	50.01 ¹	NA ²
pDP9a	46.62	NA
pJDR	NA	0.18
pJDR-3xP3G	9.84	0.05

¹Percent of total cells scanned.

²NA – Not Applicable

Table 4. Comparative levels of transcripts from pDP9a-DsRed transfected Sf9 cells.

Transcript	Copies/Cell ¹ mean (± SD)	Relative Level Transcript
28S RNA	2.55×10^5 (2.5×10^3)	1.0
BmA3EGFP	1.20×10^3 (2.2×10^2)	4.7×10^{-3}
P9DsRed	3.45×10^4 (1.9×10^3)	0.14

¹copies/cell = calculated number of copies of transcript per cell for the designated transcript determined on the basis of RT-qPCR using transcript specific primers.

Table 5. Comparative levels of transcripts from pDP9a-DsRed transfected and non-transfected Bm5 cells.

Transcript	pDP9a-DsRed Transfected Copies/Cell¹ mean (± SD)	Relative Level of Transcript	Non-Transfected Copies/Cell¹ mean (± SD)	Relative Level of Transcript
28S RNA	2.63 x 10 ⁵ (2.07 x 10 ⁴)	1.0	2.28 x 10 ⁵ (1.87 x 10 ⁴)	1.0
hsp90	2.38 x 10 ⁴ (2.08 x 10 ²)	0.1	2.51 x 10 ⁴ (1.5 x 10 ³)	0.1
P9DsRed	4.52 x 10 ⁴ (4.96 x 10 ³)	0.16	2 (0.5)	0
hr5IE1EGFP	5.03 x 10 ⁴ (2.11 x 10 ⁴)	0.19	0	0

¹copies/cell = calculated number of copies of transcript per cell for the designated transcript determined on the basis of RT-qPCR using transcript specific primers.

Figures:

Figure 1. Diagrams of the pDP9 somatic transformation vectors. The positions of the major features are shown as bars and arrows around the perimeter of the plasmid with the arrowheads oriented towards the direction of expression. The structural features of pDP9 (Panel A) including the BmA3EGFP expression cassette and pDP9a-DsRed (Panel B) including the hr5IE1EGFP expression cassette. Abbreviations: AMP = ampicillin resistance gene; BmA3 = *B. mori* actin 3 promoter; DsRed = red fluorescent protein coding sequence; EGFP = enhanced green fluorescent protein coding sequence; hr5IE1 = baculovirus hr5 enhancer/immediate early one promoter; JcESs = *Junonia coenia* enhancer sequence; JcITR = *Junonia coenia* P9 inverted terminal repeat; Kan/Neo = kanamycin/neomycin resistance gene; M13 ORI = M13 origin of replication; P9 = P9 promoter; E coli ORI = pUC19 origin of replication; SV40 polyA = SV40 polyadenylation site.

Figure 2. Expression of DsRed and GFP in pDP9a-DsRed transformed Sf9 cells. DsRed fluorescence (Panel A), GFP fluorescence (Panel B) in a population of Sf9 cells (Panel C transmitted light) 24 h after transfection. Two Sf9 cells that showed clear expression of both DsRed and GFP were marked with arrows in all three panels. Lack of observable fluorescence from either of the two marker products does not exclude expression of the gene in that cell. A scale bar of 50 μ m is shown in each panel.

Figure 3. Persistence of GFP expression in Bm5 cells transfected with pDP9a-DsRed. The percent of cells containing GFP was observed over 50 d following transfection while maintained without selection (Panel A). Following selection with G418, cells were subjected to FACScan

after 120 d and the distribution of cells expressing GFP was determined (Panel B). Q1 were cells expressing DsRed; Q2 were cells expressing both GFP and DsRed; Q3 were cells expressing GFP; and Q4 were cells that did not express either marker protein.

Figure 4. Distribution of cells expressing fluorescent proteins in Bm5 cells transformed with pDP9 and *JcDNV* vectors. GFP expressing cells in a culture of Bm5 cells transfected with pDP9a-DsRed (Panel A) or pDP9 (Panel B) and maintained for 105 d. DsRed expressing cells in a culture of Bm5 cells transfected with pJDR (Panel C) and maintained for 50 d. GFP and DsRed expressing cells (Panel D) in a culture of Bm5 cells transfected with pJDR-3xP3G and maintained for 50 d. For each panel, the Q1 sector shows the incidence of DsRed expressing cells, the Q2 sector shows the incidence of GFP and DsRed expressing cells, the Q3 sector shows the incidence of GFP expressing cells, and the Q4 sector shows the incidence of cells not expressing either fluorescent marker protein.

Figure 5. Direct PCR of genomic and plasmid DNA isolated from pDP9 transformed Sf9 cells. Lane designations: genomic DNA (Lane A) isolated from pDP9 transformed Sf9 cells; 1 Kb ladder (Lane B); plasmid DNA (Lane C) isolated from pDP9 transformed Sf9 cells; and control non-transformed Sf9 cells (Lane D). The pDP9-*JcDNV* ITR primers should produce a 1.8 kb fragment from the intact reference plasmid.