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Insect cell transformation vectors that support high level expression and promoter assessment in insect cell culture

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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.

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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) (*Jc*DNV) (Bossin et al., 2003). Transfection of *S. frugiperda* Sf9 cells with a *Jc*DNV 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 *Jc*DNV 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 *Jc*DNV 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 *Jc*DNV 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

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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 *Jc*DNV 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 *Jc*DNV 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 *Jc*DNV were individually cloned into Litmus 28i (Invitrogen|ThermoFisher Scientific, Waltham MA). A 692 bp *Bmg*BI/*Hind*III fragment that contains the P9ITR from pJDR (Bossin et al., 2007) was ligated into *Stu*I/*Hind*III digested Litmus 28i (Lit-P9ITR). Similarly, an 886 bp *Bsr*BI/*Bsr*GI fragment that contains the P93ITR from pJDR was ligated into *Acc*65I/*Zra*I digested Litmus 28i (Lit-P93ITR). A 2138 bp *BstZ*17I/*Bgl*II fragment from pSL-hr5IE1DsRed (Bossin et al., 2007) that contains hr5IE1DsRed was ligated into *Bfr*BI/*Bgl*II 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 *Bsa*I/*Dra*III 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 *Stu*I digested pSLfa1180fa (Horn and Wimmer, 2000) to produce pSL-Kan/Neo. The *Bst*Z17I/*Bgl*II fragment containing the *Bombyx mori* actin A3EGFP reporter cassette (BmA3G) from pPIGA3EGFP (Tamura et al., 2000) was cloned into *Bst*Z17I/*Bgl*II digested pSLfa1180fa to produce pSL-BmA3G. Subsequently, the BmA3G expression cassette was removed from pSL-BmA3G by digestion with *Sma*I/*Afl*II and ligated into pSL-Kan/Neo digested with *Nru*I/*Afl*II to produce pSL-Kan-BmA3G. The *Nci*I fragment containing LITR-P9 promoter was

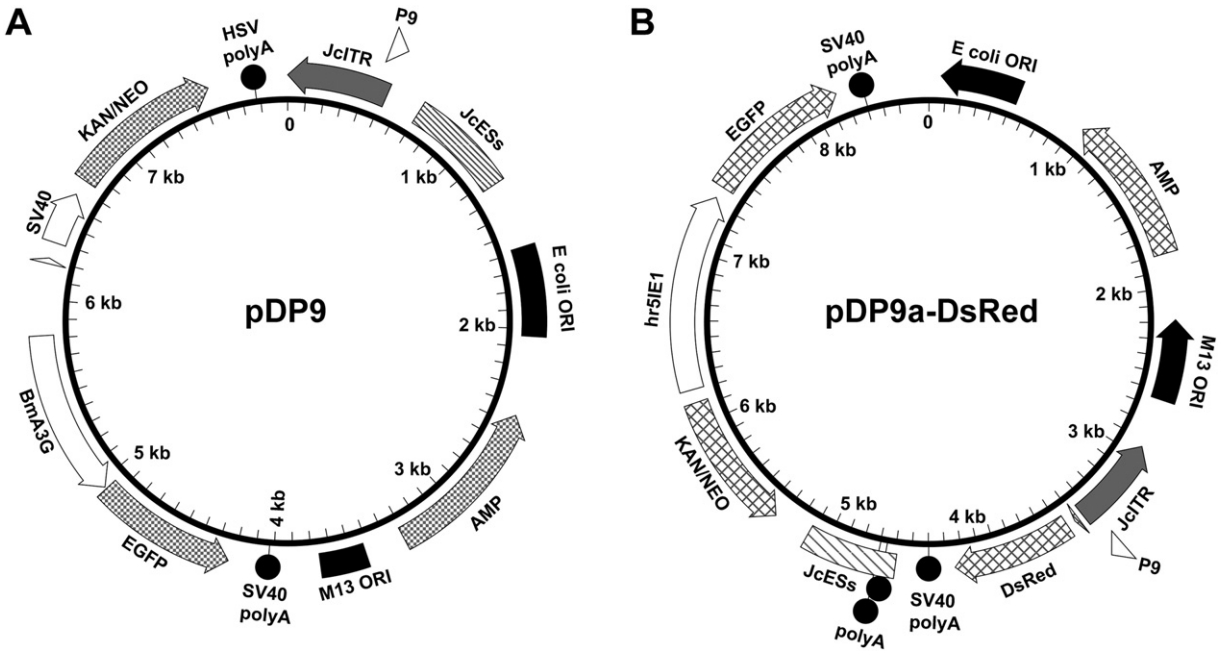

Fig. 1. Diagrams of the pDP9 somatic transformation vectors. The positions of the major features are shown as bars and arrows around the perimeter of the plasmid with the arrowheads oriented towards the direction of expression. The structural features of pDP9 (Panel A) including the BmA3EGFP expression 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; *Jc*ESs = *Junonia coenia* enhancer sequence; *Jc*ITR = *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 poly adenylation site.