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Characterization of the replication of a baculovirus mutant lacking the DNA polymerase gene

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Abstract

In a previous study, the DNA polymerase gene (*dnapol*) of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) was identified as one of six genes required for plasmid replication in a transient replication assay (M. Kool, C. Ahrens, R.W. Goldbach, G.F. Rohrmann, J.M. Vlak, Identification of genes involved in DNA replication of the *Autographa californica*, Proc. Natl. Acad. Sci. U.S.A. 91, (1994) 11212–11216); however, another study based on a similar approach reported that the virally encoded polymerase was only stimulatory (A. Lu, L.K. Miller, The roles of 18 baculovirus late expression factor genes in transcription and DNA replication, J. Virol. 69, (1995) 975–982). To reconcile the conflicting data and determine if the AcMNPV DNA polymerase is required for viral DNA replication during the course of an infection, a *dnapol*-null virus was generated using bacmid technology. To detect viral DNA replication, a highly sensitive assay was designed based on real-time PCR and SYBR green chemistry. Our results indicate that a bacmid in which the *dnapol* ORF was deleted is unable to replicate its DNA when transfected into *Spodoptera frugiperda* (Sf-9) cells, although when the *dnapol* ORF was introduced into the polyhedrin (*polh*) locus, this repaired virus could propagate at levels similar to the control virus. These results confirm that the AcMNPV-encoded DNA polymerase is required for viral DNA replication and the host DNA polymerases cannot substitute for the viral enzyme in this process.

Keywords: Baculovirus replication; AcMNPV *dnapol* knockout bacmid

Introduction

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the type member of the *Baculoviridae*, a large family of viruses containing circular, supercoiled, double-stranded DNA genomes. Infectious predominantly to certain members of the insect order *Lepidoptera*, baculoviruses have often been exploited to express eukaryotic genes in cell culture. Although the mechanism of baculovirus DNA replication is not well understood, evidence suggests replication may proceed via a rolling circle mechanism forming larger than unit length concatamers (Leisy and Rohrmann, 1993; Oppenheimer and Volkman, 1997; Wu et al., 1999). Evidence also indicates that AcMNPV DNA replication may initiate at homologous regions (*hrs*) composed of one to eight copies of an imperfect palindrome flanked by a direct repeat sequence that also serve as transcriptional enhancers in transient assays (Guarino et al., 1986; Kool et al., 1993; Pearson et al., 1992). Observations that DNA polymerase activity was induced in baculovirus-infected cells led to the discovery of the AcMNPV DNA polymerase that was identified due to highly conserved amino acid sequences found in several other viral DNA polymerases such as herpes simplex virus type 1 (HSV-1) and vaccinia virus (Tomalski et al., 1988). The *dnapol* ORF is transcribed in the early stage of infection and translated into a 114-kDa polypeptide. It has been shown to polymerize deoxyribonucleotides complimentary to single-stranded DNA templates in the absence of helix destabilizing proteins and to possess an intrinsic 3′→5′ exonuclease activity (Hang and Guarino, 1999; Mikhailov et al., 1986).

Previous investigations involving a transient replication assay identified six baculovirus genes essential for repli-

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cation of a plasmid containing the AcMNPV *hr2* as the origin of replication (Kool et al., 1994). These genes encode an activator of transcription (*ie-1*); a helicase (*p143*); a DNA polymerase (*dnapol*); and three late expression factors (*lefs*) that encode a primase (*lef-1*); a primase accessory factor (*lef-2*); and a single-stranded DNA binding protein (*lef-3*). In contrast, another group using a similar transient replication assay system was able to detect replication of a reporter plasmid containing AcMNPV *hr5* at 11% the normal level when the *dnapol* gene was omitted (Lu and Miller, 1995). The observation that suboptimal levels of replication could occur in the absence of the *dnapol* gene suggested the possibility that a host DNA polymerase might functionally interact with other viral replication factors. This explanation is in agreement with results of substitution experiments that showed the DNA polymerase from *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV), which is about 60% identical to the AcMNPV DNAPOL, and a distantly related polymerase from an ascovirus can functionally replace the AcMNPV DNA polymerase in transient replication assays (Ahrens and Rohrmann, 1996; Pellock et al., 1996). Additionally, for HSV-1 that shares many similar features to baculoviruses, the origin binding protein (UL9) has been shown to interact with the host cell DNA polymerase α suggesting its involvement in the initiation of viral replication (Lee et al., 1995).

In this report, we investigate whether replication of a *dnapol*-null virus can occur when transfected into *Spodoptera frugiperda* (Sf-9) cells. In addition, we compared the levels of replication with a 'repaired' virus in which the DNA polymerase was inserted into the polyhedrin (*polh*) locus and two other viruses that contained gene deletions of either alkaline nuclease or very late expression factor-1. These latter two viruses have been shown to be impaired for DNA replication (Okano et al., 2004; Vanarsdall et al., 2004).

Results and discussion

Construction of an AcMNPV bacmid lacking the DNA polymerase gene and construction of a 'repair' virus

In order to examine the ability of AcMNPV to replicate in the absence of the *dnapol* gene, we used bacmid technology to generate mutant viruses lacking *dnapol* or 'repaired' with the *dnapol* gene transposed into the *polh* locus. A β-glucuronidase (GUS) reporter gene under control of the early *ie-1* promoter was also transposed into the virus constructs to monitor viral infection using a chromogenic assay. The diagram in Fig. 1A outlines the organization of the control (Ac-GUS), *dnapol* knockout (*dnapol*-KO), and *dnapol* 'repair' (*dnapol*-REP) bacmids used in this study. To verify the deletion of the *dnapol* ORF in the parent virus and ensure the subsequent transposition of the GUS reporter gene and *dnapol* 'repair' fragment occurred at the correct location, PCR analysis was performed. Primers designed to amplify across the *dnapol* locus (Table 1) generated a 3.9-kb PCR product from the control bacmid containing the *dnapol* ORF, whereas the same primer set generated a 1.9-kb PCR product from the *dnapol*-KO and *dnapol*-REP bacmids (Fig. 1B). Similarly, amplification from pUC/M13 priming sites that flank the *polh* region indicated transposition of the 2.5-kb GUS fragment in all bacmid constructs as well as the 3.2-kb repair fragment present in the *dnapol*-REP bacmid (Fig. 1C). Therefore, these data confirm the insertion of the expected gene products into our bacmid constructs.

Viral growth curve analysis

To examine the ability of *dnapol*-KO and *dnapol*-REP viruses to replicate in cell culture, we transfected these virus constructs into Sf-9 cells and compared GUS expression and cytopathogenic effects (CPE) to the control virus. Whereas both the control and *dnapol*-REP virus showed widespread GUS expression and extensive CPE, cells transfected with the *dnapol*-KO virus showed isolated GUS expression from the initially transfected cells and no evidence of CPE (data not shown). To detect low levels of replication by the *dnapol*-KO virus and ensure that secondary mutations were not acquired during manipulation of the bacmids, growth curve analyses were performed. For these experiments, Sf-9 cells were transfected with bacmid DNA and at the indicated time points the titers of the supernatant were determined by an end-point dilution assay utilizing the GUS reporter gene (O'Reilly et al., 1992). For both the control and *dnapol*-REP virus constructs, a steady increase in virus production was observed throughout the time course (Fig. 2). Additionally, the *dnapol*-REP virus displayed a kinetically similar growth rate as the control virus, indicating that the *dnapol* ORF in the *polh* locus rescued the *dnapol*-KO virus. No titer was detected for cells transfected with a *dnapol*-KO virus at any time point (Fig. 2). Therefore, these results indicate that the defective phenotype of the *dnapol*-KO virus construct is directly due to the deletion of the *dnapol* ORF.

Real-time PCR analysis

Although we obtained no evidence for viral replication by the *dnapol*-KO virus by examining cells for GUS expression and virus production, this did not rule out the possibility that synthesis of viral DNA had proceeded aberrantly such that either nonviable virions were produced or were below the level detectable by titer assays. To investigate this possibility, we performed a replication assay with Sf-9 cells transfected with bacmid DNA using a quantitative and highly sensitive method based on real-time PCR and SYBR green chemistry. The strategy used to detect replicated viral DNA involved designing primers that would

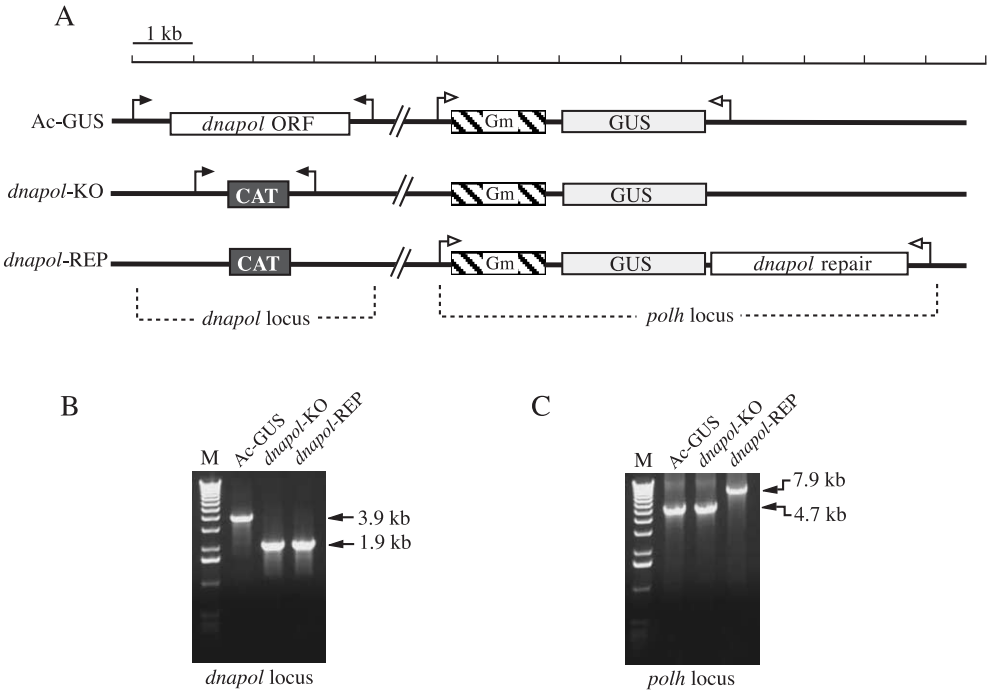

Fig. 1. Organization of the control (Ac-GUS), *dnapol* knockout (*dnapol*-KO), and *dnapol* repair (*dnapol*-REP) virus constructs derived from the commercially available bacmid bMON14272 (Invitrogen). (A) To serve as a control virus (Ac-GUS), a bacmid containing the native *dnapol* ORF was transposed with a β-glucuronidase (GUS) gene under control of the early *ie-1* promoter at the polyhedrin (*polh*) locus according to the bac-to-bac protocol (Invitrogen). A DNA polymerase knockout virus (*dnapol*-KO) was generated by transposing a GUS reporter gene into the *polh* locus as described and by replacing the *dnapol* ORF with a chloramphenicol resistance gene (CAT) via homologous recombination using the λ Red system (see Materials and methods). To serve as a repair virus (*dnapol*-REP), a genomic fragment containing the *dnapol* ORF and promoter region (*dnapol* repair) was transposed into the *polh* region of a *dnapol*-KO bacmid in addition to the GUS reporter gene. The closed arrows flanking the *polh* locus represent pUC/M13 forward and reverse primer annealing sites and the open arrows flanking the *dnapol* locus represent the annealing sites for primers 58125F and 55796R (Table 1). A 1.5-kb gentamicin resistance gene (Gm) is present at the *polh* locus as a result of transposition from the transfer plasmid. (B) Ethidium bromide-stained agarose gels of PCR products generated with primers 51825F and 55796R designed to amplify across the *dnapol* locus or with pUC/M13 forward and reverse primers that amplify across the *polh* locus of bacmid bMON14272. The templates used are indicated above the panels and the side arrows indicate the expected size of the PCR products. M represents a 1-kb ladder DNA size markers.

amplify a 100-bp genomic fragment at a region containing four *Dpn*I restriction sites (Table 1). Treatment of bacmid DNA with *Dpn*I after transfection should digest the input DNA at four positions within the target DNA and thereby prevent it from amplification in the real-time PCR reaction. For positive controls that replicate their DNA at suboptimal levels, we used AcMNPV bacmid genomes lacking either very late expression factor-1 (*vlf-1*) or alkaline nuclease (*an*). It has been shown previously that these bacmids replicate DNA only in initially transfected cells (Okano et al., 2004; Vanarsdall et al., 2004).

To quantify viral DNA replication in Sf-9 cells, a six-step standard calibration curve was generated with triplicate samples of purified bacmid DNA serially diluted from 50 to 0.0005 ng. A highly reproducible linear curve was generated when the log concentration of purified bacmid DNA was plotted against the cycle threshold allowing accurate estimation of viral DNA present in transfected cells

Table 1
Oligonucleotides used in this study

Application	Primer	Sequence
Homologous recombination	52329cat	TTTATTTTTTCATTTTATACAAACAAAATTTATACGT ATTGTTAGCACATGGTACCGTGTAGGCTGGAGCTGCT
	55333cat	CGTGTTGACGTCTGTGCCTCCATATTTGGGCCATCG CTGCATATTTAAAATACCCATATGAATATCCTCCTTAG
dnapol locus	51825F	ACACAGAATTGCGATCGTTTGAACCG
	55796R	CCAGTTCAGACTCCTCCTCGTTAGTC
dnapol repair fragment	52314Hind3	GCAAGCTTTATAATTATAAACTTTATTTTTTCAT
	55539XhoI	CGCTCGAGGTGTTGATATCTCGGCCGGCGAAATC
real-time PCR	65972F	CGTAGTGGTAGTAATCGCCGC
	66072R	AGTCGAGTCGCGTCGCTTT

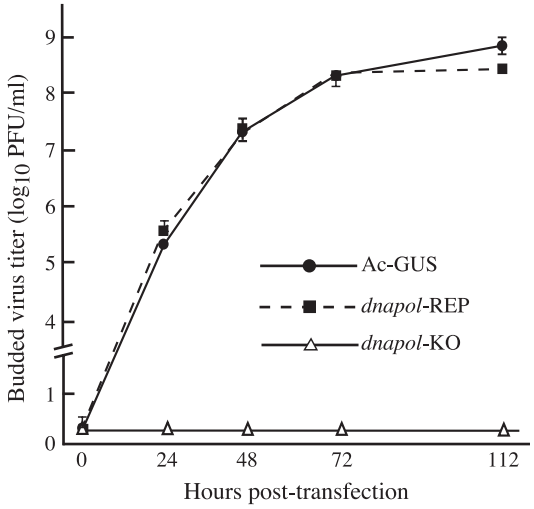

Fig. 2. Viral growth curve analysis. Sf-9 cells were transfected with 5 μg of control, *dnapol*-KO, or *dnapol*-REP bacmid DNA, and at the indicated time points, the supernatants were collected and the titer determined by a $TCID_{50}$ assay. The points indicate the averages of cells transfected in triplicate and the error bars represent the standard deviation.

(Fig. 3A). To confirm that the SYBR green fluorescence was due to its specific binding to double-stranded DNA, a dissociation curve was produced. These data showed that all the PCR products were denatured to single-stranded DNA at about 85 °C indicating specific amplification of a homogenous DNA sequence (Fig. 3B). The replication levels of our virus constructs determined by real-time PCR analysis are presented by the bar graph in Fig. 3C. As would be expected for the control virus, a steady increase in viral DNA was detected during the experiment ultimately resulting in a 400-fold increase. Analysis of the *vlf-1*- and *an*-knockout bacmids revealed a 13- and 10-fold increase in viral DNA at 48 h pt, respectively, with no further increase at the subsequent time points. As previously suggested, this block of further DNA synthesis is likely due to the inability of these bacmids to initiate secondary infection. It was previously shown using slot blot assays that these viruses could amplify DNA to about 20% the level of the control virus at 120 h pt (Okano et al., 2004; Vanarsdall et al., 2004); however, the real-time PCR assay indicated repli-

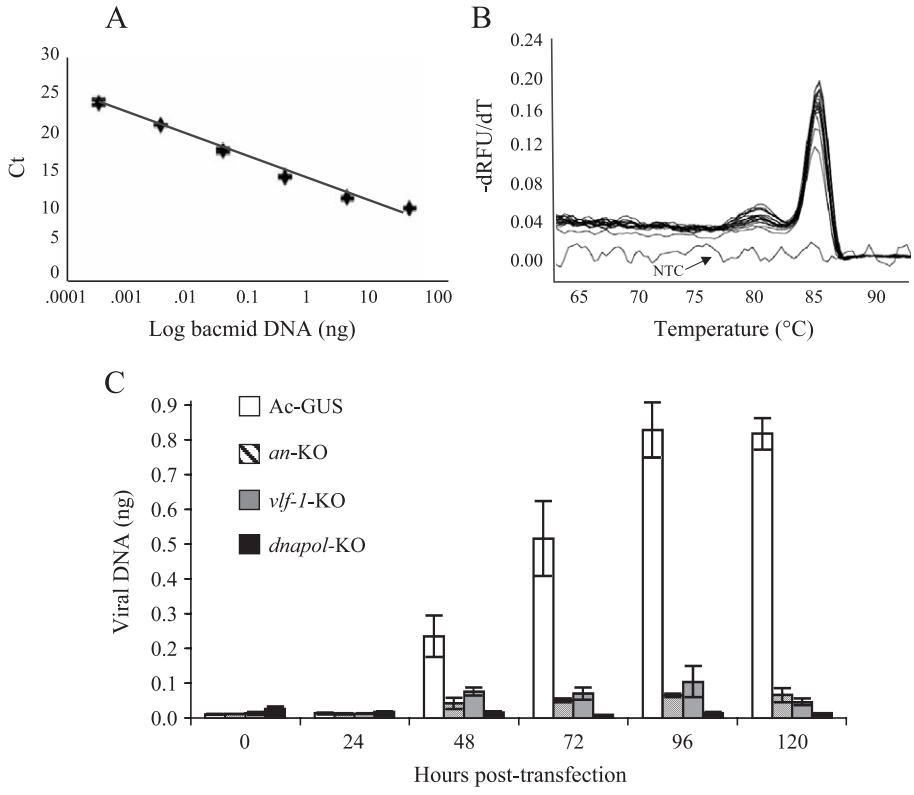

Fig. 3. Real-time PCR analysis of viral DNA replication. (A) A standard calibration curve generated from triplicate samples of purified bacmid DNA serially diluted from 50 to 0.0005 ng. The cycle threshold (Ct) corresponds to the number of cycles needed to reach a fluorescence level above the predetermined background level and has been shown to be inversely proportional to the initial amount of target DNA (correlation value = 0.998). (B) Dissociation curve of amplified PCR products. The curve was generated by subjecting PCR products to a step-wise increase in temperature and monitoring fluorescence levels. The single peak at ~85 °C indicates a single PCR product was produced and the flat line for the no template control (NTC) indicates negligible background. The *y*-axis value −dRFU/dT indicates the first negative derivative of the temperature verses fluorescence. (C) Quantitative analysis of replicated bacmid DNA detected in transfected Sf-9 cells. Total cellular DNA was isolated from triplicate transfections for each time point, digested with *Dpn*I restriction enzyme, and analyzed by real-time PCR. The bar heights indicate the averages and the error bars represent the standard deviation.

cation levels at this time point of 4% and 7% for the *vlf-1*- and *an*-knockout bacmids, respectively (Fig. 3C). We attribute the difference to the nonlinear nature of the signal on the Southern blot used in previous studies. In contrast, no increase in viral DNA replication was detected in Sf-9 cells transfected with the *dnapol*-KO virus at any time up to 120 h pt (Fig. 3C). Trace amounts of *Dpn*I-resistant PCR-amplified DNA were detectable, but since this DNA was present at 0 h pt and did not increase over time, we attribute it to low levels of hemimethylated or unmethylated (*Dpn*I resistant) DNA present in the original bacmid preparation or incomplete digestion by *Dpn*I. This was further supported by our observation that amplification of other regions of the AcMNPV genome containing fewer *Dpn*I restriction sites in the target sequence produced higher background levels (data not shown).

The fact that Lu and Miller (1995) could detect replication of their reporter plasmid at 11% the control level suggested that a host DNA polymerase has the ability to extensively synthesize nascent DNA using the origin-containing plasmid as a template. Such DNA synthesis might be initiated at random nicks in the input DNA. However, the host DNA polymerase must have copied all the *Dpn*I sites on at least one strand because the resistant DNA was the same size as the linearized input plasmid. In contrast to the transient assay, the bacmid transfection assay requires complete replication and processing of the whole genome in order to generate an infectious product. We did not detect replication of the *dnapol*-KO bacmid in the transfection assay (Fig. 2). Even if limited bacmid synthesis may have been initiated, replication was not complete. In addition, we did not detect measurable DNA synthesis in cells transfected with the *dnapol*-KO bacmid using real-time PCR analysis (Fig. 3). Therefore, our data indicate that the viral DNA polymerase is essential for AcMNPV replication and suggest that the plasmid replication observed previously by using the transient replication assay reflects an artifact of that system.

Materials and methods

Cells and antibiotics

S. frugiperda (Sf-9) cells were cultured in Sf-900 II serum-free medium (Invitrogen) with added penicillin G (50 units/ml), streptomycin (50 units/ml, Whittaker Bioproducts), and fungizone (amphotericin, 375 ng/ml, Invitrogen), as previously described (Harwood et al., 1998).

Bacmid construction, purification, and transfection

To delete the DNA polymerase gene from the AcMNPV genome propagated as bacmid bMON14272 in *Escherichia coli*, a method involving homologous recombination with the λ Red system was employed as previously described (Datsenko and Wanner, 2000; Vanarsdall et al., 2004). Briefly, a linear chloramphenicol acetyltransfease gene (CAT) was PCR amplified from plasmid pKD3 (Datsenko and Wanner, 2000) with primers 52329CAT and 55333CAT (Table 1) that contain 50 nt flanking regions homologous to the 5′ and 3′ end of the DNA polymerase ORF. Homologous recombination between the CAT gene and the *dnapol* ORF was accomplished by electroporating 500 ng of the linear CAT fragment into *E. coli* DH10B cells harboring the bacmid bMON14272 and plasmid pKD46 (Datsenko and Wanner, 2000) encoding the λ Red recombinase genes. Bacmid containing clones were selected on LB agar containing kanamycin and chloramphenicol and screened by PCR to confirm the deletion of the *dnapol* ORF. To introduce the GUS marker gene for monitoring viral replication in cell culture and to generate a *dnapol* repair virus, a method involving transposition at the *polh* gene region with the transfer plasmids pfbIEGUS and pfbIEGUS-repair was performed as previously described (Vanarsdall et al., 2004). The *dnapol* repair fragment was PCR amplified with primers 52314Hind3 and 55539XhoI (Table 1) that produce an AcMNPV genomic fragment corresponding to (nt 52,314–55,539) and include the *dnapol* ORF and promoter region.

Bacmid DNA was purified from 2-l cultures using CsCl gradients with ultracentrifugation. Equimolar amounts of purified bacmid DNA were transfected into Sf-9 cells (2×10^6) seeded in a 6-well plate using a cationic liposome method (Campbell, 1995). Bacmid DNA was mixed with 200 μl of Sf-900 II medium containing 10 μl of liposomes and incubated at 27 °C for 30–45 min. After incubation, the DNA solution was increased to 1 ml with SF 900 II medium and overlaid onto freshly plated Sf-9 cells and transferred to 27 °C for 4 h. After incubation, the transfection media were removed and the cells were replenished with 2 ml of fresh SF-900 II medium and returned to 27 °C.

Viral growth curve

To analyze the growth rate of virus constructs, Sf-9 cells were transfected with 5 μg of the appropriate bacmid DNA as described above and at the indicated time points, the supernatants were collected, and the titers determined with a $TCID_{50}$ end-point dilution assay described by O'Reilly et al. (1992) with minor modifications. At 5 days postinfection, 100 μl of cell extraction buffer (50 mM sodium phosphate, 10 mM EDTA, 5 mM β-mercaptoethanol, 0.1% sodium *n*-lauroylsarcosine, 0.1% Triton X-100) and 10 μl of a 0.7 mg/ml solution of 4-methylumbelliferyl β-D-glucuronide (4-MUG) substrate were added to each well of a 96-well plate. The plates were then incubated at 37 °C for 24 h and visualized under UV light to determine wells that were positive for viral infection.

Real-time PCR

To detect viral DNA replication by real-time PCR, primers were designed using the Primer Express software (PE Applied biosystems). The primers termed 65972F and 66072R (Table 1) correspond to AcMNPV genomic coordinates nt 65,972–65,992 and 66,054–66,072, respectively (Ayres et al., 1994), and amplify a 100-bp region within the gp41 ORF. Located in this region are four *Dpn*I restriction sites that allow discrimination between input and replicated DNA. The input bacmid DNA propagated in *E. coli* is methylated at this site by a bacteria-specific dam methylase and sensitive to *Dpn*I digestion, whereas the DNA replicated in Sf-9 cells will not be methylated at this site and will be resistant to digestion. To prepare total DNA for analysis, Sf-9 cells were transfected with 5 μg of bacmid DNA and at the indicated time points the cells were harvested into 1 ml PBS, lysed in 500 μl cell lysis buffer (10 mM Tris pH 8.0, 100 mM EDTA, 20 μg/ml RNAase A, 0.5% SDS, 20 μg of Proteinase K), and incubated overnight at 65 °C. Total DNA was phenol extracted, ethanol precipitated, and suspended in 100 μl of water. DNA concentrations were quantified with PicoGreen dsDNA Quantification Reagent (Molecular Probes) according to the manufacture's instructions. Prior to PCR, 20 ng of total DNA from each time point was digested with 10 units of *Dpn*I restriction enzyme (Fermentas) overnight in 50 μl total reaction volume. A 12.5-μl aliquot (5 ng of DNA) was removed from the digestion reaction and mixed with an equal volume of Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). PCR was performed with an ABI Prism 7000 sequence detection system with 40 cycles using the following conditions: 50 °C for 2 min, 95 °C for 2 min, and 45 cycles of 95 °C for 30 s and 60 °C for 30 s and 500 nM of each primer.

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