Autographa californica Multiple Nucleopolyhedrovirus DNA Polymerase C Terminus Is Required for Nuclear Localization and Viral DNA Replication

Guozhong Feng,^{a,b} Peter J. Krell^b

State Key Laboratory of Rice Biology, China National Rice Research Institute, Hangzhou, China^a; Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada^b

ABSTRACT

The DNA polymerase (DNApol) of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is essential for viral DNA replication. The DNApol exonuclease and polymerase domains are highly conserved and are considered functional in DNA replication. However, the role of the DNApol C terminus has not yet been characterized. To identify whether only the exonuclease and polymerase domains are sufficient for viral DNA replication, several DNApol C-terminal truncations were cloned into a *dnapol*-null AcMNPV bacmid with a green fluorescent protein (GFP) reporter. Surprisingly, most of the truncation constructs, despite containing both exonuclease and polymerase domains, could not rescue viral DNA replication and viral production in bacmid-transfected Sf21 cells. Moreover, GFP fusions of these same truncations failed to localize to the nucleus.

Truncation of the C-terminal amino acids 950 to 984 showed nuclear localization but allowed for only limited and delayed viral spread. The C terminus contains a typical bipartite nuclear localization signal (NLS) motif at residues 804 to 827 and a monopartite NLS motif at residues 939 to 948. Each NLS, as a GFP fusion peptide, localized to the nucleus, but both NLSs were required for nuclear localization of DNApol. Alanine substitutions in a highly conserved baculovirus DNApol sequence at AcMNPV DNApol amino acids 972 to 981 demonstrated its importance for virus production and DNA replication. Collectively, the data indicated that the C terminus of AcMNPV DNApol contains two NLSs and a conserved motif, all of which are required for nuclear localization of DNApol, viral DNA synthesis, and virus production.

IMPORTANCE

The baculovirus DNA polymerase (DNApol) is a highly specific polymerase that allows viral DNA synthesis and hence virus replication in infected insect cells. We demonstrated that the exonuclease and polymerase domains of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) alone are insufficient for viral DNA synthesis and virus replication. Rather, we identified three features, including two nuclear localization signals and a highly conserved 10-amino-acid sequence in the AcMNPV DNApol C terminus, all three of which are important for both nuclear localization of DNApol and for DNApol activity, as measured by viral DNA synthesis and virus replication.

The family *Baculoviridae* comprises a diverse group of insect-specific viruses and contains four genera, *Alphabaculovirus* (lepidopteran nucleopolyhedroviruses), *Betabaculovirus* (granuloviruses), *Deltabaculovirus* (dipteran baculoviruses), and *Gammapaculovirus* (hymenopteran baculoviruses) (1). Baculoviruses are characterized by a large circular double-stranded, 80- to 180-kb DNA genome packaged within a rod-shaped nucleocapsid and enclosed by a lipid envelope. Baculoviruses have a biphasic replication cycle and present two morphologically distinct virion phenotypes: budded virus (BV) early in infection and occlusion-derived virus (ODV) later in infection. The ODV nucleocapsids are enveloped and become embedded in large protein crystals as polyhedra or occlusion bodies (OBs) in the ring zone (2). BV is responsible for cell-to-cell transmission within a host, while ODVs spread virus infection from insect to insect hosts.

The molecular mechanisms of baculovirus DNA replication have been studied mostly in the alphabaculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which replicates its 134-kb double-stranded DNA (dsDNA) genome in the nucleus of infected cells following a molecular interaction between virus-encoded *trans*-acting factors and *cis*-acting sequences (3). To date, the functional roles of only some *trans*-acting factors

involved in DNA replication have been elucidated (4, 5). Transient-expression assays showed that six viral genes (*dnapol*, *p143*, *ie-1*, *lef-1*, *lef-2*, and *lef-3*) are required for virus DNA replication, suggesting that these are more directly involved in baculovirus DNA replication (6, 7). Of these, *dnapol*, *p143*, *ie-1*, and *lef-3* are essential for viral DNA replication in the AcMNPV bacmid system (8–12). *lef-11*, though not essential for viral DNA replication in transient-replication assays, is also required for viral DNA replication in the AcMNPV bacmid system (13). Three other genes, *p35*, *ie-2*, and *pe38*, stimulate viral DNA replication in transient-replication assays (6). IE-1, an early protein, is the major viral transactivator and is required for both delayed-early and late gene

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Address correspondence to Peter J. Krell, pkrell@uoguelph.ca.

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expression, including genes encoding other DNA replication proteins (10).

The highly conserved baculovirus DNApol plays a pivotal role in viral DNA replication and shows high similarity to DNApol of other DNA viruses, such as adenovirus 7 and herpes simplex virus 1, and even *Archaea* (14). The 3-kb AcMNPV DNApol open reading frame (ORF) encodes a polypeptide of 984 amino acids (aa) with a predicted molecular mass of 114.31 kDa (15). The expression of immediate-early genes, *ie-0* and/or *ie-1*, dramatically enhances *dnapol* transcription (16). *dnapol* mRNA is detectable at 2 h postinfection (hpi) and accumulates to a maximum expression level between 4 and 6 hpi, and then mRNA levels decline by 10 hpi (15). AcMNPV DNApol activity is different from that of the host, *Trichoplusia ni*, in sensitivities to heat inactivation and salt concentration (17). AcMNPV DNApol is essential for viral DNA replication and cannot be replaced by the DNApol of the host Sf21 cell (9). Previous biochemical analyses demonstrated that purified AcMNPV DNApol had significant polymerase activity and 3'-5' exonuclease activity, but no 5'-3' exonuclease activity (18). AcMNPV DNApol is highly processive with the help of LEF-3 and the viral single-strand DNA binding protein (SSB), which stimulates strand displacement (19). Two antiviral compounds, aphidicolin targeting DNApol activity and abacavir targeting the HIV reverse transcriptase, remarkably inhibited AcMNPV replication (9, 18, 20). Furthermore, aphidicolin and abacavir resistance mutants with a C543R mutation in DNApol have been recovered (9). AcMNPV DNApol is a homologue in DNApol family B (15, 21). Translated amino acid sequence analysis of AcMNPV DNApol revealed the presence of an exonuclease domain containing exonuclease (exo I) to exo III and a polymerase domain with designated regions I through VII in the following order: IV-II-VI-III-I-VII-V (Fig. 1A). These domains are shared among α -like DNApol of eukaryotes and various DNA viruses (15, 22–24). Although not demonstrated experimentally for a *Spodoptera littoralis* nucleopolyhedrovirus DNApol, both conserved domains have been postulated to be functional domains involved in substrate recognition (25). Nevertheless, studies on the herpes simplex virus DNApol confirmed that some of these conserved motifs are critical for exonuclease and polymerizing activities (24, 26, 27). Moreover, in our own studies, amino acid mutations in polymerizing motifs II and III of AcMNPV DNA polymerase affected DNA replication and viral production, which demonstrated that the motifs play an important functional role in DNA replication (9). AcMNPV DNApol is considered to regulate the fidelity of DNA replication by two different means: the polymerase domain selects the correct nucleotides and inserts them into the growing primer terminus, while the exonuclease domain proofreads and edits any mispaired nucleotides (28).

As DNApol acts in viral DNA replication, it must be translocated to the nucleus, though the mechanism is unknown. The nuclear localization of proteins is normally mediated via passive diffusion for small proteins less than 50 kDa or by nuclear localization signals (NLSs) (29). The NLSs are short peptide motifs that mediate the nuclear import of proteins by binding to cellular importins (29–32). Six different classes of NLSs, including 5 classes of monopartite NLSs and one class of bipartite NLSs, bind specifically to distinct grooves of importin- α (33). NLSs are also involved in nuclear localization of some baculovirus nuclear proteins. For example, the first NLS identified in baculoviruses was in the AcMNPV polyhedrin, in which the motif between amino acids

30 and 57 mediates its nuclear localization (34). Residues 117 to 148 comprise an NLS motif necessary to mediate *Bombyx mori* nucleopolyhedrovirus (BmNPV) ORF47 transport to the nucleus (35). Amino acids at positions 534 to 538 of AcMNPV IE-1 are the core element of an NLS, and substitution or deletion of amino acids within this region impairs nuclear import (36). The N-terminal 56-amino-acid region of AcMNPV LEF-3 is required for nuclear transport, and residues 28 to 32 form the core NLS (37, 38). The NLS motif of AcMNPV nucleocapsid protein BV/ODV-C42 is essential for its nuclear localization. Furthermore, this NLS is required for the BV/ODV-C42-mediated nuclear relocation of P78/83, a protein that is important for nuclear actin rearrangement (39). These examples demonstrate that NLSs are important for translocating many baculovirus proteins to the nucleus and, in some cases, NLS-bearing proteins act as chaperones to import other baculovirus proteins to the nucleus.

Our preliminary aim was to determine whether only the exonuclease and polymerase domains of AcMNPV DNApol are sufficient to function in DNA replication in Sf21 cells. Unexpectedly, the mutants we constructed with C-terminal truncations of DNApol that retained these domains could not rescue viral DNA synthesis and produce infectious virus. We constructed peptide fusions with enhanced GFP (EGFP; for simplicity, referred to as GFP), deletion mutations, and multiple point substitutions in the C terminus of AcMNPV DNApol, and we also generated a bacmid carrying an AcMNPV/*Choristoneura fumiferana* nucleopolyhedrovirus (CfMNPV) DNApol chimera in which the C terminus of the CfMNPV DNApol replaced that of the AcMNPV DNApol. Our results demonstrated that the AcMNPV DNA pol C terminus is important for nuclear localization, virus yield, and viral DNA synthesis in insect cells.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* clonal isolate 21 (Sf21) cells (40) were cultured in spinner flasks (Corning) containing Grace's insect medium supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (25 unit/ml), and streptomycin (25 μ g/ml). The spinner cultures were maintained at 27°C under constant stirring on a low-profile multi-stir, 4-position magnetic stirrer (Bellco Biotechnology). Viral stocks of the parental E2 strain of AcMNPV were amplified in Sf21 cells. The wild-type (WT) CfMNPV genome was used for PCR amplification of the CfMNPV sequence for DNApol aa 798 to 990 of the C terminus (41).

Construction of DNApol C-terminal truncations, peptides, and mutations in pBlue and pFAct plasmids. A series of pBluescript II SK(+) (Stratagene), pFAct (42), and bacmid based vectors were constructed. All cloned constructs were confirmed by sequencing, and bacmids were confirmed by PCR analysis. Constructs were named according to the vector used (pBlue or pFAct) or with Bac, for bacmid, and Pol refers to DNApol. For truncations, the number after Pol refers to the last amino acid of the DNApol truncation; for internal peptides, the numbers refer to the range of DNApol amino acids cloned and deletions indicate the range of DNApol amino acid numbers deleted. All fusions with GFP are designated as variations of GFP:Pol or Pol:GFP, depending on whether GFP is at the amino terminus or carboxy terminus, respectively, of DNApol or the indicated DNApol construct.

The plasmid pBlue-GFP was constructed by inserting the SacI/NotI *ie-1* promoter PCR fragment from AcMNPV *ie-1* (using primers 1 and 2 [Table 1]), the XbaI/EcoRI PCR fragment for the EGFP ORF (lacking the start codon) from a chitinase-GFP fusion (43) (using primers 3 and 4), and the HindIII/XhoI simian virus 40 (SV40) poly(A) signal PCR fragment from pFastBACHta (Invitrogen) (using primers 5 and 6) into

FIG 1 DNAPol truncations and repair virus bacmids. (A) Schematic diagram of AcMNPV DNAPol and of the C-terminal DNAPol truncations in bacmids Bac-GFP-Pol800 to Bac-GFP-Pol949 and WT repair viruses (WT^{rep}). Conserved exonuclease domains are labeled Exo I to Exo III, with corresponding DNAPol amino acid numbers above the label and polymerase domains labeled I to VII with corresponding amino acid numbers below the label. C-terminal DNAPol truncations are numbered according to the last residue of the WT DNAPol present in the mutant. (B) Schematic of cassettes with truncated *dnapol* fragments (Pol800, Pol831, Pol841, Pol878, and Pol949), *egfp* as a reporter under the *Opie1* promoter, and the adjoining *polyhedrin* (*polh*) gene under the *polh* promoter. The cassettes were inserted in the original *polyhedrin* locus of the DNAPol KO, Ac^{KO} bacmid by Tn7-mediated transposition. The WT and truncated DNAPol constructs were expressed by the *dnapol* native promoter and SV40 poly(A). (C) Schematic of truncated fragments (Pol800, Pol831, Pol841, Pol878, and Pol949) with GFP fusions following insertion into a pBluescript II(+) vector. The truncated DNAPol constructs were expressed by the *Acie-1* promoter and SV40 poly(A).

pBluescript II SK(+). In this construct, GFP lacking the start codon was driven by the AcMNPV *ie-1* promoter (*Acie1*).

Construction of independent C-terminal DNAPol truncation plasmids. To determine whether truncated DNAPol fragments could be expressed and functional in Sf21 cells, we first constructed the following

series of plasmids. pBlue-GFP-based plasmids with independent C-terminal DNAPol truncations C800 to C949 (Fig. 1A). Plasmids pBlue-Pol800, pBlue-Pol831, pBlue-Pol841, pBlue-Pol878, pBlue-Pol949, and full-length pBlue-Pol984 containing independent full-length DNAPol and C-terminal truncations were generated by inserting different C-terminal

TABLE 1 Primers used in this study

Primer no. and name	Primer sequence (5'–3') ^a	Position in genome ^b
(1) Ie-1PupSacl	CGGGAGCTCGCGAATGCAGCTGATCAC	AcMNPV (126881–26899)
(2) Ie-1PdownNotI	GAATGCGGCCGAGCTCACTTGGTTGTTTACCG	AcMNPV (127179–127197)
(3) EgfpFXbaI	GCTCTAGAGTGAGCAAGGGCGAGGAGCTGTT	4–26
(4) EgfpREcoRI	CGGAATTCCTTACTTGTACAGCTCGTCCATGCC	694–720
(5) SV40FHindIII	CCCAAGCTTGATCATAATCAGCCATACCA	Simian virus (2755–2774)
(6) SV40DXhoI	CCGCTCGAGGGGATCCAGACATGATAAGATA	Simian virus (2532–2553)
(7) DNApolP55851SacI	CGAGCTCGCGATCCTGATTAACACTTCAACGA	AcMNPV (55851–55874)
(8) DNA800EcoRI	CGGAATTCCTTACATACTAAAGCTGTAGTCGGTC	AcMNPV (52884–52905)
(9) DNAP830EcoRI	CGGAATTCCTTACCTAGCCACCGTAATCACG	AcMNPV (52794–52812)
(10) DNAP841EcoRI	CGGAATTCCTTAGATACGATCTCCGTTGCCG	AcMNPV (52731–52749)
(11) DNAP878EcoRI	CGGAATTCCTTATCGCATTTTCACTGGATCGAAC	AcMNPV (52650–52671)
(12) DNAP949EcoRI	CGGAATTCCTTAATCGTCGTCGTCACGTTTG	AcMNPV (52437–52455)
(13) DNAP984EcoRI	CGGAATTCCTTATTTTTCATTTTATACAAACAAAATTTATACG	AcMNPV (52332–52363)
(14) DNApolFNNotI	GAATGCGGCCGCGTGTGACGTCTGTGCCT	AcMNPV (55315–55333)
(15) DNA800XbaI	GCTCTAGACATACTAAAGCTGTAGTCGGTC	AcMNPV (52884–52905)
(16) DNAP830XbaI	GCTCTAGACCTAGCCACCGTAATCACG	AcMNPV (52794–52812)
(17) DNAP841XbaI	GCTCTAGAGATACGATCTCCGTTGCCG	AcMNPV (52731–52749)
(18) DNAP878XbaI	GCTCTAGATCGCATTTTCACTGGATCGAAC	AcMNPV (52650–52671)
(19) DNAP949XbaI	GCTCTAGATCGTCGTCGTCACGTTTG	AcMNPV (52437–52455)
(20) DNAP984XbaI	GCTCTAGATTTTTCATTTTATACAAACAAAATTTATACG	AcMNPV (52332–52363)
(21) DNAP52314XhoI	CCGCTCGAGTATATAATTATAAACTTTATTTTTCAT	AcMNPV (52313–52340)
(22) Ie-1PdownXbaI	GCTCTAGAAGTCACTTGGTTGTTTACCG	AcMNPV (127179–127197)
(23) EgfpFXbaI2	GCTCTAGAATGGTGAGCAAGGGCGAGGA	1–20
(24) EgfpREcoRI2	CGGAATTCCTTGTACAGCTCGTCCATGCC	697–720
(25) 804FEcoRI	CGGAATTCGATAACCCGGGCAAAAAAC	AcMNPV (52856–52874)
(26) 827RHindIII	CCCAAGCTTTTACGTAATCAGCGTCTTTTGG	AcMNPV (52803–52822)
(27) 939FEcoRI	CGGAATTCCTGCAGTGTCAAACGCCAAACG	AcMNPV (52450–52469)
(28) 948RHindIII	CCCAAGCTTTTAGTCGTCGATCAGCTTTGCG	AcMNPV (52440–52457)
(29) F-AcieIR	GAATTGTAGTCGTTGTCTGTC	AcMNPV (127464–127485)
(30) F-AcieIF	AGTTAGTTCTGCTTATTATTCG	AcMNPV (127740–127758)
(31) 804DEL1FL	TAACGTGG CATGGCATTGTAGAGAAATTTTG	AcMNPV (52799–52806)
(32) 804DEL1FS	CATGGCATTGTAGAGAAATTTTG	AcMNPV (52879–52902)
(33) 804DEL1RL	CCACGTT ATAGGTCATACTAAAGCTGTAGTCG	AcMNPV (52799–52806)
(34) 804DEL1RS	TAGGTCATACTAAAGCTGTAGTCG	AcMNPV (52776–52798)
(35) 939DEL1FL	CATCATCATCATCATTTGTCATC	AcMNPV (52418–52439)
(36) 939DEL1FS	GAAAAGAACCAGTGTAGTGA	AcMNPV (52485–52507)
(37) 939DEL1RL	GAAAAGAACCAGTGTAGTGA AAATTAACCAAAAAA	AcMNPV (52470–52484)
(38) 939DEL1RS	CATCATCATCATCATTTGTCAT CTTTTTTTGGTTAAT	AcMNPV (52470–52484)
(39) 2913FL	GCTGCGGCGCGTATGCGGCGTGTGCGGCGCGATGA AAAAATAAAGTTTATAATTATA	AcMNPV (52315–52340); mutation position (52341–52470)
(40) 2913FS	AAAAATAAAGTTTATAATTATA	AcMNPV (52315–52337)
(41) 2913RL	CATCGCGCGCGCACACCGCGCATACCGCGCGCAGCAC ATTGAGTGTCAATTTTCGCTG	AcMNPV (52371–52395); mutation position (52341–52470)
(42) 2913RS	ACATTGAGTGTCAATTTTCGCTG	AcMNPV (52374–52395)
(43) Cfpol793EcoRI	CGGAATTCAGCTTCAGCATGACGTACAAC	CfMNPV (50339–50359)
(44) Cf49639 XhoI	CCGCTCGAGGACTGGCACCGACAGGTA	CfMNPV (49639–49656)

^a The restriction enzyme sites, if restriction enzymes were used, are indicated in the primer name and are underlined in the primer sequence. For primers 804DEL1FL, 804DEL1RL, 939DEL1RL, 939DEL1RS, 2913FL, and 2913RL, boldface represents the region deleted or mutated by using these primers.

^b For sequences from AcMNPV and CfMNPV, the position in the genome refers to nucleotide numbers in the complete genomes of AcMNPV (NC_001623.1) and CfMNPV (NC_004778.3), respectively. For sequences from SV40, the position refers to nucleotide numbers in the complete genome of SV40 (NC_001669.1), and for the egfp sequence, to nucleotide numbers in the sequence with accession number U55761.1.

dnapol PCR fragments (using primers 7 to 13) into the SacI/EcoRI site of pBlue-GFP (Fig. 1A and B). The full-length DNAPol and truncations were under control of the native *dnapol* promoter and SV40 poly(A) signal. The resulting plasmids contained the DNAPol but lacked any GFP sequences. These plasmids were then digested with SacI/XhoI and subcloned into the pFAct-GFP vector (42), creating pFAct-GFP-Pol800, pFAct-GFP-Pol831, pFAct-GFP-Pol841, pFAct-GFP-Pol878, pFAct-GFP-Pol949, and full-length pFAct-GFP-Pol984. These transfer vectors contained GFP under the *Orgyia pseudotsugata* MNPV *ie-1* promoter (Opie1), full-length DNAPol or truncations under the native AcMNPV DNAPol pro-

motor and polyhedrin under the *polh* promoter. These were used to generate the corresponding bacmids.

Construction of fusion proteins between GFP and C-terminal truncations. The importance of C-terminal sequences of DNAPol in virus replication was also assessed by use of DNAPol-GFP fusions. Moreover, this allowed for studies on the intracellular localization of different C-terminal truncations of DNAPol. Truncated *dnapol* PCR NotI/XbaI fragments that lacked the *dnapol* promoter (primers 14 to 20) were inserted into the NotI/XbaI sites of pBlue-GFP. This generated DNAPol C-terminal truncation-GFP fusions pBlue-Pol800:GFP, pBlue-Pol831:GFP,

pBlue-Pol841:GFP, pBlue-Pol878:GFP, pBlue-Pol949:GFP, and full-length pBlue-Pol984:GFP (DNAPol:GFP signifies a DNAPol truncation fusion with GFP at the C terminus). The DNAPol truncation GFP fusions were under the control of *Acie1* (Fig. 1C). These plasmids were digested with *SacI/XhoI* and subcloned into the pFACt vector containing a functional *polyhedrin* gene (42), generating pFACt-Pol800:GFP, pFACt-Pol831:GFP, pFACt-Pol841:GFP, pFACt-Pol878:GFP, pFACt-Pol949:GFP, and full-length pFACt-Pol984:GFP (Fig. 1C), and were used to generate the corresponding bacmids.

Construction of fusion peptides between GFP and putative NLSs.

Constructs expressing fusion peptides between GFP and each of the two putative NLS motifs were generated using the following strategy. The AcMNPV *ie-1* promoter *SacI/XbaI* PCR fragment (generated using primers 1 and 22) and *egfp* (lacking the termination codon) containing an *XbaI/EcoRI* PCR fragment (generated using primers 23 and 24) were inserted into pBluescript II SK(+) to create pBlue-*Acie1-egfp*. The *EcoRI/XhoI* PCR fragments encoding the C terminus, including the *dnapol* native poly(A) signal and amino acids 804 to 984 (AcMNPV nucleotide sequence positions 52314 to 52874; generated using primers 21 and 25) and amino acids 939 to 984 (AcMNPV nucleotide sequence 52314 to 52469; generated using primers 21 and 27) were cloned into the *EcoRI/XhoI* site of pBlue-*Acie1-egfp*, creating pBlue-GFP:Pol804–984 and pBlue-GFP:Pol939–984, respectively. Plasmids were then constructed as GFP:NLS fusions for the aa 804 to 827 putative bipartite NLS and aa 938 to 948 putative monopartite NLS, respectively, as follows. The *XbaI/HindIII* PCR fragments of fusion peptides between GFP and the NLS motif from pBlue-GFP:Pol804–984 (generated using primers 23 and 26) and pBlue-GFP:Pol939–984 (generated using primers 23 and 28) were inserted into the *XbaI/HindIII* sites of the pBlue-GFP vector, replacing the resident EGFP with the GFP-NLS fusions under the control of *Acie1* and the poly(A) signal of SV40 to generate expression vectors pBlue-GFP:Pol804–827 and pBlue-GFP:Pol938–948, respectively.

Deletions and substitutions in pBlue and pFACt plasmids. Internal deletions and alanine substitutions in DNAPol fused to GFP were generated by site-directed, ligase-independent mutagenesis (44, 45) using pBlue-Pol949:GFP and pGEM-WT (9), respectively, as targets for mutagenesis. The internal deletions in the DNAPol C terminus were generated using primers 31 to 34 for deletion of aa 804 to 827 and primers 35 to 38 for deletion of aa 939 to 948, resulting in pBlue-Pol Δ 804–827:GFP and pBlue-Pol Δ 939–948:GFP, respectively. Primers 39 to 42 were used for alanine substitution mutations in pGEM-WT to generate pGEM-Pol10A; the plasmid was digested with *SacI/XhoI* and subcloned into pFACt-GFP, creating pFACt-GFP-Pol10A.

Construction with the AcMNPV C terminus replaced by CfMNPV.

To swap the AcMNPV DNAPol C-terminal fragment (aa 801 to 984) with the C-terminus aa 798 to 990 fragment from CfMNPV, the *EcoRI/XhoI* CfMNPV C-terminal DNAPol fragment was PCR amplified (using primers 43 and 44) from the CfMNPV genome (for the C-terminus nucleotide sequence, positions 49639 to 50359). This fragment was cloned into *EcoRI/XhoI* site of pBlue-Pol800, creating pBlue-Ac800:CfPol798–990, and the resulting plasmid was digested with *SacI/XhoI*. The chimeric fragment was then subcloned into pFACt-GFP, creating pFACt-GFP-Ac800:CfPol798–990.

Bacmid constructions. An AcMNPV *dnapol* knockout bacmid (Ac^{KO}), a *dnapol*-null bacmid with the *egfp* reporter (Ac^{KO}GFP), and WT *dnapol* repair bacmid (WT^{rep}) were constructed by Feng (9).

Other bacmids were generated from various pFACt constructs with a functional *polyhedrin* gene as described in the Bac-to-Bac expression manual (Invitrogen). Two series of bacmids were constructed. In one series, DNAPol truncations were expressed independently of a GFP reporter, and in the second series, DNAPol truncations were expressed as a fusion with GFP; all were cloned into the Ac^{KO} bacmid. To generate bacmids expressing DNAPol truncations and GFP independently, the Tn7 cassettes from pFACt-GFP transfer vectors with a GFP reporter and different C-terminal truncations, pFACt-GFP-Pol800, pFACt-GFP-Pol831,

pFACt-GFP-Pol841, pFACt-GFP-Pol878, pFACt-GFP-Pol949, and pFACt-GFP-Pol984, were transposed into the Ac^{KO} bacmid (9), creating the bacmids Bac-GFP-Pol800, Bac-GFP-Pol831, Bac-GFP-Pol841, Bac-GFP-Pol878, Bac-GFP-Pol949, and full length Bac-GFP-Pol984, respectively. To generate bacmids expressing DNAPol truncations as a GFP fusion the Tn7 cassettes from pFACt transfer vectors with different C-terminal truncation fusions with GFP (Fig. 1C), pFACt-Pol800:GFP, pFACt-Pol831:GFP, pFACt-Pol841:GFP, pFACt-Pol878:GFP, pFACt-Pol949:GFP, and pFACt-Pol984:GFP were also transposed into the Ac^{KO} bacmid, creating the bacmids Bac-Pol800:GFP, Bac-Pol831:GFP, Bac-Pol841:GFP, Bac-Pol878:GFP, Bac-Pol949:GFP, and full length Bac-Pol984:GFP, respectively. To generate bacmid Bac-GFP-Pol10A, in which the codons for the C-terminal consensus 10 aa, from aa 972 to 981, were converted to alanine codons, plasmid pFACt-GFP-Pol10A was transposed into the Ac^{KO} bacmid. To express the N-terminal 800 aa of AcMNPV DNAPol fused to the C-terminal aa 798 to 990 of the CfMNPV DNAPol, the pFACt vector pFACt-GFP-Ac800:CfPol798–990 was transposed to Ac^{KO} bacmid, generating Bac-GFP-AcPol800:CfPol798–990.

Quantitative real-time PCR. Total intracellular DNA was extracted using the QIAamp DNA blood minikit (Qiagen). After determining the DNA concentration via a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies), real-time quantitative PCR (qPCR) was performed as described previously (9) with the Rotor-Gene 6000 series (Corbett Robotics) and using SYBR Green 1 dye chemistry (Roche) and primer sequences 29 and 30 (Table 1).

Transfection. Bacmid DNA was purified from 500-ml LB cultures by using a Qiagen midi-plasmid kit, and concentrations were determined spectrophotometrically by using a NanoDrop ND-1000 apparatus. Sf21 cells were plated at a density of 1×10^6 cells per well in six-well plates. Cells were transfected with 2 μ g of bacmid DNA by using Cellfectin (Invitrogen) according to the manufacturer's protocol. Cells were incubated in the presence of the transfection mixture for 5 h, at which time the transfection mixture was removed and replaced with fresh Grace's medium (9).

Viral growth curves. To assess the ability of different C-terminal DNAPol truncations to support virus production, viral growth curves were generated for the truncation and repair mutants. Sf21 cells (1×10^6) were transfected in triplicate with 2.0 μ g of the appropriate bacmid DNA by using Cellfectin. Cell monolayers were incubated for 5 h posttransfection (hpt), rinsed twice with Grace's medium, and replenished with 2 ml of fresh Grace's medium supplemented with 10% FBS. Virus supernatants were collected from extracellular medium at the indicated time points. At different times posttransfection, viral yields were determined by a 50% tissue culture infective dose (TCID₅₀) endpoint dilution assay on Sf21 cells, and virus titers were calculated by the Reed-Muench method (46). For all transfections, time zero (0 hpi) was considered to be when the monolayer was replenished with fresh medium after the transfection solution had been aspirated from the wells. Each single-step growth curve experiment was replicated three times.

Confocal microscopy. The transfected cells were washed twice with 2 ml of phosphate-buffered saline (PBS) after 24 hpt. For nuclear staining, 4',6-diamidino-2-phenylindole (DAPI) at 20 μ g/ml was added to the cells for 15 min. The dye solution was replaced with PBS to remove the stain. Cells were imaged on a Leica SP5 confocal laser scanning microscope (CLSM) with a 63 $\times$ dipping lens for live cells, excitation with a 488-nm laser with collection between 500 and 523 nm, 1,024 by 1,024 pixels, 200 Hz, and with a line average of 3 and frame average of 1 for live cells.

RESULTS

Effects of C-terminal DNAPol truncations on virus production and viral DNA replication. To determine if the conserved exonuclease and polymerase domains alone were sufficient for viral DNA replication, a variety of DNAPol C-terminal truncation bacmids retaining both domains were constructed. C-terminal-truncated DNAPols retaining amino acids 1 to 800, 1 to 831, 1 to 841, 1 to 878, or 1 to 949 or full-length, aa 1 to 984 (Fig. 1A), were

inserted into Ac^{KO}, creating DNAPol replacement bacmids Bac-GFP-pol800, Bac-GFP-Pol831, Bac-GFP-Pol841, Bac-GFP-Pol878, Bac-GFP-Pol949, and repair bacmid Bac-GFP-Pol984 (WT^{rep}). The truncated DNAPols were expressed under the control of the native AcMNPV *dnapol* promoter and SV40 poly(A) signal, and GFP was expressed independently from the *Opie1* promoter and used as a reporter (Fig. 1B). Following bacmid transfection, microscopy showed reporter GFP fluorescence spreading to neighboring cells by 48 hpt and in almost all cells for the repair bacmid WT^{rep} at 72 hpt (data not shown). However, GFP fluorescence did not spread for bacmids Bac-GFP-Pol800, Bac-GFP-Pol831, Bac-GFP-Pol841, and Bac-GFP-Pol878 even by 144 hpt. Nevertheless, for Bac-GFP-Pol949 at 144 hpt, some spread of GFP fluorescence was observed though very limited (data not shown), showing the importance of the C terminus in virus replication.

To evaluate the importance of the DNAPol C terminus (aa 801 to 984) in viral production, DNA replication, and intracellular localization, truncated DNAPols were fused to the GFP tag and reintroduced into Ac^{KO}, creating replacement bacmids Bac-Pol800:GFP, Bac-Pol831:GFP, Bac-Pol841:GFP, Bac-Pol878:GFP, Bac-Pol949:GFP, and full-length Bac-Pol984:GFP (Fig. 1C). WT^{rep} with full-length DNAPol and an independently expressed GFP reporter was used as a positive control (9). Ac^{KO}GFP, the AcMNPV *dnapol* knockout bacmid with the reporter *egfp*, was a negative control. All these bacmids were transfected into Sf21 cells, and virus replication and spread were monitored by GFP fluorescence. There was no difference in the numbers of GFP-positive cells by 24 hpt between the bacmid-transfected monolayers, indicating equivalent transfection efficiencies (Fig. 2A). For both Bac-Pol984:GFP and WT^{rep}, there was an increased spread of fluorescence to neighboring cells by 48 hpt, and by 72 hpt fluorescence was observed in almost all cells (data not shown). By 96 and 144 hpt, GFP fluorescence for both Bac-Pol984:GFP and WT^{rep} spread completely from the initially transfected cells to adjacent cells. Bac-Pol984:GFP, expressing full-length DNAPol fused to GFP, showed virus spread and polyhedron production identical to that of WT^{rep}, which indicated that DNAPol fusion to GFP did not adversely affect the function of DNAPol. The number of GFP-positive cells did not increase for any of Bac-Pol800:GFP, Bac-Pol831:GFP, Bac-Pol841:GFP, Bac-Pol878:GFP, and Ac^{KO}GFP up to 96 or 144 hpt, suggesting that there was no spread of the virus from the initially transfected cells. Nevertheless, a limited amount of localized spread was observed for Bac-Pol949:GFP, but only by 144 hpt (Fig. 2A). Occlusion bodies were detected microscopically in both Bac-Pol984:GFP and WT^{rep} transfected cells by 96 hpt, while no occlusion bodies were found for any of the DNAPol truncation bacmids or Ac^{KO}GFP, even by 144 hpt (Fig. 2B). This suggested that any of the C-terminal truncation fragments containing just the conserved exonuclease and polymerase domains (aa 1 to 800) were insufficient for virus production and release and occlusion body production.

Virus spread and polyhedron formation for the DNAPol:GFP fusions showed the same results as DNAPols expressed independently of GFP. Both sets of bacmids indicated that the DNAPol C terminus, beyond aa 801, may play an important role at least in viral production, perhaps through lack of viral DNA replication when the C terminus is deleted.

Viral growth curves were used to evaluate the effects of DNAPol truncation-GFP fusions on virus production. Cells were transfected with 2 μ g of bacmid DNA of either Bac-Pol800:GFP,

Bac-Pol831:GFP, Bac-Pol841:GFP, Bac-Pol878:GFP, Bac-Pol949:GFP, Bac-Pol984:GFP, WT^{rep}, or Ac^{KO}GFP. The virus growth curves showed a steady increase in virus production for Bac-Pol984:GFP and WT^{rep} following transfection, while the virus titers of the different truncations or Ac^{KO}GFP did not increase at any detectable level over the 120 h posttransfection (Fig. 2C). These data revealed that the constructs containing the C-terminal truncated fragments could not produce infectious virus.

We also performed a qPCR assay to monitor the accumulation of intracellular total viral DNA in Sf21 cells. This assay was restricted to a single replication cycle over the first 24 hpt. Cells were transfected with bacmids Bac-Pol800:GFP, Bac-Pol831:GFP, Bac-Pol841:GFP, Bac-Pol878:GFP, Bac-Pol949:GFP, full-length Bac-Pol984:GFP, WT^{rep}, and Ac^{KO}GFP. Except for Bac-Pol984:GFP and WT^{rep}, which increased about 28- and 24-fold, respectively, there was no detectable increase over 24 hpt in intracellular viral DNA synthesis for the other bacmids (Fig. 2D). Thus, none of the truncated DNAPols could rescue viral DNA replication over 24 hpt or virus production over 120 hpt in transfected cells. That there was no viral DNA replication for these truncated DNAPols suggested that late gene expression was aborted so that polyhedra would not be produced, resulting in lack of OB production.

Confocal microscopy of intracellular localization of DNAPol:GFP truncations. To follow intracellular localization, bacmids expressing DNAPol and various C-terminal DNAPol truncations fused to a GFP tag were transfected into Sf21 cells. Fluorescence microscopy showed that at even up to 120 hpt the truncated DNAPols, Bac-Pol800:GFP, Bac-Pol831:GFP, Bac-Pol-841:GFP, and Bac-Pol878:GFP, remained cytoplasmic, while GFP fluorescence could be observed exclusively in the nuclei of Bac-Pol949:GFP- and Bac-Pol984:GFP (WT)-transfected Sf21 cells (data not shown).

To determine if DNAPols with certain truncations, in the absence of virus infection, could at least be transported to nuclei, expression plasmids for the truncated DNAPols fused to GFP, pBlue-Pol800:GFP, pBlue-Pol831:GFP, pBlue-Pol-841:GFP, pBlue-Pol-878:GFP, pBlue-Pol949:GFP, and full-length pBlue-Pol984:GFP were transfected into Sf21 cells. Using confocal fluorescence microscopy, green fluorescence was observed by 24 hpt, but only in the cytoplasm for pBlue-Pol800:GFP, pBlue-Pol831:GFP, pBlue-Pol841:GFP, and pBlue-Pol-878:GFP, indicating that the proteins were expressed but excluded from the nucleus (Fig. 3A to D). However, exclusive nuclear localization was observed for pBlue-Pol949:GFP and pBlue-Pol984:GFP by colocalization of green (GFP) and blue (DAPI) nuclear fluorescence, suggesting that these DNAPols were transported into the nucleus (Fig. 3E and F). These results paralleled those of bacmids for the same constructs. Furthermore, the same expression plasmids were transfected into Sf21 cells, except in the presence of WT virus infection, and this experiment had identical results with respect to nuclear localization of the different DNAPol:GFP constructs. These data demonstrate that nuclear localization of certain DNAPol truncations is wholly dependent on the C terminus and independent of virus infection.

Bioinformatic analysis of AcMNPV and other baculovirus DNAPol NLSs. Clearly, DNAPol needs to be imported into the nucleus, as it is responsible for intranuclear viral DNA replication. We speculated that the inability of nuclear translocation of AcMNPV DNAPol when lacking certain lengths of the C terminus is due to lack of an NLS, or another nuclear targeting signal in the

FIG 2 GFP fluorescence and virus production analysis of C-terminal-truncated DNAPol:GFP fusion bacmids and WT^{rep} and Ac^{KO}GFP bacmid in Sf21 cells. (A) GFP fluorescence of C-terminal truncation bacmids and WT^{rep} and Ac^{KO}GFP bacmids in Sf21 cells. Fluorescence images of monolayers of cells transfected with WT^{rep}, Ac^{KO}GFP, Bac-Pol800:GFP, Bac-Pol831:GFP, Bac-Pol841:GFP, Bac-Pol878:GFP, Bac-Pol949:GFP, and Bac-Pol984:GFP at 24, 96, and 144 hpt (top, middle, and bottom rows, respectively). Ac^{KO}GFP was used as a DNAPol negative control, and WT^{rep} was used as a positive control. All images were taken at the same magnification. (B) Light microscopy images of monolayers of cells transfected with WT^{rep}, Ac^{KO}GFP, Bac-Pol800:GFP, Bac-Pol831:GFP, Bac-Pol841:GFP, Bac-Pol878:GFP, Bac-Pol949:GFP, and Bac-Pol984:GFP at 144 hpt. (C) Monolayers were transfected with 2 μ g of the indicated bacmid DNAs, Ac^{KO}GFP, or WT^{rep} bacmid. The production of infectious BV was determined in TCID₅₀ endpoint dilution assays. (D) AcMNPV DNAPol accumulation kinetics of the WT^{rep}, Ac^{KO}GFP, Bac-Pol800:GFP, Bac-Pol831:GFP, Bac-Pol841:GFP, Bac-Pol878:GFP, Bac-Pol949:GFP, and Bac-Pol984:GFP in independently transfected Sf21 cells. Total intracellular DNA was extracted from transfected cells at 0 hpt and 24 hpt, and AcMNPV DNA copy numbers were determined by qPCR. Bars in the panels represent standard deviations determined from three independent replicates.

C terminus. Consequently, we searched for relevant domains through online resources (<http://hits.isb-sib.ch/cgi-bin/PFSCAN> and http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). Based on NLS classification (33), the AcMNPV DNAPol 16-amino-acid sequence 804- DNP GK K R K S T D D N E G P S P K R R V

IT-827 in the C terminus fits the consensus of the bipartite classical [(R/K) (R/K)X₁₀(R/K)_{>3-5}; underlined] NLS (Fig. 4A) (33, 47). In addition, a classical monopartite NLS consensus sequence (underlined; KR[K/R]R) (33) was found further downstream within the 10-amino-acid stretch 939-CSVKRKRDDDD-948.

FIG 3 Nuclear localization of C-terminal-truncated DNAPol via pBlue transient-expression plasmids. Fluorescence localization of DNAPol-GFP fusion proteins was performed by using transient-expression plasmids following transfection of Sf21 cells at 24 hpt. Nuclei were stained with DAPI, and cells were visualized by confocal microscopy. Cells were transfected by pBlue-Pol800:GFP (Pol800, A1 to A5), pBlue-Pol831:GFP (Pol831, B1 to B5), pBlue-Pol841:GFP (Pol841, C1 to C5), pBlue-Pol878:GFP (Pol878, D1 to D5), pBlue-Pol949:GFP (Pol949, E1 to E5), and pBlue-Pol984:GFP (Pol984, F1 to F5). Photographs were taken via light microscopy (A1 to F1), GFP green fluorescence (A2 to F2), and blue DAPI fluorescence (A3 to F3). Merged green and blue fluorescence images (A4 to F4) are in the column labeled G+D. Light microscopy and green and blue fluorescence images were also merged (A5 to F5) in the column labeled L+G+D.

Bioinformatic analysis was also used to compare NLS contents and relative locations in the DNAPols of other baculoviruses. The DNAPols of many baculoviruses in all four genera have at least one bipartite or one or more monopartite NLSs. DNAPols of some baculoviruses have only one of either type, yet some do not have any easily identifiable NLS in their DNAPol. The DNAPols of six group I alphabaculoviruses, including AcMNPV, *Bombyx mori* NPV (BmNPV), and CfMNPV, have both one classic bipartite NLS and one monopartite NLS with the monopartite NLS sequence being of class 1 or 4 (Table 2). Of other group I alphabaculovirus DNAPols, those for *Anthereae pernyi* NPV (AnpeNPV), *Orgyia pseudotsugata* MNPV (OpMNPV), and *Rachiplusia ou* MNPV (RoMNPV) have a single bipartite NLS, while those for *Anticarsia gemmatilis* MNPV (AgMNPV) and *Choristoneura fumiferana* defective NPV (CfDEFNPV) have a double bipartite NLS. The *Choristoneura occidentalis* NPV DNAPol has a single monopartite NLS, while that for *Maruca vitrata* NPV (MaviNPV) has two monopartite NLSs. DNAPols of *Epiphyas postvittana* NPV (EppoNPV) and *Hyphantria cunea* NPV (HcNPV) did not show

consensus with any known NLS classes. NLSs of all group I alphabaculoviruses are located in the DNAPol C terminus (Table 2).

Seven group II alphabaculoviruses, specifically, *Ectropis obliqua* NPV (EcobNPV), *Helicoverpa armigera* NPV-A (HaNPV-A), *Helicoverpa armigera* NPV NNg1 (HaNPV NNg1), *Helicoverpa armigera* NPV G4 (HaNPV G4), *Helicoverpa zea* single NPV (HzSNPV), *Lymantria dispar* MNPV (LdMNPV), and *Spodoptera litura* NPV (SpltNPV) contain a single monopartite NLS. DNAPols of another 21 group II alphabaculoviruses did not contain sequences easily identifiable as known NLSs. From this we speculated that only one classic monopartite NLS motif or a new class of NLS, not yet identified, exists for DNAPols in some of the group II alphabaculoviruses. This comparison suggested that DNAPol NLSs in alphabaculovirus group I are different from those in group II.

Among the betabaculoviruses, the DNAPol from only the granulovirus *Plutella xylostella* GV (PxyGV) contains a bipartite NLS; none of the other betabaculoviruses showed any evidence of NLSs in their DNAPols. Similarly, classical NLSs in the DNAPols were not identified from among either deltabaculoviruses or gammabaculoviruses (Table 2).

Functional characterization of AcMNPV DNAPol NLSs. The prediction of monopartite and bipartite NLS sequences in the AcMNPV DNAPol did not prove they are functional as NLSs. As reported by Kosugi et al. (33), there are also nonfunctional sequences that match such a consensus. Thus, it was important to determine that these AcMNPV DNAPol sequences can function as NLSs. To determine if the two putative NLSs mediate nuclear localization, we chose to generate GFP tag peptides representing the putative bipartite NLS at aa 804 to 827 (DNPGKKRKSTDDN EGPSPKRRVIT; pBlue-GFP:Pol804-827) and the putative monopartite NLS at aa 939 to 948 (CSVKRRKDDDD; pBlue-GFP:Pol939-948) with GFP. The resultant expression plasmids were then transfected into Sf21 cells. As shown by others, when GFP was expressed alone, a diffuse signal was observed in both the cytoplasm and nucleus of Sf21 cells (Fig. 5A). The partial nuclear localization is likely due to the small size, 27-kDa, of GFP, enabling it to pass through the nuclear pore complex via passive diffusion. Independent of virus infection, the fluorescence signals of both GFP:Pol804-827 and GFP:Pol939-948 fusions showed exclusive nuclear localization (Fig. 5B and C). Compared to GFP alone, the exclusive nature of the nuclear fluorescence signals of the GFP-tagged peptides aa 804 to 827 and aa 939 to 948 indicated that each putative NLS-bearing peptide contributed to highly efficient nuclear translocation of the fused GFP.

While this suggested that both putative NLSs were independently functional in nuclear localization of GFP, the GFP fusion proteins, being small (less than 40 kDa), may have diffused into the nucleus but were then retained there, rather than gaining access through active nuclear localization. To test the requirement of the two potential NLSs for nuclear localization of AcMNPV DNAPol, we also generated deletion mutants of each of the putative DNAPol NLSs. For this, pBlue-Pol949:GFP, the largest DNAPol truncation that still showed exclusive nuclear localization (Fig. 3E), was used as a template to construct DNAPol949:GFP deletion mutants for either DNAPol Δ 804-827, which lacks only the classic bipartite NLS (amino acids 804 to 827) or DNAPol Δ 939-948, which lacks only the monopartite NLS. By 24 hpt of Sf21 cells, GFP fluorescence for both pBlue-Pol949 Δ 804-827:GFP and pBlue-Pol949 Δ 939-948:GFP was localized exclu-

TABLE 2 Predicted NLSs in baculovirus DNAPols^a

Virus group and name	NLS or NLS-like amino acid sequence	Size (aa)	Relative position	NLS classification		Accession no.
				Bipartite	Monopartite	
Alphabaculoviruses group I						
<i>Antheraea pernyi</i> NPV	DAAAP <u>K</u> RRAAEADDEPPA <u>K</u> RRVVT	920	759–782	Bipartite	—	YP_611056.1
<i>Anticarsia gemmatilis</i> NPV	KAN <u>K</u> RRANEADEAPTLA <u>K</u> RRVVT <u>K</u> RKAQDNKNEENC <u>K</u> KRKTP	991	800–822 932–950	Bipartite Bipartite	— —	YP_803459.1
<i>Autographa californica</i> NPV	DNP <u>G</u> K <u>K</u> RKSTDDNEGSP <u>K</u> RRVIT CSV <u>K</u> RKRDDDD	984	804–827 939–948	Bipartite —	— Class 1	AAA46692.1
<i>Bombyx mandarina</i> NPV	DNP <u>G</u> K <u>K</u> RKSADDNNEGSP <u>K</u> RRVIT CSV <u>K</u> RKRDDDD	989	804–828 940–949	— —	— Class 1	YP_002884294.1
<i>Bombyx mori</i> NPV	DNP <u>G</u> K <u>K</u> RKSADDNNEGSP <u>K</u> RRVIT CGV <u>K</u> RKRDDDD	988	804–828 940–949	Bipartite —	— Class 1	BAA03756.1
<i>Choristoneura fumiferana</i> MNPV	<u>R</u> PT <u>K</u> RRVVTV <u>K</u> RVTLIKINAAY <u>K</u> RK	990	812–821 920–934	— Bipartite	Class 4 —	NP_848372.1
<i>Choristoneura fumiferana</i> DEF MNPV	KAN <u>K</u> RRADEADETPTPA <u>K</u> RRVVT <u>K</u> RKAQNSKDEGN <u>C</u> K <u>K</u> RK	993	800–822 932–948	Bipartite Bipartite	— —	NP_932670.1
<i>Choristoneura murinana</i> NPV	AACKRKAPKD	991	929–938	—	ND	YP_008992181.1
<i>Choristoneura occidentalis</i> NPV	<u>R</u> PT <u>K</u> RRVVTV	990	812–821	—	Class 4	YP_008378589.1
<i>Choristoneura rosaceana</i> MNPV	<u>K</u> RQILSDNDDVACN <u>K</u> RARAE	989	930–949	Bipartite	—	YP_008378438.1
<i>Epiphyas postvittana</i> NPV	DEPAT <u>K</u> KRRVI KRVTLIKINAAY <u>K</u> RK	960	808–818 920–934	— Bipartite	ND —	NP_203227.1
<i>Hyphantria cunea</i> NPV	ASRKRKAUSD	986	941–950	—	ND	YP_473273.1
<i>Maruca vitrata</i> MNPV	PSPP <u>K</u> RRVITV CGV <u>K</u> RKRDDDD	980	814–824 935–944	— —	Class 4 Class 1	YP_950779.1
<i>Orgyia pseudotsugata</i> MNPV	KIAT <u>K</u> RKAPSASDAS <u>G</u> K <u>R</u> ARK	985	929–949	Bipartite	—	NP_046226.1
<i>Plutella xylostella</i> MNPV	DNP <u>G</u> K <u>K</u> RKSTDDNEGSP <u>K</u> RRVIT CSV <u>K</u> RKRDDDD	985	804–827 939–948	Bipartite —	— Class 1	YP_758532.1
<i>Rachiplusia ou</i> MNPV	DNP <u>G</u> K <u>K</u> RKSTDDNEGSP <u>K</u> RRVIT	985	804–827	Bipartite	—	NP_703055.1
<i>Thysanoplusia orichalcea</i> NPV	DNP <u>G</u> K <u>K</u> RKSNNDDGPA <u>P</u> <u>K</u> RRVIT CGV <u>K</u> RKRDDND	986	805–829 941–950	Bipartite —	— Class 1	YP_007250472.1
Alphabaculoviruses group II						
<i>Adoxophyes honmai</i> NPV	—	1,019	—	—	—	NP_818705.1
<i>Adoxophyes orana</i> NPV	—	1,019	—	—	—	YP_002300573.1
<i>Agrotis ipsilon</i> MNPV	—	1,043	—	—	—	YP_002268136.1
<i>Agrotis segetum</i> NPV	—	1,025	—	—	—	YP_529770.1
<i>Buzura suppressaria</i> NPV	—	1,056	—	—	—	YP_009001835.1
<i>Chrysodeixis chalcites</i> NPV	—	1,048	—	—	—	YP_249662.1
<i>Clanis bilineata</i> NPV	—	1,060	—	—	—	YP_717605.1
<i>Ecotropis obliqua</i> NPV	SKLKKRKND	1,065	865–873	—	Class 1	YP_874251.1
<i>Euproctis pseudoconspersa</i> NPV	RSEYKAKRDQQGPGSFLYHLYDTLQNAVKRIAN	1,067	652–684	ND	—	YP_002854676.1
<i>Helicoverpa armigera</i> NPV (2)	—	1,020	—	—	—	NP_203622.1
<i>Helicoverpa armigera</i> NPV-A	TSA <u>K</u> K <u>R</u> KTT	1,020	829–837	—	Class 1	AEN03990.1
<i>Helicoverpa armigera</i> NPV NNgl	TSA <u>K</u> K <u>R</u> KTT	1,020	829–837	—	Class 1	BAG74634.1
<i>Helicoverpa armigera</i> M NPV	—	1,000	—	—	—	YP_002332643.1
<i>Helicoverpa armigera</i> NPV G4	TSA <u>K</u> K <u>R</u> KTT	1,020	829–837	—	Class 1	NP_075136.1
<i>Helicoverpa zea</i> SNP	TSA <u>K</u> K <u>R</u> KTT	1,020	829–837	—	Class 1	NP_542692.1
<i>Hemileuca</i> sp. NPV	TSLKKRKSA	1,087	891–899	—	ND	YP_008378282.1
<i>Leucania separata</i> nuclear NPV	—	1,053	—	—	—	YP_758381.1

(Continued on following page)

TABLE 2 (Continued)

Virus group and name	NLS or NLS-like amino acid sequence	Size (aa)	Relative position	NLS classification		Accession no.
				Bipartite	Monopartite	
<i>Lymantria dispar</i> MNPV	TTAK <u>RRKEED</u>	1,013	827–836	—	Class 1	BAA02036.1
<i>Lymantria xylinia</i> MNPV	RKRAFNEAPDANTIDLSQLKRTVPD	1,023	498–523	ND	—	YP_003517819.1
<i>Mamestra brassicae</i> MNPV	—	1,000	—	—	—	YP_009011170.1
<i>Mamestra configurata</i> NPV-A	RKRNYDGDSVDSQNVDLTKLQRKP	998	487–510	ND	—	NP_613198.1
<i>Mamestra configurata</i> NPV-B	—	1,000	—	—	—	AAM95100.1
<i>Orgyia leucostigma</i> NPV	—	1,072	—	—	—	YP_001650977.1
<i>Spodoptera exigua</i> MNPV	—	1,063	—	—	—	NP_037853.1
<i>Spodoptera frugiperda</i> MNPV (2)	GRKRTFDQMN	1,040	494–503	—	—	AFH59036.1
<i>Spodoptera litura</i> NPV	PPAK <u>RRAMP</u>	1,022	829–837	—	Class 2	NP_258337.1
<i>Spodoptera litura</i> NPV II	—	1,054	—	—	—	YP_002332794.1
<i>Trichoplusia ni</i> SNPV	—	1,052	—	—	—	YP_308944.1
Betabaculoviruses						
<i>Adoxophyes orana</i> GV	—	1,031	—	—	—	NP_872548.1
<i>Agrotis segetum</i> GV	RSSPKRKLYDE	1,145	20–30	—	ND	YP_006243.1
<i>Closteria anastomosis</i> GV	SLKRRAEELTDETPKFIKFD	1,036	3–23	ND	—	YP_008720044.1
<i>Choristoneura occidentalis</i> GV	—	1,044	—	—	—	YP_654511.1
<i>Cryptophlebia leucotreta</i> GV	—	1,045	—	—	—	NP_891948.1
<i>Cydia pomonella</i> GV	LSKRKAEGVEYEPVAVKAIKLS	1,051	3–23	ND	—	NP_148895.1
<i>Epinotia aporema</i> GV	EVPKKRNAAAMAANDDESPPVKTKRVD	1,068	29–55	ND	—	YP_006908614.1
<i>Helicoverpa armigera</i> GV	ELAFKRKLPSNSETRGIKKHFN	1,098	1069–1089	ND	—	YP_001649116.1
<i>Phthorimaea operculella</i> GV	—	1,021	—	—	—	NP_663268.1
<i>Pieris rapae</i> GV	LFKRKLDEPPTDHTLVKAIKLS	1,046	4–25	ND	—	YP_003429417.1
<i>Plutella xylostella</i> GV	FKTMK <u>RRASVVVNNNNNNKKRKL</u>	979	958–978	Bipartite	—	NP_068312.1
<i>Pseudaletia unipuncta</i> GV	ELAFKRKLNSSEAKGIKKAFT	1,094	1064–1084	ND	—	YP_003422477.1
<i>Spodoptera litura</i> GV	—	1,035	—	—	—	YP_001257052.1
<i>Xestia c nigrum</i> GV	ELAFKRKLPSSETRGIKKHFT	1,098	1069–1089	ND	-	AAC06350.1
Deltabaculovirus						
<i>Culex nigripalpus</i> NPV	—	1,138	—	—	—	AAK13281.1
Gammabaculovirus						
<i>Neodiprion abietis</i> NPV	—	919	—	—	—	YP_667860.1
<i>Neodiprion lecontei</i> NPV	—	923	—	—	—	YP_025217.1
<i>Neodiprion sertifer</i> NPV	—	913	—	—	—	YP_025135.1

* The prediction was performed using online analysis (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>, http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) and the classification of NLSs (31, 46). The amino acids underlined show the predicted core amino acid sequences. The GenBank accession number is listed for each virus. ND, not identified as a strict NLS but had a strong basic characteristic. —, no easily identified NLS was found.

production. Cells were transfected with 2 µg of bacmid DNA of either WT^{rep}, Bac-GFP-pol10A, or Ac^{KO}GFP and monitored for virus production for up to 144 hpt. Only WT^{rep} showed a steady increase in the level of virus production for up to 120 hpt, with a small decrease by 144 hpt. However, no BV production was detected at any time point up to 144 hpt for Bac-GFP-pol10A- or Ac^{KO}GFP-transfected cells (Fig. 6B). The levels of viral DNA synthesis over the first 24 hpt for WT^{rep}, Bac-GFP-pol10A, and Ac^{KO}GFP were also determined. DNA isolated from WT^{rep}-, Bac-GFP-pol10A-, and Ac^{KO}GFP-transfected Sf21 cells at 24 hpt was subjected to real-time PCR with primers targeting AcMNPV *ie-1*. Neither Bac-GFP-pol10A nor Ac^{KO}GFP showed any increase in viral DNA level during 24 h. By contrast, the levels of intracellular DNA for WT^{rep} increased about 12-fold over that at the zero time point (Fig. 6C). The difference in viral DNA content between Bac-GFP-pol10A and WT^{rep} indicated that the C-terminal conserved region is essential for viral production and virus DNA synthesis. Nevertheless, that some spread was observed for the alanine substitutions at 144 hpt suggested that some delayed low level of viral

DNA synthesis and virus production may have occurred but were below the level of detection.

The C-terminus DNAPol of CfMNPV can replace that in AcMNPV. CfMNPV and AcMNPV are both alphabaculovirus group I viruses (14). Though the CfMNPV DNAPol is homologous to that of AcMNPV, the C-terminal amino acid sequence identity (between residues 801 to 984 for AcMNPV and 787 to 990 for CfMNPV) is only 57%. Bioinformatic analysis revealed that the CfMNPV DNAPol C terminus also contains two putative NLSs but they are distinct from those of AcMNPV DNAPol and in the reverse orientation. The NLS at aa 812 to 821 (RPTKRRV VTV) matches the class 4 monopartite consensus [(R/P)XXXR (K/R)VTV)] (33). Downstream of this monopartite NLS is the sequence KRVTLIKINAAYKRRK, at aa 920 to 934, which is consistent with the classic bipartite NLS [(R/K) (R/K)X₁₀(R/K)_{>3-5}]. The CfMNPV DNAPol also contains the AcMNPV DNAPol C-terminal consensus sequence, at aa 972 to 981, shown to be critical for AcMNPV replication.

To determine if the CfMNPV DNAPol C terminus could re-

FIG 5 Functional analysis of putative NLSs in the DNAPol C terminus performed with pBlue transient-expression plasmids. Localization of the indicated DNAPol-GFP fusion peptides was performed by using transient-expression plasmids in transfected Sf21 cells at 24 hpt. Nuclei were stained with DAPI and visualized via confocal microscopy. Cells were transfected with *egfp*-only plasmid (A1 to A5) or with EGFP-tagged DNAPol plasmids pBlue-*egfp*:Pol804-827 (Pol804-827; B1 to B5), *egfp*:Pol939-948 (Pol939-948; C1 to C5), or with EGFP-tagged DNAPol deletions pBlue-PolΔ804-827:GFP (PolΔ804-827; D1 to D5), pBlue-PolΔ939-948:GFP (PolΔ939-948; E1 to E5). The constructs were expressed by an *Acie-1* promoter and SV40 poly(A). Photographs were taken at 24 hpt by using light microscopy (A1 to E1), GFP fluorescence (A2 to E2), or blue DAPI staining (A3 to E3). Green and blue fluorescence images were merged (A4 to E4) in the column labeled G+D, and light and green and blue fluorescence images were merged (A5 to E5) in the column labeled L+G+D. All images are at the same magnification. Compare with Fig. 3F for the full-length pBlue-Pol984:GFP as a positive control.

place that of AcMNPV, CfMNPV *dnapol* nucleotides 2392 to 2970 (for aa 798 to 990), including the two CfMNPV DNAPol NLSs, the DNAPol C-terminal consensus, and *dnapol* poly(A) site, was cloned to replace the C terminus of AcMNPV after aa 800 (Fig. 7A) and cloned into the AcMNPV polyhedrin locus, creating the GFP-expressing Bac-AcPol800:CfPol798-990.

Ac^{KO}GFP, Bac-AcPol800:CfPol798-990, and WT^{rep} bacmids were transfected into Sf21 cells. WT^{rep} was the positive control, and Ac^{KO}GFP was the negative control. GFP fluorescent cells were monitored for virus infection and spread. At 24 hpt, only individual fluorescent cells were found, and there was no obvious difference among these transfections, indicating equivalent transfection efficiencies (Fig. 7B). By 72 and 120 hpt, GFP was expressed in almost all the cells for Bac-AcPol800:CfPol798-990 and WT^{rep}, whereas there was no increase in the number of fluorescent cells for Ac^{KO}GFP from 24 hpt throughout transfection. Light microscopy showed occlusion body formation for both Bac-AcPol800:CfPol798-990- and WT^{rep}-transfected cells at 120 hpt. However, no occlusion bodies were detected for Ac^{KO}GFP (Fig. 7B). To further characterize the ability of CfMNPV C-terminal DNAPol to replace that of AcMNPV, we also prepared virus growth curves

in a TCID₅₀ endpoint dilution assay to determine the BV titers. The bacmids were transfected with 2 μg bacmid DNA of either Ac^{KO}GFP, Bac-AcPol800:CfPol798-990, or WT^{rep} and monitored for up to 120 hpt, and the supernatants were collected at the indicated times. Sf21 cells transfected with Bac-AcPol800:CfPol798-990 and WT^{rep} revealed a steady increase in the level of viral production, and there were no significant differences between the two groups for up to 120 hpt (Fig. 7C). In contrast, no extracellular virus was detectable at any time for Ac^{KO}GFP-transfected cells. These data suggested that the C-terminal substituted repair virus could produce infectious virus and the yield was comparable with that of the native AcMNPV DNAPol WT^{rep}.

These results indicated that the 193-aa CfMNPV C-terminal DNAPol sequence, which contains two NLSs and the conserved 10 aa at the C terminus, can replace the C-terminal 184 aa of the AcMNPV DNAPol to rescue virus production, and presumably viral DNA replication in the *dnapol* knockout AcMNPV bacmid.

DISCUSSION

Viral DNAPol is critical for viral DNA replication, and the DNAPol protein is highly conserved in all baculoviruses. Viral DNA replication is generally associated with DNAPol exonuclease and polymerase domains. The exonuclease domain proofreads and edits out any mispaired nucleotides, and the polymerase domain selects the correct nucleotides and incorporates them into the growing primer terminus (28). However, the C-terminal region of the baculovirus DNAPol responsible for DNA replication has been only poorly characterized. In the present study, we investigated the functional role of the AcMNPV DNAPol C terminus by constructing several bacmids in which the native DNAPol was replaced with C-terminal-truncated DNAPols in a *dnapol*-null AcMNPV bacmid. As we wished to follow the effects on DNAPol by using GFP as a reporter or with DNAPol:GFP fusions with the various DNAPol truncations, we first ensured that the WT DNAPols expressed as an independent DNAPol or as a DNAPol:GFP fusion were competent for viral DNA synthesis and virus production. When WT DNAPol, under control of its native promoter, was cloned into a bacmid coexpressing GFP under the Opie1 promoter, there was normal virus spread, viral DNA synthesis, and virus replication. Similarly, bacmids expressing full-length DNAPol fused to GFP (as DNAPol:GFP) under the native *dnapol* promoter also showed normal virus spread, DNA synthesis, and virus replication. These findings suggest that GFP, either as an independent fluorescent reporter or as a fused fluorescent tag, did not interfere with DNAPol function and allowed for a normal virus replication cycle. However, none of the bacmids expressing the C-terminal truncations, either alone or as GFP fusions, although all retained both the exonuclease and polymerase domains, could rescue virus replication or produce infectious BV in transfected Sf21 cells. This indicated that sequences in the C terminus might also be needed for a functional DNAPol. Consequently, bioinformatic analysis showed that the C-terminal AcMNPV DNAPol encodes two putative NLSs which could be responsible for nuclear translocation of the AcMNPV DNAPol. Amino acids 804 to 827, DNPGRKRKSTDDNEGSPKRRVIT, have a 16-aa NLS with consensus to the classic bipartite NLS sequence ([K/R]₂[X]₁₀₋₁₂[K/R]_{>3-5}), while the 10-aa NLS at aa 939 to 948 (CSVKRRKDDDD) corresponds to the classical monopartite consensus sequence (K[K/R]X[K/R]). Furthermore, we identified a highly conserved baculovirus DNAPol C-terminal sequence at aa

FIG 6 GFP fluorescence and virus production analysis of bacmids of alanine-replaced aa 972 to 981 as well as Ac^{KO}GFP and WT^{rep} bacmids in Sf21 cells. (A) Fluorescence and light microscopy images of monolayers of cells transfected with Ac^{KO}GFP, Bac-GFP-Pol10A, and WT^{rep} at 24, 96, and 144 hpt. Ac^{KO}GFP was used as a negative control, and WT^{rep} was a positive control. (B) Monolayers were transfected with 2 μ g of the bacmid DNAs. Infectious BV titers were determined in TCID₅₀ endpoint dilution assays. (C) Viral DNA accumulation kinetics following independent transfections of Sf21 cells with bacmids Bac-GFP-Pol10A, Ac^{KO}GFP, or WT^{rep}. Total intracellular DNA was extracted at 0 and 24 hpt, and AcMNPV DNA copy numbers were determined by qPCR of Acie1. Bars in the panels represent standard deviations determined from three independent replicates.

972 to 981 that is required for DNA replication and viral production.

AcMNPV DNAPol C-terminal truncation bacmids Bac-GFP-Pol800, Bac-GFP-Pol831, Bac-GFP-Pol841, and Bac-GFP-Pol878 and the corresponding GFP-fused truncations of DNAPol were unable to spread virus infection after transfection into Sf21 cells and were unable to replicate viral DNA or produce infectious virus. Of the C-terminal truncation mutants, only Pol949 (or GFP: Pol949), which localized to the nucleus, showed any evidence of viral spread, but even that was only very limited and somewhat delayed. If there was any increase in DNA replication or virus production for the Pol949 truncations, it was below the limit of detection. Confocal microscopy analysis showed that following transient transfection, the GFP-fused C-terminal DNAPol truncation peptide lacking both NLSs expressed in plasmid pBlue-Pol800:GFP was excluded from the nucleus, as were all of the Pol:GFP truncations, although all included one putative NLS (aa 804 to 827) expressed by plasmids pBlue-pol831:GFP, pBlue-pol841:GFP, and pBlue-pol878:GFP. However, the Pol-GFP fusion, which included both putative NLSs expressed by pBlue-

Pol949:GFP, localized exclusively to the nucleus. Furthermore, results with independent peptide fusions of the two AcMNPV NLS domains with GFP and the two corresponding, independent NLS DNAPol deletion mutations demonstrated that each AcMNPV NLS was independently functional in nuclear localization but that neither NLS on its own was sufficient for nuclear localization of DNAPol. Nuclear localization could be rescued if the missing NLS was repaired by an addition to the C terminus, but not if plasmids for both deletion mutants were cotransfected together. These data demonstrate that both NLSs are needed for nuclear localization of AcMNPV DNAPol and that they can only act if both are in *cis*. As DNAPol is a relatively large, 984-aa protein, perhaps multiple nuclear localization signals are necessary for its nuclear translocation.

NLSs were common features identified in many, but not all, DNAPols in a survey of DNAPols from 63 baculoviruses with complete genomes sequenced. Some DNAPols had several or at least one easily identified NLSs, but several had no easily recognizable NLS, suggesting that novel NLSs might exist for some nuclear baculovirus proteins, like DNAPol for nuclear localization in in-

FIG 7 GFP fluorescence and viral production analysis of DNAPol C-terminal substitution bacmid, WT^{rep}, and Ac^{KO}GFP bacmid in Sf21 cells. (A) Schematic of AcMNPV C-terminal DNAPol (amino acids 801 to 984) replaced with that of CfMNPV (amino acids 798 to 990). (B) GFP fluorescence images of monolayers of cells transfected with bacmid Ac^{KO}GFP, Bac-GFP-AcPol800:CfPol798-990, or WT^{rep} at 24, 72, and 120 hpt. (C) Monolayers were transfected with 2 µg of Ac^{KO}GFP, Bac-GFP-AcPol800:CfPol798-990, or WT^{rep} bacmid DNA, and BV production was determined in a TCID₅₀ assay.

sect cells. Alternatively, those nuclear proteins lacking any NLS might localize to the nucleus through a chaperone-mediated process. There are six classes of NLSs, including five monopartite NLSs, KR(K/R)R or K(K/R)RK for class 1, (P/R)XXKR(K/R)xq for class 2, KRX(W/F/Y)XXAF for class 3, (R/P)XXKR(K/R)X for class 4, and LGKR(K/R)(W/F/Y) for class 5. Class 6 comprises two consensus sequences of the bipartite NLS KRX₁₀₋₁₂K(KR)(KR) or KRX₁₀₋₁₂K(KR)X(K/R), where X represents any amino acid (32, 33, 48, 49). Any of the six classes of NLS in a protein binds a groove in importin-α and, along with an adapter protein, forms a ternary complex of importin-α–importin-β₁–cargo in the cytoplasm in the importin-α/β pathway (33). Importin-β₁ docks the complex to the nuclear pore complex. The complex of importin-α–importin-β₁–cargo disassembles in the nucleus by binding of RanGTP to importin-β₁ (29, 50). It is not yet known if the two DNAPol

NLSs we identified can bind to importin-α in insect cells. Thus, further analysis of AcMNPV DNAPol NLS interactions with host proteins is important to our understanding of the mechanism of nuclear protein import in baculovirus-infected cells.

To date, the importance of a C terminus for viral DNA replication has been reported only for the HSV DNAPol. The C-terminal sequence of HSV-1 DNAPol interacts with UL42, and the activity of UL42 is mediated by amino acid residues located in the C-terminal DNAPol (51, 52). Sequence alignment analysis showed that the C-terminal DNAPol of group I alphabaculoviruses, including CfMNPV, similarly contains a highly conserved region. Multiple alanine substitution analysis of the DNAPol conserved region demonstrated that amino acids 972 to 981 (NNTYK FCLYK) is important for viral production and viral DNA replication. It is not known if the C-terminal amino acid sequence of

AcMNPV DNApol participates in interactions with other proteins which might play an important role in DNA replication.

As CfMNPV DNApol similarly has two NLSs and the conserved C-terminal 10-aa sequence, we wished to see if these C-terminal features, even in a virus from a different group I alphabaculovirus, could replace the AcMNPV DNApol C terminus. As demonstrated, the C-terminal 193 aa from CfMNPV DNApol could functionally replace the C-terminal 184 aa from AcMNPV DNApol. This suggests that it is the NLSs and C-terminal conserved domain features that are important for nuclear localization and DNApol function, rather than the amino acid sequence *per se*. Analysis of protein interactions between the C terminus of AcMNPV DNApol and other viral or host proteins is required to further explore its functional role.

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