Virus Research 266 (2019) 52–57

Contents lists available at ScienceDirect

Virus Research

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

The deficiency in nuclear localization signal of Neodiprion lecontei nucleopolyhedrovirus DNA polymerase prevents rescue of viral DNA replication and virus production in *dnapol*-null Autographa californica multiple nucleopolyhedrovirus

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ARTICLE INFO

Keywords:
Baculovirus
DNA polymerase
Nuclear localization signal
DNA replication

ABSTRACT

DNA polymerase (DNApol) is highly conserved in baculovirus and is required for viral DNA replication. However, little is known about gammabaculovirus DNApol. Here DNApol of the gammabaculovirus Neodiprion lecontei nucleopolyhedrovirus (NeleNPV) was cloned into a *dnapol*-null alphabaculovirus AcMNPV bacmid, creating Bac-GFP-AcΔPol-NlPol. The resulting recombinant bacmid did not spread to neighboring cells, virus growth curve and real-time PCR revealed that NeleNPV *dnapol* substitution did not rescue AcMNPV DNA replication and virus production. Immunofluorescence microscopy revealed that NeleNPV DNApol was expressed but could not localize to the nucleus. Subsequently NeleNPV DNApol was fused to SpltNPV DNApol nuclear localization signal (NLS) and the fused DNApol could import into nucleus. The NLS-fusing NeleNPV DNApol was further transposed into the *dnapol*-null AcMNPV bacmid, creating Bac-GFP-AcΔPol-HA:NlPol^{NLS}. The recombinant virus could replicate and produce infectious virus in Sf9 cells, albeit at reduced levels compared to wild type AcMNPV. Taken together, our results suggested that the NLS deficiency of NeleNPV DNApol blocked viral DNA replication and production of infectious virus in *dnapol*-null AcMNPV bacmid.

The family *Baculoviridae* comprises a diverse group of insect-specific DNA viruses that are pathogenic to arthropods, these viruses are divided into four genera: *Alphabaculovirus* (lepidopteran-specific NPV), *Betabaculovirus* (lepidopteran-specific GV), *Gammabaculovirus* (hymenopteran-specific NPV) and *Deltabaculovirus* (dipteran-specific NPV) (Jehle et al., 2006a,b; Miele et al., 2011). Baculoviruses have a biphasic replication cycle and produce two different types of infectious virus particles. Budded viruses (BV) establish an early systemic infection for cell-to-cell transmission within a susceptible host while occlusion-derived viruses (ODV) spread virus infection from insect to insect hosts later in infection (Federici, 1997). Baculoviruses replicate their circular, double-stranded DNA genomes in the nucleus of infected cells following a molecular interaction between virus-encoded *trans*-acting factors and *cis*-acting DNA sequences (Okano et al., 2006).

DNA polymerase (DNApol) is highly conserved in baculovirus and plays a crucial role in viral DNA replication (Kool et al., 1994; Lu and Miller, 1995). The molecular mechanism of baculovirus DNA replication has been studied in Autographa californica multiple nucleopolyhedrovirus (AcMNPV), the type species of the genus *Alphabaculovirus*. AcMNPV DNApol is a family B DNApol homologue (Braithwaite and Ito, 1993; Tomalski et al., 1988). AcMNPV DNApol contains an exonuclease (exo) domain including exo I to III and a polymerase domain with designated regions I through VII (Feng et al., 2012). These domains are shared among α-like DNApols of eukaryotes and various DNA viruses (Chaeychomsri et al., 1995; Hwang et al., 2004). The fidelity of DNA replication is regulated by two 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 (Blanco et al., 1991).

Baculovirus DNApols share many common features, such as amino acid (aa) sequence conservation in seven polymerase motifs and three exonuclease motifs (Huang and Levin, 2001). Previous results showed that the DNApol from AcMNPV could be substituted by DNApols from alphabaculovirus Choristoneura fumiferana MNPV (CfMNPV) and Spodoptera litura NPV (SpltNPV) (Chen et al., 2016; Feng and Krell, 2014), but not by that of betabaculovirus Pieris rapae granulovirus

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https://doi.org/10.1016/j.virusres.2019.04.005
Received 22 January 2019; Received in revised form 9 April 2019; Accepted 12 April 2019
Available online 13 April 2019
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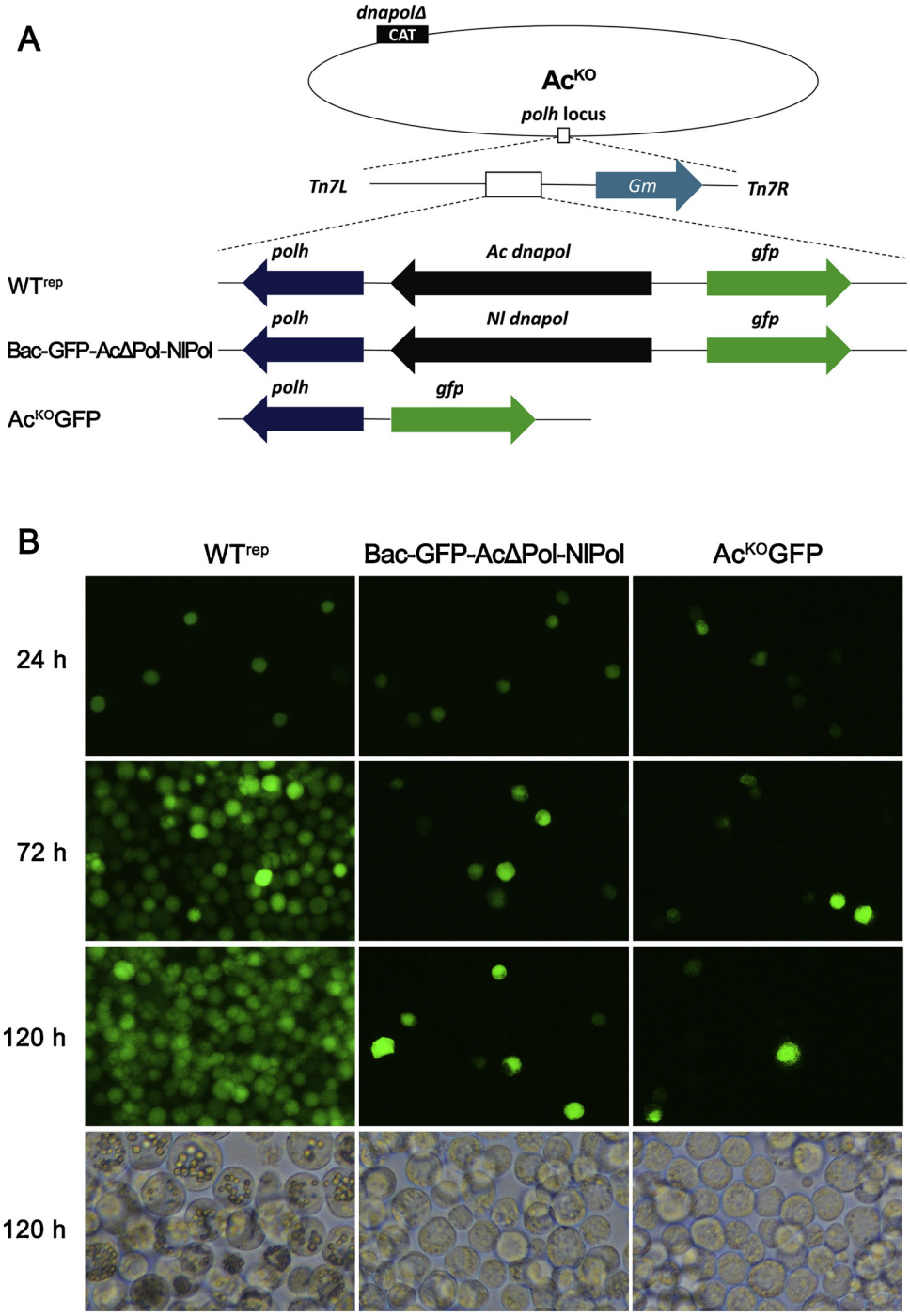

Fig. 1. Bacmid construction and microscopic analysis of bacmid transfected cells. (A) Schematic of cassettes with *egfp* as a reporter under the *Opie1* promoter, *dnapol* expressed by the AcMNPV *dnapol* promoter and *dnapol* native poly(A) from NeleNPV, and the adjoining *polyhedrin* (*polh*) gene under the control of the *polh* promoter. The cassettes were inserted into the original *polyhedrin* locus of the DNApol KO (Ac^{KO} bacmid) by Tn7-mediated transposition. (B) Fluorescence microscopy images of monolayers of cells transfected with WT^{rep}, Bac-GFP-AcΔPol-NlPol and Ac^{KO}GFP bacmids at 24, 72 and 120 hpt and light microscopy images were acquired for transfection at 120 hpt. WT^{rep} was used as a positive control and Ac^{KO}GFP was a negative control.

(PiraGV) (Chen et al., 2017). In this study, we further examine whether DNApol from gammabaculovirus Neodiprion lecontei nucleopolyhedrovirus (NeleNPV) could be functional as AcMNPV DNApol. NeleNPV *dnapol* nucleotides (15806–18577) and a polyadenylation site under the control of the native AcMNPV *dnapol* promoter were inserted into a *dnapol*-null AcMNPV bacmid into the *polyhedrin* locus, creating the *dnapol* replacement bacmid Bac-GFP-AcΔPol-NlPol. The constructs were confirmed by PCR. Ac^{KO}GFP was used as a negative control and WT^{rep} was a positive control (Fig. 1A). Bac-GFP-AcΔPol-NlPol, Ac^{KO}GFP and WT^{rep} bacmids were transfected into Sf9 cells, and virus replication and spread were monitored by GFP fluorescence throughout a bacmid transfected monolayer. The numbers of GFP-positive cells for all three bacmids at 24 h post transfection (hpt) were similar, suggesting equivalent transfection efficiencies (Fig. 1B). There was an increased spread of fluorescence to neighboring cells for the WT^{rep} at 48 hpt and by 72 hpt GFP fluorescence was observed in almost all cells transfected by WT^{rep}. By 120 hpt, fluorescence spread completely from the initially transfected cells to adjacent cells in the monolayer for the WT^{rep}. However, the number of GFP-positive cells did not increase with time for Ac^{KO}GFP or Bac-GFP-AcΔPol-NlPol even by 120 hpt, indicating that there was no virus spread from the initially transfected cells for these viruses (Fig. 1B). Occlusion bodies were observed microscopically in WT^{rep} at 72 hpt while no occlusion bodies were observed for Ac^{KO}GFP or Bac-GFP-AcΔPol-NlPol, even by 120 hpt (Fig. 1B). These results suggest that the NeleNPV *dnapol* was not able to repair the AcMNPV *dnapol* knockout.

The effect of DNApol replacement on virus replication was evaluated by viral growth curves. Cells were transfected with 2 μg of

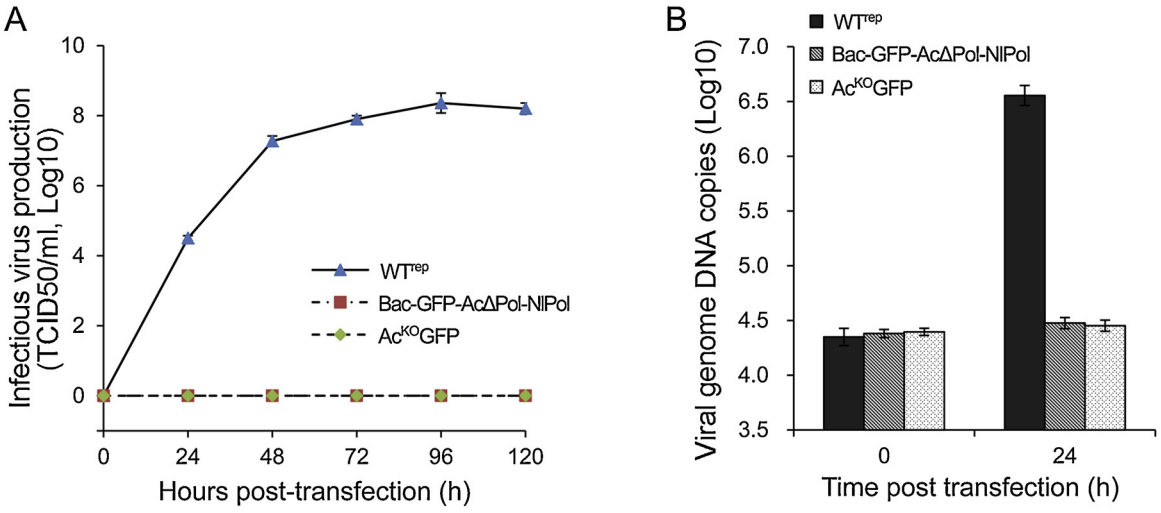

Fig. 2. Virus production and viral DNA synthesis of WT^{rep}, Bac-GFP-AcΔPol-NlPol and $Ac^{KO}GFP$ bacmids. (A) Monolayers were transfected with 2 ug of bacmid DNAs, WT^{rep}, Bac-GFP-AcΔPol-NlPol and $Ac^{KO}GFP$. The production of infectious BV was determined by a $TCID_{50}$ endpoint dilution assays. (B) DNA accumulation kinetics of the WT^{rep}, Bac-GFP-AcΔPol-NlPol and $Ac^{KO}GFP$. Total intracellular DNA was extracted from transfected cells at 0 hpt and 24 hpt. The bacmid copy numbers were determined by qPCR using AcMNPV *gp41* as the target. Bars in the panels represent standard deviation determined from three independent replicates.

bacmid DNA of Bac-GFP-AcΔPol-NlPol, $Ac^{KO}GFP$ or WT^{rep}. Viral yields were determined by a 50% tissue culture infective dose ($TCID_{50}$) endpoint dilution assay on Sf9 cells. The virus growth curves showed a steady increase in virus production for WT^{rep} following transfection, while the titers of Bac-GFP-AcΔPol-NlPol or $Ac^{KO}GFP$ did not increase at any detectable level even up to 120 hpt (Fig. 2A). These results suggest that viruses with NeleNPV *dnapol* instead of AcMNPV *dnapol* were not infectious.

We also performed a quantitative PCR to monitor the accumulation of intracellular total viral DNA in Sf9 cells over a single replication cycle up to 24 h post transfection. Cells were seeded at a density of 1×10^5/ml, after which monolayers were transfected with Bac-GFP-AcΔPol-NlPol, $Ac^{KO}GFP$ or WT^{rep}. The level of viral DNA for WT^{rep} increased about 158-fold over 24 hpt while there was no detectable increase in intracellular viral DNA levels for Bac-GFP-AcΔPol-NlPol or $Ac^{KO}GFP$ over the same time course (Fig. 2B). Thus, NeleNPV DNApol bacmid Bac-GFP-AcΔPol-NlPol could not rescue viral DNA replication or virus production over 120 hpt in transfected cells. Deficiency of viral DNA replication would be expected to impact late gene expression which could lead to the abrogation of polyhedra production as shown by lack of occlusion bodies visible by light microscopy (Fig. 1B).

As baculovirus DNApol is responsible for intranuclear viral DNA replication, it needs to be translocated into the nucleus. To determine if NeleNPV DNApol could localize to nuclei, plasmid pBlue-HA:NlPol was transfected into Sf9 cells and DNApol of NeleNPV was monitored for immunofluorescence using mouse monoclonal anti-HA antibody conjugated with the Alexa 594 (red) goat anti-mouse antibody. Using confocal fluorescence microscopy, red immunofluorescence was observed in cytoplasm for pBlue-HA:NlPol at 24 hpt, indicating that the NeleNPV DNApol was expressed in plasmid transfected cells. Furthermore, exclusive cytoplasm localization was identified for pBlue-HA:NlPol by colocalization of red immunofluorescence and Hoechst blue fluorescence, suggesting that NeleNPV DNApol could not be imported into the nucleus (Fig. 3A). The same plasmid was transfected into cells in the presence of WT virus infection again showing expression of NeleNPV DNApol was exclusive cytoplasm localization (Fig. 3A). Western blot analysis was also performed using mouse monoclonal anti-HA antibody, the results confirmed NeleNPV DNApol was expressed (Fig. 3C). These results demonstrated that NeleNPV DNApol was expressed in transfected cells, but it could not localize to the nucleus and this localization was independent of virus infection.

To further demonstrate that NeleNPV DNApol could be expressed and localized to cytoplasm in transfected cells, we cloned NeleNPV *dnapol* with an HA tag into *dnapol*-null AcMNPV bacmid, creating Bac-AcΔPol-HA:NlPol. Following transfection of the recombinant bacmid Bac-AcΔPol-HA:NlPol into Sf9 cells, red fluorescence was observed in cytoplasm using confocal fluorescence microscopy at 24 hpt, indicating that NeleNPV DNApol was expressed in Sf9 cells. Colocalization of red immunofluorescence and Hoechst blue fluorescence demonstrated that NeleNPV DNApol could only localize to the cytoplasm (Fig. 3A).

NeleNPV DNApol could not localize to nucleus, and previous prediction in baculovirus DNApols showed that NeleNPV DNApol did not have a nuclear localization signal (NLS) (Feng and Krell, 2014). To make it import into nucleus, NeleNPV DNApol was fused to SpltNPV DNApol NLS (QEPPAKRARMPT), resulting in a plasmid pBlue-HA:$NlPol^{NLS}$. Plasmid pBlue-HA:$NlPol^{NLS}$ was transfected into Sf9 cells and DNApol of NeleNPV was monitored for immunofluorescence. Confocal fluorescence microscopy showed that red immunofluorescence was observed for pBlue-HA:$NlPol^{NLS}$ at 24 hpt, indicating that the NeleNPV DNApol was expressed in plasmid transfected cells. Furthermore, exclusive nuclear localization was identified for pBlue-HA:$NlPol^{NLS}$ by colocalization of red immunofluorescence and Hoechst blue fluorescence, suggesting that NeleNPV DNApol could localize to nucleus (Fig. 3B). The same plasmid was transfected into Sf9 cells in the presence of WT virus infection again, the results show that NeleNPV DNApol was exclusive nuclear localization (Fig. 3B). Western blot analysis confirmed the expression of HA:$NlPol^{NLS}$ using mouse monoclonal anti-HA antibody (Fig. 3D). These results demonstrate that NeleNPV DNApol fused to SpltNPV NLS could localize to the nucleus and this localization was independent of virus infection.

The recombinant NeleNPV DNApol, HA:$NlPol^{NLS}$, was also cloned into *dnapol*-null AcMNPV bacmid, creating Bac-AcΔPol-HA:$NlPol^{NLS}$. The *polyhedrin* were also cloned into the same locus of the bacmid. Bac-AcΔPol-HA:$NlPol^{NLS}$ was transfected into Sf9 cells to monitor DNApol localization. Confocal fluorescence microscopy showed that red immunofluorescence was observed at 24 hpt, indicating that NeleNPV DNApol was expressed in Sf9 cells. Colocalization of red immunofluorescence and Hoechst blue fluorescence demonstrated that NeleNPV DNApol could import into nucleus (Fig. 3B).

To determine if NLS fused NeleNPV DNApol could replace that of AcMNPV, fusion protein HA:$NlPol^{NLS}$ was reinserted into *dnapol*-null AcMNPV bacmid, creating Bac-GFP-AcΔPol-HA:$NlPol^{NLS}$. The *polyhedrin* and *gfp* were also cloned into the same locus of the bacmid. $Ac^{KO}GFP$, Bac-GFP-AcΔPol-HA:$NlPol^{NLS}$ and WT^{rep} bacmids were

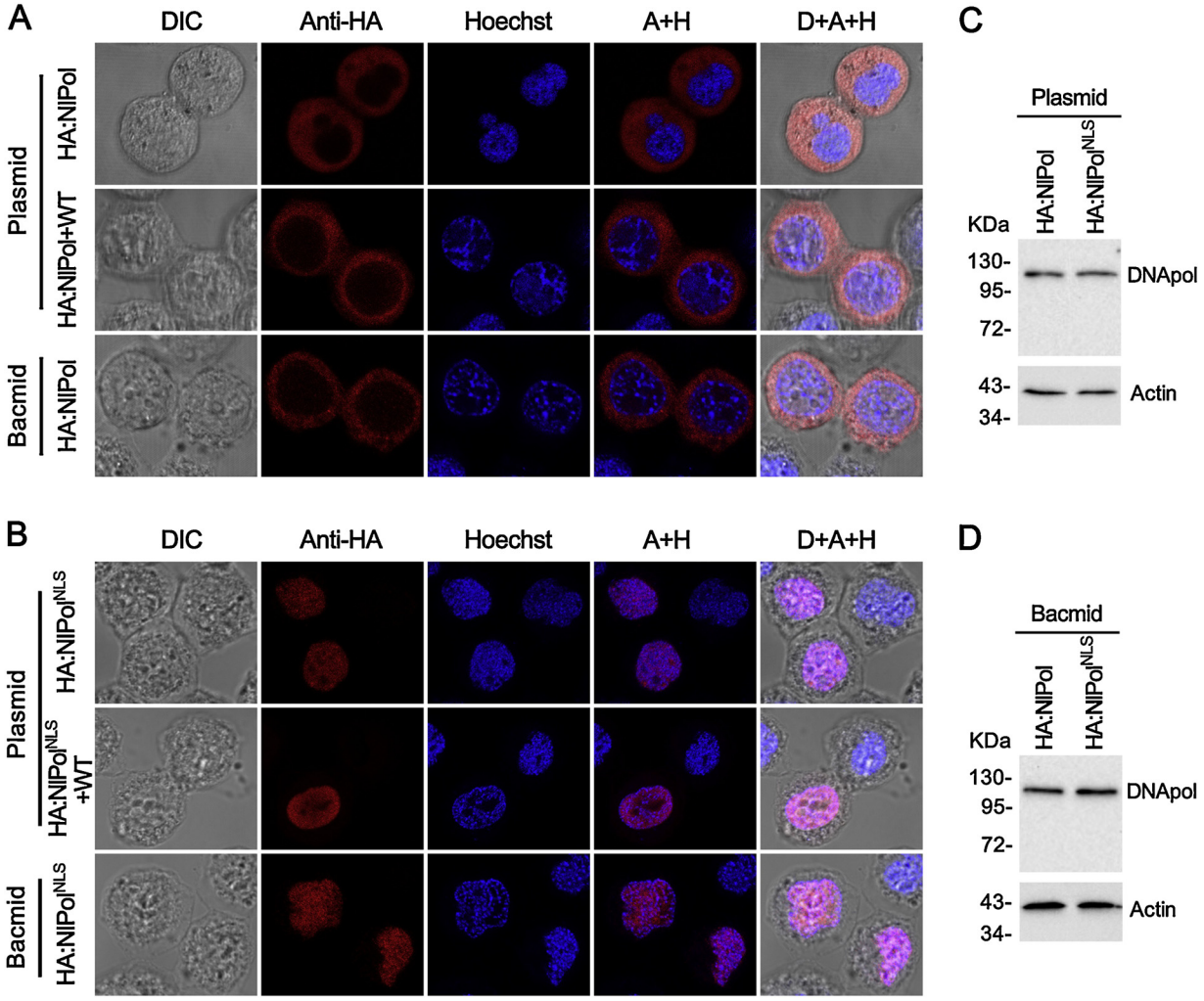

Fig. 3. Localization of NeleNPV DNApol. (A) Localization of HA tagged NeleNPV DNApol. Sf9 cells were transfected with NeleNPV DNApol expression plasmid (pBlue-HA:NlPol) or bacmid (Bac-AcΔPol-HA:NlPol). Plasmid or bacmid was transfected in the presence of WT virus infection or not. (B) Localization of NeleNPV DNApol fused to SpltNPV NLS. Sf9 cells were transfected with NLS-fusing NeleNPV DNApol expression plasmid, pBlue-HA:Nlpol^{NLS}, or bacmid, Bac-AcΔPol-HA:NlPol^{NLS}. Plasmid was transfected in the presence of WT virus infection or not. Cells were treated with mouse monoclonal anti-HA antibody, and incubated with the Alexa 594–conjugated goat anti-mouse antibody. Red immunofluorescence was observed at 24 hpt under confocal microscopy. Nuclei were stained with Hoechst 33342, and DNApol protein was stained with anti-HA. Merged red and blue fluorescence images are in the column labeled A + H. Light microscopy and red and blue fluorescence images were also merged in the column labeled D + A + H. (C) Expression of HA tagged NeleNPV DNApol in plasmid transfected cells. Sf9 cells were transfected for 24 hpt with plasmids, pBlue-HA:NlPol and pBlue-HA:NlPol^{NLS}. Lysates were subjected to immunoblot analysis using anti-FLAG monoclonal antibody, β-actin was used as a control for protein loading. (D) Expression of HA tagged NeleNPV DNApol in bacmid transfected cells. Bacmids, Bac-AcΔPol-HA:NlPol and Bac-AcΔPol-HA:NlPol^{NLS}. Lysates were subjected to immunoblot analysis using anti-FLAG monoclonal antibody, β-actin was used as a control for protein loading.

transfected into Sf9 cells. Ac^{KO}GFP was the negative control and WT^{rep} was served as a positive 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. 4A). By 72 hpt, GFP was expressed in almost all the cells for WT^{rep}, whereas GFP fluorescence spread from the initially transfected cells to adjacent cells for Bac-GFP-AcΔPol-HA:NlPol^{NLS}. There was no increase in the number of fluorescent cells for Ac^{KO}GFP from 24 hpt throughout transfection. Polyhedron were observed in 0.8% Bac-GFP-AcΔPol-HA:NlPol^{NLS}-transfected cells and 81.2% WT^{rep}-transfected cells at 120 hpt. However, no occlusion bodies were detected for Ac^{KO}GFP (Fig. 4A).

Virus growth curves were also used to determine the ability of NeleNPV fused DNApol to replace that of AcMNPV. Cells were transfected with 2 μg bacmid DNAs of either Ac^{KO}GFP, Bac-GFP-AcΔPol-HA:NlPol^{NLS} and WT^{rep}. The supernatants were collected at the indicated times. The virus growth curve showed a steady increase in virus production for Bac-GFP-AcΔPol-HA:NlPol^{NLS} and WT^{rep}, but the BV titers of Bac-GFP-AcΔPol-HA:NlPol^{NLS} was 3162-fold lower than that of WT^{rep} at 120 hpt (Fig. 4B). However, the titer of Ac^{KO}GFP did not increase at any detectable level even at 120 hpt.

The level of viral DNA synthesis over the first 24 hpt for Ac^{KO}GFP, Bac-GFP-AcΔPol-HA:NlPol^{NLS} and WT^{rep} were also determined. DNAs from Ac^{KO}GFP, Bac-GFP-AcΔPol-HA:NlPol^{NLS} and WT^{rep}-transfected cells at 24 hpt were subjected to real-time PCR with primers targeting AcMNPV *gp41*, Ac^{KO}GFP did not show any increase in viral DNA level during 24 h. By contrast, there were approximately 2.33 logs and 0.77 logs increase in viral DNA accumulation over the level at time zero for WT^{rep} and Bac-GFP-AcΔPol-HA:NlPol^{NLS}, respectively. The level of DNA for WT^{rep} was 35.3-fold higher than that of Bac-GFP-AcΔPol-HA:NlPol^{NLS} (Fig. 4C). These results indicated that the replacement of DNApol of AcMNPV with NLS fused NeleNPV DNApol allowed for viral DNA replication and production of infectious virus but with a lower yield than that of the wild type.

We earlier showed that the DNApols of alphabaculovirus group I

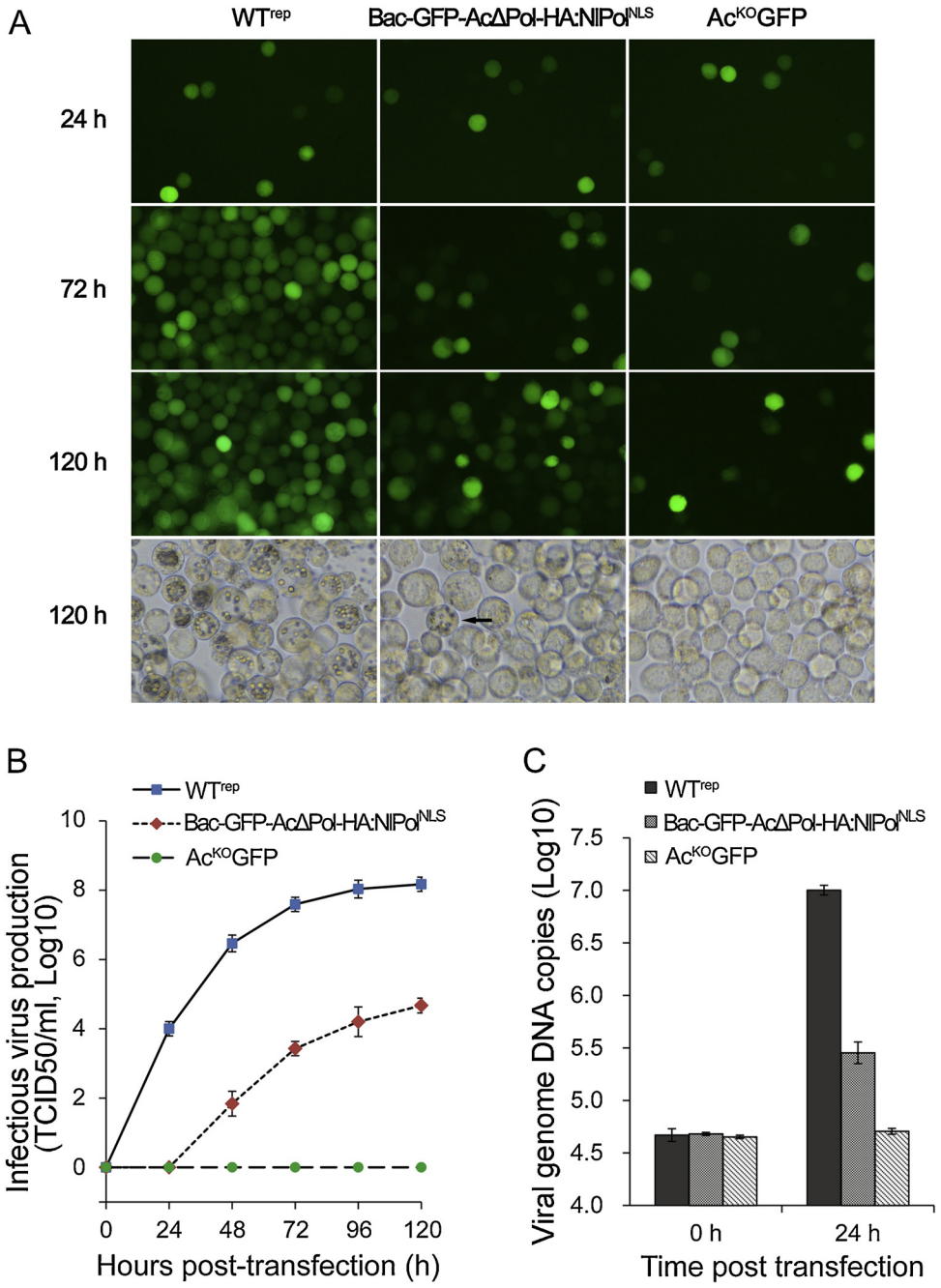

Fig. 4. GFP fluorescence and virus production analysis of WT^{rep}, Bac-GFP-AcΔPol-HA:NlPol^{NLS} and Ac^{KO}GFP bacmids in Sf9 cells. (a) GFP fluorescence images of monolayers of cells transfected with WT^{rep}, Bac-GFP-AcΔPol-HA:NlPol^{NLS} and Ac^{KO}GFP at 24, 72 and 120 hpt and light microscopy images were taken for transfection at 120 hpt. (b) Virus growth curves. Cells were transfected with 2 μg bacmid DNAs, the resulting supernatants were used to determine the yield of infectious BV. (c) Intracellular viral DNA accumulation for WT^{rep}, Bac-GFP-AcΔPol-HA:NlPol^{NLS} and Ac^{KO}GFP in Sf9 cells. Total intracellular DNA was extracted from transfected cells at 0 hpt and 24 hpt. Viral DNA copy numbers were then determined by qPCR using AcMNPV *gp41* as the target. Bars in the panels represent standard deviations determined from three independent transfections.

CfMNPV and group II SpltNPV, could substitute for the DNApol of AcMNPV and that both DNApols could localize to the nucleus (Chen et al., 2016; Feng and Krell, 2014). PiraGV DNApol, a DNApol from betabaculovirus, could be imported into nucleus, albeit it could not rescue virus replication in a *dnapol*-null AcBacmid (Chen et al., 2016). In the present study using a gammabaculovirus, the replacement of NeleNPV DNApol resulted in total abrogation of AcMNPV DNA replication and viral spread, demonstrating that viral DNA synthesis was blocked for Bac-GFP-AcΔPol-NlPol in transfected cells. NeleNPV DNApol fused to SpltNPV DNApol NLS partially rescued *dnapol*-null AcMNPV bacmid infection in host cells, but at low BV levels compared to wild type AcMNPV. Compared to 63% identity between AcMNPV and CfMNPV DNApols as well as 45% identity between AcMNPV and SpltNPV DNApols, NeleNPV DNApol showed an overall amino acid sequence identity of only 26% with that of AcMNPV. Baculovirus DNApol contains N terminus, exonuclease domain and polymerase domain and C terminus. The exonuclease domain and polymerase domain are required for viral DNA replication and are highly conserved in all baculovirus DNApols. However, previous results showed that the N and C terminal domains of AcMNPV DNApol are also important for viral DNA replication and infectious virus production (Feng and Krell, 2014; Chen et al., 2018). Four conserved motifs of the N terminus of alphabaculovirus and betebaculovirus DNApols were important for DNA replication (Chen et al., 2018), the sequence aa 972 to 981 (NNTYKFCLYK) is highly conserved in C terminus of group I alphabaculovirus DNApol and is critical for viral DNA replication (Feng and Krell, 2014). AcMNPV is a member of group I alphabaculovirus, and AcMNPV DNApol contains the five motifs, while these motifs could not be found in gammabaculoviurs NeleNPV DNApol. Thus, NeleNPV DNApol of Bac-GFP-AcΔPol-HA:NlPol^{NLS} could not completely match replication machinery of AcMNPV in Sf9 cells, which resulted in low replication efficiency and reduction in BV production.

Baculoviruses replicate their genomes in nucleus of transfected cells. DNApol must be transported into the nuclei of host cells. Many large proteins like DNApol (≥50 kDa) are imported into nuclei by a nuclear localization signal (Goldfarb et al., 2004). However, some proteins do not contain nuclear localization signal, they could be co-transported into nucleus by interaction with nuclear-targeted proteins. AcMNPV single-stranded DNA binding protein LEF-3 mediates the nuclear localization of the helicase P143 (Wu and Carstens, 1998). Nucleocapsid protein BV/ODV-C42 mediates the nuclear entry of P78/83, and the NLS motif of C42 was essential for this process (Wang et al., 2008). In this study, we could not identify a nuclear localization signal for NeleNPV DNApol. Immunofluorescence analysis confirmed that NeleNPV DNApol was localized to cytoplasm of transfected cells for Bac-AcΔPol-NlPol and pBlue-HA:NlPol (Fig. 3A), while DNApol is important for viral DNA replication, it funtions as replicase in nucleus of host cells. It is possible that DNApol is localized to nucleus by interaction with nuclear-targeted proteins of NeleNPV, further experiment is necessary to demonstrate nuclear localization of NeleNPV DNApol in baculovirus-infected cells.

In conclusion, we constructed a mutant alphabaculovirus AcMNPV bacmid, Bac-GFP-AcΔPol-NlPol in which the AcMNPV DNApol was replaced by a DNApol from the gammabaculovirus NeleNPV. Our results demonstrated that NeleNPV DNApol was expressed but could not be imported into the nucleus, and the NeleNPV *dnapol* substitution abrogated AcMNPV DNA replication and virus production. We further demonstrated the replacement of NeleNPV DNApol fused to SpltNPV DNApol NLS could partially rescue viral DNA replication and virus production in *dnapol*-null AcMNPV bacmid. Thus, the deficiency in NLS of NeleNPV DNApol blocked viral replication and production of infectious virus in *dnapol*-null AcMNPV bacmid.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by a National Natural Science Foundation of China (grant numbers 31572006, 31701847), Innovation Project of Chinese Academy of Agricultural Sciences and the Central Level Public Interest Research Institute for Basic R & D Special Fund Business (grant number 2017RG002-7) and Innovation Project of Chinese Academy of Agricultural Sciences.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.04.005.

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