Rescue of dnapol-null Autographa californica multiple nucleopolyhedrovirus with DNA polymerase (DNApol) of Spodoptera litura nucleopolyhedrovirus (SpltNPV) and identification of a nuclear localization signal in SpltNPV DNApol

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DNA polymerase (DNApol) is highly conserved in all baculoviruses and plays an essential role in viral DNA replication. It determines the fidelity of baculovirus DNA replication by inserting the correct nucleotides into the primer terminus and proofreading any mispaired nucleotides. DNApols of groups I and II of the genus Alphabaculovirus in the family Baculoviridae share many common structural features. However, it is not clear whether a group I Autographa californica multiple nucleopolyhedrovirus (AcMNPV) DNApol can be substituted by a group II NPV DNApol. Here we report the successful generation of AcMNPV dnapol-null virus being rescued by a group II Spodoptera litura NPV (SpltNPV) dnapol (Bac-AcDPol : Slpol). Viral growth curves and quantitative real-time PCR showed that the dnapol replacement reduced the level of viral production and DNA replication of Bac-AcDPol : Slpol compared with WTrep, a native dnapol insertion in an AcMNPV dnapol-null virus. Light microscopy showed that production of occlusion bodies for Bac-AcDPol : Slpol was reduced. We also identified a nuclear localization signal (NLS) for the SpltNPV DNApol C terminus at residues 827–838 by mutational analysis and confocal microscopy. Multiple point substitution of SpltNPV DNApol NLS abrogated virus production and viral DNA replication. Overall, these data suggested that the NLS plays an important role in SpltNPV DNApol nuclear localization and that SpltNPV DNApol cannot efficiently substitute the AcMNPV DNApol in AcMNPV.

INTRODUCTION

Baculoviridae is a family of large, rod-shaped, enveloped, circular dsDNA viruses that are pathogenic to insects and contains four genera, Alphabaculovirus (lepidopteran baculoviruses), Betabaculovirus (granuloviruses), Deltabaculovirus (dipteran baculoviruses) and Gammabaculovirus (hymenopteran baculoviruses). Baculoviruses have a biphasic replication cycle producing budded virus (BV) early in infection and occlusion-derived virus (ODV) later in infection (Federici, 1997). These viruses replicate their 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).

Baculovirus DNA polymerase (DNApol) is highly conserved and plays an essential role in viral DNA replication (Kool et al., 1994). The 3 kb Autographa californica multiple nucleopolyhedrovirus (AcMNPV) DNApol ORF encodes a polypeptide of 984 aa with a predicted molecular mass of 114.31 kDa (Tomasaki et al., 1988). Previous biochemical analysis demonstrated that purified DNApol from the group I alphabaculovirus AcMNPV had significant DNApol activity and 3′–5′ exonuclease activity, but no 5′–3′ exonuclease activity (Hang & Guarino, 1999; Miller et al., 1981). AcMNPV DNApol is a family B DNApol homologues (Braithwaite & Ito, 1993;
Tomalski et al., 1988). Based on the translated amino acid sequence analysis, AcMNPV DNApol contains both exonuclease and polymerase domains. These domains are shared among α-like DNApol of eukaryotes and other DNA viruses (Chaeychomsri et al., 1995). 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 mispaired nucleotides (Blanco et al., 1991).

Like all eukaryotic DNApol, baculovirus DNApol have to localize to the nucleus. The nuclear localization of proteins is normally mediated via passive diffusion for small proteins (<50 kDa) or by an energy-dependent process by nuclear localization signals (NLSs) for larger ones (≥50 kDa) (Macara, 2001)). It is common that proteins containing NLSs are transported to the nucleus by nuclear import proteins. Classic NLSs (cNLSs) include five classes of monopartite and one class of bipartite NLSs (Kosugi et al., 2009). These cNLSs are bound by the adaptor protein importin-α and, along with importin-β, form a ternary complex. Importin-β docks the complex to the nuclear pore complex and mediates nuclear entry (Macara, 2001; Weis, 2003). NLSs of some baculovirus proteins are also involved in nuclear localization. The polyhedrin motif between aa 30 and 57 mediates nuclear translocation; the sequence KRKK (aa 32–35) is the minimal component for its nuclear localization (Jarvis et al., 1991). Two basic clusters at residues RKRR (aa 117–120) and RKRLK (aa 144–148) compose NLS core elements, which are necessary for the transport of Bombyx mori nucleopolyhedrovirus ORF47, a protein involved in cell cycle regulation, to the nucleus (Guo et al., 2010). The residues KVNRR (aa 534–538) of AcMNPV IE-1 form the core element of an NLS, and substitution or deletion of amino acids within this region impairs its nuclear localization (Olson et al., 2002). The N-terminal 56 aa of AcMNPV LEF-3 is required for nuclear import of P143; the residues KIREN (aa 28–32) form the core NLS (Au et al., 2009; Chen & Carstens, 2005).

The group I DNApol from Choristoneura fumiferana multiple nucleopolyhedrovirus can substitute for that of another group I alphabaculovirus, AcMNPV (Feng & Krell, 2014). Since DNApol have high amino acid sequence conservation between group I and group II alphabaculoviruses and share many common structural features, such as an exonuclease domain containing exonuclease I (exo I) to exo III and a polymerase domain with designated regions I through VII (Bulach et al., 1999; Huang & Levin, 2001), we speculated that AcMNPV DNApol could be substituted by DNApol in group II NPVs such as Spodoptera litura nucleopolyhedrovirus (SpltNPV). To address this hypothesis, we generated an AcMNPV-based bacmid carrying a SpltNPV DNApol (Fig. 1). Transfection of SpltNPV dnapol-rescued bacmid (Bac-AcΔPol:Slpol) showed that the SpltNPV dnapol replacement could rescue AcMNPV, but that the SpltNPV dnapol substitution reduced AcMNPV DNA replication and viral production. Interestingly, light microscopy showed that the production of occlusion bodies (OBs) for Bac-AcΔPol:SlPol was also reduced. Through peptide fusions with enhanced green fluorescent protein (eGFP) and point mutations in the NLS regions of SpltNPV DNApol we identified an NLS in SpltNPV DNApol. Multiple point substitution of the NLS region completely abrogated virus production and viral DNA replication. Our results suggested that SpltNPV DNApol has an NLS that plays an important role in SpltNPV DNApol nuclear localization and that SpltNPV DNApol could rescue infectivity of a dnapol-null AcMNPV but with decreased DNA replication, BV and polyhedra production.

**RESULTS**

**SpltNPV dnapol can rescue a dnapol-null AcMNPV bacmid**

To determine if alphabaculovirus group I AcMNPV DNApol could be substituted by that of an alphabaculovirus group II SpltNPV, SpltNPV dnapol nucleotides 66 926 to 66 994 (for aa 1–1022) and the native poly(A) site under the control of AcMNPV ie-1 promoter were cloned into the dnapol-null AcMNPV bacmid polyhedrin locus, creating the GFP-expressing Bac-AcΔPol:SlPol locus. The constructs were confirmed by PCR analysis (Fig. 1).

$Ac^{KO}$GFP, Bac-AcΔPol:SlPol and WT$^{rep}$bacmids were transfected into SF9 cells; $Ac^{KO}$GFP (a dnapol-null virus) was the negative control, and WT$^{rep}$ was used as a positive control. GFP-fluorescent cells were monitored for virus production and spread. At 24 h post-transfection (p.t.), only individual GFP-fluorescent cells were found for all three bacmid-transfected monolayers, indicating equivalent transfection efficiencies (Fig. 2a). By 72 h p.t., GFP was expressed in almost all the cells for WT$^{rep}$ while there was some spread of fluorescence to neighbouring cells for the
Bac-AcΔPol:SlPol. Only a limited spread of fluorescence was observed, with GFP-positive cells being found either individually or in small clusters of two or three cells each for AcKO-GFP (Fig. 2a). By 120 h p.t., GFP fluorescence spread completely from the initially transfected cells to adjacent cells for WTrep, and GFP was expressed in most of the cells for the Bac-AcΔPol:SlPol, while there was no increase in the number of fluorescent cells for the AcKO-GFP (Fig. 2a). These results suggested that SpltNPV dnapol was able to rescue a dnapol-null AcMNPV bacmid, but the virus spread was somewhat delayed compared with WTrep.

**SplitNPV dnapol substitution decreased virus replication and OB production**

The decreased viral spread was also reflected in viral growth curves. Cells were transfected by AcKO-GFP, Bac-AcΔPol:SlPol and WTrep bacmids. The extracellular supernatants were collected at the indicated times and BV titres were assayed by a TCID50 endpoint dilution assay in which signs of infection were determined by GFP fluorescence. Detection of extracellular virus for Bac-AcΔPol:SlPol was delayed about 24 h compared with WTrep. There was a steady increase in the level of virus production following transfections with Bac-AcΔPol:SlPol and WTrep but by 120 h p.t. the titres of the Bac-AcΔPol:SlPol and WTrep were about 5.47 and 8.48 logs, respectively; there was a difference of 1000-fold. In contrast, BV was not detectable at any time point up to 120 h p.t. for AcKO-GFP, as previously described (Feng et al., 2012) (Fig. 2b). These results revealed that the SpltNPV dnapol repair bacmid produced infectious virus but with a much lower yield than that of the AcMNPV dnapol repair bacmid.

A quantitative PCR assay was performed to monitor the accumulation of intracellular viral DNA and restricted to a single replication cycle over the first 24 h post-infection (p.i.). Sf9 cells were seeded at a density of 1×10^5, after which monolayers were infected by Bac-AcΔPol:SlPol and WTrep viruses at an m.o.i. of 10. By 24 h p.i., the level of intracellular viral DNA was determined from three independent replicates. The arrows in (d) showed production of occlusion bodies for Bac-AcΔPol:SlPol.
viral DNA for WT<sup>rep</sup> had increased about 4 logs over that at time zero while there was only about a 1.55 log increase for Bac-Ac<sub>C</sub>pol: SlPol (Fig. 2c). The difference between Bac-Ac<sub>C</sub>pol: SlPol and WT<sup>rep</sup> suggested that the virus with the replacement of SpltNPV dna<sub>p</sub> was somewhat compromised in viral DNA replication.

By light microscopy, we initially noticed a difference in OBs for WT<sup>rep</sup> and Bac-Ac<sub>C</sub>pol: SlPol. WT<sup>rep</sup> and Bac-Ac<sub>C</sub>pol: SlPol were generated in a common bacmid background; the only difference was the source of the dna<sub>p</sub>. To determine if SpltNPV DNApol substitution affected the formation and production of polyhedra, WT<sup>rep</sup>, Bac-Ac<sub>C</sub>pol: SlPol and Ac<sub>K</sub>GFP bacmids were transfected into Sf9 cells. Similarly, cells were infected with the corresponding virus at an m.o.i. of 1. About 6% of the cells transfected with the WT<sup>rep</sup> bacmid synthesized OBs at 72 h p.t. By 120 h p.t., OBs were detected in 83% of WT<sup>rep</sup>-transfected cells (Fig. 2d). The OBs in the transfected cells were released to the medium for WT<sup>rep</sup> at 144 h p.t. Polyhedra were detected in 85% of WT<sup>rep</sup>-infected cells at 96 h p.i. (Fig. 2d). In contrast, OBs were observed in fewer than 1% of Bac-Ac<sub>C</sub>pol: SlPol-transfected cells at 120 h p.t., while they were observed in only about 1.2% of Bac-Ac<sub>C</sub>pol: SlPol-infected cells at 96 h p.i. (Fig. 2d). Both the transfection and infection results revealed that the DNApol substitution affected production of OBs.

Furthermore, we also constructed bacmids with AcMNPV and SpltNPV dna<sub>p</sub> under control of the AcMNPV dna<sub>p</sub> promoter, resulting in WT<sup>rep</sup>-PA<sub>p</sub> and Bac-Ac<sub>C</sub>pol: SlPol-PA<sub>p</sub>. Cells were transfected with 2 µg bacmid DNA of either WT<sup>rep</sup>-PA<sub>p</sub> or Bac-Ac<sub>C</sub>pol:SlPol-PA<sub>p</sub>. Viral growth curves revealed a steady increase in virus production for both bacmids (data not shown). By 120 h p.t., the titres of the WT<sup>rep</sup>-PA<sub>p</sub> and Bac-Ac<sub>C</sub>pol: SlPol-PA<sub>p</sub> were about 8.16 and 5.22 logs, respectively. OBs were detected in 81.5% of WT<sup>rep</sup>-PA<sub>p</sub>-transfected cells, while 0.7% of the cells transfected with the Bac-Ac<sub>C</sub>pol: SlPol-PA<sub>p</sub> bacmid synthesized OBs. A quantitative PCR assay was also performed to monitor the accumulation of intracellular total viral DNA over the first 24 h p.i. Cells were infected at an m.o.i. of 10. The intracellular DNA increased about 3.76 and 1.68 logs at 24 h p.i. for WT<sup>rep</sup>-PA<sub>p</sub> and Bac-Ac<sub>C</sub>pol: SlPol-PA<sub>p</sub>, respectively. The results showed that these bacmids are similar to those of the AcMNPV ie-1 promoter in DNA replication, virus production and OB formation. Together, these data demonstrated that the SpltNPV dna<sub>p</sub> substitution reduced viral DNA replication and OB formation.

**Comparative analysis of alphabaculovirus group I and II DNApol**

Replacement of AcMNPV dna<sub>p</sub> with SpltNPV dna<sub>p</sub> resulted in lower AcMNPV virus production and less viral DNA replication, suggesting that SpltNPV DNApol is functionally weaker than that of AcMNPV. To identify any amino acid sequence differences that might explain this, a comparative analysis was performed between alphabaculovirus group I and II DNApol. All the alphabaculovirus DNApol revealed the presence of both exonuclease and polymerase domains (Huang & Levin, 2001). Further, all the exonuclease domains included three conserved motifs (exo I to III), and the polymerase domains showed a set of seven motifs (IV-II-VI-III-I-VII-V), including AcMNPV DNApol and SpltNPV DNApol (Huang & Levin, 2001) (Fig. S1, available in the online Supplementary Material).

The C terminus of alphabaculovirus group I DNApol plays an important role for virus production and DNA synthesis (Feng & Krell, 2014), and it contains two types of motifs, NLS and conserved region NNTYKFCLYK. NLS is responsible for DNApol nuclear localization; the DNApol of alphabaculovirus group I have at least one bipartite or one more monopartite NLS. Conserved region NNTYKFCLYK is required for DNA replication and virus production. This motif is present in all alphabaculovirus group I DNApol (Feng & Krell, 2014). While DNApol of only seven group II alphabaculoviruses contain only a single monopartite NLS, including SpltNPV DNApol, other DNApol of alphabaculovirus group II do not contain sequences identifiable as known NLSs (Feng & Krell, 2014). The sequence NNTYKFCLYK was not found for any group II alphabaculovirus DNApol.

**Intracellular localization of SpltNPV DNApol C terminus**

As DNApol is essential for viral genome replication, it must be translocated into the nucleus. The nuclear localization is normally mediated by NLSs for proteins more than 50 kDa, like DNApol (Macara, 2001), and NLSs are often distributed in either the N or C terminus of proteins, with basic amino acids being important components of NLSs (Marfori et al., 2012). We scanned the SpltNPV DNApol and found that the C terminus (aa 759–1022) may be important for nuclear translocation of SpltNPV DNApol (Fig. S1). To determine if the SpltNPV DNApol C terminus could localize to nuclei, different truncated SpltNPV DNApol C termini were fused to GFP, comprising Slpol759-1022, Slpol801-1022, Slpol827-1022, Slpol839-1022, Slpol886-1022, Slpol913-1022, Slpol943-1022 and Slpol965-1022 (Fig. 3a–h). These expression plasmids were transfected into Sf9 cells. By 24 h p.t., GFP fluorescence was observed exclusively in the nucleus of Sf9 cells by confocal microscopy for pBlue-egfp: Slpol759-1022, pBlue-egfp: Slpol801-1022, pBlue-egfp: Slpol827-1022, pBlue-egfp: Slpol839-1022, pBlue-egfp: Slpol913-1022 and pBlue-egfp: Slpol943-1022 (Fig. 3a–c, f–g), indicating that these GFP fusion proteins were transported into the nucleus. However, when GFP alone was expressed, GFP fluorescence localized in both the cytoplasm and nucleus as it did for pBlue-egfp: Slpol965-1022 (Fig. 3h, i). Surprisingly, green fluorescence was observed exclusively in the cytoplasm for pBlue-egfp: Slpol839-1022 and pBlue-egfp: Slpol886-1022 (Fig. 3d, e). These results confirmed that GFP nuclear localization was dependent on the SpltNPV DNApol C terminus and independent of virus infection. These results also suggested that aa 801–1022 are sufficient to target GFP exclusively to the nuclei and that the
The C-terminal 222 aa (aa 801–1022) of SpltNPV DNApol is sufficient for nuclear localization. However, the precise location of the NLS was still elusive. 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), showing that SpltNPV DNApol contains a 12 aa stretch at aa 827–838 in the C terminus (Fig. 4a). Based on NLS classification, the sequence QEPPAKRARMPT fits the consensus of classic monopartite class 2 NLS [(P/R)XXKRX(K/R)] (Kosugi et al., 2009).

In keeping with this prediction, when this region was deleted from the C terminus in aa 839–1022 and 886–1022, nuclear localization was not observed (Fig. 3d, e). Nevertheless, the prediction of aa 827–838 as a functional NLS

Identification and functional mapping of SpltNPV DNApol NLS

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In keeping with this prediction, when this region was deleted from the C terminus in aa 839–1022 and 886–1022, nuclear localization was not observed (Fig. 3d, e). Nevertheless, the prediction of aa 827–838 as a functional NLS
sequence in the SpltNPV DNAPol, as reported by Kosugi et al. (2009), does not prove that it is. To further confirm the putative NLS is responsible for the nuclear localization of SpltNPV DNAPol, we constructed a series of GFP-fused peptides encompassing aa 801–838, 801–813, 827–838 and 814–838 (Fig. 4b–e). The resultant plasmids, pBlue-egfp: Slpol801–838, pBlue-egfp: Slpol801–813, pBlue-egfp: Slpol827–838 and pBlue-egfp: Slpol814–838, were transfected into Sf9 cells. pBlue-GFP was used as a GFP control. A diffuse signal was observed in both cytoplasm and nucleus of Sf9 cells for pBlue-GFP and pBlue-egfp: Slpol801–813 (Fig. 4c, f). However, the fluorescence of fusion proteins GFP: Slpol801–838, GFP: Slpol827–838 and GFP: Slpol814–838 showed exclusive nuclear localization (Fig. 4b, d, e). Apart from GFP alone, the GFP-tagged peptide GFP: Slpol827–838 was the smallest peptide tested, which contributed to highly efficient nuclear translocation. These results suggested that the peptide aa 827–838 is a functional NLS.

Since these GFP fusion proteins were small (less than 40 kDa), they might have diffused into the nucleus and then been retained there, rather than gaining access through active nuclear localization. To further verify that aa 827–838 was a domain responsible for nuclear localization of SpltNPV DNAPol, we constructed multiple point substitution plasmids pBlue-HA: SlpolMut827–838, in which all 12 aa were collectively replaced with alanine at positions 827–838.
of an haemagglutinin (HA)-tagged SpltNPV DNApol (Fig. 4h). Expression plasmids pBlue-HA : Slpol\textsuperscript{Mut827-838} and full-length pBlue-HA : Slpol were transfected into Sf9 cells. Green fluorescence was localized exclusively in the nucleus for pBlue-HA : Slpol (Fig. 4g). In contrast, when the HA : Slpol\textsuperscript{Mut827-838} plasmid was transfected into Sf9 cells, GFP fluorescence was found to be enriched exclusively in the cytoplasm, indicating that the substitution mutations of NLS codons abrogated nuclear localization of SpltNPV DNApol. Furthermore, the same expression plasmids were transfected into Sf9 in the presence of WT virus infection; this experiment had identical results with respect to nuclear localization of constructs (data not shown). These results indicated that although the putative NLS independently allowed nuclear localization of GFP, inactivation of the NLS in SpltNPV DNApol led to its nuclear exclusion in transfected Sf9 cells. These results further demonstrated that the motif aa 827–838 is a functional NLS.

**Analysis of point mutations in SpltNPV DNApol NLS motif**

To investigate which amino acids in the NLS motif are critical for nuclear localization, single point mutations were used to identify them. Since prolines or basic amino acids are important for classic class 2 NLS function, we performed a proline and basic amino acid scan within the aa 827–837 NLS motif in a GFP-tagged aa 801–838 DNApol fragment (Fig. 5a). Single amino acid substitutions with alanine within the NLS were generated using pBlue-egfp : Slpol801-838 as template for mutagenesis, respectively, resulting in pBlue-egfp : Slpol801-838\textsuperscript{K809A}, pBlue-egfp : Slpol801-838\textsuperscript{K810A} and pBlue-egfp : Slpol801-838\textsuperscript{K811A}, pBlue-egfp : Slpol801-838\textsuperscript{P829A}, pBlue-egfp : Slpol801-838\textsuperscript{K832A}, pBlue-egfp : Slpol801-838\textsuperscript{R833A} and pBlue-egfp : Slpol801-838\textsuperscript{R835A} (Fig. 5a–c). These transient expression plasmids were transfected into Sf9 cells. By 24 h p.t., GFP fluorescence for all transfected plasmids was localized exclusively in the nucleus (Fig. 5a–c), demonstrating that none of these single amino acid substitutions abrogated nuclear localization of GFP. These data confirmed that although the NLS motif 827QEPPKARMMPT838 matches the classic monopartite class 2 NLS, neither P829 nor P830 on its own is important for GFP nuclear localization. Neither K832, R833 nor R835 individually is critical for GFP nuclear localization.

We also generated multiple point mutations of prolines or basic amino acids within the 801–838 fragment and examined their effects on nuclear transport of the short peptide. For proline, arginine or lysine scanning mutagenesis of the NLS motif and upstream regions, we replaced multiple amino acids in the motif with alanines. DNA sequencing confirmed that these corresponding nucleotide sequences were substituted. The expression plasmids containing multiple point mutations – pBlue-egfp : Slpol801-838\textsuperscript{Mut809-811} (K809A, K810A and K811A), pBlue-egfp : Slpol801-838\textsuperscript{Mut829-830} (P829A and P830A) and pBlue-egfp : Slpol801-838\textsuperscript{Mut832-835} (K832A, R833A and R835A) – were transfected into Sf9 cells. Analysis of nuclear localization for these plasmids showed that GFP was exclusively localized in the nucleus for pBlue-egfp : Slpol801-838\textsuperscript{Mut809-811} and pBlue-egfp : Slpol801-838\textsuperscript{Mut829-830} (Fig. 5i, g), indicating that mutations to these amino acid had no significant effect on nuclear localization of the aa 801–838 peptide. In contrast, GFP fluorescence was localized in both nucleus and cytoplasm for pBlue-egfp : Slpol801-838\textsuperscript{Mut832-835} (Fig. 5h), suggesting that the collective substitution of these particular amino acids significantly impaired the nuclear localization.

To further demonstrate whether the basic amino acids are important for nuclear import, plasmids containing similar substitutions in full-length SpltNPV DNApol fused to HA were used. pBlue-HA : Slpol\textsuperscript{Mut809-811} (K809A, K810A and K811A), pBlue-HA : Slpol\textsuperscript{Mut829-830} (P829A and P830A) and pBlue-HA : Slpol\textsuperscript{Mut832-835} (K832A, R833A and R835A) (Fig. 5i–k) were constructed with multiple mutations in SpltNPV DNApol. These plasmids were transfected into Sf9 cells. By 24 h p.t., green fluorescence was visualized in nuclei for pBlue-HA : Slpol\textsuperscript{Mut809-811} and pBlue-HA : Slpol\textsuperscript{Mut829-830} (Fig. 5i, j). In contrast, GFP fluorescence was observed exclusively in the cytoplasm for pBlue-HA : Slpol\textsuperscript{Mut832-835} (Fig. 5k). These results suggested that the amino acids at positions 832–833 and 835 were critical for nuclear localization while, as shown for the corresponding peptide (Fig. 5a, b), P829 or P830 was not similarly K809, K810 or R811 was also not critical for nuclear localization of HA-tagged DNApol.

**Substitution of SpltNPV DNApol NLS abrogated virus production and viral DNA replication**

To determine if the SpltNPV DNApol NLS was critical for AcMNPV virus production and viral DNA replication, all 12 aa of this region were collectively substituted by alanines. The mutated SpltNPV DNApol was inserted into Ac\textsuperscript{KCO}, creating bacmids Bac-AcApol : Slpol\textsuperscript{Mut827-838} (Fig. 6a). The SpltNPV DNApol was expressed under the control of the AcMNPV ie-1 promoter and SV40 poly(A) signal, and GFP was expressed independently from the Opie1 promoter and used as a reporter. Ac\textsuperscript{KCO}GFP was used as a negative control and WT\textsuperscript{rep} was a positive control. The bacmids Bac-AcApol : Slpol\textsuperscript{Mut827-838}, Ac\textsuperscript{KCO}GFP and WT\textsuperscript{rep} were transfected into Sf9 cells. Reporter GFP-fluorescent cells were monitored for virus infection and spread. At 24 h p.t., only individual fluorescent cells were found, and there was no obvious difference among these transfections, indicating equivalent transfection efficiencies (Fig. 6b). By 72 h p.t., GFP fluorescence had spread from the initially transfected cells to adjacent cells for the repair bacmid WT\textsuperscript{rep}, whereas there was no spread of GFP fluorescence for either the Bac-AcApol : Slpol\textsuperscript{Mut827-838} or Ac\textsuperscript{KCO}GFP bacmids. By 120 h p.t., fluorescence was observed in almost all cells for the repair bacmid WT\textsuperscript{rep}. However, there was no increase in the number of fluorescent cells for Ac\textsuperscript{KCO}GFP and Bac-AcApol : Slpol\textsuperscript{Mut827-838} throughout the transfection (Fig. 6b). By 120 h p.t., light microscopy showed the presence of OBs for WT\textsuperscript{rep}-transfected cells but not for Ac\textsuperscript{KCO}GFP and Bac-
AcΔpol: Slpol\textsuperscript{Mut827-838} (Fig. 6b). These results revealed that the collective substitution of all aa 827–838 within the NLS of SpltNPV DNApol abrogated virus infection.

Growth curves were determined by a TCID\textsubscript{50} endpoint dilution assay for the three bacmids. Cells were transfected with 2 µg bacmid DNA of either Ac\textsuperscript{KO}GFP, Bac-Ac\textsuperscript{D}pol : GFP or Bac-Ac\textsuperscript{ΔNLS}P829A P830A R835A K832A R833A K832A K833A P829A K830A K809A K810A K811A R835A R833A (Fig. 6b). These results revealed that the collective substitution of all aa 827–838 within the NLS of SpltNPV DNApol abrogated virus infection.
Slpo\textsuperscript{Mut827–838} or \textsuperscript{WT} and monitored for up to 120 h p.t. The supernatants were collected at the indicated times. The results showed a steady increase in the level of virus production for \textsuperscript{WT} for up to 120 h p.t. (Fig. 6c). However, no extracellular virus was detectable at any time for either Ac\textsuperscript{KO}GFP or Bac-Ac\textsuperscript{D}pol : Slpo\textsuperscript{827–838} (Fig. 6c). These data indicated that the NLS identified at aa 827–838 in SpltNPV DNApol is critical for virus production.

**DISCUSSION**

Baculoviruses replicate their genomes in the nuclei of infected cells and viral DNApol plays a crucial role in viral DNA synthesis (Kool et al., 1994). The AcMNPV DNApol could be functionally substituted by that of *Choristoneura fumiferana* multiple nucleopolyhedrovirus (Feng & Krell, 2014), indicating that a group I NPV DNApol can be a functional analogue of that of another group I virus, AcMNPV. In the present study, we found that viral propagation could be rescued by substitution of the native AcMNPV dnapol with that of a group II SpltNPV dnapol gene in the group I dnapol-null AcMNPV bacmid (Bac-Ac\textsuperscript{D}pol : Slpo\textsuperscript{827–838} and \textsuperscript{WT} bacmids at 24, 72 and 120 h p.t. Ac\textsuperscript{KO}GFP was a negative control and \textsuperscript{WT} was used as a positive control. (c) Cells were transfected with 2 \( \mu \)g bacmid DNAs and infectious BV titres were determined by TCID\textsubscript{50} endpoint dilution assays.

**Fig. 6.** Bacmid construct, GFP fluorescence and virus production analysis of AcMNPV bacmids with alanine-substituted residues 827–838 in SpltNPV DNApol (all 12 aa residues were collectively substituted by alanine). (a) Schematic of cassettes with 12-alanine-substituted SpltNPV DNApol at aa 827–838, egfp as a reporter under the *Opie1* promoter, dnapol expressed by the AcMNPV ie-1 promoter and native poly(A), 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\textsuperscript{KO} bacmid) by Tn\textsuperscript{7L}-mediated transposition. (b) Fluorescence and light microscopy images of monolayers of cells transfected with Ac\textsuperscript{KO}GFP, Bac-Ac\textsuperscript{D}pol : Slpo\textsuperscript{Mut827–838} and \textsuperscript{WT} bacmids at 24, 72 and 120 h p.t. (c) Cells were transfected with 2 \( \mu \)g bacmid DNAs and infectious BV titres were determined by TCID\textsubscript{50} endpoint dilution assays.
contains at least one NLS and another highly conserved region, both important for viral production and DNA replication (Feng & Krell, 2014). AcMNPV and SplTNPV are both alaphaculoviruses, with 45% identity between the two DNApolps. Though the SplTNPV DNApol C terminus revealed the presence of an NLS, sequence alignment analysis showed that the DNApol C terminus of SplTNPV lacked the motif NNTYKFCLYK, which is highly conserved in the group I DNApolps (Fig. 3) (Feng & Krell, 2014). It is this difference that might have affected baculovirus replication and led to a low efficiency for viral DNA replication.

SplTNPV DNApol might also affect OB formation. Previous proteomics analysis showed that DNApol is one of the protein components of the baculovirus ODV (Braunagel et al., 2003; Deng et al., 2007), demonstrating that DNApol could have a direct effect on ODV formation and production. Evidence from point mutations of AcMNPV DNApol confirmed the association between DNApol and OB production (Feng et al., 2012). In this work, the SplTNPV DNApol replacement of the AcMNPV DNApol led to less OB production than with WT rep (Fig. 2d). SplTNPV DNApol differs from AcMNPV DNApol (Fig. S1), and it might not efficiently match the polymerase machinery of AcMNPV DNApol-mediated viral DNA replication, which results in both less viral DNA and reduced late gene expression, consequently resulting in fewer OBs. Further analysis will be necessary to confirm this hypothesis.

Baculovirus protein NLSs are important for nuclear transport (Au et al., 2009; Chen & Carstens, 2005; Guo et al., 2010; Jarvis et al., 1991; Olson et al., 2002; Wang et al., 2008). In this report, we observed that the amino acid sequence at positions 827–838 mediated transport of GFP to the nucleus (Fig. 4d) and that multiple point substitutions of SplTNPV DNApol within this NLS region abrogated nuclear translocation (Fig. 5h, k), demonstrating that this sequence, 827QEPPAKRARMPT838, is a functional NLS. Moreover, these substitutions in the NLS region resulted in failure of viral spread and BV production (Fig. 6), indicating that this NLS is important to the overall virus replication cycle. Together, these results demonstrated that the aa 827–838 NLS is essential for translocating SplTNPV DNApol to the nucleus, allowing a normal virus replication cycle.

Our bioinformatic analysis showed that SplTNPV DNApol has a 12 aa stretch in the C terminus, and the sequence 827QEPPAKRARMPT838 matches the consensus of the monopartite NLS class 2 (Fig. 4a). Both single point mutations for either P829A or P830A (Fig. 5a, b) and multiple point substitutions (Fig. 5g, j) at these two positions failed to abrogate nuclear localization, indicating that neither proline is important for nuclear translocation, while the two prolines are critical for nuclear localization in classic class 2 NLSs (Kosugi et al., 2009). Thus, the core sequence QEPPAKRAMPPT is different from a classic class 2 NLS and could represent a new type of NLS.

Six classes of NLSs specifically bind a groove in importin α and, along with importin β, form a ternary complex in the cytoplasm (Kosugi et al., 2009). cNLSs are composed of either a single cluster of basic amino acids (monopartite NLS) or two clusters of basic amino acids separated by a 10–12 aa linker (bipartite NLS). Importin α has a highly structured domain composed of ten tandem armadillo (ARM) repeats. The long groove, consisting of ARM repeats 2–4 and 7–9, can accommodate monopartite cNLS peptides or a single bipartite cNLS peptide (Conti et al., 1998; Fontes et al., 2000; Goldfarb et al., 2004; Herold et al., 1998). In this work, we identified an essential basic core sequence KRAR within the NLS, and this sequence completely fits the KRX(K/R) motif in the basic amino acid region of the cNLS class 2 (Fig. 4a). This suggested that the NLS in SplTNPV DNApol could dock to importin α and mediate nuclear targeting in insect cells. Further analysis of NLS interactions with importin α is required to understand the mechanism of baculovirus protein nuclear translocation.

It is noteworthy that fusion peptides 839–1022 and 886–1022 were excluded from the nucleus (Fig. 3d, e) while short C-terminal peptides 943–1022 and 913–1022 appeared have nuclear localization (Fig. 3f, g). This suggested that SplTNPV DNApol may shuttle between nucleus and cytoplasm. Nucleocytoplasm transport of viral proteins was found in some other viruses (Chen et al., 2001; Kalland et al., 1994; Mears & Rice, 1998; Phelan & Clements, 1997); these viral proteins were confirmed to contribute to viral mRNA export (Juillard et al., 2009; Ruvolo et al., 2001; Sandri-Goldin, 1998; Taniguchi et al., 2014). It is not known if SplTNPV DNApol plays a role in nucleocytoplasm transport; further analysis of SplTNPV DNApol is important to our understanding of its functional role.

In summary, we successfully generated a SplTNPV dnaPol-substituted AcMNPV and analysed its biological properties. We demonstrated that the DNA replication ability of SplTNPV DNApol was lower than that of AcMNPV DNApol in Sf9 cells and that substituting of the DNApol led to a reduction of BV and OB production. We also identified an NLS in the C terminus of SplTNPV DNApol with a core 827QEPPAKRARMPT838, and demonstrated that the NLS is required for SplTNPV DNApol nuclear translocation and viral DNA replication.

**METHODS**

**Cells and viruses.** Spodoptera frugiperda clonal isolate 9 (S9) (Vaughn et al., 1977) cells were cultured in Grace's insect medium supplemented with 10% FBS (Invitrogen), penicillin (25 units ml⁻¹) and streptomycin (25 µg ml⁻¹). The S9 cells were maintained at 27°C. The WT Spodoptera litura nucleopolyhedrovirus (SplNPV) genome was used for PCR amplification of SplTNPV dnaPol (Pang et al., 2001).

**Replacement of AcMNPV DNApol with SplTNPV DNApol.** To determine whether SplTNPV dnaPol could rescue an AcMNPV dnaPol-null mutant, the plasmid pGEM-Spol was constructed. Previous results showed that the promoters of AcMNPV dnaPol and ie-1 had no obvious difference in expression of dnaPol (Feng & Krell, 2014). The AcMNPV ie-1 promoter was used to express dnaPol for transfection and bacmid construction in this study. Firstly the AcMNPV ie-1 promoter and
SpltNPV dnapol with its native poly(A) signal were PCR amplified using primers 1/2 and 3/4 (see Table S1), respectively. Secondly the PCR products of the ie-1 promoter and SpltNPV dnapol with native poly(A) signal were fused by a PCR-based DNA-assembly procedure, named double-joint (DJ) PCR, using primers 1 and 4 (Yu et al., 2004). The resulting PCR product was inserted into pGEM-T to generate pGEM-Spol. The construct was digested with SacI/PstI and subcloned into pFACT-GFP, creating pFACT-GFP-Spol, which was used to generate the corresponding bacmid.

Construction of independent DNApol C-terminal plasmids. To follow the SpltNPV DNApol C-terminal localization, the plasmid pBlue-gfp-Spol759-1022 was constructed. The SacI/NotI ie-1 promoter PCR fragment from AcMNPV ie-1 was PCR amplified using primers 1 and 5, and the NotI/Xhol fragment for the EFGP ORF (lacking the stop codon) from a chitinase-GFP fusion (Hodgson et al., 2011) was also amplified, using primers 6 and 7. The resulting two PCR fragments were inserted into pBlueScript II SK(+) using primers 31 and 39. The fragment containing the SpltNPV DNApol C-terminus (aa 759–1022) fused with its native poly(A) signal was PCR amplified using primers 4 and 8, and the PCR fragment was cloned to the Xhol/PstI sites of pBlue-iec-GFP, creating pBlue-gfp-Spol759-1022.

To determine whether different SpltNPV DNApol C-terminal fragments could be functional in Sf9 cells, we constructed a series of pBlue-GFP-based plasmids with different lengths of the C-terminal fragments (Fig. 3a–h). The plasmids were generated using the deletion strategy based on site-directed, ligase-independent mutagenesis (Chiu et al., 2004); pBlue-gfp-Spol759-1022 was used as a template for mutagenesis. Each reaction contained four primers, and primers 9 and 10 were used in all reactions. Another two special primers are listed below for the corresponding plasmid. The upstream deletions in SpltNPV DNApol C terminus were generated using primers 11 and 12 for deletion of aa 801–838, primers 13 and 14 for deletion of aa 759–826, primers 15 and 16 for deletion of aa 759–838, primers 17 and 18 for deletion of aa 759–885, primers 19 and 20 for deletion of aa 759–912, primers 21 and 22 for deletion of aa 759–942, and primers 23 and 24 for deletion of aa 759–964, resulting in pBlue-gfp-Spol801-1022, pBlue-gefp-Spol827-1022, pBlue-gefp-Spol839-1022, pBlue-gefp-Spol866-1022, pBlue-gefp-Spol913-1022, pBlue-gefp-Spol943-1022 and pBlue-gfp-Spol965-1022, respectively.

Construction of fusion peptides. A GFP expression cassette plasmid, pBlue-iec-gfp-SV40, was constructed by inserting the SacI/Xhol AcMNPV ie-1 promoter PCR fragment (using primers 1 and 25), the GFP ORF with its native poly(A) signal were PCR amplified using primers 28 and 29) into pBluescript II SK(+). The resulting construct was digested with SacI/PstI and subcloned into pFAcT-GFP, generating pFAcT-GFP-Spol. This pFACT plasmid was used to generate the corresponding bacmid.

Bacmid constructions. An AcMNPV dnapol knockout bacmid (AcKO), a dnapol-null bacmid with the egfp reporter (AcEGFP) and WT dnapol repair bacmid (WTdnapol) had already been constructed by Feng et al. (2012). Other bacmids were generated from various pFACT constructs as described in the Bac-to-Bac expression manual (Invitrogen). The TN7 cassettes from pFACT-GFP transfer vectors with a GFP reporter and different SpltNPV DNApol, pFACT-GFP-Spol and pFACT-gfp-Spol814-838 were transposed to AcKO bacmid, creating Bac-AcSpol: Spol and Bac-AcΔpol: Spol801-838, respectively.

Quantitative real-time PCR. Total intracellular DNA was extracted using the QiAamp DNA blood mini kit (Qiagen). After determining the DNA concentration using a NanoDrop spectrophotometer (NanoDrop Technologies), quantitative real-time PCR was performed as described previously (Feng et al., 2012) with the CFX96 Real-Time System (Bio-Rad) using SYBR Green 1 dye chemistry (Roche) and primer sequences F-Acie1R (GAATTGATGCTGGTGGTCGTC) and F-Acie1F (AGTATTGTCGTCTATTATTCG).

Transfection. Bacmid DNA was purified from 300 ml Luria-Bertani (LB) cultures using a Qiagen midi-plasmid kit, and concentrations were determined spectrophotometrically by using a NanoDrop ND-3300. Sf9 cells were plated at a density of 1 × 10⁶ cells per well in six-well plates. Cells were transfected with 2 μg 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 (Feng et al., 2012).

Viral growth curves. To assess the ability of SpltNPV dnapol replacement to support virus production, viral growth curves were generated for AcMNPV dnapol-null virus mutants repaired with SpltNPV dnapol, Sf9 cells (1 × 10⁶) were transfected in triplicate with 2.0 μg of the appropriate bacmid DNA using Cellfectin. Cell monolayers were incubated for 5 h p.t., rinsed twice with Grace’s medium, and replenished with 2 ml fresh Grace’s medium supplemented with 10% FBS. Virus supernatants were collected from extracellular medium at the indicated time points. At different times post transfection, viral yields were determined by a TCID₅₀ endpoint dilution assay on Sf9 cells and virus titres were calculated by the Reed–Muench method (O’Reilly et al., 1992). Signs of infection were determined by GFP fluorescence. For all transfections, time zero (0 h p.t.) 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. S9 cells (5 x 10^4) were seeded onto 35 mm dishes to attach for 5 h. Cells were transfected with 1 µg plasmid DNA by using Cellfectin (Invitrogen). Monolayers were washed three times in PBS and overlaid with 2 ml PBS before imaging. The staining and imaging were carried out as described previously (de Jong et al., 2011; Feng & Krell, 2014).

For GFP and GFP fusion peptides, at 24 h p.t. images were acquired with a Zeiss LSM 700 confocal microscope using ZEN 2011 software (Zeiss). For nuclear staining, DAPI at 20 µg ml^-1 was added to the cells for 15 min and then the dye solution was replaced by PBS. Cells were imaged on a Zeiss LSM 700 confocal microscope with ZEN 2011 software (Zeiss) for fluorescent imaging.

For HA: Slpol, at 24 h p.t. the transfected cells were washed once in PBS, and incubated with 4 % paraformaldehyde in PBS for 15 min. The fixed cells were washed three times in 2 ml PBS followed by permeabilization in PBS supplemented with 0.3 % Triton X-100 for 15 min. Cells were then blocked for 1 h in blocking buffer (PBS with 2 % BSA), and incubated at 4 °C overnight with mouse monoclonal anti-HA antibody (1:1000) (Abmart). Monolayers were washed three times with blocking buffer for 10 min each time, and incubated with an Alexa 488-conjugated goat anti-mouse antibody (1:400) (Molecular Probes) for 1 h. Cells images were viewed on a Zeiss LSM 700 confocal microscope with ZEN 2011 software (Zeiss); nuclear staining was carried out as described above.

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