Expression, purification and characterization of the Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) DNA polymerase and interaction with the SpliNPV non-hr origin of DNA replication

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The DNA polymerase from Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) was expressed in, and purified from, prokaryotic and eukaryotic expression systems. While less protein was obtained from the E. coli expression system, SpliNPV DNAPOL purified from E. coli displayed similar biochemical characteristics to DNAPOL expressed in, and subsequently purified from, insect cells (Sf9) using a baculovirus expression system. Biochemical analyses suggested that the DNA polymerase and the 3′–5′ exonuclease activities are intrinsic to the protein. Deletion of the first 80 amino acid residues from the N terminus of the DNAPOL affected neither the DNA polymerase nor the exonuclease activities of the enzyme. Replication products from single-stranded M13 DNA demonstrated that the DNA synthesis activity of SpliNPV DNAPOL is highly processive. Transient expression assays with a set of deletion clones containing the putative SpliNPV non-hr origin of DNA replication permitted functional characterization of sequence elements within the origin fragment. Purified SpliNPV DNAPOL stimulated origin-dependent DNA replication in a cell-free replication assay.

Introduction

Baculoviruses are large double-stranded DNA viruses that only infect invertebrates. The family Baculoviridae consists of two genera, Nucleopolyhedrovirus (NPV) and Granulovirus (GV) (Volkman et al., 1995). NPV genomes contain two distinct sets of sequences, homologous regions (hr) and non-homologous regions (non-hr), which are believed to act as the origins of viral DNA replication (Kool et al., 1995; Lu et al., 1997). In addition, nine virus-encoded proteins (dnapol, p143, lef-1, lef-2, lef-3, ie-1, ie-2, p35 and pe-38) have been shown to be involved in the transient replication of plasmids containing viral origin sequences (Kool et al., 1995; Lu et al., 1997). All NPV genomes sequenced to date encode a single DNA polymerase gene. Nucleopolyhedroviruses (NPVs) and Granuloviruses (GVs) share a number of conserved amino acid residues in their DNA polymerases. The NPV DNAPOLs contain conserved motifs similar to those found in mammalian DNA polymerases (Huang & Levin, 2001). One of our interests is directed toward structure–function aspects that are unique to SpliNPV DNAPOL.

Baculoviruses

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DNAPOL interact specifically with cis-acting sequence elements within the SpliNPV non-hr origin of DNA replication? In this report, we have expressed the SpliNPV dnapol gene product using both prokaryotic and eukaryotic expression systems. We have characterized the functional activities of the full-length DNAPOL and a mutant DNAPOL, in which the first 80 amino acid residues were deleted. Using the SpliNPV non-hr origin of replication and a set of serial deletion clones as templates, we also demonstrated that SpliNPV DNAPOL can stimulate origin-dependent DNA replication in a cell-free system.

Methods

**Cells and virus.** S. frugiperda (Sf9) cells were obtained commercially (PharMingen). SpliNPV isolate M-2 was obtained from G. Crozier, Station de Recherches de Pathologie Comparée, INRA-CNRS, F-30380 Saint-Christol-Les-Ales, France. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (O’Reilly et al., 1992).

**PCR and cloning of the SpliNPV dnapol gene.** Based on DNA sequence analysis of SpliNPV dnapol (Huang & Levin, 2001), we designed two primers that amplify the full-length SpliNPV dnapol (Table 1). In order to amplify a deletion mutation without the first 80 amino acids of the full-length DNAPOL, we employed an additional 39-mer forward primer (Table 1). These primers were designed to create an insert with a BamHI and a NotI site on the amino and carboxyl termini of the DNA polymerase gene, respectively. All amplifications were carried out using Tfu DNA polymerase following the manufacturer’s instructions (Stratagene). The amplification products (3 kb for full-length and 2.8 kb for the Δ80 mutant) were gel purified, digested with BamHI and NotI, and ligated into vectors pGEX-5X-1 (Pharmacia) or pFastBacHTc (baculovirus expression system; Life Technologies) following the manufacturer’s instructions.

**Expression and purification of SpliNPV DNAPOL in E. coli.** PCR products containing the full-length SpliNPV dnapol and the Δ80 mutant genes were introduced into pGEX-5X-1 to create fusion proteins in which the amino termini of the DNAPOL polypeptides were linked, in-frame, with the carboxyl termini of glutathione S-transferase (GST). The expression construct was transformed into E. coli BL21 cells. The transformed BL21 cells were grown to an OD600 of 0.6 in LB media containing 50 μg/ml ampicillin. Protein production was then induced by addition of IPTG to a final concentration of 0.4 mM. After 1 h of induction at 37 °C, the cells were harvested by centrifugation for 5 min at 1000 g in a Sorvall GSA rotor at 4 °C. Cell pellets were then frozen in liquid nitrogen.

All procedures were carried out at 4 °C unless otherwise specified. The bacterial cell pellets were thawed and resuspended in 10 vols (mass to volume) of lysis buffer (20 mM Tris–HCl pH 8.0, 250 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 60 μg/ml lysozyme, 0.2 mM PMSF and 0.7 μg/ml leupeptin). The lysate was sonicated on ice until it lost viscosity and then centrifuged for 1 h at 4000 g in a Beckman SS34 rotor. The pellet and supernatant were analysed via 8% SDS–PAGE. Fusion proteins were purified by affinity chromatography using glutathione–Sepharose 4B contained in GST Purification Modules (Pharmacia). Cleavage of the desired proteins from GST was achieved using a site-specific protease, Factor Xa. Fusion proteins were detected using an immunoassay provided in the GST Detection Module, following the manufacturer’s protocol.

**Expression and purification of SpliNPV DNAPOL in Sf9 cells.** The full-length SpliNPV dnapol and the Δ80 mutant gene products were hyper-expressed in Sf9 cells from a recombinant AcMNPV using the Bac-to-Bac Expression System (Life Technologies). Two recombinant plasmids, pFastBacHT-DNAPOL and pFastBacHT-Δ80DNAPOL, were constructed by subcloning the full-length dnapol and the Δ80 mutant PCR products into the BamHI and NotI sites of pFastBacHTc. Recombinant bacmids and AcMNPV were subsequently prepared according to the manufacturer’s instructions. Sf9 cells were infected at an m.o.i. of 10 and cells were harvested at various time points between 24 and 72 h post-infection (p.i.). The recombinant proteins, designated HisDNAPOL and His-del80DNAPOL, respectively, were purified on Ni–NTA agarose from Sf9 cells infected with recombinant baculovirus. Expression and purification of the fusion proteins were monitored by SDS–PAGE analysis (Laemmli, 1970). The purified polymerases were stored at −80 °C in buffer containing 65% glycerol.

**SDS–PAGE and Western blot analysis.** Protein concentrations were determined by the Bradford method (Bradford, 1976) with BSA as a standard. For Western blotting, proteins were electrophoresed by SDS–PAGE and transferred to Immobilon-NC membranes (Millipore). The membranes were blocked with 5% nonfat milk and probed with a 1:5000 dilution of primary rabbit anti-His polyclonal antibodies. Membranes were then incubated with a 1:2000 dilution of goat anti-rabbit IgG–horseradish peroxidase conjugates, and developed using Enhanced Chemiluminescence (Amersham) substrates.

**DNA templates.** Singly primed single-stranded M13mp18 was made by annealing 25 pmol of M13 universal forward primer (5′ TGTTAACGACGCCAGT 3′) to 2.5 pmol of single-stranded M13

Table 1. Primers used for PCR amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Length</th>
<th>Sequence*</th>
</tr>
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<tbody>
<tr>
<td>DNAPOL-F</td>
<td>26</td>
<td>5′ GACTGGATCCCATATGAAGGTGAAG 3′</td>
</tr>
<tr>
<td>DNAPOL-R</td>
<td>36</td>
<td>5′ GACCGCGCCGCCCGCCGCAGTTTGAAGG 3′</td>
</tr>
<tr>
<td>DNAPOL-Δ80</td>
<td>39</td>
<td>5′ GACCGGATCCAGAAACAGATCGTTGCGCCCAATTGTGCAG 3′</td>
</tr>
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</table>

* Primers were designed to create a BamHI or NotI restriction endonuclease site (underlined) to facilitate cloning.
DNA. The mixtures, containing 50 mM Tris–HCl (pH 7.5), 5 mM MgCl₂ and 100 mM NaCl in 50 µl, were heated to 100 °C and slowly cooled to room temperature. To prepare template for exonuclease assays, reaction mixtures (50 µl) containing 50 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 5 mM MgCl₂, 0.4 mM each of dCTP, dGTP and dTTP, 20 µCi [³²P]dATP (3000 Ci/mmol), 500 µg/ml activated calf thymus DNA and 10 units of Klenow enzyme were incubated at 37 °C for 30 min, quenched by addition of 2.5 µl 0.5 M EDTA, and chilled on ice. DNA substrate was extracted twice with phenol–chloroform–isoamyl alcohol and purified by gel filtration using TE buffer.

DNA polymerase assay. Reaction mixtures contained 50 mM Tris–HCl, pH 8.0, 50 µg BSA, 0.5 mM dithiothreitol, 15 mM MgCl₂, 200 mM KCl, 0.1 mM each of dCTP, dGTP and dTTP, 0.0125 mM dATP, 0.5 µCi [³²P]dATP (3000 Ci/mmol), 25 µg activated calf thymus DNA (Sigma) and purified DNA polymerase in a reaction volume of 100 µl. After incubation at 37 °C for 30 min, the reactions were stopped by addition of 100 µl 25 mM EDTA, 25 mM sodium pyrophosphate and 50 µg/ml of salmon sperm DNA followed by 1 ml 10% trichloroacetic acid (TCA). After 10 min on ice, the mixture was filtered through GF/C glass filters. The filters were washed twice with 2 ml 1 M HCl, 0.05 M sodium pyrophosphate, rinsed with ethanol, dried and counted in a liquid scintillation counter. One unit of DNA polymerase activity was defined as the amount of enzyme required to incorporate 1 nmol [³²P]dNTP into acid-insoluble material/min at 37 °C.

DNA polymerase activity was also determined in a liquid scintillation counter. One unit of DNA polymerase activity was defined as the amount of enzyme required to incorporate 1 nmol [³²P]dATP into acid-soluble material in 30 min at 37 °C.

DNA polymerase activity was also evaluated in the presence of various concentrations of MgCl₂, KCl and (NH₄)₂SO₄. The effect of aphidicolin was examined by addition of various concentrations of this DNA polymerase inhibitor (Pedrali-Noy & Spadari, 1980; Sheaff et al., 1991). When inhibition of DNA polymerase activity by aphidicolin was measured, the concentration of dCTP was lowered to 10 µM. Thermostability of the DNA polymerase activity was examined by incubation of the SpliNPV DNApol at 50 °C for various lengths of time prior to its addition to the reaction mixture. All reactions were repeated in triplicate. Statistical analyses (ANOVA) were calculated using Microsoft Excel 97.

For DNA synthesis on singly primed, single-stranded circular M13 template, reaction mixtures (50 µl) contained 20 fmol substrate DNA, 20 mM Tris–acetate (pH 7.3), 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, 0.5 mM ATP, 60 µM each of dGTP, dATP and dTTP, 20 µM [³²P]dCTP (3000 Ci/mmol), 50 µg/ml BSA and 200 fmol purified DNA polymerase. The reaction was incubated at 37 °C and terminated by addition of an equal volume of stop buffer (1% SDS, 40 mM EDTA, 60 µg/ml sonicated calf thymus DNA). The reaction products were precipitated with ethanol, resuspended in 20 µl sample buffer (0.1 M NaOH, 5% glycerol, 1 mM EDTA, 0.025% bromocresol green) and separated on a 1% alkaline agarose gel as described (Sambrook et al., 1989). Dried gels were exposed and visualized by autoradiography.

3′–5′ exonuclease assay. Exonuclease activity was determined in the absence of dNTPs under the conditions of the DNA polymerase assay. Reaction mixtures (100 µl) were incubated for 30 min at 37 °C with 25 µg activated calf thymus DNA containing 0.15 µg (6.7 × 10⁶ c.p.m./µg) of [³²P]labelled exonuclease substrate. Reactions were terminated by chilling on ice and by adding 20 µl 0.25 M EDTA, pH 8.0, 5 mg/ml BSA, and 20 µl 100% TCA. After centrifugation (13,000 g, 30 min, 4 °C) the radioactivity of supernatant fractions (100 µl) was determined in a liquid scintillation counter. One unit of 3′–5′ exonuclease activity was defined as the amount of enzyme required to release 1 pmol [³²P]dCTP into acid-soluble material in 30 min at 37 °C.

The 3′–5′ exonuclease activity was also evaluated in the presence of various concentrations of MgCl₂, KCl, (NH₄)₂SO₄ and aphidicolin. The thermostability of the exonuclease activity was examined as outlined above. All reactions were carried out in triplicate. Statistical analyses were carried out as above.

Plasmid construction and transient replication assays. Identification and DNA sequence analysis of the SpliNPV non-λ origin of DNA replication were reported previously (Huang & Levin, 1999). To identify the minimal origin sequence, we constructed a set of unidirectional nested deletions of the origin sequence using the Exo Mung Bean Deletion Kit (Clontech). Transient replication assays were performed as described (Huang & Levin, 1999). Briefly, plasmid DNAs were transfected into 5 × 10⁶ Sf9 cells by calcium phosphate precipitation. After 24 h at 27 °C, cells were infected with SpliNPV. Cells were harvested and DNA was extracted following the protocol of Sarisky & Hayward (1996). To test for replication in cells, DNA was digested with HindIII to linearize the plasmid, and with DpnI, which cleaves in the sequence 5′-CAGT-3′ but only if the A is methylated. Plasmids that replicate in Sf9 cells due to the presence of a viral origin of DNA replication no longer possess the E. coli pattern of methylation at the DpnI site and are therefore resistant to DpnI cleavage.

After electrophoresis on 0.7% agarose gels, the DNA was transferred to nylon membranes (Hybond-N, Amersham) and hybridized with [³²P]CTP-labelled pBluescript II DNA. Replicated plasmid DNAs are detected on Southern blots as HindIII linearized molecules. Plasmid DNAs that do not replicate retain the E. coli pattern of DpnI methylation and are detected on Southern blots as a ladder of fragments. All transient replication assays were repeated at least three times.

Preparation of cell extracts and cell-free replication. Nuclear extracts from SpliNPV-infected Sf9 cells were prepared as reported previously (Huang & Levin, 1999). Total protein concentrations were determined by the Bradford method. The extracts were frozen in liquid nitrogen and stored at −80 °C. The cell-free replication assay was adapted from methods described by Stillman & Gluzman (1985). Briefly, a 25 µl reaction mixture contained 40 ng DNA templates and 100 µg of nuclear protein extract prepared from SpliNPV-infected Sf9 cells in 20 mM HEPES buffer, pH 7.5, 200 µM each UTP, GTP and CTP, 4 mM ATP, 100 µM each dATP, dGTP and dTTP, 25 mM dCTP, 40 mM phosphocreatine and 100 µg/ml creatine phosphokinase (Sigma). Purified SpliNPV DNApol (250 ng) was added to one set of reactions, while a second set was conducted in the absence of purified SpliNPV DNApol. The reaction mixtures were preincubated for 1 h at 37 °C, and then 2.5 µCi of [³²P]dCTP (3000 Ci/mmol) was added to the reactions. Incubation was continued for another hour at 37 °C. Reactions were terminated by adding 200 µl 20 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.1% SDS and 20 µg/ml RNase A, followed by incubation at 37 °C for 15 min. Protease K was added to 200 µg/ml and incubation was continued for another 30 min at 37 °C. The reaction mixtures were extracted with phenol and chloroform–isoamyl alcohol (24:1), precipitated with 2.5 M ammonium acetate, 95% ethanol, and analysed by 0.8% agarose gel electrophoresis. Gels were dried, exposed, and quantified using a PhosphorImager (Molecular Dynamics).

Results

Overexpression and purification of SpliNPV DNAPOL and the Δ80 mutant proteins

The full-length SpliNPV dnapol ORF encodes a 998 amino acid polypeptide (DNAPOL) with a predicted molecular mass of 116 kDa. The deletion mutant dnapol ORF encodes a 918 amino acid polypeptide (DNAPOL Δ80).
products containing the dnapol and Δ80 mutant genes were cloned into pFastBacHTc, which incorporates an amino-terminal extension of 20 amino acids containing a six-histidine affinity tag. Approximately 3.5 mg of recombinant proteins were purified, in a one-step Ni–resin affinity chromatography, from a 500 ml Sf9 culture. The purification systems yielded single Coomassie blue-stained species of 110 and 96 kDa on SDS–PAGE for proteins expressed both in E. coli (Fig. 1A) and in Sf9 cells (Fig. 1B), respectively. While less protein was obtained from the E. coli expression system, the expressed proteins from the two different expression systems showed essentially the same biochemical characteristics (see below).

DNA polymerase activity

Comparison studies revealed that the specific activities of proteins prepared using the two different systems were indistinguishable (Table 2). The DNAPOLs were most active at pH 7.5 (data not shown). The enzyme required moderate concentrations of divalent cations for activity. Maximum DNA polymerase activity was observed at MgCl₂ concentrations of 10–20 mM, while a concentration of 100 mM MgCl₂ was inhibitory (Fig. 2A). No activity was detected in the absence of divalent cations. Maximum SpliNPV DNAPOL polymerase activity was observed at KCl concentrations of 100–200 mM. The polymerase activity was greatly reduced at a KCl concentration of 300 mM and was inhibited at higher salt concentrations (Fig. 2B). Only 20% of residual activity could be detected in the presence of 400 mM KCl. While the SpliNPV DNAPOL was active at 100 mM (NH₄)₂SO₄, only 40% activity could be detected in the presence of 300 mM (NH₄)₂SO₄ (Fig. 2C). Higher concentrations of ammonium ions strongly inhibited the enzymes (data not shown). The differences in DNA polymerase activity observed with 10 mM and 20 mM MgCl₂ as well as with 100 mM and 200 mM KCl, were not statistically significant.

The polymerase activity of the SpliNPV DNAPOL was inhibited by aphidicolin. Forty percent of the original activity was detected in the presence of 0.25 µg/ml aphidicolin, and 20% was detected in the presence of 0.5 µg/ml aphidicolin (Fig. 2D). The heat sensitivity was also examined (Fig. 2E). The enzymes displayed similar heat sensitivities, losing approximately 80% activity after incubation at 50 °C for 9 min (Fig. 2E). Thus enzyme preparations from both bacterial and baculovirus expression systems showed the same pattern of polymerase catalytic activity, suggesting that the first 80 amino acids are not essential for in vitro DNA polymerase activity. The specific activities of the full-length His-DNAPOL and His-del80DNAPOL were 6890 ± 8 U/mg and 6743 ± 42 U/mg (Table 2), respectively.

3′–5′ exonuclease activity

The 3′–5′ exonuclease activities of the full-length

Table 2. Specificity of SpliNPV DNAPOL

<table>
<thead>
<tr>
<th>Expression system/protein</th>
<th>Activity (U/mg ± SD)</th>
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<tr>
<td></td>
<td>DNA polymerase</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
</tr>
<tr>
<td>DNAPOL</td>
<td>6532 ± 32</td>
</tr>
<tr>
<td>Δ80DNAPOL</td>
<td>606 ± 27</td>
</tr>
<tr>
<td>Baculovirus</td>
<td></td>
</tr>
<tr>
<td>His-DNAPOL</td>
<td>6890 ± 8</td>
</tr>
<tr>
<td>His-del80DNAPOL</td>
<td>6743 ± 42</td>
</tr>
</tbody>
</table>

The Bac-to-Bac Expression System (Life Technologies) was used to obtain larger and more concentrated amounts of DNA polymerase with potentially higher specific activity. PCR

Fig. 1. Expression of the SpliNPV DNAPOL and the Δ80 mutant. (A) Coomassie brilliant blue R-250-stained SDS–PAGE analysis of the recombinant full-length DNAPOL (lane 1) and the Δ80 mutant (Δ80DNAPOL, lane 2) proteins overexpressed in E. coli and purified to near homogeneity after digested with Factor Xa; (B) Coomassie brilliant blue R-250-stained SDS–PAGE analysis of the recombinant full-length DNAPOL (His-DNAPOL, lane 1) and the Δ80 mutant (His-del80DNAPOL) (lane 2) proteins overexpressed in Sf9 cells, purified to near homogeneity using Ni–NTA agarose. M, molecular mass standard (kDa).

amin acid polypeptide (Δ80DNAPOL) with a predicted molecular mass of 96 kDa (Huang & Levin, 2001). SDS–PAGE analyses indicated that the SpliNPV DNAPOL and the Δ80 mutant proteins have apparent molecular masses of 110 kDa and 96 kDa, respectively (Fig. 1), which correspond well with the predicted molecular masses based on the amino acid sequences. The concentrations of the DNAPOL and Δ80DNAPOL proteins, purified from 500 ml bacterial cultures, were 0.5 and 0.7 mg, respectively. The specific activities of the proteins were 6532 ± 32 (1 SD) U/mg and 6063 ± 27 U/mg (Table 2), respectively, with activated calf thymus DNA as a template.

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Characterization of SpliNPV DNA polymerase

Fig. 2. Recombinant SpliNPV DNAPOL DNA polymerase activity. Effects of different components and conditions were assayed using activated calf thymus DNA as a template. The standard assays as described in the text were carried out with 0–2 U of purified DNA polymerase in the presence of various concentrations of (A) MgCl₂, (B) KCl, (C) (NH₄)₂SO₄, in the presence of (D) aphidicolin at different concentrations, or (E) preincubated at 50 °C for various lengths of time. ▲, DNAPOL; ●, △80POL; ▼, His-DNAPOL; ■, △His-DNAPOL.

DNAPOL and the △80 mutants from the two expression systems were high. The 3′→5′ exonuclease activity required Mg²⁺ ions (Fig. 3A). Peak exonuclease activity was observed at MgCl₂ concentrations of 10–20 mM (Fig. 3A) and at KCl concentrations of 100–200 mM (Fig. 3B). The differences in exonuclease activity observed with 10 and 20 mM MgCl₂, as well as with 100 and 200 mM KCl, were not statistically significant. The inclusion of 200 mM (NH₄)₂SO₄ reduced exonuclease activity of all four enzymes by 30% (Fig. 3C).

Aphidicolin, which was shown to be an inhibitor of the polymerase activity of SpliNPV, also affected the 3′→5′ exonuclease activity (Fig. 3D). Exonuclease activity was reduced to 30% of the original level at 0.25 µg/ml aphidicolin, and to approximately 10% at 0.5 µg/ml aphidicolin. The results suggest that the same aphidicolin-sensitive enzymatic system is responsible for both the polymerase activity and the exonuclease activity. The exonuclease activities of the SpliNPV full-length DNAPOL and the △80 mutants were inactivated by heat with a nearly identical pattern (Fig. 3E). The enzymes lost 50% of the exonuclease activity after a 3 min preincubation at 50 °C. The exonuclease activity from all preparations demonstrated a similar pattern of enzymatic activity, suggesting that the exonuclease activity is intrinsic to SpliNPV DNAPOL, and that the first 80 amino acids are not required for the activity.

Replication of singly primed single-stranded M13 DNA

Having observed that the DNAPOL utilized gapped, activated calf thymus DNA as primer-templates, we further investigated the ability of DNAPOL to replicate single-
stranded DNA. The 18-mer oligonucleotide M13 DNA primer was incubated with single-stranded M13 DNA, purified DNA polymerase from the baculovirus expression system, and three deoxyribonucleoside triphosphates. Radiolabelled M13 DNA products were detected after 3 min of incubation. A strong signal was detected by 5 min after addition of dCTP, indicating that the SpliNPV DNA POL synthesized DNA molecules of approximately 7200 nucleotides, corresponding to the entire length of M13 DNA (Fig. 4). The singly primed replication assay using single-stranded M13 DNA revealed that the Δ80 mutant was also capable of synthesizing a 7200 nucleotide replication product (data not shown). These results demonstrated that the DNA synthesis activity of SpliNPV DNA polymerase is processive.

**Transient replication assays with SpliNPV non-hr origin deletion clones**

Transient replication assays with SpliNPV non-hr deletion constructs allowed us to map the minimal essential domain for origin activity. A schematic diagram of the sequence elements identified within the 344 bp E4 fragment containing the SpliNPV non-hr origin of DNA replication is presented in Fig. 5(A). Deletion clone Δ12 (315 bp) contained all sequence elements downstream (3′) of the first repeat sequence (R1 = 5′ CGATGGTGCTT 3′). Deletion clone Δ14 (259 bp) contained all sequence elements 3′ of the putative USP site. Deletion clone Δ15 (234 bp) contained all sequence elements 3′ of the A+T-rich sequence. Deletion clone Δ23 (158 bp) lacked all
sequence elements 5′ of the 22 bp imperfect palindrome (P2 = 5′ CGG1GATCTGGCCAGATCgCCG 3′). Deletion clone Δ33 (81 bp) lacked all sequence elements 5′ of a 9 bp repeat sequence (R2 = 5′ CCGTCGCCG 3′) and the two putative GATA sites.

Transient replication assays with plasmids containing either the full-length E4 non-hr origin fragment or the Δ12 fragment resulted in equally strong replication products (Fig. 5B). The Δ12 fragment contained all sequence elements except the most 5′ R1 repeat sequence. Transient replication assays with plasmids containing the Δ14 and Δ15 fragments resulted in replication products that were slightly less strong than those observed with the E4 and Δ12 plasmids. The Δ14 fragment lacked the two R1 repeat sequences, the R2 repeat sequence, the two putative SP1 binding sites and the putative USP binding site, while the Δ14 fragment lacked all these elements plus the P2 imperfect palindrome sequence. The plasmid containing the Δ23 fragment, which lacked all sequence elements 3′ of the A+T-rich/NFIII site sequences, replicated very poorly, and the plasmid containing the Δ33 fragment did not replicate at all. These data suggest that the Δ23 fragment contains sequence elements that support minimal transient replication, and that the minimal essential domain for SpliNPV origin of replication activity can be mapped to a region that is approximately 158 bp in size within the SpliNPV non-hr origin fragment. Control experiments with pBlueScriptII plasmids that did not contain the SpliNPV non-hr origin fragment were cleaved by DpnI, as expected, indicating that they did not replicate in SpliNPV-infected Sf9 cells (data not shown; see Huang & Levin, 2001).

Replication of the non-hr origin of SpliNPV with DNAPO and nuclear extracts

Using the SpliNPV non-hr origin fragment (E4) and the set of origin deletion clones, we established a cell-free DNA replication system for SpliNPV. Origin-dependent DNA replication was observed with nuclear extracts prepared from SpliNPV-infected Sf9 cells plus purified SpliNPV DNAPO expressed from recombinant AcMNPV in Sf9 cells (Fig. 5C). Replication products were strongest with the E4 fragment containing the full-length SpliNPV non-hr origin. As observed with in the transient replication assays, the plasmid containing the Δ23 fragment supported minimal replication in the presence of nuclear proteins and the strength of the replication product signal increased as the length of the origin fragment increased. Plasmids containing the Δ14 and Δ15 fragments supported replication better than the plasmid containing the Δ23 fragment, but the strength of the replication products from these plasmids was less than that generated when the plasmid containing the E4 fragment was used as template. No replication signals were detected when plasmids either containing the Δ33 fragment or without origin-specific elements (pBlueScriptII) were used as template. A very low level of origin-dependent DNA replication was observed with nuclear extracts prepared from SpliNPV-infected Sf9 cells in the absence of purified SpliNPV DNAPO (Fig. 5D).

These experiments suggest that origin-dependent DNA replication occurred in both the transient replication assays and in cell-free replication assays using nuclear extracts prepared from SpliNPV-infected cells. Nuclear protein extracts prepared from SpliNPV-infected Sf9 cells supported a low level of origin-dependent DNA replication. The addition of purified SpliNPV DNAPO to the reaction mix dramatically increased the strength of the replication product signal detected. Moreover, the strength of this origin-dependent replication signal was a function of specific DNA sequence elements within the origin fragments. Deletion of the 22 bp palindromic sequence (P2) overlapping the putative NFIII transcription factor binding site abolished in vitro replication, and the strength of the replication products increased dramatically when the A+T-rich/NFIII sequences were included in the cloned fragments.

Discussion

The properties of the full-length SpliNPV DNAPO, and a mutant DNAPO in which the first 80 amino acid residues were deleted, were analyzed after purification from either E. coli or Sf9 cells. Assays using gapped, activated calf thymus DNA as a template showed that the SpliNPV DNAPO polypeptide contained both DNA polymerase and 3′–5′ exonuclease activities, and that deletion of the first 80 amino acid residues did not affect its enzymatic activities. Maximum polymerase activity of SpliNPV DNAPO was observed with 10–20 mM MgCl₂ and 100–200 mM KCl. The 3′–5′ exonuclease activity was maximal in the same MgCl₂ and KCl concentration ranges. The enzymes were sensitive to the polymerase inhibitor aphidicolin, as well as to heat treatment. The enzymatic activities and biochemical properties of SpliNPV DNAPO are
Fig. 5. Transient replication and \textit{in vitro} origin-dependent DNA replication assays. (A) Schematic diagram showing the structure of the non-\textit{hr} origin of SpliNPV (E4) fragment and the deletion clones. A plus (+) or a minus (−) indicates the replication of the various plasmids in transient replication assays. The parent plasmid was pBlueScript KS(+) +. Plasmids were named for the fragments that they contain. The plasmid names (and lengths of the cloned fragments that they contain) are: lane (1) ∆33 – 69 bp; lane (2) ∆23 – 144 bp; lane (3) ∆15 – 189 bp; lane (4) ∆14 – 244 bp; lane (5) ∆12 – 289 bp; and lane (6) E4 – 344 bp. (B) Replication of plasmids containing the E4 fragment, which carries the SpliNPV non-\textit{hr} origin of DNA replication, or containing deletion clones of the E4 fragment, in transient replication assays. Plasmids are named for the fragments they contain, as indicated in (A). (C) \textit{In vitro} replication, using plasmid templates containing the E4 fragment or deletion clones of the E4 fragment, by nuclear protein extracts prepared from SpliNPV-infected Sf9 cells, in the presence of 250 ng of purified SpliNPV DNAPOL (from Sf9 cells infected with the full-length recombinant AcMNPV, pFastBacHT-DNAPOL). Plasmids are named as above. pBS – pBlueScript KS(+). (D) \textit{In vitro} replication, using plasmid templates containing the E4 fragment or deletion clones of the E4 fragment, by nuclear protein extracts, in the absence of purified SpliNPV DNAPOL. Plasmids are named as above. pBS – pBlueScript KS(+). Digitized images captured by the PhosphorImager (Molecular Dynamics Corp.) were assembled using Adobe Photoshop 5.0. Cell-free replication assays done with nuclear protein extracts prepared from mock-infected Sf9 cells did not produce a detectable signal (data not shown).
very similar to the DNAPOLs of AcMNPV and Bombyx mori NPV (Mikhailov et al., 1986; Hang & Guarino, 1999; McDougal & Guarino, 1999).

Our observations that the N-terminal truncated form of SpliNPV DNAPOL (Δ80DNApol) possesses the same biochemical characteristics in vitro as full-length DNAPOL appear to be consistent with a study of mammalian DNAPol δ (Schumacher et al., 2000). When DNA synthesis was measured using a purified DNAPol δ, the full-length and ΔNPol δ (missing the first 80 amino acids) were equally active, suggesting that the 80 amino acids at the N terminus do not function in catalysis per se (Schumacher et al., 2000). Further functional analyses, however, indicated that the holoenzyme containing ΔNPol δ was significantly less efficient and slower than that containing full-length Pol δ, suggesting that the N-terminal part of Pol δ is involved in interactions with other proteins, such as SSB and RPC (Schumacher et al., 2000). While the mammalian Pol δ is about 100 amino acids longer than the SpliNPV DNAPOL, the first 80 amino acid residues of the two sequences aligned when subject to Clustal W analysis (data not shown). Further investigation is needed to address the importance of the first 80 amino acid residues of the SpliNPV DNAPOL during viral DNA replication in vivo.

Two types of cis-acting elements which function as viral origins of DNA replication have been identified in NPVs (hr and non-hr elements) by use of transient replication assays in which plasmid DNAs replicate in the presence of virus in NPV-infected insect cell lines (Kool et al., 1995). Non-hr origins of DNA replication (non-hr oris) have been described from AcMNPV (Kool et al., 1994), Orgyia pseudotsugata MNPV (Pearson et al., 1993), Spodoptera exigua MNPV (Heldens et al., 1997) and Spodoptera littoralis NPV (Huang & Levin, 1999). The non-hr ori of AcMNPV (the HindIII-K fragment) is enriched in defective AcMNPV genomes (Lee & Krell, 1994) and appears to function as an origin of DNA replication during AcMNPV infection in vivo (Habib & Hasnain, 2000). Thus, there is evidence that suggests that non-hr oris do play an important role in NPV replication, but the molecular mechanisms of this function are not known.

Non-hr oris have sequence elements that are similar to those found in origins of DNA replication of many eukaryotic DNA viruses (Kool et al., 1994, 1995; DePamphilis, 1996). These comprise both a core element, which often consists of direct repeats and/or imperfect palindrome sequences, flanked by an A + T-rich region, and one or more auxiliary components that are composed of transcription-factor binding sites and/or promoter elements. The core element is absolutely required for replication. The presence of the auxiliary elements can stimulate the initiation of replication, but are not essential for it and are dispensable (DePamphilis, 1996). In order for viral DNA synthesis to begin, sequence-specific recognition events, mediated by one or more initiator proteins (origin-binding proteins) encoded by the virus, are usually required (DePamphilis, 1996). Following the initiation events, the origin-binding proteins recruit other replication proteins to the initiation site to unwind DNA, to synthesize new DNA primers and to elongate the synthesized DNA from both strands.

We conducted both transient replications assays and in vitro replication assays with purified SpliNPV DNAPOL using a set of deletion clones of the E4 fragment containing the putative SpliNPV non-hr ori as template. The results of these experiments suggest that a putative origin core element is located in a region between the two HindIII sites on the E4 fragment. This region contains a 22 bp imperfect palindrome sequence (5’- CGGtGATCTGGCCAGATCgCCG 3’), which overlaps a putative NFI transcription-factor binding site. Deletion of this region abolished both plasmid replication in the transient replication assays and ori-dependent replication in the in vitro replication assays with purified SpliNPV DNAPOL. Based on these results, we hypothesize that the 22 bp imperfect palindrome sequence and/or the putative NFI binding site constitute the core element of the SpliNPV non-hr ori and that this sequence represents an origin-binding protein site.

Our analyses indicated that the A + T-rich sequence upstream of the 22 bp imperfect palindrome sequence also plays an important role in origin activity. Deletion of the A + T-rich sequence dramatically decreased replication in both the transient and in vitro replication assays. The region at the 5’ end of the E4 fragment contains many repeat sequences and putative transcription-factor binding sites. Deletion of this region had little effect on replication in either the transient or the in vitro replication assays, and it may represent an auxiliary component that is not essential for origin activity.

In conclusion, our data suggest that the SpliNPV E4 fragment contains sequence elements that can support plasmid replication in transient replication assays and that purified SpliNPV DNAPOL can utilize these elements to synthesize DNA in vitro, in a sequence-dependent manner. While our data do not demonstrate conclusively that the SpliNPV E4 fragment functions as an origin of DNA replication during virus replication in vivo, they do suggest that there are specific interactions between SpliNPV DNA POL and sequences within the putative SpliNPV non-hr element.

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References

