Purification of *Autographa californica* nucleopolyhedrovirus DNA polymerase from infected insect cells

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*Autographa californica* nucleopolyhedrovirus (AcMNPV) DNA polymerase was purified from virus-infected cells using conventional chromatographic methods. The enzymatic activity of fractions eluting from single-stranded agarose gels was found to exactly coincide with a single polypeptide with an apparent molecular mass of approximately 110 000 Da on denaturing polyacrylamide gels stained with Coomassie blue. This purification scheme resulted in a 228-fold purification of AcMNPV DNA polymerase with recovery of 3–5% of the initial activity. The specific activity of the most purified fraction of DNA polymerase was 5000 units/mg, which is sufficiently high to eliminate the possibility that contaminants significantly contribute to the polymerase activity. Preparations of purified DNA polymerase had 3′–5′ exonuclease activity, but no 5′–3′ exonuclease activity. The proofreading activity was apparently an intrinsic property of the enzyme as the ratio of nuclease activity to polymerase activity was constant throughout purification. Using a singly-primed M13 DNA template, RF-II DNA was detected within 3 min, indicating a polymerization rate of 40 nt/s. The effects of several DNA polymerase inhibitors on the enzymatic activity of purified DNA polymerase were also determined.

Introduction

The genome of *Autographa californica* nuclear polyhedrovirus (AcMNPV) is a double-stranded, supercoiled DNA molecule of 134 kb that potentially encodes 154 proteins (Ayres *et al*., 1994). The viral genome consists of unique sequences, with the exception of regions of homologous sequences (*hrs*) that are interspersed along the length of the genome (Cochran & Faulkner, 1983). Each *hr* contains from two to eight 30 bp imperfect palindromes with naturally occurring EcoRI sites at their core. The *hrs* function as enhancers of early viral gene expression (Guarino *et al*., 1986; Guarino & Summers, 1986). The *hrs* also serve as origins of DNA replication in transient replication assays (Kool *et al*., 1993; Pearson *et al*., 1992).

To identify the viral genes required for baculovirus DNA replication, a transient complementation assay (Kool *et al*., 1994; Lu & Miller, 1995) was used. These experiments revealed that proteins encoded by six viral genes (*dna pol, p143, lef-1, lef-2, lef-3* and *ie1*) are essential for replication of plasmids containing a viral origin of replication. In addition, products of the *ie2, pe38, p35* and *lef-7* genes stimulated the level of DNA replication. Thus far, the functions of only a few of these genes are known.

The *ie1* gene encodes the major transactivator of delayed early gene expression (Guarino *et al*., 1986; Guarino & Summers, 1987; Ross & Guarino, 1997), so it is essential for replication at least partly due to its role in transcription. In addition, it is possible that IE1 plays a direct role in viral DNA replication because it binds to the *hr* elements (Guarino & Dong, 1994). *lef-3* encodes a single-stranded DNA-binding protein (Hang *et al*., 1995), and is also involved in the nuclear localization of P143 (Wu & Carstens, 1998). The P143 gene was identified as part of a temperature-sensitive screen for DNA negative mutants (Lu & Carstens, 1991). P143 is a candidate helicase based on amino acid sequence similarity, though enzymatic activity has yet to be demonstrated. The stimulatory proteins IE2 and PE38 probably function by increasing the expression of IE1 and other essential DNA replication proteins (Carson *et al*., 1991; Lu & Carstens, 1993). P35 suppresses apoptosis thus increasing the viability of transfected cells during the transient replication assays (Clem
& Miller, 1993). The functions of lef-1, lef-2 and lef-7 are unknown.

The AcMNPV dna pol gene was first identified by using an oligonucleotide probe corresponding to an amino acid sequence that is conserved among other viral DNA polymerases (Tomalski et al., 1988). Additional transcriptional mapping of dna pol indicated that it was transcribed during the early phase of infection, which is consistent with its proposed function. The identification of the viral DNA polymerase gene confirmed earlier biochemical studies on DNA polymerase activity in infected cells. These data indicated that DNA polymerase activity is significantly enhanced during the course of viral infection. Furthermore, chromatographic separation of polymerases from control and infected cells identified a unique activity from the infected cells (Miller et al., 1979; Wang & Kelly, 1983). However, the AcMNPV DNA polymerase has not been purified to homogeneity nor have experiments been performed that conclusively link the biochemical activity to the virus gene.

As part of our continuing effort to elucidate the molecular mechanism of baculovirus DNA replication, we decided to purify AcMNPV DNA polymerase and analyse the purified enzyme by examining the kinetics of its replication of single-stranded DNA templates.

**Methods**

**DNA polymerase purification.** *Spodoptera frugiperda* (Sf9) cells were cultured and infected according to the procedures previously described (Summers & Smith, 1987). Five litres (1 x 10^8 cells/ml) of Sf9 cells were infected with wild-type AcMNPV at an m.o.i. of 10 and harvested by centrifugation at 16 h post-infection. After washing with cold PBS, the cell pellet (approx. 6 ml) was resuspended in 1 packed cell volume (PCV) of hypotonic buffer, lysed with the addition of an equal volume of 3 M NaCl, and incubated on ice for 1 h. The cytosolic and nuclear extracts were clarified by centrifugation at 80,000 g for 1 h, and the resulting supernatant fractions were collected and separately dialysed four times against 1 l of buffer A (20 mM potassium phosphate, pH 7.5, 5 mM KC1, 1.5 mM MgCl2, 10 mM dithiothreitol, 1 µg leupeptin/ml, 0.5 mM PMSF, 1% aprotinin). The cells were allowed to swell on ice for 10 min and were lysed with a Dounce homogenizer. The nuclear and cytosolic fractions were separated by low-speed centrifugation (2000 g). The supernatant containing the cytosolic fraction was saved, and NaCl was added to a final salt concentration of 100 mM. The nuclear pellet (approx. 4 ml) was resuspended in 1 PCV of hypotonic buffer, lysed with the addition of an equal volume of 3 M NaCl, and incubated on ice for 1 h. The cytosolic and nuclear extracts were clarified by centrifugation at 100,000 g for 1 h, and the resulting supernatant fractions were collected and separately dialysed four times against 1 l of buffer A (20 mM KH2PO4, pH 7.2, 1 mM EDTA, 50 mM KC1, 10 mM β-mercaptoethanol) containing 10% glycerol. The precipitates that formed during dialysis were removed by centrifugation and the supernatants were stored at −80°C.

Nuclear extract (20 ml) was loaded in two 10 ml batches onto a 5 ml heparin affinity column (Bio-Rad) connected to a Pharmacia FPLC system. The column was washed with 20 ml buffer A and eluted with a 20 ml linear gradient from 0 to 0.5 M KC1 in buffer B. The flow rate was 1 ml/min and the fraction size was 1 ml. Fractions containing viral DNA polymerase activity were pooled, dialysed against buffer A containing 10% glycerol and applied to a 5 ml DEAE-Blue column (Bio-Rad) equilibrated in the same buffer. Proteins were eluted with a 25 ml linear salt gradient from 0.05 to 0.5 M KC1 in buffer B. Peak fractions of DNA polymerase activity were pooled and dialysed against buffer A. Flowthrough fractions containing enzyme activity were also pooled and loaded on the same column and eluted as before. Peak DNA polymerase activity fractions were pooled, dialysed with buffer A and combined with the previous DEAE fractions before loading onto a Mono Q HR 5/5 anion-exchange column (Pharmacia) and eluted with a 20 ml linear salt gradient from 0.05 to 0.5 M KC1 in buffer A. Fractions containing DNA polymerase activity were pooled, dialysed against buffer A and loaded onto a 1 ml single-stranded (ss)DNA–agarose column (BRL). The column was eluted with an increasing salt gradient from 0 to 1 M KC1 in buffer A. During purification, all fractions were assayed for DNA polymerase activity and peak fractions were analysed by SDS-PAGE. Silver staining of SDS-PAGE gels was performed according to standard procedure (Harlow & Lane, 1988). Protein concentrations were determined by the Bradford (1976) method using BSA as a standard.

**Protein fragmentation and mass analysis.** Purified DNA polymerase was digested with trypsin and mass analysis was conducted at the Protein/Peptide Micro Analytical Laboratory (California Institute of Technology). Mass spectrometry was performed on a PerSeptive Biosystems Elite MALDI TOF. Tryptic peptide masses were used by MOWSE to search a peptide mass database constructed from a theoretical trypsin digest of all proteins in the OWL database (Pappin et al., 1993). Search parameters used were a molecular mass filter of 25%, a 3 Da peptide mass tolerance and a partial cleavage score factor of 0.4.

**DNA templates.** Singly-primed single-stranded DNA templates were produced by hybridization of 25 pmol of synthetic oligonucleotide primer with 2.5 pmol of the appropriate phage DNA (M13mp19 or φX174) in a reaction volume of 50 µl containing 50 mM Tris–HCl (pH 7.5), 5 mM MgCl2 and 100 mM NaCl. The hybridization mixtures were incubated at 90 °C for 5 min before cooling slowly to room temperature for 1 h. Excess primer was removed by spin chromatography through Sephadex G-50. The oligonucleotide used for the M13 template was universal M13 (−40) sequencing primer, 5'-GTTTTCCCAGTCAGAC-3', while the φX174 primer was 5'-GGCGGATAACGATACCATGACC-3', which is complementary to nucleotides 2067 to 2045 of φX174 DNA.

**Measurements of DNA polymerase activity.** During purification, DNA polymerase activity was assayed using activated calf thymus DNA as primer–template. Typically, 50 µl reaction mixtures contained 20 mM Tris–acetate (pH 7.3), 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, 0.1 mM each dGTP, dATP and dTTP, 0.01 mM [γ-32P]dCTP (3000 Ci/mmol), 100 µg/ml BSA and 0.2 mg/ml activated calf thymus DNA (Sigma) as substrate. Each fraction was titrated to identify the linear range of activity. The reaction mixtures were incubated at 37 °C for 15 min and spotted onto glass-fibre filters, which were then washed extensively with 5% trichloroacetic acid (TCA) and 1 M HCl rinsed with ethanol and dried. Filter-bound radioactivity was determined by Cerenkov counting. The specific activity of the input dCTP was also calculated by Cerenkov counting an aliquot of each reaction. One unit of enzyme activity was defined as the amount of enzyme required to incorporate 1 nmol [γ-32P]dNTP into acid-insoluble material/min at 37 °C.

For DNA synthesis on singly-primed M13 single-stranded circular template, reaction mixtures (50 µl) contained 20 to 50 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 dGTP, dATP and dTTP, 20 µM [γ-32P]dCTP (300 Ci/mmol), 50 µg/ml BSA and 10 to 200 fmol purified DNA polymerase. The reactions were incubated at
37 °C and terminated by the addition of an equal volume of stop buffer (1% SDS—40 mM EDTA—60 µg sonicated calf thymus DNA/ml). The reaction products were precipitated with ethanol, resuspended in 20 µl 0 M NaOH—5% glycerol—1 mM EDTA—0.025% bromocresol green sample buffer and separated on a 1% alkaline agarose gel as described (Sambrook et al., 1989). For autoradiography, dried gels were exposed to X-ray film overnight at −80 °C.

### Measurements of 3′–5′ exonuclease activity
3′–5′ exonuclease activity was assayed in a 50 µl reaction containing 20 mM Tris–acetate (pH 7.5), 75 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, 100 µg/ml BSA and 5000 to 10 000 c.p.m. of 3′-end labelled DNA template. The template was prepared by digestion of 1 µg pUC18 DNA with XmaI, and radiolabelling at the 3′ end with the Klenow fragment of E. coli DNA polymerase I in the presence of [α-32P]dCTP (3000Ci/mmol) as described (Sambrook et al., 1989). The reactions were initiated by addition of 5 or 10 µl aliquots of column fractions and incubated at 37 °C for 30 min. The reaction mixtures were precipitated with 1 ml ice-cold 5% TCA, and then filtered onto a glass-fibre filter. The acid-insoluble radioactivity collected on the filter and was then washed with 5 ml 5% TCA. The acid-soluble material washed through the filter and was collected directly into a scintillation vial. The total amount of radioactivity (soluble plus insoluble) was determined by Cerenkov counting. One unit of enzyme activity was defined as the amount of enzyme required to release 1 pmol [α-32P]dCTP into acid-soluble material in 30 min at 37 °C.

### Results

#### Purification of DNA polymerase from AcMNPV-infected cells

AcMNPV DNA polymerase was purified from 5 l of Spodoptera frugiperda cell cultures harvested at 16 h post-infection, at which time DNA polymerase activity reaches a maximum level (data not shown). Although prior experiments based on whole-cell extracts indicated that a virus-specific DNA polymerase activity was induced after virus infection (Miller et al., 1979; Wang & Kelly, 1983), the subcellular localization of the viral enzyme had not previously been determined. To identify the best starting material for purification of AcMNPV DNA polymerase, we prepared both cytosolic and high-salt-extracted nuclear fractions from insect cell cultures as previously described (Hang et al., 1995). We then performed DNA polymerase assays using activated calf thymus DNA as substrate. The results indicated that in uninfected cells, the level of DNA polymerase activity was equally distributed in the nuclear and cytosolic fractions (Fig. 1). Although DNA polymerase is a nuclear protein, cytoplasmic extracts of mammalian cells contain significant amounts of DNA polymerases that have leaked from the nucleus. Therefore, DNA replication extracts are always made from cytosolic extracts (Brush et al., 1995) and purification of DNA polymerases usually start with cytosolic extracts (Wang et al., 1995; Chui & Linn, 1995). In infected cells, the total DNA polymerase activity was approximately 4-fold higher than in the uninfected cells. Furthermore, it was 3 times higher in the nuclear fraction than in the cytosolic fraction. These results suggested that the majority of viral DNA polymerase was present in the nuclei of infected cells and its activity was considerably higher than the host-cell DNA polymerase. Therefore, nuclear extracts prepared from infected cells were used as starting material for purification of AcMNPV DNA polymerase.

In order to purify the DNA polymerase, we combined several different conventional chromatographic methods including ion-exchange and affinity chromatography. During the course of purification, DNA polymerase activity was monitored by following the incorporation of acid-insoluble radioactivity using activated calf thymus DNA as primer–template. The relative purity and peptide compositions of column fractions were examined by electrophoresis on denaturing 8% polyacrylamide gels. Uninfected-cell nuclear extracts were included as a negative control in the column purification steps to exclude the possibility of purification of host-cell DNA polymerase.

The final step in our purification was affinity chromatography on ssDNA–agarose. The DNA polymerase activity of fractions eluting from ssDNA–agarose was quantified using activated calf thymus DNA as primer–template (Fig. 2B). Fractions across the peak of activity were assayed by SDS–PAGE and stained with Coomassie blue. A single polypeptide with an apparent molecular mass of 110 000 Da was found to increase and decrease concomitant with the peak of enzymatic activity (Fig. 2A). Fraction 4 was contaminated with a lower molecular mass protein, but fractions 5 and 6 were apparently homogeneous with respect to the 110 000 Da band. This purification scheme resulted in a 228-fold purification of AcMNPV DNA polymerase with recovery of 35% of the
Fig. 2. Purification of AcMNPV DNA polymerase (A). Peak fractions from a Mono Q column containing DNA polymerase activity was loaded onto a 1 ml ssDNA–agarose column. Proteins were eluted with a step gradient of increasing salt. Proteins were analysed by SDS–PAGE, followed by stained with Coomassie brilliant blue. Lane 1 contains protein molecular markers, and the sizes of relevant bands in kDa are shown on the left. (B) Aliquots of the corresponding fractions were assayed for DNA polymerase activity.

We cannot, however, entirely eliminate the possibility that our preparations of AcMNPV DNA polymerase contain submolar amounts of contaminating proteins.

The observed molecular mass of 110,000 Da is in close agreement with the predicted value (114,000) calculated from the deduced amino acid sequence of the AcMNPV dna pol gene (Tomalski et al., 1988). This suggests that the purified enzyme is the protein product of the previously identified dna pol gene. To confirm this hypothesis, the purified protein was digested with trypsin and the peptides were analysed by mass spectroscopy to determine their molecular masses. The masses of nine tryptic peptides were entered into the MOWSE database searching program (Pappin, 1993). The database matches the peptide fingerprints of an unknown protein with the predicted fingerprints of all ORFs in the OWL database. The program identified a significant match with the AcMNPV DNA polymerase protein (Table 2), concurring with the hypothesis that the purified protein was virus encoded.

### Intrinsic 3′–5′ exonuclease activity

As part of the functional characterization of AcMNPV DNA polymerase, peak fractions from each column purification step were also analysed for 3′–5′ exonuclease and 5′–3′ exonuclease activity. In these assays, 3′ or 5′ end-labelled DNAs were used as substrates and the exonuclease activity was determined by the release of acid-soluble radioactivity from labelled templates. 3′–5′ exonuclease activity was detected in the pooled fractions of each column, which is consistent with a previous report of partially purified Bombyx mori nucleopolyhedrovirus (BmNPV) DNA polymerase (Mikhailov et al., 1986). This 3′–5′ exonuclease activity is probably an intrinsic property of AcMNPV DNA polymerase since the ratio of the exonuclease activity to the polymerase activity remained relatively constant through all stages of purification.

### Table 1. Purification of DNA polymerase from AcMNPV-infected cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg protein)</th>
<th>Recovery (%)</th>
<th>Relative purification (-fold)</th>
<th>Specific activity (units/mg protein)</th>
<th>Recovery (%)</th>
<th>Relative purification (-fold)</th>
<th>Ratio DNApol/Exo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear extract</td>
<td>192</td>
<td>23.6</td>
<td></td>
<td></td>
<td>1.7</td>
<td>48</td>
<td>2.4</td>
<td>68.8</td>
</tr>
<tr>
<td>Heparin</td>
<td>40</td>
<td>62</td>
<td>54</td>
<td>2.6</td>
<td>12.0</td>
<td>25.6</td>
<td>7.2</td>
<td>54</td>
</tr>
<tr>
<td>DEAE-Blue</td>
<td>7.4</td>
<td>130</td>
<td>21</td>
<td>5.7</td>
<td>52.3</td>
<td>6.3</td>
<td>31.4</td>
<td>60.2</td>
</tr>
<tr>
<td>Mono Q</td>
<td>0.42</td>
<td>628</td>
<td>5.8</td>
<td>26.9</td>
<td>426.2</td>
<td>3.7</td>
<td>256</td>
<td>62.6</td>
</tr>
<tr>
<td>ssDNA–agarose</td>
<td>0.03</td>
<td>5332</td>
<td>3.5</td>
<td>228</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One unit of DNA polymerase activity was defined as the amount of enzyme required to incorporate 1 nmol [α-32P]dNTP into acid-insoluble material/min at 37 °C.

† One unit of 3′–5′ exonuclease activity was defined as the amount of enzyme required to release 1 pmol [α-32P]dCTP into acid-soluble material in 30 min at 37 °C.
purification (Table 1), although both the total polymerase and exonuclease activities decreased during the course of purification. However, the 3′–5′ exonuclease activity in the purified fraction is apparently not as stable as the polymerase activity, since exonuclease activity was lost more rapidly during storage than was polymerase activity (data not shown). 5′–3′ exonuclease activity was observed in the initial stages of purification, but it was not detectable after the DEAE-Blue column (data not shown), suggesting that this activity is not an intrinsic property of the viral DNA polymerase.

Effects of different metabolic drugs on DNA polymerase activity

In order to gain more insight into the molecular mechanisms of the viral DNA polymerase, we also investigated the effects of several metabolic drugs on the activity of the purified protein. Aphidicolin is a mycotoxin that was previously shown to inhibit the activity of baculovirus DNA polymerase (Miller et al., 1979; Wang & Kelly, 1983). In agreement with these results, our experiment using purified viral DNA polymerase also showed that aphidicolin can effectively inhibit its activity (Fig. 3). An inhibitor constant (K_i) of 0.6 µM for aphidicolin was determined from a Dixon plot of the inhibition data. Cytosine-β-d-arabinofuranoside 5′-triphosphate (AraCTP) also had a marked inhibitory effect, with a K_i of 4.2 µM. In addition, 5′-bromo-2′-deoxyuridine 5′-triphosphate (BdUTP) and phosphonoacetic acid (PAA) also inhibited the purified viral DNA polymerase activity. The K_i values of these inhibitors were 118 µM for BdUTP and 76 µM for PAA.

Utilization of a singly-primed single-stranded DNA template

Most DNA polymerases efficiently utilize gapped DNAs, such as activated calf-thymus DNA, as primer–template as described in Methods. The indicated drugs were included in the reaction mixtures at the indicated concentrations. Percentage of DNA polymerase activity was determined using untreated enzyme as standard.

**Table 2. Mass spectrophotometric analysis of tryptic fragments of DNA polymerase**

<table>
<thead>
<tr>
<th>Mass (Da)</th>
<th>D (Da)*</th>
<th>DNA polymerase peptide sequence consistent with mass</th>
<th>DNA polymerase amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>4975.0</td>
<td>2.5</td>
<td>MFNLQHFENDFELVDMTLTGIMPVLSNYDIETHSDGHNMK</td>
<td>166–208</td>
</tr>
<tr>
<td>2165.2</td>
<td>−0.85</td>
<td>FAQYKGPFINTSADTFR</td>
<td>13–31</td>
</tr>
<tr>
<td>2121.5</td>
<td>−2.19</td>
<td>VENQLNNTISSYLYGENK</td>
<td>349–336</td>
</tr>
<tr>
<td>1667.2</td>
<td>1.56</td>
<td>VHMQTPFPEGAYMR</td>
<td>134–147</td>
</tr>
<tr>
<td>1169.3†</td>
<td>1.7</td>
<td>VLQFYFKVK</td>
<td>56–64</td>
</tr>
<tr>
<td>3391.2, 2572.8, 1424.6, 1392.6‡</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Difference between measured mass and calculated mass of DNA polymerase.
† Peptide mass results from partial cleavage.
‡ Masses not attributed.

**Fig. 3.** Effects of nucleoside analogues on the activity of purified viral DNA polymerase. DNA polymerase activities were assayed using activated calf thymus DNA as primer–template as described in Methods. The indicated drugs were included in the reaction mixtures at the indicated concentrations. Percentage of DNA polymerase activity was determined using untreated enzyme as standard.
Fig. 4. Utilization of singly-primed single-stranded templates. (A) Rate of polymerization. A standard replication assay (700 µl) containing 600 fmol singly-primed M13 ssDNA and 400 fmol AcMNPV DNA polymerase was incubated at 37 °C. At the indicated times 50 µl aliquots were removed and the reactions were terminated by addition of an equal volume of stop buffer (1% SDS–40 mM EDTA–60 µg sonicated calf thymus DNA/ml). The reaction products were precipitated with ethanol, resuspended in sample buffer, separated on a 1% alkaline agarose gel and visualized by autoradiography. (B) Comparison of reaction products generated by 0–5 units AcMNPV DNA polymerase (lane 1), T4 DNA polymerase (lane 2) or E. coli DNA polymerase I (lane 3). The migration of λ DNA markers is shown on the left of each panel, and the position of single-stranded linear M13 DNA is indicated on the right. All of the markers are migrating at the position of single-stranded DNA because it is an alkaline agarose gel and, therefore, the DNAs are denatured.

M13 DNA was detected. This band is frequently observed in these types of assays and is due to self-priming of broken circles (Challberg & Englund, 1987). Hairpin priming of a broken circle would produce a double-stranded copy of M13 with a hairpin at one end; subsequent denaturation produces a single strand that is twice the length of M13.

The rapid rate of polymerization indicates that AcMNPV DNA polymerase can efficiently copy a single-stranded template. Furthermore, the absence of shorter extended products suggests that the polymerase does not pause at regions of secondary structure. Some enzymes, like T4 DNA polymerase (Challberg & Englund, 1979) are not capable of strand-displacement and so they pause at specific sites that have been shown to correlate with predicted hairpins in the template DNA (Fig. 4B, lane 2). However, E. coli DNA polymerase I, a strand-displacing enzyme, easily replicates through these hairpin structures. The activity of the baculovirus enzyme is more similar to the E. coli enzyme than to the T4 protein.

Discussion

As part of our continuing effort to functionally characterize the essential baculovirus replication proteins, we report here the purification of AcMNPV DNA polymerase from infected insect cells. Nuclear extracts prepared from 5 l of cells were subjected to conventional column chromatography and the viral DNA polymerase was purified to a high specific activity. The most purified fraction had a specific activity 228-fold higher than the starting nuclear extract, and was obtained with a 3.5% yield (Table 1). When electrophoresed on an SDS gel, the most purified fraction showed a single polypeptide band with an apparent molecular mass of 110000 Da. When fractions from the final column were assayed for DNA polymerase activity, the activity exactly coincided with the peak of protein indicating that the protein detected was responsible for the enzymatic activity observed.

Several lines of evidence suggest that the purified DNA polymerase is virus encoded. One, its apparent molecular mass is in close agreement with the predicted molecular mass of the protein putatively encoded by the previously identified AcMNPV dna pol (Tomalski et al., 1988). Two, uninfected-cell nuclear extracts had much lower DNA polymerase activity and cellular DNA polymerases have molecular masses and subunit compositions different from the single polypeptide identified here (Kornberg & Baker, 1992). Furthermore, mass spectrophotometric analysis of tryptic peptides generated from the purified protein was consistent with the identification of the dna pol gene product. Five of the masses measured were matched to predicted tryptic fragments of the protein putatively encoded by the AcMNPV dna pol gene. The presence of unassigned fragments is not unusual as many proteins generate tryptic fragments that cannot be matched to the predicted proteins; these could be due to naturally occurring post-translational modifications or chemical modifications that can occur during purification. It is possible that the protein sequence of the E2 isolate of AcMNPV that we used is somewhat different than the L1 isolate sequenced by
Tomalski et al. (1988) or the C6 isolate sequenced by Ayres et al. (1994).

Our purification results indicate that AcMNPV-infected insect cells are a good source material for purification of viral DNA polymerase since it was expressed at high levels and localized to the nuclei during infection. Compared to the previously published DNA polymerase purification data for Trichoplusia ni nucleopolyhedrovirus (TnNPV) and BmNPV (Miller et al., 1979; Wang & Kelly, 1983), the relative purification as well as the final product yield were significantly improved in this report.

The presence of 3′–5′ exonuclease activity is a common feature of viral DNA polymerases as it is required for proofreading. Therefore, we tested whether this activity was associated with the baculovirus DNA polymerase. Our data suggest that 3′–5′ exonuclease activity is tightly associated with AcMNPV DNA polymerase activity since the activities copurified through all column purification steps and the ratio of exonuclease activity to polymerase activity remained relatively constant during purification (Table 1). The enzymatic data are consistent with the presence of three highly conserved segments (Exo I, II and III) near the N terminus; this region of homology is proposed to be a general 3′–5′ exonuclease active site conserved among many prokaryotic, eukaryotic and viral DNA polymerases (Bernad et al., 1989). Our data confirm a previous report on the related baculovirus BmNPV (Mikhailov et al., 1986). However, our results more convincingly show that 3′–5′ exonuclease activity and DNA polymerase are integrally associated because the BmNPV experiments were performed with partially purified polymerase preparations. Similar assays with a 5′-labelled probe suggest that 5′–3′ exonuclease activity is not an integral component of the AcMNPV polymerase. This is consistent with the lack of a conserved 5′–3′ exonuclease motif (Lopez et al., 1989) in the predicted amino acid sequence of AcMNPV DNA polymerase.

To further characterize the purified viral DNA polymerase, we tested the effect of different metabolic drugs on its activity. Aphidicolin is a tetracyclic diterpenoid antibiotic and has been shown to be a potent inhibitor of eukaryotic DNA polymerase α and δ (Spadari et al., 1982) and the DNA polymerases encoded by several large DNA viruses such as herpes and vaccinia (Pedrali-Noy & Spadari, 1980; Spadari et al., 1982). We showed that aphidicolin is an efficient inhibitor of purified AcMNPV DNA polymerase, in agreement with the previous report (Wang & Kelly, 1983) of inhibition by aphidicolin of TnNPV DNA polymerase. We also decided to test the effect of AraCTP on DNA polymerase activity because of inconsistencies in the literature. Previous reports (Kelly, 1981; Kelly & Lescott, 1976) showed that AraC blocked viral DNA replication and late viral gene expression in virus-infected cells. However, Rice & Miller (1986) reported that AraC was an inefficient inhibitor of viral DNA synthesis in AcMNPV-infected cells. We found that AraCTP was a potent inhibitor of the purified AcMNPV DNA polymerase, suggesting that the results of Rice & Miller may be due to problems with uptake of the drug into cells or with synthesis of the triphosphate rather than a lack of incorporation of AraCTP into DNA. The inhibitory effect of AraCTP is generally believed to be due to the incorporation of the arabinosides into DNA, where they distort the primer–template and block further DNA synthesis by chain termination (Kornberg & Baker, 1992). Another metabolic drug we showed to have an inhibitory effect on viral DNA polymerase is BdUTP, which contains bromine in the 5′-position. Incorporation of BdUTP into DNA as a nucleoside analogue is known to cause replication errors and alter the recognition of specific replication signals. In addition, we showed that PAA could also inhibit the purified AcMNPV DNA polymerase. PAA is an inorganic pyrophosphate analogue that selectively inhibits the DNA polymerase encoded by herpes simplex and vaccinia viruses, and was not previously shown to affect baculovirus DNA polymerase.

To better define the molecular mechanisms of viral DNA replication, we performed replication assays on singly-primed single-stranded templates. We found that AcMNPV DNA polymerase could effectively copy long single-stranded DNA templates such as singly-primed M13 and φX174 DNA. The rate of polymerization was 40 nt/s, and there was little evidence of pausing at regions known to contain hairpins. The lack of pausing indicates that the AcMNPV DNA polymerase could replicate through regions of secondary structure in the apparent absence of single-stranded DNA-binding proteins (SSB). Although we cannot be certain that our purified DNA polymerase preparation contained no minor contaminants, it is highly unlikely that enough SSB could be present to unwind all of the hairpins in M13 DNA because SSBs need to be added in a high enough molar ratio to coat the DNA template. The viral SSB is LEF-3 which binds 9 or 10 nucleotides of single-stranded DNA (Hang et al., 1995). Therefore, more than 725 mol LEF-3 per mol of DNA polymerase would be required to coat the DNA and unwind regions of secondary structure. This amount of protein could not be present in our preparations and remain undetected by either silver stain or Coomassie stain. Host SSBs or the viral DNA-binding protein (Mikhailov et al., 1998) could also be present as minor contaminants, but it is also highly unlikely that they could significantly affect the character of the DNA template at submolar concentrations.

This work was supported by the National Science Foundation (MCB 95-06233). Mass spectroscopy was performed with equipment purchased under NIH grant RR11292.

References


Received 31 March 1999; Accepted 14 May 1999