Characterization of DNA Polymerase Activity in *Trichoplusia ni* Cells Infected with *Autographa californica* Nuclear Polyhedrosis Virus

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**SUMMARY**

Nuclei isolated from *Trichoplusia ni* cells (TN-368) infected with *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were used to study the replication of viral DNA. Based on DNA : DNA hybridization data, virus-specific DNA polymerase activity was insensitive to aphidicolin and novobiocin and was inhibited by ddTTP and N-ethylmaleimide. The data indicate that the AcMNPV-specific DNA polymerase is a γ-like DNA polymerase which was first detected at 5 h post-inoculation.

The *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is an insect pathogen classified in subgroup A of the *Baculoviridae*. The viral genome is a 128 kilobase pairs (kbp), double-stranded, circular, supercoiled DNA (Summers *et al.*, 1980; Cochran *et al.*, 1982). Although it is well documented that virus replication is restricted to the nuclei of host cells (Kelly, 1981), information concerning DNA replication and transcription during the infection process is limited. Because of the large size of baculovirus genomes, it is suspected that a DNA-dependent DNA polymerase and/or a DNA-dependent RNA polymerase is coded by the virus genome. However, only circumstantial evidence has been obtained for virus-coded polymerases (Miller *et al.*, 1981; Wang & Kelly, 1983; Fuchs *et al.*, 1983). DNA polymerase activity associated with the occluded form of *Galleria mellonella* NPV was reported (Ryndich *et al.*, 1975).

Miller *et al.* (1981) reported a new DNA polymerase in tissue extracts of *Trichoplusia ni* larvae at 4 days post-inoculation (p.i.) with AcMNPV. The distinctive properties of the enzyme were based on the percentage inhibition or stimulation of activity following heat treatment and the addition of KCl, NaCl or phosphate to the assay mixture.

The DNA polymerase activities associated with whole cell extracts from *T. ni* NPV-infected and control *Spodoptera frugiperda* tissue culture cells have been studied by Kelly (1981) and by Wang & Kelly (1983). The DNA polymerase activity associated with infected cells was preferentially inhibited by aphidicolin when compared to control cell samples. Using larval tissue extracts, Miller *et al.* (1981) observed undifferentiated aphidicolin inhibition of polymerase activity (based on percentage inhibition) associated with control and infected samples and concluded that an ε-like DNA polymerase directed viral DNA replication. Both studies showed that total DNA polymerase activity increased dramatically in infected cells. Mikhailov *et al.* (1986a, b, c) reported changes in DNA polymerase activities in pupae and tissue culture cells of *Bombyx mori* after infection with *Bombyx mori* NPV. They reported a new DNA polymerase and increased DNA polymerase α and β activities. On the basis of primer : template preference, they found no evidence for increased levels of γ DNA polymerase activity. All of these studies used semi-purified enzyme preparations and their results indicate a complex regulation of the relative activities of several DNA polymerase species in the infected tissues as measured by TCA-precipitable products.

The present study documents the properties of the AcMNPV-specific, DNA-dependent...
DNA polymerase(s) in isolated nuclei from synchronously infected *T. ni* cells. In order to evaluate only virus-specific DNA replication, nucleic acid hybridization procedures were employed, thereby discriminating between viral and non-viral DNA replication products. Template specificities, time of detection p.i. and sensitivity to salts and inhibitors were investigated as a means of characterizing the DNA polymerase(s) and their induction during the infection cycle.

The *T. ni* cell line (TN-368) was grown in modified TNMFH medium (Wood, 1980a) at 26 °C. The 1-A clone of the AcMNPV (Wood, 1980b) was used in all of the experiments and was propagated as described by Wood & Speyer (1978). Cells in the logarithmic phase of growth were inoculated in flasks at 2 × 10⁵ cells/ml and infected at a multiplicity of 10 p.f.u. per cell. Flasks were gently rocked for 1 h at room temperature. The time immediately following rocking and removal of inoculum was denoted as zero time p.i. Nuclei were isolated from inoculated and mock-inoculated TN-368 cells according to Wood *et al.* (1982) except that the hypotonic TMN-buffer contained 0.75 mM-MgCl₂ and 0.14 mM-NaCl. The nuclei were free of cytoplasmic membranes as judged by phase microscopy. Nuclei were stored at −70 °C or used immediately. After freezing and thawing the nuclei were clumped and therefore were briefly sonicated before use. Virus was extracted from occlusion bodies purified from infected *T. ni* larvae as described by Wood (1980b). The AcMNPV DNA was extracted as outlined by Burand *et al.* (1980).

The optimal conditions used in the standard assay for DNA synthesis by intact nuclei *in vitro* were the following (listed as final concentrations in 50 µl): 100 mM-Tris–HCl pH 7.7; 2.5 mM-dithiothreitol; 6-25 mM-MgCl₂; 75 mM-KCl; 0.3 mM-dATP, -dCTP, and -dGTP; 0.003 mM-dTTP; 1 µCi [³²P]dTTP (triethylammonium salt, sp.act. 3000 Ci/mmol; Amersham). Gapped calf thymus DNA (2.5 µg/sample) was prepared according to Spanos & Hübscher (1983). All reactions were initiated by the addition of 5 × 10⁴ nuclei in 25 µl. Incubation was at 30 °C for the indicated times. Inhibitors of polymerase activity were added prior to the addition of nuclei. The reaction was terminated by adding 400 µl ice-cold 10% (w/v) TCA, 1% sodium pyrophosphate and 25 µg yeast RNA (2 h at 4 °C). The precipitates were collected on no. 25 glass-fibre filters (Schleicher & Schüll), washed with 5% (w/v) TCA and absolute ethanol, dried and counted in a Beckman LS 5801 liquid scintillation counter.

For slot blot hybridization experiments, the reaction volume was increased threefold. After 30 min incubation, SDS was added to 1% (w/v) and DNA was extracted with phenol and precipitated with ethanol. Viral or control DNA (1.5 µg per slot) were immobilized on nitrocellulose paper (Schleicher & Schüll, 0.45 µm, BA85 in a MiniFold II) as described by Wood *et al.* (1982). Southern blots were prepared as described by Summers *et al.* (1980). Prehybridization and hybridization buffers (Wood *et al.*, 1982) contained 50% (v/v) formamide. The [³²P]labelled DNA probes were sheared and heated to 100 °C for 2 min and incubated with the filters for 48 h at 37 °C. They were then washed three times at room temperature with 2 × SSC (1 × SSC is 0.15 M-NaCl plus 0.015 M-sodium citrate) containing 1% (w/v) SDS, followed by two washes at 42 °C with 0.1 × SSC, 0.1% (w/v) SDS. Autoradiograms of the filters were prepared with Kodak XRP-1 X-ray film at −70 °C with a Cronex intensifying screen (DuPont).

Incorporation of deoxyribonucleotides into DNA by DNA polymerases was optimized for pH, temperature, salt concentration, metal ion requirements and nucleotide concentration (see above). Magnesium chloride was necessary for optimal DNA synthesis in isolated nuclei at a concentration of 6-25 mM. Manganese chloride (6-25 mM) could be substituted for MgCl₂ without loss of activity. The incorporation of [³²P]dTMP under optimal conditions was proportional to the amount of sample protein and labelled precursor added and was linear for 30 min at 30 °C. The DNA polymerase activity was enhanced 30-fold at 23 h p.i. (280600 TCA-precipitable c.p.m.) as compared to uninfected cells (9120 c.p.m.). No differences were detected between fresh, intact nuclei and frozen and thawed, sonicated nuclear samples.

The data in Table 1 confirm that the product synthesized in the DNA polymerase reaction system was DNA. The product was sensitive to DNase I, but not to RNase A. Sodium pyrophosphate inhibited DNA synthesis, as did heat treatment of the nuclei. Since ethidium bromide (0.4 mg/ml) completely inhibited the DNA polymerase reaction, terminal transferase activity was not a contributing factor in the assay. Inhibition of DNA replication by aphidicolin
Short communication

Fig. 1. Effect of aphidicolin on the incorporation of \[^{32}\text{P}]\text{dTMP}\ into TCA-precipitable DNA isolated at 22 h p.i. from AcMNPV-infected TN-368 nuclei. Each sample contained 7.5 \times 10^6 nuclei.

Fig. 2. Slot blot hybridization of 1.5 \mu g immobilized AcMNPV DNA to DNA synthesized \textit{in vitro} by AcMNPV-infected TN-368 nuclei, that were isolated 24 h p.i. and incubated in the presence (lane 2) or absence (lane 1) of 20 \mu g/ml aphidicolin. Equal proportions of \[^{32}\text{P}]\text{-labelled product DNA containing 27249 c.p.m.}/\mu l in the presence and 45690 c.p.m.}/\mu l in the absence of aphidicolin were hybridized. The volumes (in \mu l) are as follows: (A) 0.033, (B) 0.05, (C) 0.10, (D) 0.20, (E) 0.4, (F) 0.8, (G) 1.6, (H) 3.2.

Table 1. \textit{Effect of inhibitors on polymerases in nuclei isolated from AcMNPV-infected TN-368 cells}

<table>
<thead>
<tr>
<th>Inhibiting treatment</th>
<th>DNA polymerase*</th>
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<tbody>
<tr>
<td>Standard</td>
<td>100 (128,212 c.p.m.)</td>
</tr>
<tr>
<td>40 \mu g/ml DNase I</td>
<td>7</td>
</tr>
<tr>
<td>40 \mu g/ml RNase A</td>
<td>100</td>
</tr>
<tr>
<td>25% (w/v) sodium pyrophosphate</td>
<td>1</td>
</tr>
<tr>
<td>Heat treatment (100 °C/5 min)</td>
<td>3</td>
</tr>
<tr>
<td>0.4 mg/ml ethidium bromide</td>
<td>0</td>
</tr>
<tr>
<td>20 \mu g/ml aphidicolin</td>
<td>16</td>
</tr>
<tr>
<td>5 mM-N-ethylmaleimide</td>
<td>20</td>
</tr>
<tr>
<td>50 \mu M-ddTTP</td>
<td>3</td>
</tr>
<tr>
<td>1 mM-novobiocin</td>
<td>139 (37†)</td>
</tr>
</tbody>
</table>

* Activity is expressed as % of c.p.m. incorporated per 5 \times 10^4 standard nuclei sample. Nuclei were isolated 22 h p.i. as described above.
† Nuclei isolated from mock-inoculated TN-368 cells.

was concentration-dependent with 50% inhibition observed at 0.2 \mu g/ml and 84% at 20 to 50 \mu g/ml (Fig. 1). The bimodal shape of the curve may reflect the difference in aphidicolin sensitivity between host- and virus-induced DNA polymerase (Fig. 1). Although the aphidicolin solvent dimethyl sulphoxide (DMSO) is known to have an effect on mammalian DNA polymerase \(\alpha\) (Lee & Toomey, 1986), 50% DMSO had no effect in our assay system.

To investigate the nature of the DNA polymerase which was inhibited at 20 \mu g/ml
In the presence of aphidicolin, we examined the DNA product synthesized in the presence of aphidicolin. Nuclei were incubated in the presence or absence of 20 μg/ml aphidicolin and the labelled DNA was extracted. Equal proportions of the labelled DNA samples were hybridized to constant amounts of AcMNPV DNA. As shown in Fig. 2, there was essentially no difference in intensity of the hybridization between corresponding aphidicolin plus and minus samples. Therefore, although total DNA synthesis decreased approximately 84% in the presence of aphidicolin, viral DNA synthesis did not decrease.

N-Ethylmaleimide (NEM), an inhibitor of α and γ DNA polymerases, at 5 mM inhibited the DNA polymerase activity by 80% and no virus-specific products were detected. DNA polymerase activity was inhibited 97% by 50 μM-ddTTP, an inhibitor of β and γ DNA polymerases, in nuclei from infected cells but only 50% when assayed in nuclei from mock-inoculated cells. In the presence of ddTTP, virus-specific products were not detected by DNA-DNA hybridization. The α DNA polymerase (Edenberg, 1980) and topoisomerase inhibitor novobiocin (1 mM) inhibited the DNA polymerase activity 63% in mock-inoculated cell nuclei, but that in virus-infected cell nuclei was slightly stimulated. In contrast, 2 mM-spermine enhanced the DNA polymerase activity in mock-inoculated cells by 515% while inhibiting that of infected cells by only 64%.

To determine whether DNA polymerases in nuclei of AcMNPV-infected TN-368 cells could synthesize DNA sequences on heterologous template DNA, the isolated nuclei were incubated in the presence of saturating levels of heterologous DNA (Fig. 3). The DNA polymerase activity in infected nuclei at 23 h p.i., but not in control nuclei (data not shown), synthesized AcMNPV DNA as well as sequences complementary to *Lymantria dispar* NPV (LdMNPV) DNA, *Heliothis armigera* granulosis virus DNA and herring sperm DNA. The limited amount of hybridization detected between 32P-labelled AcMNPV DNA and LdMNPV DNA (Fig. 3) was expected because all the NPVs tested share homologous polyhedrin gene sequences (Smith & Summers, 1982). Due to the endogenous template in infected cells, DNA products which hybridized to AcMNPV DNA were detected when no DNA was added to the reaction mixture (Fig. 3).

To examine virus-specific replication during the course of infection, virus-specific DNA polymerase activity was assayed in the presence of aphidicolin in nuclei which were isolated at different times p.i. DNA sequences complementary to AcMNPV DNA were first detected at 5 h p.i. (Fig. 4), which coincides with the onset of viral DNA replication in whole cells (Wood et al., 1982). The slight decrease in hybridized product detected at 7 h p.i. in the presence of aphidicolin may reflect a contribution by an α DNA polymerase early in the replication cycle. Supplementing these reactions with AcMNPV DNA gave identical results, indicating that enough template DNA had accumulated for replication to proceed at early stages of the infection process.

Since the viral DNA templates were not gapped, the enzymic activity measured was probably either α- or γ-like in its ability to initiate replication as opposed to the repair activity of β-like DNA polymerases (Kornberg, 1980). This was consistent with the absence of detectable levels of hybridizable DNA products from infected nuclei in the presence of NEM. Beta- and γ-like polymerases from mammals and *Drosophila* are insensitive to aphidicolin and are strongly inhibited by ddTTP (Kornberg, 1980; Sakaguchi & Boyd, 1985). Based on DNA-DNA hybridizations (Fig. 2, 4), the replication of viral DNA was not inhibited by aphidicolin. The aphidicolin-insensitive DNA polymerase activity in infected cells was 4.8-fold (16% of a 30-fold increase) more active than the total DNA polymerase activity associated with uninfected cells. Accordingly, it must play the major role in viral DNA replication. On the basis of the lack of sensitivity to aphidicolin and novobiocin as well as the sensitivity to NEM and ddTTP, we conclude that the polymerase involved in viral DNA replication is a γ-like polymerase. The stimulation of activity by the addition of salts and heat inactivation (Kornberg, 1980; Wernette & Kaguni, 1986) reported by Miller et al. (1981) support this conclusion. Although γ DNA polymerases are usually found in low levels in the nucleus (Kornberg, 1980), baculovirus infections, like those of adenovirus (Oguru et al., 1984), apparently induce high levels of γ DNA polymerase(s). An aphidicolin-sensitive DNA polymerase is involved with the synthesis of
Fig. 3

Fig. 3. DNA synthesis in nuclei of TN-368 cells isolated 23 h after infection with AcMNPV. The following templates were added to the reaction mixture at 60 µg/ml: AcMNPV DNA (Ac), LdMNPV (Ld), Heliothis armigera granulosis virus (HaG) and herring sperm DNA (HS). The 32P-labelled products were hybridized to the DNA immobilized on nitrocellulose filters, as indicated. AT-DNA, Added template DNA; IM-DNA, immobilized DNA.

Fig. 4

Fig. 4. Slot blot hybridization of 1.5 µg immobilized AcMNPV DNA to DNA synthesized in vitro by AcMNPV-infected TN-368 nuclei that were isolated at various times p.i. and incubated in the presence (lane 2) or absence (lane 1) of 20 µg/ml aphidicolin. *, Samples from mock-inoculated TN-368 nuclei to which 50 µg/ml AcMNPV DNA was added in the presence (lane 2) or absence (lane 1) of 20 µg/ml aphidicolin.

interior fragments of the adenovirus DNA located approximately 20 to 30% from each terminus, while an aphidicolin-insensitive DNA polymerase is implicated in initiation and elongation of other regions of adenovirus DNA (Oguru et al., 1984). Without purified enzyme preparations it is not possible to determine whether there is a differential aphidicolin sensitivity as found with adenovirus replication.

We have recently developed a DNA-dependent replication system from infected TN-368 cell nuclei by removing endogenous DNA on a DEAE-cellulose column in essentially the same manner as described for a template-dependent soluble extract containing vaccinia virus RNA polymerase (Rohrmann & Moss, 1985). In this system the inhibition studies described above were completely identical (results not shown), indicating that our results were not an artifact of the nuclei system.

Novobiocin, an inhibitor of DNA topoisomerase I activity, stimulates the replication of AcMNPV DNA but inhibits DNA synthesis in nuclei of healthy cells (Table 1). Apparently, the
DNA polymerase induced by AcMNPV infection does not require unwinding of the DNA template for replication to proceed.

In conclusion, we have confirmed earlier evidence that the DNA polymerase which directs AcMNPV DNA replication is either a novel, virus-induced, modified or normally undetectable host enzyme. The induction of an additional 30-fold increase in total DNA polymerase activity associated with AcMNPV-infected cells as compared to control cells is of interest since host DNA synthesis is interrupted following infection (Wood et al., 1982). We have extended previous characterizations using additional DNA polymerase inhibitors combined with hybridization studies. The results strongly suggest the induction of a γ-like polymerase by AcMNPV.

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REFERENCES


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