Baculovirus Replication: Stimulation of Thymidine Kinase and DNA Polymerase Activities in Spodoptera frugiperda Cells Infected with Trichoplusia ni Nuclear Polyhedrosis Virus

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SUMMARY

Trichoplusia ni multiply enveloped nuclear polyhedrosis virus stimulates thymidine kinase and DNA polymerase activities in infected cells. The kinetics of induction and sensitivity of induction to cytosine arabinoside indicate that they represent early functions in infected cells. The basic characteristics of the induced enzymes are defined.

INTRODUCTION

Baculoviruses are large DNA-containing viruses which replicate in the nucleus of invertebrate cells. The viruses contain supercoiled DNA of mol. wt. about $8 \times 10^7$ (Bud & Kelly, 1977) and the multiply enveloped nuclear polyhedrosis virus of Trichoplusia ni used in this study has a genome size of $8.3 \times 10^7$ (Bud & Kelly, 1980). Little is known about the replication of baculoviruses in molecular terms. The viruses sequentially induce 30 to 35 polypeptides in infected cells (Carstens et al., 1979); it has been suggested that at least four phases (named α, β, γ and δ from earliest to latest) of induction of polypeptides occur in a cascade fashion (Kelly & Lescott, 1980) typical of a number of large DNA viruses (Honess & Roizman, 1974; Elliott & Kelly, 1980). When baculovirus DNA replication is prevented, the induction of virus-specific polypeptides fails to progress beyond immediate early α or early β polypeptide synthesis (Kelly & Lescott, 1980). Virus DNA synthesis occurs from about 6 to 8 h after infection (Tjia et al., 1979; D. C. Kelly & M. D. Ayres, unpublished observations).

In a previous paper, we have shown that a number of non-structural polypeptides (i.e. polypeptides not detected in virus particles or polyhedra) are induced in baculovirus-infected cells (Kelly & Lescott, 1980). This paper reports that thymidine kinase and DNA polymerase activities, candidate functions for non-structural polypeptides, are stimulated in baculovirus-infected cells. These two enzyme activities were investigated to provide markers for non-structural functions in future genetic studies of these viruses. The basic biochemical properties of these two activities are reported in this paper.

METHODS

Virus and cells. Spodoptera frugiperda cells (Vaughn et al., 1977) were grown in BML/TC10 medium (Gardiner & Stockdale, 1975). T. ni multiply enveloped nuclear polyhedrosis virus (Faulkner & Henderson, 1972) was grown and titrated in S. frugiperda cells as previously described (Kelly & Lescott, 1980; Brown et al., 1978).

Preparation of cell extracts for enzyme assay. Cells were infected at a multiplicity of 50 p.f.u./cell, $10^6$ and $10^7$ cells being used for most experiments. Routinely, cells were harvested at 12 h after infection by removing medium, washing in ice-cold PBS and finally resuspending...
in cold sterile distilled water (8 × 10^5 cells/ml). Cells were disrupted for 10 min in a sonicating water bath (Kerry Ultrasonics, Hunting Gale, U.K.) at 4 °C. This provided the crude extract for the determination of DNA-dependent DNA polymerase activity. To provide material to assay thymidine kinase activity the extract was overlaid with water-saturated liquid paraffin and centrifuged at 100 000 g for 45 min at 4 °C; the top 75% of the supernatant was removed and used for assay. Extracts were used immediately, or stored at −20 °C and thawed once before assay.

**Determination of thymidine kinase activity.** The assay was based on that for herpes simplex virus-stimulated thymidine kinase activity described by Klemperer et al. (1967). The optimum conditions used to assay the virus-induced enzyme and uninfected cell enzyme activities were as follows. The reaction mixture (100 µl) contained cell 100 000 g supernatant (16.5 µg protein), 8.3 µM-2-14C-thymidine (0.05 µCi; The Radiochemical Centre, Amersham, U.K.), 24.5 mM-ATP, 0.3 mM-MgCl2 and 0.05 mM-tris-HCl pH 7.5. Uninfected cell extracts contained 1 mM-MgCl2 and were buffered at pH 8.5. The reaction mixtures were made up at 4 °C. The mixtures were incubated for 90 min at 28 °C and the reaction was terminated by boiling for 2 min and then cooling quickly on ice. Denatured material was removed by centrifuging at 1000 g for 10 min.

Thymidine was separated from thymidine monophosphate by the chromatographic method described by Sheinin (1966). Forty-five µl of the reaction mixture was applied to a 2.5 × 46 cm DE81 cellulose paper (Whatman) 12.5 cm from the leading edge. The sample was allowed to dry and the labelled thymidine was removed from the phosphorylated derivatives by descending chromatography in 1 mM-ammonium formate for 5 h. The phosphorylated labelled material remaining at the origin was assayed by drying the strips at 60 °C for 30 min, cutting out 4 × 2.5 cm area at the origin, and then determining the amount of radioactivity by liquid scintillation spectrometry. This measurement was corrected by deducting the blank value found for an unincubated reaction mixture.

**Determination of DNA-dependent DNA polymerase activity.** The reaction mixture was a modified form of a method used to analyse DNA-dependent DNA polymerase activity in herpes simplex virus-infected BHK cells (A. Buchan, unpublished observations). The mixture (280 µl) contained supernatant from sonicated cell extracts (37.5 µg protein), 17 mM-tris-HCl buffer pH 7.5, 89 mM-(NH4)2SO4, 26.5 mM-KCl, 2.8 mM-MgCl2, 0.36 mM-EDTA, 3.6 mM-dithiothreitol, 180 µM-dATP, 180 µM-dGTP and 180 pM-dCTP, 1.79 µM-TTP, 0.72 mg nicked DNA primer and 24.4 µM-methyl-3H-thymidine 5-triphosphate (0.5 µCi) (The Radiochemical Centre, Amersham). Uninfected cell extracts were assayed in the presence of 1-4 mM-MgCl2 and 14 mM-KCl. The nicked salmon sperm DNA primer was prepared by incubating 1 ml salmon sperm DNA solution (1 mg/ml) with 50 µl DNase I from bovine pancreas (0-2 mg/ml) (Sigma, London) for 10 min at 37 °C; the reaction was stopped by heating at 72 °C for 10 min.

The reaction mixture was made up at 4 °C, incubated for 90 min at 28 °C, cooled quickly on ice, and the reaction was stopped by adding 10 ml flocculation mixture. The flocculation mixture consisted of 205 mM-perchloric acid, 1 mM-sodium pyrophosphate, 0-41 mM-thymidine and 100 µg heat-denatured calf thymus DNA carrier in 10 ml. Flocculation proceeded overnight at 4 °C. The precipitate was collected by filtering through a glass fibre filter disc which was washed with 10 ml vol. of 0.2 M-perchloric acid (four times), ethanol (three times) and ether (once). The filter discs were dried in vials and the radioactivity determined by liquid scintillation spectrometry.

**Liquid scintillation spectrometry.** Radioactivity was measured by placing strips or discs containing sample into 10 ml toluene containing 4 g 2,5-diphenyloxazole and 0-5 g 1,4-di-2-(5-phenyloxazolyl)-benzene/l. The samples were read in a Packard Tricarb liquid scintillation counter.
Enzyme induction by a baculovirus

Table 1. Inhibition of thymidine kinase activity in T. ni-infected and uninfected S. frugiperda cell extracts by inhibitors of nucleic acid synthesis

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Infected cell extract (K/)*</th>
<th>Uninfected cell extract (K/)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromodeoxyuridine</td>
<td>2.31 ( \mu M )</td>
<td>—</td>
</tr>
<tr>
<td>Fluordeoxyuridine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cytosine arabinoside</td>
<td>1.11 ( \mu M )</td>
<td>—</td>
</tr>
<tr>
<td>Thymidine triphosphate</td>
<td>1.05 ( \mu M )</td>
<td>—</td>
</tr>
</tbody>
</table>

\*\( K_i \), the dissociation constant of the enzyme inhibitor complex, was determined from graphs based on the equation of Lineweaver & Burk as shown by Dawes (1969). The results were obtained at a substrate concentration of 16.7 \( \mu M \).—C-thymidine.

† No inhibition.

**Determination of protein.** Protein was estimated by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

**RESULTS**

**Basic characteristics of thymidine kinase activities in infected and uninfected cells**

The optima for assay of the enzyme activities with respect to pH, temperature and Mg\(^{2+}\) ion concentrations are as indicated in Methods and show differences between those of infected and uninfected cells in pH and divalent cation requirements. At these optimum conditions, using Mg\(^{2+}\) as the divalent cation, the activities were proportional to the amount of protein in the assay (from 1 to 100 \( \mu g \)), and to the time of incubation (up to 150 min for the infected extract and 90 min for the uninfected extract). The two enzyme activities differed in their affinity for substrate as their differing \( K_m \) shows (5.56 \( \mu M \) for the infected cell extract and 12.5 \( \mu M \) for the uninfected cell extract). Monovalent cation requirements were investigated using Na\(^+\), K\(^+\) and NH\(_4\)\(^+\) requirements in the 0 to 20 \( mM \) range (in 2 \( mM \) intervals) and no stimulation was detected in the presence or absence of divalent cations for both activities. Mercaptoethanol (1%, v/v) had no effect on kinase activities. Treatment of the enzyme extracts with 0.1% (w/v) SDS or heat treatment at 100 °C for 2 min totally destroyed enzyme activities.

**Effect of inhibitors of nucleic acid synthesis on thymidine kinase activities in infected and uninfected cells**

Addition of bromodeoxyuridine, fluorodeoxyuridine, cytosine arabinoside and thymidine triphosphate (TTP) in the range 0 to 300 \( \mu M \) (in 10 \( \mu M \) intervals) had little effect on uninfected cell extracts. Addition of these four drugs to the virus-infected cell extracts showed that all except fluorodeoxyuridine inhibited enzyme activity (Table 1).

**Stimulation of thymidine kinase activity during the growth cycle of T. ni nuclear polyhedrosis virus**

Fig. 1 shows the pattern of thymidine kinase stimulation at various times after infection of S. frugiperda cells by T. ni nuclear polyhedrosis virus, in the presence and absence of cytosine arabinoside. The enzyme was stimulated by 6 h after infection, reached a plateau level at 8 to 9 h and remained elevated for up to 24 h. The enzyme was also stimulated in cells treated with cytosine arabinoside to inhibit formation of polypeptides synthesized after DNA replication.
Fig. 1. Stimulation of thymidine kinase activity in *S. frugiperda* cells during the growth of *T. ni* nuclear polyhedrosis virus in the presence and absence of cytosine arabinoside. O—O, Normal virus stimulation; •—•, virus stimulation in the presence of cytosine arabinoside; △—△, uninfected cell activity; ▲—▲, uninfected cell activity in the presence of cytosine arabinoside.

Table 2. Substrate requirements and effect of inhibitors of nucleic acid synthesis on DNA polymerase activity in *T. ni* nuclear polyhedrosis virus-infected and uninfected *S. frugiperda* cell extracts

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Treatment</th>
<th>Infected cell extract</th>
<th>Uninfected cell extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Complete reaction mixture</td>
<td>10.4 (100%)*</td>
<td>1.4 (100%)*</td>
</tr>
<tr>
<td></td>
<td>— nicked DNA</td>
<td>0.0 (0%)</td>
<td>0.0 (0%)</td>
</tr>
<tr>
<td></td>
<td>— dATP</td>
<td>1.1 (11%)</td>
<td>0.7 (49%)</td>
</tr>
<tr>
<td></td>
<td>— dCTP</td>
<td>4.5 (43%)</td>
<td>0.5 (33%)</td>
</tr>
<tr>
<td></td>
<td>— dGTP</td>
<td>1.7 (17%)</td>
<td>0.0 (0%)</td>
</tr>
<tr>
<td></td>
<td>— dATP, dCTP, dGTP</td>
<td>0.0 (0%)</td>
<td>0.0 (0%)</td>
</tr>
<tr>
<td>2</td>
<td>Complete reaction mixture</td>
<td>10.6 (100%)</td>
<td>1.4 (100%)</td>
</tr>
<tr>
<td></td>
<td>1 µg actinomycin D/ml</td>
<td>10.4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>20 µg rifampicin/ml</td>
<td>10.0</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>20 µg phosphonoacetic acid/ml</td>
<td>10.1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>20 µg camptothecin/ml</td>
<td>9.7</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>1 µg sodium pyrophosphate/ ml</td>
<td>0.3</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Specific activity is nmol *H*-TTP incorporated into an acid product/µg cell protein/h at 28 °C.

Basic characteristics of DNA polymerase activities in infected and uninfected cells

The optima for assay of the enzyme activities with respect to pH, temperature, Mg$^{2+}$ ion and K$^+$ ion concentrations are as indicated in Methods, and (NH$_4$)$_2$SO$_4$ was essential for the reaction. At these optimum conditions the reactions were proportional to the amount of protein in the assay (from 1 to 200 µg) and to the time of incubation (up to 240 min). The reaction required all nucleotide triphosphates and a DNA primer to proceed efficiently (Table 2). The reaction of both enzymes was inhibited by the addition of sodium pyrophosphate. The product of the reaction was susceptible to degradation by DNase.

Effect of inhibitors of nucleic acid synthesis on DNA polymerase activities in infected and uninfected cells

Table 2 shows the effect of adding rifampicin, actinomycin D, phosphonoacetic acid and camptothecin to the basic DNA polymerase reaction. None of the drugs inhibited either reaction.
Enzyme induction by a baculovirus

\[ 2 \text{C-thymidine phosphorylated/\mu g cell protein h (mol)} \]

Fig. 2. Stimulation of DNA polymerase activity in *S. frugiperda* cells during the growth of *T. ni* nuclear polyhedrosis virus in the presence and absence of cytosine arabinoside. O--O, Normal virus stimulation; ●--●, virus stimulation in the presence of cytosine arabinoside; △--△, uninfected cell activity, •--•, uninfected cell activity in the presence of cytosine arabinoside.

**Stimulation of DNA polymerase activity during the growth cycle of *T. ni* nuclear polyhedrosis virus**

Fig. 2 shows the pattern of stimulation of DNA polymerase activity at various times after infection. The enzyme was stimulated by 5 h after infection, reaching maximum synthesis at 10 to 12 h and remaining at elevated levels thereafter. The DNA polymerase and thymidine kinase assays were performed on extracts obtained in one experiment.

**DISCUSSION**

*T. ni* nuclear polyhedrosis virus stimulates thymidine kinase and DNA polymerase activities in infected *S. frugiperda* cells. In the case of thymidine kinase, the virus-induced enzyme differed biochemically from the uninfected cell enzyme, although the differences between the two DNA polymerase activities were less marked. Crude cell extracts were used to determine these properties and as yet no concrete evidence that the enzyme activities are virus-coded has been obtained. It is probable that future genetic studies, or in the case of thymidine kinase, investigations with enzyme-deficient cells, will conclusively show whether baculovirus-induced enzymes are virus-coded. By analogy with other large DNA viruses (Cohen, 1968; Keir, 1968) it is probable that baculoviruses possess the genetic information for these enzymes. However, the demonstration that papovaviruses stimulate enzymes concerned with nucleic acid synthesis while not possessing the genetic information for them (Kit, 1967), indicates that the inference for baculoviruses should be treated with caution. Nevertheless, for the purpose of this discussion it is assumed that the enzymes are virus-coded.

Both enzymes are stimulated in the presence of cytosine arabinoside which markedly depresses \( \gamma \) and \( \delta \) polypeptide synthesis in baculovirus-infected cells (Kelly & Lescott, 1980), indicating that the enzymes are probably \( \alpha \) or \( \beta \) polypeptides. From the timing of their induction they are probably \( \beta \) polypeptides since the enzymes are stimulated from 5 to 6 h after infection coincident with the onset of \( \beta \) polypeptide synthesis (Kelly & Lescott, 1980), and preceding virus DNA synthesis (from 6 to 7 h after infection with this virus).

Thymidine kinase stimulated in baculovirus-infected cells was markedly different to the uninfected cell enzyme, indicating that a novel enzyme was induced in the cells. The two enzymes differed in their susceptibility to inhibitors of nucleic acid synthesis. Interestingly, TTP inhibits only the virus-stimulated enzyme, whereas the cell enzyme is not inhibited and
so presumably is not subject to feedback inhibition. The phenomenon is the converse of that observed with herpes simplex virus-induced thymidine kinase where the virus enzyme is not inhibited by TTP and the cell enzyme is (Klemperer et al., 1967). The induced baculovirus enzyme has a greater affinity for its substrate than has the cell enzyme, as shown by their respective Michaelis constants. The baculovirus-induced enzyme is inhibited by SDS and by heat treatment, but not mercaptoethanol and so preparative gel systems to produce purified enzyme would require a non-denaturing system.

DNA polymerase activity stimulated in baculovirus-infected cells was not markedly different to the cell enzyme, and these results do not show that a novel enzyme is induced in infected cells, nor do they invalidate such a suggestion. Both enzyme activities required a DNA primer, were inhibited by pyrophosphate, and the product was degraded by DNase, consistent with the enzyme being a DNA-dependent DNA polymerase, as was the failure of rifampicin and actinomycin D to block the reaction. Phosphonoacetic acid, a drug which inhibits the replication of a number of large viruses by specifically inhibiting the virus-induced polymerase (Gerstein et al., 1975; Overby et al., 1974; Moreno et al., 1978; Elliott et al., 1980; Bolden et al., 1975), failed to inhibit the baculovirus-stimulated enzyme and this may explain why the virus is able to induce late polypeptides in infected cells and to replicate normally in cells treated with this drug (Kelly & Lescott, 1980).

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REFERENCES


Enzyme induction by a baculovirus


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